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Key words: benign prostatic hyperplasia, innate immunity, malignancies, NKT cells, prostate cancer, regulatory T cells

Received January 19, 2015.

Abstract

Background and Purpose: *Regulatory T cells (Tregs) and NKT cells are two subpopulations of T lymphocytes that independently regulate innate and adaptive immunity, but there is some evidence for cross-talk between Tregs and NKT cells, which allow a new immunoregulatory networks. Activated NKT cells may modulate quantitatively and qualitatively the function of Tregs through IL-2-dependent mechanisms, while Tregs can suppress the proliferation, cytokine release and cytotoxic activity of NKT cells by cell-contact-dependent mechanisms. Tregs may control tumor expansion at the priming, as well as the effector's phase of T immune responses. Tumor cells provide antigenic stimulation of T cells and interact with the tumor-infiltrated innate immune cells secreting cytokines that are crucial for T-cell differentiation.*

Patients and Methods: *In this study we examined the prostate tissue infiltrating lymphocytes of patients with prostate cancer (PCa) and benign prostatic hyperplasia (BPH) by flow cytometric technique (FACSCalibur) for determine the number of T, B, NK, NKT and Tregs and investigate the local regulatory immunosurveillance which allows the tumor's immune-escape.*

Results: *Our results have shown the statistically significantly elevated number of Tregs in prostatic tissue and slightly diminished percentage of NKT cells in prostate cancer patients in comparison to patients with benign prostatic hyperplasia.*

Conclusion: *Although the exact mechanism is still unknown, increased infiltration of prostate tissue with T regulatory cells seems that stimulate the tumor to secrete factors (chemokines) that attract these cells in the tissue of the prostate where they achieve their anti-tumor effect and thus may contribute the tumor progression.*

INTRODUCTION

Over the last few years, many studies using human tissue showed a possible role of the immune system in the pathogenesis of BPH and PCa *(1-9)*. Epithelial cell turnover is affected by chronic inflammation, increasing on that way the risk of malignancies on 15% worldwide which may be attributed to infection agents *(3)*. The inflammatory mi-

croenvironment includes the action of many different cells like macrophages and activated T lymphocytes that may release angiogenic factors, chemokines and proteases. Acute and chronic inflammation of the urogenital system leads to accumulation of immunocompetent cells in the prostate, mainly T lymphocytes and macrophages *(4)*. These cells then secrete numerous cytokines (IL-2, IFN-gamma, IL-6, IL-8, IL-15), which participate in pathological changes, as well as, in the activation of infiltrating lymphocytes which are characteristic for BPH and PCa *(10)*. Secretion of stimulatory or inhibitory cytokines by infiltrating lymphocytes or neoplastic cells significantly affects the development or suppression of the occurrence of BPH and PCa. Although many studies pointed to the potential role of infiltrating T lymphocytes in the development and progression of BPH and PCa, the role of other immunocomponent cells, primarily regulatory T cells (Tregs) and NKT cells in the pathogenesis of BPH and PCa, still remains unclear. Regulatory T cells (Tregs) represent a diverse subpopulation of T lymphocytes that have a possibility to promote their actions by different mechanisms to regulate pathogenic and autoreactive immune responses *(11, 12)*. Naturally occurring Tregs are endogenous and mature within the thymus, while the adaptive Tregs are maturated from naive T cells and may differentiate into type 1 regulatory (TR1) cells, which have a crucial role in autoimmune disease and are characterized by producing anti-inflammatory cytokine IL-10 and subpopulation with characteristic phenotype CD4+CD25+FoxP3+ that converse from peripheral memory T cells. Adaptive Tregs with defined immunosuppressive properties represent Th3 cells and secrete transforming growth factor (TGF-beta). Double negative CD4−CD8−T cells which express the gamma/delta TCR chain represent a subset of natural killer T cells and Th2 cells with possibility to suppress autoimmunity. Another small population of T cells (TCR-γδ+ T cells) has γ and δ TCR chains, differs from αβ+ T cells and seems to has a possibility to act as professional antigen-presenting cells (APC) and regulatory cells. These γδ+ Tregs are presented in peripheral tissues, digestive tract and solid tumor, but it is still not clear their function and presence in tumor sites *(13)*. Natural Tregs (CD25+CD4+) express different very important molecules for their function [Foxp3, cytotoxic lymphocyte-associated antigen-4 (CTLA-4), IL-2 receptor (IL-2R), glucocorticoid-induced tumor necrosis factor related protein (GITR)], which are involved in their development, activation and survival *(14)*. Manipulation with the activation or blocking of these molecules may induce different immunological response of tumor immunity or autoimmune disease. On the other side, genetic deficiency of some of these molecules induces severe autoimmune diseases. Foxp3 represents a new transcription factor of the forkhead/winged-helix family and first was found as the defective gene in X-linked recessive mutant with lethality in hemizygous males, associated with overproduction of proinflamma-

tory cytokines. In humans mutation of FoxP3 gene induces rare, but severe syndrom IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), accompanied with autoimmune disease (type I diabetes mellitus, autoimmune thyroiditis, allergic dermatitis, inflammatory bowel disease and severe infections) *(15, 16)*. Moreover, some of these molecules are used to detect natural Tregs and their kinetics in different physiological and pathological conditions *(17, 18)*. NKT cells represent a unique subpopulation of T cells and NK cells, sharing common markers characteristic for NK cells and T cells. In contrast to conventional T lymphocytes, NKT cells do not perform their activities through actions with peptide antigen presented by classical MHC-class I or II molecules, but recognizes glycolipids antigen presented by nonclassical antigen–presenting molecule CD1d *(19-21)*.

SUBJECT AND METHODS

Tissue samples

The study protocols was approved by Ethics Committee of the Medical Faculty, University of Rijeka and written informed consent was obtained for each patient included in the study. Patients' data and tissue samples were acquired in accordance with the published International Health Guidelines outlined in the declaration of Helsinki.

Prostate tissue samples were collected from 20 patients (ages 62-73; mean 67 years old) undergoing radical prostatectomy in the Clinic of Urology, Clinical Hospital Centre Rijeka, Croatia. Histopathological analysis of prostate tissue samples confirmed that all samples were carcinomas with a differentiation grade according to Gleason of 6 – 9. The tissues with BPH were acquired form 20 patients (ages 56-70; mean 63 years old) who underwent transvesical prostatectomy. Due to ethical reasons healthy prostate tissue were not obtained for enzymatic digestion of the prostate tissue. Prostate tissue from two healthy men was obtained during autopsy, paraffin embedded and used for immunoflurescence staining.

None of the patients included in the study were previously treated with immunosuppressive or radiation therapy and had any immunological disease, acute or chronic inflammatory disease.

Isolation of prostate mononuclear cells

Prostate tissue samples obtained at surgery were cut into small pieces and digested with 0.1% collagenase type IV (Sigma-Aldrich, Taufkirchen, Germany) on a magnetic stirrer for 90 minutes at 37ºC. After the digestion, the cell suspension was passed through 100 mm nylon mesh (Becton Dickinson, Franklin Lakes, USA) to remove tissue debris, overlaid on Lymphoprep (Nycomed

Pharma AS, Oslo, Norway) and centrifuged for 20 minutes at 600 g. The prostate mononuclear cells were collected from the interface, washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Auckland, NZ) and used for further experiments. The viability of the isolated mononuclear cells was over than 95% assessed with propidium iodide 0.5 μg/ml/10^6 cells (Sigma-Aldrich, Taufkirchen, Germany) and flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

Antibody staining and flow cytometry analysis

Surface staining of prostate mononuclear cells were performed for 30 min at 4 °C with different combination of fluorescein isothiocyanate (FITC)-conjugated anti-CD3 monoclonal antibody (mAb, mouse UCHT1, IgG_1), FITC-conjugated anti-CD4 mAb (RPA-T4, IgG1), Phycoerithryn PE-conjugated anti-CD4 mAb (mouse RPA-T4, IgG1), PE-conjugated anti-CD56 mAb (mouse B159, IgG1), PE labelled anti-CD8 mAb (mouse RPA-T8, IgG1) and allophycocyanin (APC)-conjugated anti-CD25 mAb (BC96, IgG1). Isotype match antibodies were used to set negative controls for each class of antibody used. All the antibodies were provided from BD Biosciences, Erembodegen, Belgium and used at the concentration of 20 μl/10^6 cells, unless otherwise specified. Intracellular staining for FoxP3 was performed on the cells previously stained with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 mAbs, and consequent-ly fixed and permeabilized using fixation/permeabilisation kit (BD Biosciences) according to the manufacturer's instructions. The cells were than labelled with PE-conjugated mAb against FoxP3 (PCH101, IgG2a) or its isotype control for 30 min at 4°C. Labelled cells were fixed with 2% paraformaldehyde pH 7.4 (Kemika, Zagreb, Croatia) for 20 min at room temperature, washed twice in Phosphate Buffered Saline (PBS) [NaCl 8 g, KCl 0.2 g, Na_2H-$PO_4 x12H_2O$ 2.87 g and KH_2PO_4 0.2 g (all from Kemika, Zagreb, Croatia) dissolved in 1 L of distilled water] and analyzed by flow cytometry. Dead cells were excluded by propidium iodide (0.5 μg/ml) and total of 10,000 cells was acquired by flow cytometry.

Statistical analyses

Statistical analysis was done using data analysis software system Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). Data are presented as median value and 25-75% ($25^{th} - 75^{th}$ percentile). Outlier results are also shown. The difference between groups was calculated with Mann–Whitney U-test non-parametric test and the difference was significant at $p < 0.05$.

RESULTS

To investigate the prevalence and distribution of certain lymphocyte subpopulations (T lymphocytes and their subpopulations, NK cells and NKT cells) in the prostate tissue of patients with BPH and PCa, we labeled paraffin preparations of prostate tissue of patients with
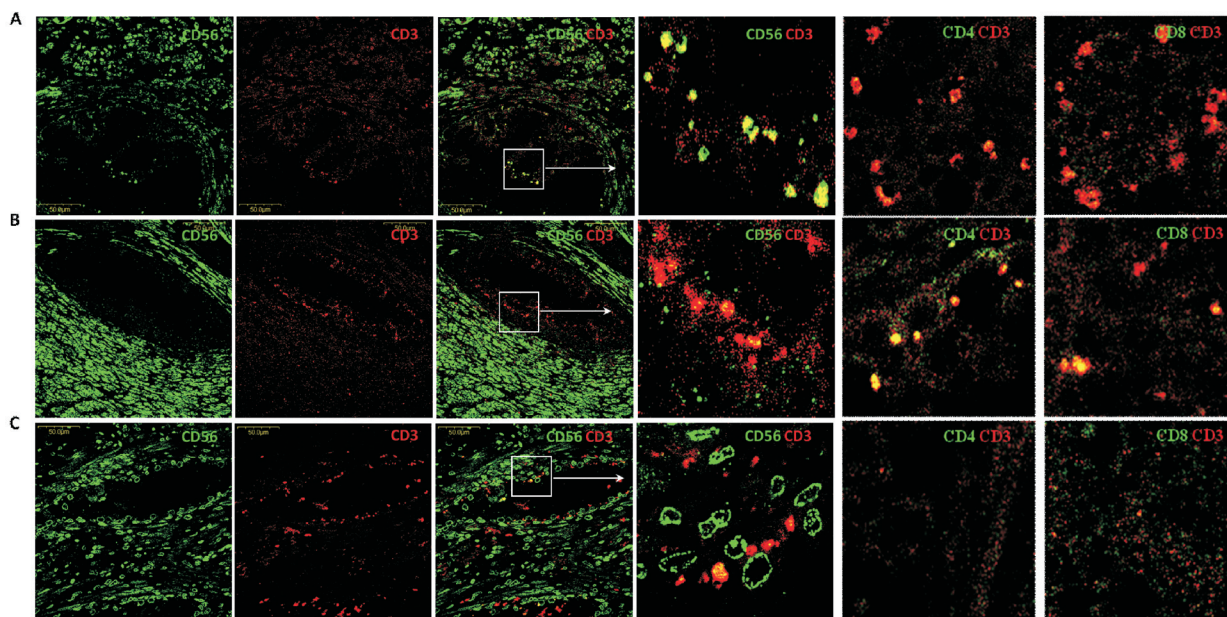

Figure 1. *Expression and localization of CD3+ CD56+ ,CD4+ and CD8+ cells in BPH and PCa. After deparaffinization of the sections, either control prostate tissue (A), BPH (B) or PCa (C), immunofluorescence assay was performed. Sections were incubated with primary antibodies against CD3, CD56, CD4 and CD8 over night. Then, the sections were stained with secondary reagents.*
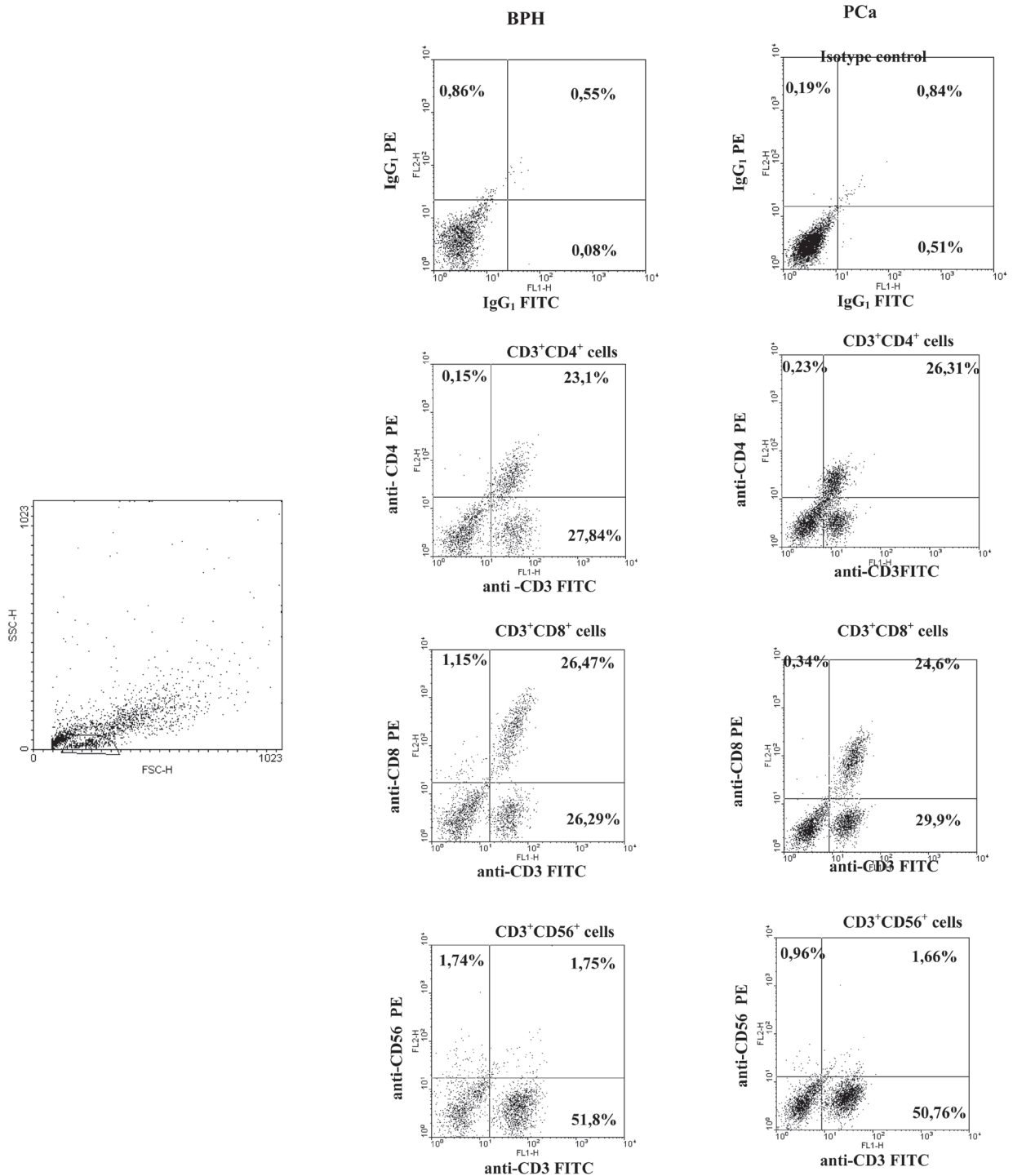
Figure 2. *The representative phenotypic profile of CD4/CD8 (dot plots) in prostatic tissue of patients with benign prostatic hyperplasia and prostate cancer.*

BPH and PCa and prostate tissue without pathological changes (control tissue prostate), with fluorescent labeled antibodies against CD3, CD4, CD8 and CD56 molecules and analyzed on a confocal microscope. In Figure

1 may be clearly observed infiltration of stromal NK cells (green fluorescence) in prostate tissue of patients with BPH (Figure 1B) and PCa (Figure 1C) and in control prostate tissue (Figure 1A). Infiltration of stromal NK
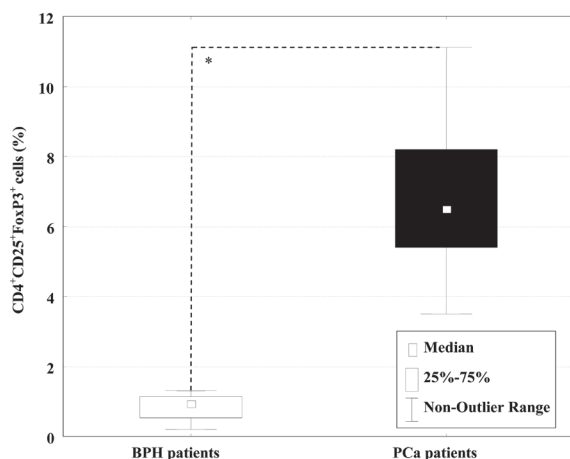
Figure 3. *Proportion of Tregs (CD4 + CD25 + FoxP3 +) in patients with BPH and Pca.*

cells in patients with PCa as in control prostate tissue was significantly lower than in patients with BPH. CD3 + cells were found only slightly in the stroma, but in epithelium considerably more in the control prostate tissue and in prostate tissue from patients with BPH and PCa. Colocalizations CD3 + and CD56 + cells was observed in the epithelium of the prostate tissues of patients with BPH and PCa only slightly, while in the control epithelial tissue of the prostate were clearly expressed. Colocalizations CD3 + and CD4 + and CD3 + and CD8 + cells was observed in the epithelium of the prostate tissues of patients with BPH, but not in prostate tissue from patients with PCa.

Figure 2 has shown a representative flow cytometry analysis of prostate tissue infiltrating lymphocytes of patients with prostate cancer (PCa) and benign prostatic hyperplasia (BPH).

Proportion of regulatory T cells (CD4 + CD25 + FoxP3 +) was significantly higher in patients with PCa compared to the proportion of Tregs in the prostate tissue of patients with BPH (Figure 3).
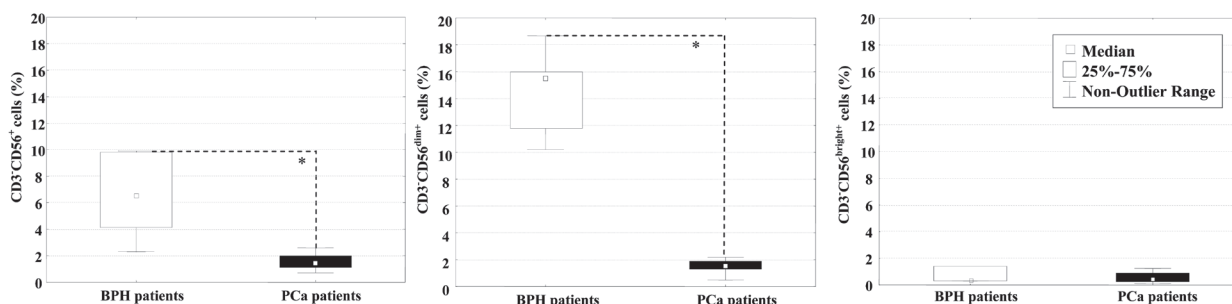
NK cells (CD3-CD56+) were statistically significantly augmented in patients with BPH in comparison with patients with PCa, as well as the percentage of CD3-CD56dim+ cells, while in subpopulation of CD3-CD56bright+ was not statistically significantly differences (Figure 4).

Comparing the proportion of NKT cells in prostatic tissue of patients with BPH and PCa we noted less, but not statistically significantly reduced proportion of NKT cells in prostatic tissue of patients with PCa in comparison with patients who suffer of BPH:

DISCUSSION

Chronic inflammation has been shown may play a key role in the pathogenesis of BPH and could be associated with the development of PCa. Numerous studies in animal models and humans have shown that the chronic inflammation is one of the important factors in the development of cancers such as pancreatic cancer, colorectal cancer or gastric cancer *(22-24)*. Infiltration of tumor cells correlates with positive outcome of treatment. In patients with ovarian and colorectal cancer have been shown lower density of CD3 + cells in the tissue around the tumor, what may reduce the survival of these patients compared to patients in whom high density of infiltrating CD3 + cells were noticed *(25, 26)*. Although in the tissue of the prostate cancer was observed infiltration of lymphocytes, outcome of these patients does not correlate with the density of their infiltration *(27, 28)*. In our studies we observed an infiltration of prostate tissues of patients with PCa with T lymphocytes, NK cells and NKT cells, but their proportion was smaller in comparison with the frequency of these cells in prostate tissue in patients with BPH, especially we found a small proportion of NK cells and their CD3-CD56dim + subpopualation in prostate tissue of patients with PCa. This small proportion of NK cells, particularly its cytotoxic CD3-CD56dim + subpopulation, could be one possible reason for the progression of PCa. It seems that the infiltration of T lymphocytes in prostate tissue of patients with PCa, what we observed in our study, may be associated with slower progression of tumor growth. A similar observation was no-
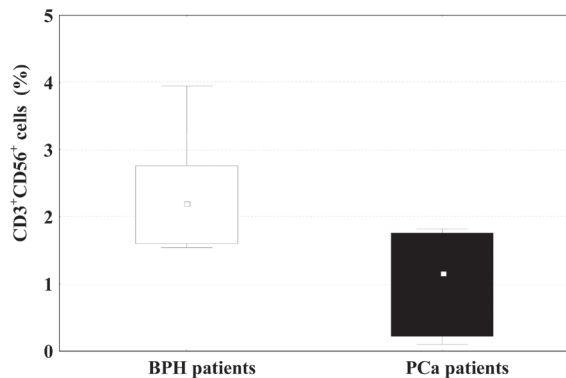

Figure 4. *NK cells (CD3-CD56+) and its subpopulation of CD3-CD56dim+ cells and CD3-CD56bright+ in patients with BPH and Pca.*

Figure 5. *The proportion of NKT cells in prostatic tissue of patients with BPH and Pca.*

ticed in patients with small cell lung cancer, where a large number of tumor infiltrating lymphocytes, especially CD3 + CD8 + cells was observed in patients with markedly smaller tumor size *(29)*. However, since the cells were also found in the tissue of BPH, the question still stays: are these infiltrating lymphocytes in the prostate tissue of patients with PCa reflection of nonspecific chronic inflammation or are involved in the immune response against tumor.

The important role in the progression of PCa may have an increased proportion of CD4+ CD25+FoxP3+ regulatory T cells in the prostate tissue and in peripheral blood of patients with PCa *(30)*. Many studies have shown that CD4+ T regulatory cells that constitutively express the high affinity receptor for IL-2 (CD25) and the transcription factor FoxP3 play an important role in the suppression of effective anti-tumor immune response. Numerous studies have shown a high level of CD4+CD25+FoxP3+ regulatory T cells in patients with hematological malignancies *(31)*, in patients with lung cancer, ovarian cancer *(32-34)*, in patients with melanoma *(35)*, colorectal cancer *(36-38)*, as well as in patients with squamous cell carcinoma of the head and neck *(39)*, hepatocellular carcinoma *(40)*, breast cancer and carcinoma of the pancreas *(41)*. There are differences between secretions of innate immune cells during different pain management techniques in patients after colorectal cancer surgery *(42, 43)*. In our research we have found increased levels of Tregs in prostate tissue, as well as in peripheral blood *(30)*. These results clearly indicate that increased levels of T regulatory cells in patients with PCa may stimulate the spread of tumors at the system level. On local level, increased infiltration of prostate tissue with T regulatory cells were found in patients with PCa. Another possible reason for ineffective anti-tumor activity of prostatic infiltrating lymphocytes of patients with PCa would be inadequate expression and regulation of cytotoxic molecules such as perforin. Perforin is known as a rapid mediator with cytotoxic activities which perform their effect by forming

pores on the surface of the target (infected or tumor) cells. In our research we did not find statistically significant differences in the expression of perforin in the peripheral blood of patients with PCa compared to patients with BPH or control group, but we noticed extremely low expression of perforin in lymphocyte subpopulations in prostatic tissue of patients with PCa *(44)*. It is assumed that the local microenvironment is responsible for its expression and regulation.

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