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Cross talk between NKT and regulatory T cells (Tregs) in prostatic tissue of patients with benign prostatic hyperplasia and prostate cancer

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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, whichhave 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 proinflammatory 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 37°C. 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 consequently 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, $Na_2HPO_4x12H_20$ 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

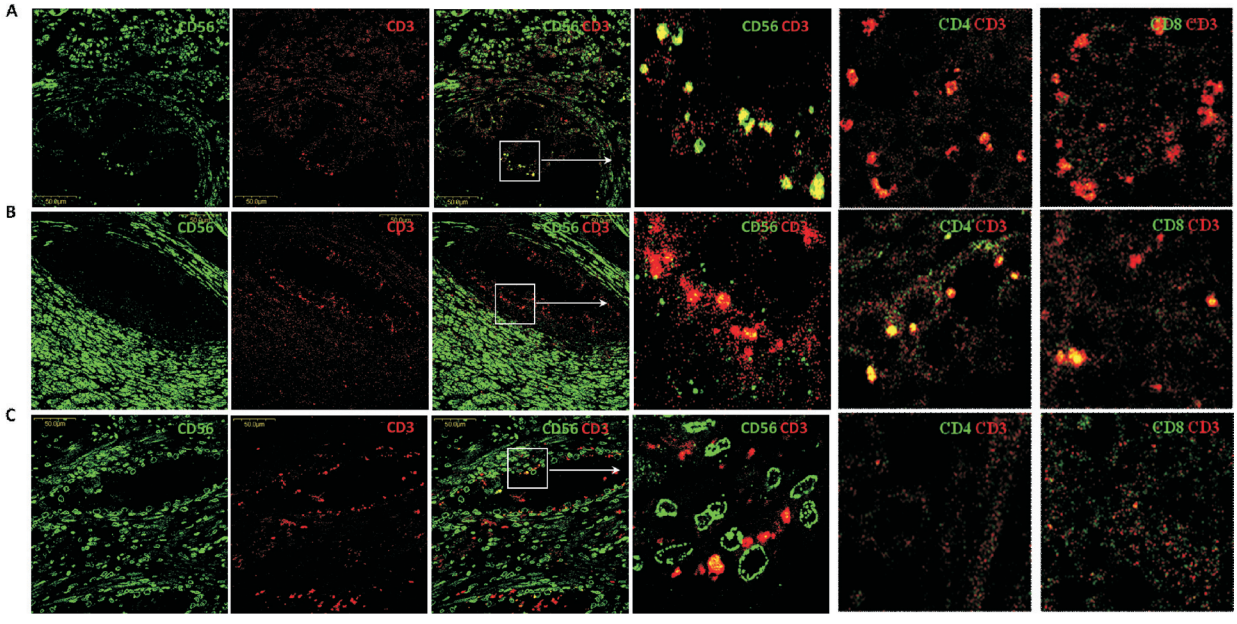

Figure 1. *Expression and localization of CD3+ CD56+ ,CD4+ and CD8+ cells in BPH and PCa. After deparaffinization of the sections, either control prostate tissue (A), BPH (B) or PCa (C), immunofluorescence assay was performed. Sections were incubated with primary antibodies against CD3, CD56, CD4 and CD8 over night. Then, the sections were stained with secondary reagents.*

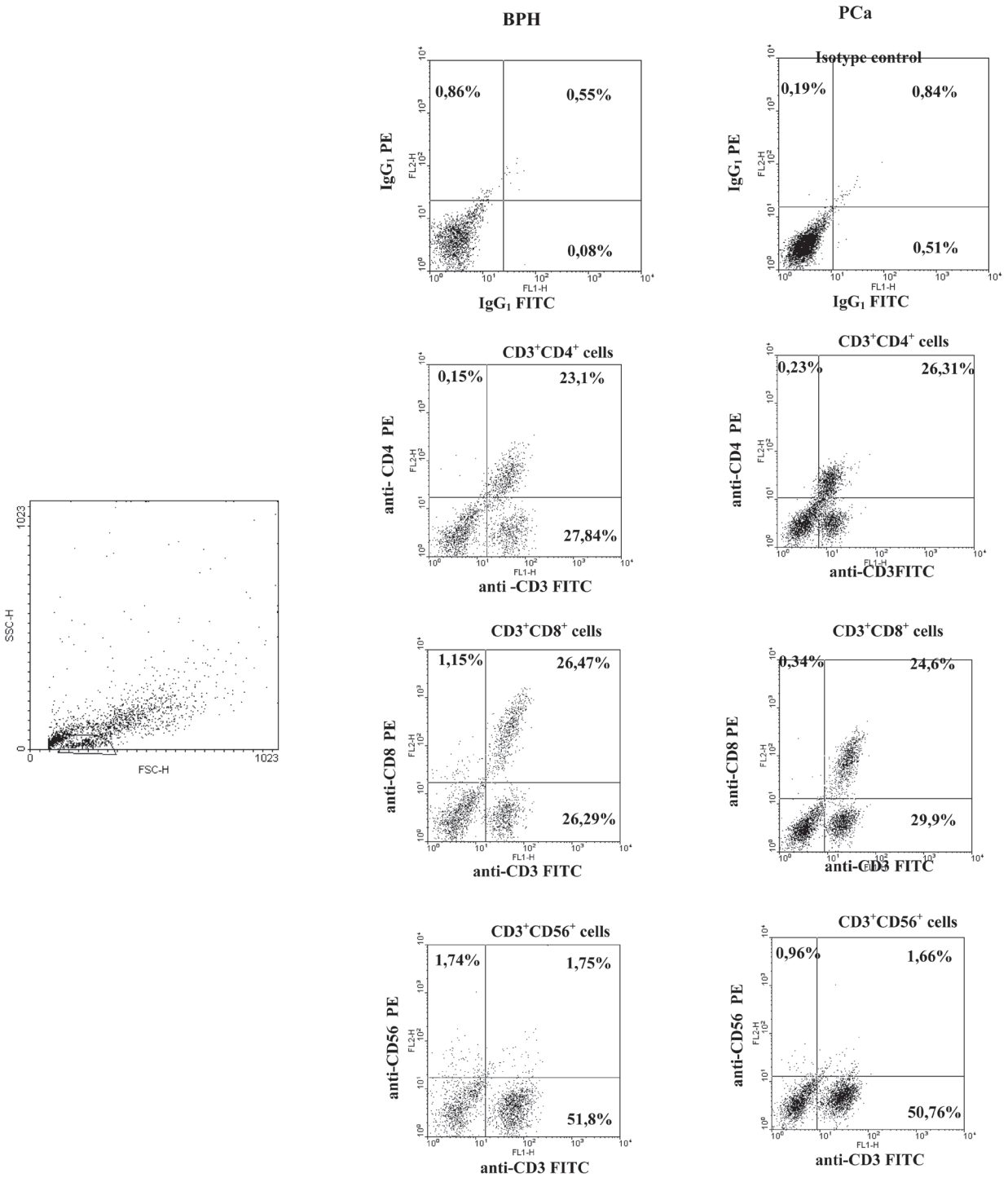

Figure 2. *The representative phenotypic profile of CD4/CD8 (dot plots) in prostatic tissue of patients with benign prostatic hyperplasia and prostate cancer.*

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

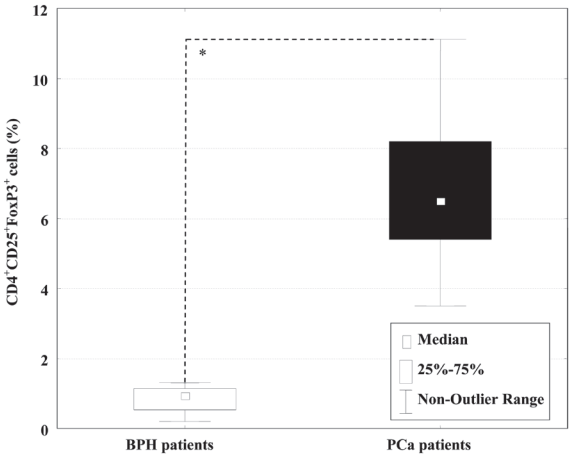

Figure 3. *Proportion of Tregs (CD4 + CD25 + FoxP3 +) in patients with BPH and Pca.*

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. Co-localizations 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 $CD3^{-}CD56^{dim+}$ cells, while in subpopulation of $CD3^{-}CD56^{bright+}$ 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-

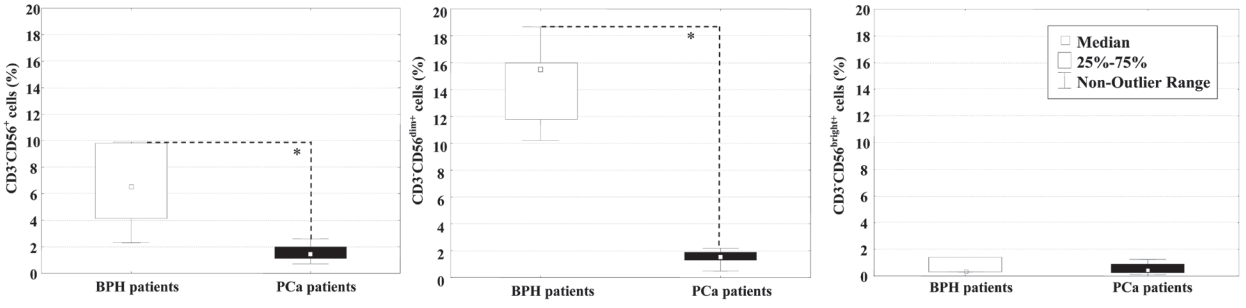

Figure 4. *NK cells (CD3-CD56+) and its subpopulation of CD3-CD56dim+ cells and CD3-CD56bright+ in patients with BPH and Pca.*

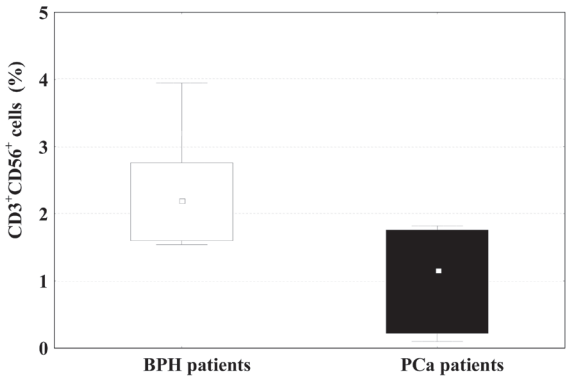

Figure 5. *The proportion of NKT cells in prostatic tissue of patients with BPH and Pca.*

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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REFERENCES

1. KRAMER G, STEINER G E, HANDISURYA A, STIX U, HAITEL A, KNERER B, GESSL A, LEE C, MARBERGER M 2002 Increased expression of lymphocyte-derived cytokines in benign hyperplastic prostate tissue, identification of the producing cell types, and effect of differentially expressed cytokines on stromal cell proliferation. *The Prostate 52:* 43-58
2. KONIG J E, SENGE T, ALLHOFF E P, KONIG W 2004 Analysis of the inflammatory network in benign prostate hyperplasia and prostate cancer. *The Prostate 58:*121
3. KUPER H, ADAMI H O, TRICHOPOULOS D 2000 Infections as a major preventable cause of human cancer. *J Intern Med 248:* 171–183
4. ADLER A 2007 Mechanisms of T Cell Tolerance and Suppression in Cancer Mediated by Tumor-Associated Antigens and Hormones. *Current Cancer Drug Targets 7(1):* 3-14
5. LEE K L, PEEHL D M 2004 Molecular and cellular pathogenesis of benign prostatic hyperplasia. *J Urol 172(5):* 1784-91
6. SETIADY Y Y, OHNO K, SAMY E T, BAGAVANT H, QIAO H, SHARP C, SHE J X,TUNG K S 2006 Physiologic self antigens rapidly capacitate autoimmune disease-specific polyclonal CD4+ CD25+ regulatory T cells. *Blood 107(3):* 1056-62
7. STEINER G E, STIX U, HANDISURYA A, WILLHEIM M, HAITEL A, REITHMAYR F, PAIKL D, ECKER R C, HRACHOWITZ K, KRAMER G, LEE C, MARBERGER M 2003 Cytokine expression pattern in benign prostatic hyperplasia infiltrating T cells and impact of lymphocytic infiltration on cytokine mRNA profile in prostatic tissue. *Lab Invest. 83(8):* 1131-46
8. LUM H E, MILLER M, DAVOL P A, GRABERT R C, DAVIS J B, LUM L G 2005 Preclinical studies comparing different bispecific antibodies for redirecting T cell cytotoxicity to extracellular antigens on prostate carcinomas. *Anticancer Res 25(1A):* 43-52
9. ZHANG Q, JANG T L, YANG X, PARK I, MEYER R E, KUNDU S, PINS M, JAVONOVIC B, KUZEL T, KIM S J, VAN PARIJS L, SMITH N, WONG L, GREENBERG N M, GUO Y, LEE C 2006 Infiltration of tumor-reactive transforming growth factor-beta insensitive CD8+ T cells into the tumor parenchyma is associated with apoptosis and rejection of tumor cells. *The Prostate 66(3):* 235-47
10. KRAMER G, MARBERGER M 2006 Could inflammation be a key component in the progression of benign prostatic hyperplasia? *Curr Opin Urol 16:* 25-9
11. LUNDBERG A M, HANSSON G K 2010 Innate immune signals in atherosclerosis. *Clin Immunol 134:* 5
12. QIN F X 2009 Dynamic behavior and function of Foxp3+ regulatory T cells in tumor bearing host. *Cell Mol Immunol 6:* 3
13. WANG RF 2006 Functional control of regulatory T cells and cancer immunotherapy. *Semin Cancer Biol 16:* 106

14. YAMAGUCHI T, SAKAGUCHI S 2006 Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol 16:* 115

15. PIERSMA S J, WELTERS M J P, VAN DER BURG S H 2008 Tumor-specific regulatory T cells in cancer patients. *Hum Immunol 69:* 241

16. WANG R F 2006 Regulatory T cells and innate immune regulation in tumor immunity. *Springer Semin Immunopathol 28:* 17

17. KOSMACZEWSKA A, CISZAK L, POTOCZEK S, FRYDECKA I 2008 The significance of Treg cells in defective tumor immunity. *Arch Immunol Ther Exp (Warsz) 56:* 181

18. CHATTOPADHYAY S, CHAKRABORTY N G, MUKHERJI B 2005 Regulatory T cells and tumor immunity. *Cancer Immunol Immunother 54:* 1153

19. MOCELLIN S, ROSSI C R, NITTI D 2004 Cancer vaccine development: on the way to break immune tolerance to malignant cells. *Exp Cell Res 299:* 267

20. BERZOFSKY J A, TERABE M 2008 NKT cells in tumor immunity: opposing subsets define a new immunoregulatory axis. *J Immunol 6:* 3627

21. TERABE M, BERZOFSKY J A 2007 NKT cells in immunoregulation of tumor immunity: a new immunoregulatory axis. *Trends Immunol 28:* 491

22. GIEHL K, BACHEM M, BEIL M, BÖHM B O *et al.* 2011 Inflammation, Regeneration, and Transformation in the Pancreas: Results of the Collaborative Research Center 518 (SFB 518) at the University of Ulm. *Pancreas 40:* 489-502

23. O'CALLAGHAN D S, O'DONNELL D, O'CONNELL F, O'BYRNE K J 2010 The role of inflammation in the pathogenesis of non-small cell lung cancer. *J Thorac Oncol 5:* 2024-36

24. ORBELL J, WEST N J 2010 Improving detection of colorectal cancer. *Practitioner 254:* 17-21

25. ZHANG L, CONEJO-GARCIA J R, KATSAROS D *et al.* 2003 Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med 348:* 203-13

26. GALON J, COSTES A, SANCHEZ-CABO F, KIRILOVSKY A *et al.* 2006 Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science 313:* 1960-4

27. VESALAINEN S, LIPPONEN P, TALJA M, SYRJÄNEN K 1994 Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. *Eur J Cancer 30:* 1797-803

28. MCARDLE P A, CANNA K, MCMILLAN D C, MCNICOL A M, CAMPBELL R, UNDERWOOD M A 2004 The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer. *Br J Cancer 91:* 541-3

29. EEROLA A K, SOINI Y, PÄÄKKÖ P 2000 A high number of tumor-infiltrating lymphocytes are associated with a small tumor size, low tumor stage, and a favorable prognosis in operated small cell lung carcinoma. *Clin Cancer Res 6:* 1875-81

30. SOTOSEK S, SOTOSEK TOKMADZIC V, MRAKOVCIC-SUTIC I, ILIC TOMAS M, DOMINOVIC M, TULIC V, SUTIC I, MARICIC A, SOKOLIC J, SUSTIC A 2011Comparative study of frequency of different lymphocytes subpopulation in peripheral blood of patients with prostate cancer and benign prostatic hyperplasia. *Wien Klin Wochnenschr 123(23-24):* 718-25

31. BEYER M, KOCHANEK M, DARABI K *et al.* 2005 Reduced frequencies and suppressive function of CD4+CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood 106:* 2018-25

32. MOTTA M, RASSENTI L, SHELVIN B J *et al.* 2005 Increased expression of CD152 (CTLA-4) by normal T lymphocytes in untreated patients with B-cell chronic lymphocytic leukemia. *Leukemia 19:* 1788-93

33. YANG Z Z, NOVAK A J, STENSON M J, WITZIG T E, ANSELL S M 2006 Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+ T cells in B-cell non-Hodgkin lymphoma. *Blood 107:* 3639-46

34. WOO E Y, CHU C S, GOLETZ T J *et al.* 2001 Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res 61:* 4766-72

35. JAVIA L R, ROSENBERG S A 2003 CD4+CD25+ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. *J Immunother 26:* 85-93

36. SASADA T, KIMURA M, YOSHIDA Y, KANAI M, TAKABAYASHI A 2003 CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer 98:* 1089-99

37. MRAKOVCIĆ-SUTIĆ I, BACIĆ D, GOLUBOVIĆ S, BACIĆ R, MARINOVIĆ M 2011 Cross-talk between NKT and regulatory T cells (Tregs) in modulation of immune response in patients with colorectal cancer following different pain management techniques. *Coll Antropol 35 (2):* 57-60

38. BACIĆ D, URAVIĆ M, BACIĆ R, SUTIĆ I, PETROSIĆ N 2011 Augmentation of regulatory T cells (CD4+CD25+Foxp3+) correlates with tumor stage in patients with colorectal cancer. *Coll Antropol 35 (2):* 65-8

39. SCHAEFER C, KIM G G, ALBERS A, HOERMANN K, MYERS E N, WHITESIDE T L 2005 Characteristics of CD4+CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer. *Br J Cancer 92:* 913-20

40. HOECHST B, ORMANDY L A, BALLMAIER M *et al.* 2008 A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. *Gastroenterology 135:* 234-43

41. LIYANAGE U K, GOEDEGEBUURE P S *et al.* 2006 Increased prevalence of regulatory T cells (Treg) is induced by pancreas adenocarcinoma. *J Immunother 29:* 416-24

42. GOLUBOVIC V, GOLUBOVIC S, SOTOSEK-TOKMADZIC V, MRAKOVCIC-SUTIC I 2009 Immune response in patients with cancer pain. *Period biol 111 (2):* 223-225

43. GOLUBOVIC S, GOLUBOVIC V, SUTIC I, PAVISIC V, SUSTIC A, MRAKOVCIC-SUTIC I 2013 Early immunological events in postoperative epidural/intravenous analgesia after colorectal cancer resection. *Period biol 115 (2):* 231-233

44. TOKMADŽIĆ V S, TOMAŠ M I, SOTOŠEK S, LAŠKARIN G, DOMINOVIĆ M, TULIĆ V, DORĐEVIĆ G, SUSTIĆ A, MRAKOVČIĆ-ŠUTIĆ I 2011 Different perforin expression in peripheral blood and prostate tissue in patients with benign prostatic hyperplasia and prostate cancer. *Scand J Immunol 74(4):* 368-76