

Colonic carcinoma microenvironment: immunohistochemical frequency and distribution of CD3, CD8, FOXP3 infiltrating T lymphocytes

Micromediul carcinomului de colon: frecvența și distribuția imunohistochimică a limfocitelor T CD3, CD8, FOXP3 infiltrante

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Abstract

Cancer pathogenesis is fundamentally dependent on tumor cell interactions with microenvironmental components. These interactions may either restrain tumor progression or promote it. Inflammatory and immune stromal cell component, particularly lymphocytic reactions, plays decisive roles at different stages of tumor development by generating a microenvironment that could be supportive for growth and invasion and/or development of anti-tumor response. It is well recognized that cytotoxic CD8⁺ T and regulatory FOXP3⁺ lymphocytes constitute important effector mechanisms of tumor immune surveillance. However, their potential prognostic influence in colorectal cancer remains controversial. We assessed infiltration of CD3⁺, CD8⁺, and FOXP3⁺ T lymphocytes within tumor tissue, invasive front, advancing tumor margin and normal colonic tissue of 69 colon tumors and correlated their distribution with clinical stage. Our study shows that tumor stage progression associate a stromal traffic of T lymphocytes and positioning depending on development of the malignant process and suggests a complex diversity of cellular interplay in the frame of cancer microenvironment.

Keywords: colon cancer, tumor microenvironment, tumor infiltrating T lymphocytes, T regulatory cells, tumor stage.

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Rezumat

Patogeneza cancerului este fundamental dependentă de interacțiunile celulei tumorale cu componentele de micromediu. Aceste interacțiuni pot, fie să restrângă progresia tumorală, ori s-o promoveze. Componenta celulară stromală inflamatorie și imună, în particular reacțiile limfocitice, joacă roluri decisive în diferite stadii ale dezvoltării tumorale prin generarea unui micromediu care poate fi un suport pentru creștere și invazie, sau/și dezvoltarea unui răspuns anti-tumoral. Este bine cunoscut că limfocitele T CD8⁺ citotoxice și FOXP3⁺ reglatoare constituie importante mecanisme efector ale supravegherii imune tumorale. Cu toate acestea potențialul lor influență prognostică în cancerul colorectal rămâne controversată. Am evaluat infiltrarea țesutului tumoral, frontului invaziv, marginii în progres a tumorii și țesutului colonic normal cu limfocite T CD3⁺, CD8⁺, FOXP3⁺, în 69 tumori de colon și am corelat distribuția lor cu stadiul clinic. Studiul nostru arată că progresia stadială asociază un trafic stromal al limfocitelor T și o poziționare dependentă de intensitatea procesului malign sugerând o diversitate complexă a relațiilor celulare reciproce în cadrul micromediului tumoral.

Cuvinte cheie: cancer de colon, micromediu tumoral, limfocite T infiltrante intratumorale, celule T reglatorii, stadiu tumoral.

Background

Human colorectal cancer tissues are infiltrated by various immune/inflammatory cells, usually along the invasive front and advancing tumor margin. Although these responses were considered rather non-specific it seems now that these cellular accumulations, particularly lymphocytic reactions, can be measured as independent prognostic factors for a better survival.

Immunohistochemical subset analyses have generally revealed that the number of T lymphocytes is important. These data suggest that, to some extent, there is anti-tumor immunity in human colorectal carcinoma (1, 2).

Colorectal cancers become clinically malignant after cancer cells infiltrate through the *muscularis mucosae* in the submucosa (3, 4). With this invasion, various host reactions take place, which include desmoplasia of the stroma (fibrogenic reaction) and various immune/inflammatory responses that are more concentrated along the invasive margin (tumor-host interface) (5).

Tumor-infiltrating lymphocytes (TILs) seem to be more prominent in the early stages (Dukes A and B) and decrease in the advanced stages. The TILs concept has then extended to include lymphoid aggregates that are formed in the proximity of the invasive margin, termed "Crohn's-like lymphoid reaction".

When assessing immune responses in pa-

tients with colorectal carcinoma, researchers focus on both intraepithelial (i.e., lymphocytes within cancer cell nests or lymphocytes infiltrating neoplastic epithelium) and stromal/*lamina propria* CD8⁺ T cells. In most colorectal cancers, cancer tissues are infiltrated by few lymphocytes. Lymphocytes and other inflammatory cells that infiltrate the area along the invasive front and advancing tumor margin are changing from a thick continuous pattern to a nearly indistinct one. Although the number of stromal CD8⁺ T cells is larger at the invasive front, it does not stand for a significant survival prognostic factor in contrast with the number of intraepithelial tumor-infiltrating CD8⁺ T lymphocytes, judged as having favorable effect on overall survival time (6-14).

A link between metastatic colon cancer and a weaker immune response to the tumor, as defined by the frequency of tumor infiltrating CD3⁺ T cells, was reported. Some results concluded that the density of T cells in and around colorectal tumors proved to be a more efficient prognostic marker than the UICC TNM classification (14).

However, to understand the balance of immune responses, it is important to analyze both effector and immunosuppressive cells. For example, CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Treg) are considered important for tumor immune evasion. Treg cells were noticed in the mucosal lymphoid follicles of both the normal and malignant colon. However, the number of

Treg cells in the tumor stroma was larger than the one in the normal colon *lamina propria*. Thus, Treg cells could both suppress the induction of an immune response in the lymphoid tissue and inhibit proliferation of effector cytotoxic cells within the tumor stroma (15-17).

In this study we focused on analyzing the distribution of immune cells expressing CD3⁺, CD8⁺, FOXP3⁺ phenotypes within stromal-parenchymal tumor compartments of colon adenomas and carcinomas in an attempt to detect T cell functional disposition according the type and evolution of disease.

Materials and methods

The study batch was made of 69 patients admitted in the Piatra-Neamt County Hospital between December 2007 and September 2009. The biopsy specimens collected were processed in the Pathology Department of the hospital. Tumor specimens were collected and examined based on patients' informed consent and the immunohistochemistry study performed in the Laboratory of Immunology and Genetics, St. Spiridon University Hospital Iasi, Romania. Tumor colorectal specimens were selected for the morphologic and immunohistochemical interpretation of immune/inflammatory microenvironment after surgery. Immune/inflammatory infiltrate was evaluated in four target areas defined using standard criteria (18): (1) glandular and stromal areas of the tumor, (2) on the invasive front, the most active tumor-host interface, (3) advancing tumor margin bordering adjacent non-tumor colon epithelium, and (4) in a normal colonic specimen.

Each tumor sample was fixed in 10% formaldehyde neutral buffer solution for 24 hours, embedded in paraffin and cut for 4 μ m-thick successive sections, stained with haematoxylin-eosin (HE) and Van Gieson trichrome or subjected to immunohistochemical (IHC) staining.

The following protocol was applied: deparaffination, hydration of the sections, antigen retrieval (Dako S1700 retrieval solution, 20-minute retrieval at 96°C).

Individual sections were then incubated with monoclonal antibodies diluted in ADBR (Antibody Diluent with Background Reducing) solution: CD3, 1/2 dilution (clone UCHT1/T3-4B5/F7.2.38, code N1580, ready to use (RTU), Dako), CD8, 1/2 dilution (clone DK25 C8/144B, RTU, Dako), FOXP3, dilution 1/150 (clone 236A/E7, Santa Cruz Biotechnology code 56680). Positive control (and dose optimization) for antibodies was performed on tonsil tissue.

Incubation with primary antibody was performed overnight, at 4°C. Peroxidase was blocked with 3% hydrogen peroxide for 15 minutes. The incubation time for secondary biotinylated antibody (Biotinylated Link Dako) was 1 hour at room temperature. Then, after 30 minute incubation at room temperature with Streptavidin HRP enzyme (Dako), the reaction was developed for 5-6 minutes with 1/50 3-3'-DAB diluted chromogen substrate (Dako), while examining the cross-section under the microscope in real time in order to notice the immunostaining. The brown reactive end product stained the membrane for CD3 and CD8 proteins and the nuclei for FOXP3. Counterstaining was performed with Meyer haematoxylin, for 30-60 seconds (Dako).

Staining evaluation was done using a motorized stage Zeiss Observer Z1 microscope. Medium magnification (20x objective), individual field of view images captured with a CMOS-colour camera (PixelLink) were software stitched in order to create whole-section pictures (TissueFacs software) for both IHC staining and standard haematoxylin-eosin paired slides that were used for pathological evaluation and contextual identification of the previously mentioned four types of target areas analyzed. Immunostained cells were counted in five randomly selected fields of view (FOV) of each area type under study in individual slides, followed by FOV averaging. Cell counts are indicating the lymphocytes density of a 0.307 mm² 20X FOV size.

Statistical analysis was performed by t-Test and Pearson Correlations using SPSS 13.

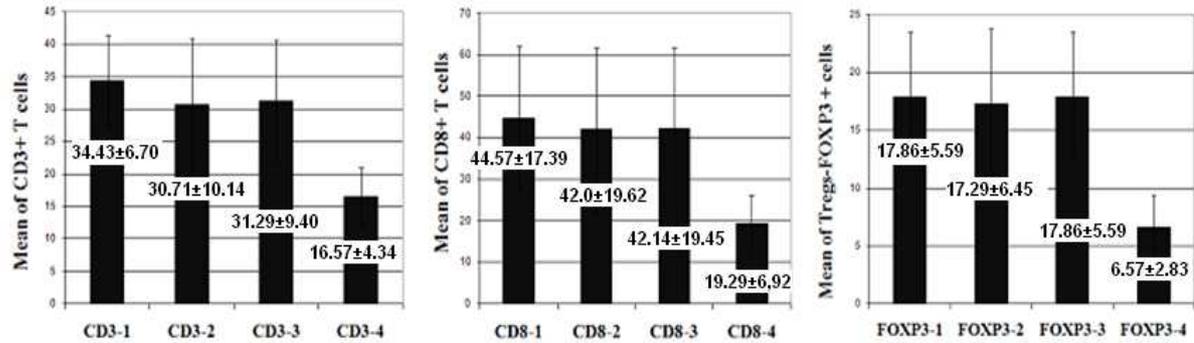


Figure 1. Graphs of FOV averages (mean ± SD) for CD3, CD8, FOXP3 markers indicate statistic significant differences ($p < 0.001$) among adenoma delineated areas - (1) adenoma tissue, (2) peripheral adenoma area, (3) adjacent normal tissue - and (4) normal colonic specimen.

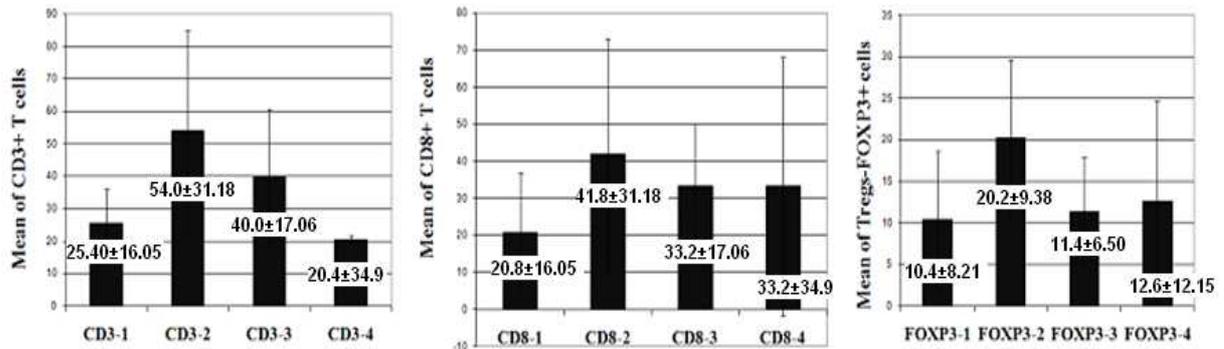


Figure 2. Stage I tumors, where 80% are identified as T2N0M0 and G1 neoplasms concentrate the expression of CD3⁺, CD8⁺ and FOXP3⁺ cell phenotypes at the invasive front of the tumor (2), tumor advancing borders (3) and normal colonic tissue (4) (Mean ± SD).

Significance thresholds for registered differences were as follows: $p > 0.05$ non-statistically significant, $p < 0.05$ statistically significant, $p < 0.01$ strong statistic significance and $p < 0.001$ highly statistic significance.

Results

Of 69 cases analyzed for CD3⁺, CD8⁺, FOXP3⁺ T cell frequency 21 were adenomas, and 48 adenocarcinomas with a TNM disease staging of 15 in stage I, 24 stage II and 9 in stage III.

The markers under study showed for adenomas a similar evolution for CD3⁺, CD8⁺ and FOXP3⁺ T cells that register a significantly higher plotting for FOV averages in the adeno-

matous tissue, peripheral adenoma area or adjacent normal tissue than for at distance normal epithelial tissue of excised specimen ($p < 0.001$). Statistic significance and positive Pearson correlations between markers indicate on one hand that statistically CD8 or FOXP3 cells are part of CD3 population (T cells) and on the other that the increase/decrease in the cell number of one sub-population is parallel with the others. Hence, increase of CD3⁺ cell FOV average in adenoma tissue associate with an increase in the normal epithelium, while increase of CD8⁺ expressing cells in the same adjacent normal tissue entails the increase of FOXP3⁺ cells. (Figure 1).

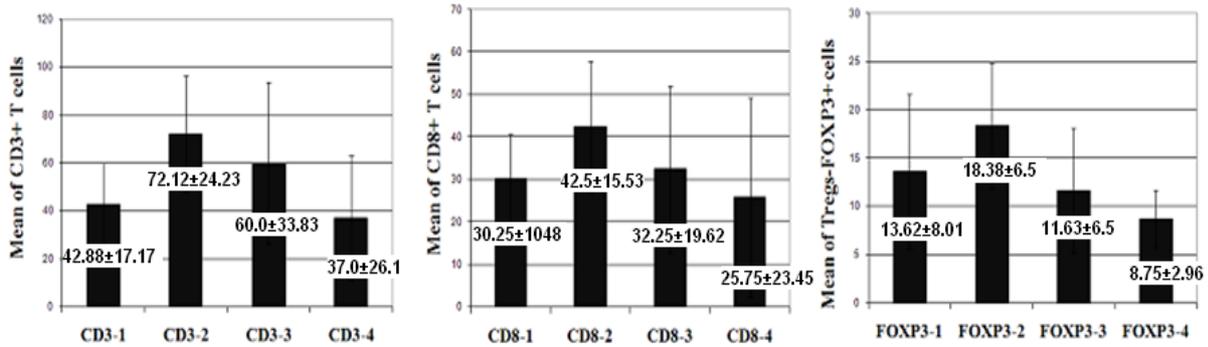


Figure 3. Stage II tumors, 24 patients, 75% included as T3N0M0G1 neoplasms maintain the appearance of an active presence of CD3⁺, CD8⁺ and FOXP3⁺ cell phenotypes at the invasive tumor front (2) and transition margin from florid tumor to normal colonic epithelia (3), although some mean values (±SD) do not reach always significant differences to tumor tissue (1) or to normal colonic specimen (4).

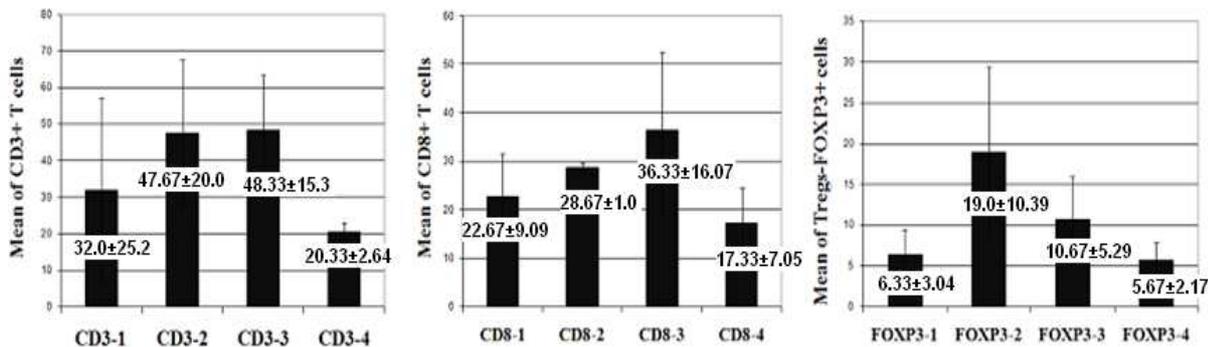


Figure 4. Stage III colonic neoplasm associate a tendency of CD3⁺ and CD8⁺ T cells to expand along the tumour advancing borders (3) matching or exceeding the invasive front (2); FOXP3 mean value (±SD) fall down in glandular and stromal areas of the tumour (1), not changing the appearance at the invasive front (2) (p=0.002) and moving down through the invasive front to tumour adjacent to normal colonic epithelium (3) (p=0.037) and normal colonic tissue (4) (p=0.001).

For Stage I adenocarcinomas immunostaining average values show a different dispersion among delineated fields: The invasive front displays a significant statistic accumulation of CD3⁺, CD8⁺, or FOXP3⁺ T cells, whereas no difference was registered for the same immune markers between areas of full tumor tissue and morphologically normal epithelium (p>0.05). For CD3⁺ T cells the registered mean values decrease progressively along the invasive front to advancing tumor margin (p=0.027) and to the apparently normal colonic specimen (p=0.003), with a positive correlations between the overt tumor and distant normal colonic

epithelium (r=0.637, p=0.011). However, the CD8⁺ and FOXP3⁺ T cells take a different distribution. Hence, mean marker values counted in selected fields under investigation indicate that the CD8⁺ T cells have a rather motionless appearance from invasive front to tumor borders (p>0.05) and further to morphologically normal epithelium (p>0.05). As for the mean of FOXP3⁺ counted cells a significant statistic expansion was registered when passing from the tumor tissue to the tumor invasive front (p<0.001 with positive correlations r=0.680, p=0.005), while without significance between tumor, its margin and colon at distance (p>0.05) (Figure 2).

Pearson positive correlations between markers and examined areas indicate a similar evolution at all levels, except when comparing CD3⁺ T cells with those that express FOXP3 in normal colonic epithelia that show weak differences ($p=0.035$) and negative correlation ($r = -0.571$, $p=0.026$) suggesting that CD3 rising associate with decline of FOXP3 expression.

The behavior of T cells identified as CD3⁺ cells in stage II colon adenocarcinomas concerning distribution and FOV averages is essentially the same as in stage I. Among stage II carcinomas 75% were T3N0M0G1 tumors and the highest expression of the above mentioned marker is present in the invasion front of the tumor-host interface. T CD8⁺ and FOXP3⁺ cells change distribution and indicate a tendency of decreasing average values from invasion front to advancing tumor border and normal epithelia although with weak or even absent significance among fields mean values (*Figure 3*). Pearson correlations are moderately or weakly negative when comparing some areas and markers. Thus, if we assume an expansion of CD3⁺ T cells at the invasive front then we expect numerical decrease in the normal colonic epithelium ($r=-0.682$, $p<0.001$). Reduction of CD8⁺ T cells mean value within the tumor would be accompanied by an increased level in the normal colon ($r=-0.644$, $p=0.001$). FOXP3⁺ T cells growth inside the tumor will involve a mean value decrease in the area of morphological transition from florid tumor to normal epithelia ($r=-0.501$, $p=0.013$) (*Figure 3*).

As for FOXP3⁺ and CD8⁺ T lymphocytes by pair Pearson analysis it looks that growth of FOXP3⁺ T cell at the invasive front and at distance in normal epithelium is followed by a parallel decrease of CD8⁺ cells in normal colonic mucosa ($r=-0.415$, $p=0.044$) and within the tumor ($r=-0.497$, $p=0.014$).

Stage III colonic tumors (T4N1M0G1, T3N1M0G2, T3N2M0G2 equally represented among 9 cases of the present study) indicate a change of T cell functional profile as reflected by

averaging CD3⁺, CD8⁺, and FOXP3⁺ immunostained cells in each of four areas under study. Thus, CD3⁺ T cells show a similar increase of the mean value at the invasive front and tumor border but concurrently a more prominent rise of CD8⁺ cells at tumor margin although not yet significant when comparing with the invasive front ($p>0.05$). Also FOXP3 marker fall down inside the neoplastic tissue mass, still lasting as a high statistically significant mean value on the invasive front of the tumor and shifting down from this region to adjacent non-tumor border and normal colonic specimen.

Pearson negative correlations are expanding among areas of interest for CD8, FOXP3 markers, or by mostly all pair analysis of FOXP3/CD8. Therefore it follows that agglomeration of CD8⁺ T cells at the invasive front is accompanied by a decrease of mean value within the tumor ($r=-0.992$, $p<0.001$), while increasing numbers of FOXP3⁺ cells inside the tumor will be associated with a decrease at the limits towards non-tumor colonic epithelia ($r=-0.715$, $p=0.031$). As for by pair Pearson correlation, nearly all instances of FOXP3/CD8 analysis show a negative correlation, then a reverse movement of the two markers in the frame of investigated areas. It is worth to mention that for stage III all negative correlations are statistically very strong (*Figure 4*).

Discussions

Tumors are heterogeneous population and growth and invasion involve largely the combined kinetic interactions of these cells with the extracellular matrix and stromal cells that confines tumor microenvironment.

The overall phenotype of a developing neoplasm is not determined only by the evolutionary competition among malignant clones but, to a large extent, by the tumor stromal tissue. Tumor cells and stromal cells undergo a stepwise co-evolution and phenotype transition successfully generating particular molecular markers eventually with diagnostic and therapeutic aptitudes. Inflam-

matory and immune stromal cell component play important cooperating roles by generating a microenvironment that could be supportive for growth and metastasis and/or development of anti-tumor response. This double way behavior is consistent with the concept that the immune system plays an important role in counteracting foreign antigens and also in regulating tissue homeostasis. The leukocyte network participating in this process could be considered as a physiological unit of tissue control. Immune pathology represents a deviated immune physiology from controlling proliferation, differentiation, and preservation of structure and function of a certain tissue (19).

Considering T cells as an important component of this control network and tumor-host interplay, the aim of the study was to examine infiltration of CD3⁺, CD8⁺ and FOXP3⁺ lymphocytes in colorectal cancer and adenomas, and their tissue distribution.

In this context we can show that tumor stage progression associates with a shifting of T cells that entails, with high probability, a focalized functional interplay at the level of invasive front of tumor parenchyma, advancing tumor margin and at distance normal colonic epithelial tissue (Figure 5).

Immune T cell infiltration in adenomas prevail in the adenomatous tissue, or at the normal epithelial limit, but is significantly less represented at a distance, in the normal colonic epithelium ($p < 0.001$). From CD3 T cell population either CD8⁺ or FOXP3⁺ phenotypes have an identical distribution.

The accumulation of CD3⁺ T cells and functional profiles of CD8 and FOXP3 markers in adenomatous benign tumor could reflect the intervention of adaptive component of the tissue immune control unit (19) either as support for a sustained proliferative process and/or an antigenic activation process. In this context FOXP3⁺ stained cells could represent the induction of regulatory functions that have the role to temperate the inflammatory T cells proliferation. However, expression of FOXP3 transcription factor as a key marker for T regulatory cells in humans remains controver-

sial. FOXP3 is transiently expressed in TCR activated human CD4 and CD8 T cells but is silenced over time and upon progression of cellular division (20, 21). T cell activation, CD4⁺ T cells in particular, is associated with the expression of FOXP3 in effectors/memory T cells without detectable regulatory function when present at physiologically relevant ratios (22). Thus, FOXP3 marker in adenomas could be associated with a stimulated condition of CD3⁺/CD8⁺ infiltrated cells rather than representing an inhibitory regulation process. This issue should be further investigated.

The T cell infiltrates in stage I colon cancer take different profiles. The mean calculated values of CD3⁺ cells are higher at the invasive front and tumor margins than within florid tumor or normal colonic specimen. This could represent a different functional interplay of T cells at different levels of tumor development and progression, the invasive front being from far the most active region. CD8 marker is well represented in the same areas but also in the normal epithelium around the tumor tissue suggesting a possible influx of cytotoxic effector lymphocytes. FOXP3⁺ cells have a similar profile as CD8⁺ lymphocytes. Expression could follow both regulatory function and an activated state of T cells under a gradient of antigenic stimulation. FOXP3 induction in naive CD4⁺ or CD8⁺ T cells following low dose antigenic stimulation, under vague immunogenic conditions, demonstrated that cells that proliferate the slightest induce FOXP3 more efficiently than those that divide vigorously (23, 24). Therefore, it seems probable that the activated state of the *foxp3* locus is progressively lost in rapidly dividing cells. Within the tumor microenvironment conditions might change from a region to the other and create the possibility that T regulatory differentiation is set in motion by uneven developmental conditions of the tumor mass. Moreover, recently published data assert an association between a high frequency of tumor-infiltrating FOXP3⁺ T regulatory cells and improved survival in colon cancer patients (25, 26).

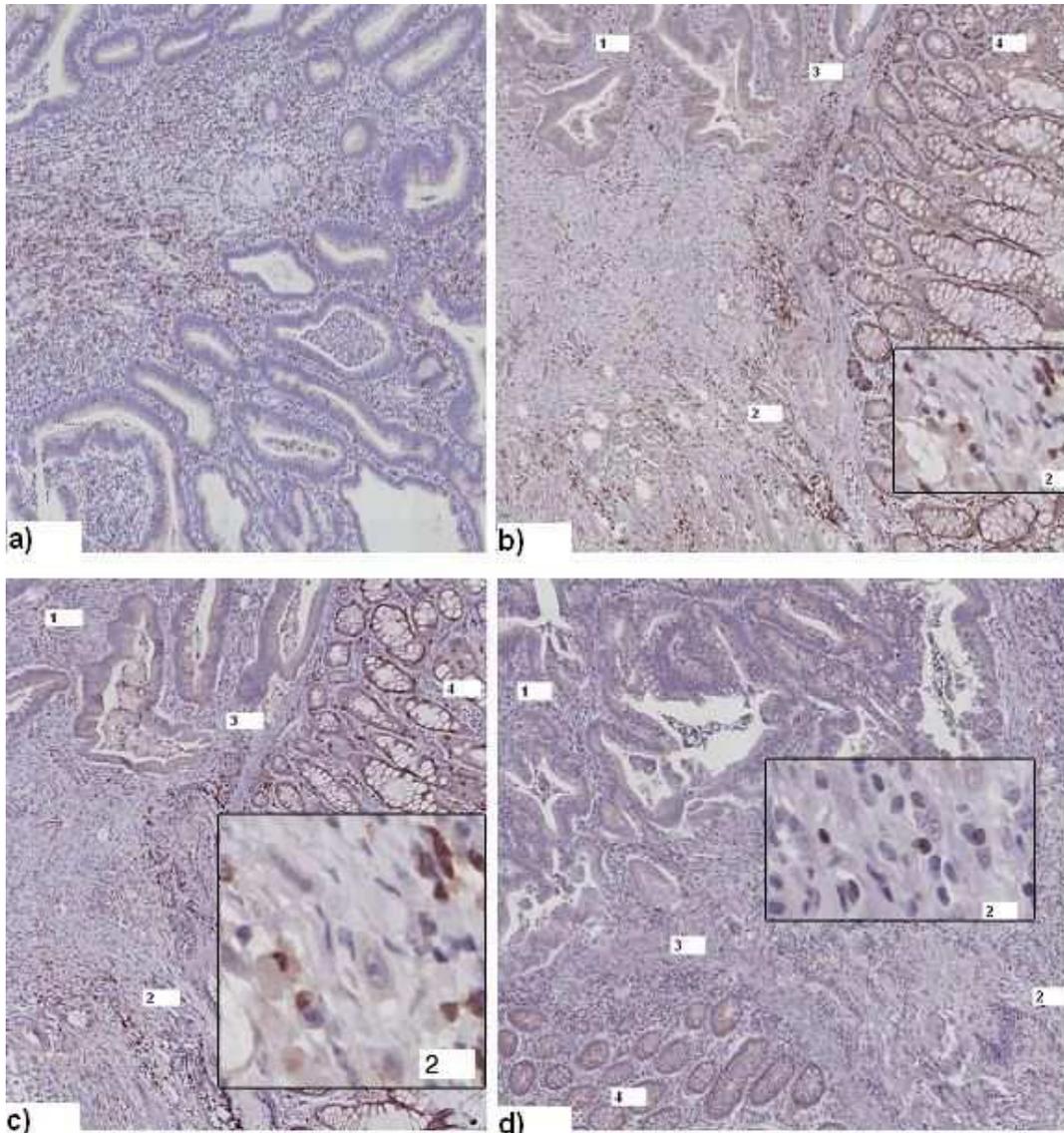


Figure 5. Evaluation of benign lesion (adenomatous polyp) with a significant CD3⁺ cell population (a) and malignant lesion (colonic carcinoma, Stage II) immunostained for CD3 (b), CD8 (c), and FOXP3 (d) in glandular and stromal areas of the tumor (1), on the invasive front (2), advancing tumor margin bordering adjacent non-tumor colon epithelium (3), and normal colonic epithelium (4). Stitched FOVs at 20x and inserts at 40x.

Stage II colon cancer keeps for infiltrating lymphocytes the same shape at the invasive front and margin of the tumor, but their count gets higher inside the tumor or in the normal epithelium suggesting that tumor-host interplay is changing. This will be more evident in stage III of disease where plots of mean calculated values

for CD3⁺ and CD8⁺ lymphocytes have a tendency of growth at the tumor margins heralding, with great probability, the enlargement of invasive front. FOXP3 expression changes entirely the pace, decreasing within the tumor, at tumor borders and in normal colonic epithelium. It retains a residual high value at the invasive front.

It is of notice that reports were published, describing the expression of FOXP3 outside the T-cell lineage and providing evidence that FOXP3 transcription factor is up-regulated by a variety of solid tumors. Depending on the nature of the tumor FOXP3-expressing cells could effectively suppress T-cell proliferation and, in some cases, were even more potent than T regulatory lymphocytes (27, 28).

In conclusion, our study shows that tumor stage progression associate a stromal traffic of T lymphocytes and positioning depending on development of the malignant process and suggests a complex diversity of cellular interplay in the frame of cancer microenvironment. Functional studies of tumor-infiltrating FOXP3⁺ T lymphocytes are needed to clarify their promotion and role in stromal response to malignant proliferation.

Disclosures

The authors declare no conflict of interest.

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