

Immunohistochemical Investigation of the role of Foxp3+ T regulatory cells in patients with Inflammatory Bowel Disease complicated with infectious intestinal presence of Cytomegalovirus

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Abstract

Aim: We attempted to study whether the presence of T-regulatory cells in tissue obtained from patients with an inflammatory bowel disease augment when Cytomegalovirus coexists in the bowel. Experimental data were analysed using statistical methods and were combined with bibliographical references in an attempt to investigate the role of T-reg cells in immunodeficient patients with or without CMV infection.

Materials and Methods: Sixty-one cases of inflammatory bowel disease were divided into two groups. The first one included patients with either Crohn's disease or ulcerative colitis that co-existed with CMV bowel infection, whereas the second group (control group) consisted of patients with inflammatory bowel disease without CMV infection. Formalin-fixed, paraffin-embedded tissues were immunohistochemically elaborated with CMV-specific polyclonal antibodies and the stained sections were microscopically evaluated in order to define Foxp3 protein levels and the attendance of Tregs in specific tissues.

Results: Statistical analysis of the evaluated samples revealed statistically significant correlation between CMV's presence and the number of Tregs in bowel tissue. There was no evidence that CMV is related with acute phases of inflammatory bowel disease. Tregs were diminished in patients with disease in recession. Eosinophils attendance was also examined and the results proved statistically significant correlation between the number of eosinophils and Tregs number in the examined samples.

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Conclusions: Our investigation provides strong evidence that the increase of Treg population in colon mucosa is probably an underlying defensive mechanism of the human organism in order to control and suppress the local inflammatory reaction caused by infectious factors in immune deficient patients. Further study could help to shed light on Tregs' suppressive potency and its potential value in clinical therapeutics.

Introduction

Regulatory T cells (Treg) develop mainly in the thymus gland (most specifically called natural Tregs –nTregs) and conduce to the development of responses to self antigens, allergens and commensal microbiota, as well as immune homeostasis to infectious factors and tumors. Various tissue-specific inflammatory responses depend on the quantity and correspondence of Tregs.[1] Function and proliferation of Treg cells can indirectly be measured by detecting forkhead box protein 3 (Foxp3), which is a transcriptional factor specific for regulatory T cells. Treg cells deficiency due to mutations of the FOXP3 gene leads to immune mediated inflammatory diseases. [2]

CD4⁺CD25⁺Foxp3⁺ regulatory cells (Tregs) constitute 5-10% of CD4⁺ T cells and produce various anti-inflammatory cytokines, such as IL-10, TGF- β and IL-35. Tregs suspend rather than promote the action of local immune cells in a contact-mediated manner. Suppressive function of Tregs is performed by various different mechanisms. First of all, as mentioned above, Tregs produce suppressive cytokines, such as IL-10 and TGF- β . In addition to this, high affinity IL-2 receptor is expressed by Tregs, so Tregs can bind to T cell growth factor IL-2 from cell environment blocking up the advance of local inflammation. Futhermore, Tregs may promote cell lysis via perforin and granzymes A and B pathways. Alteration of the function and maturation of dendritic cells is also mediated from Tregs for immune homeostasis. In addition

to previous procedures, many other mechanisms have also been described in international bibliography. [3]

As far as it concerns patients with Inflammatory bowel disease (IBD), although it is already known that a downregulation of the immune system exists, the participation and role of human intestinal Foxp3⁺ Tregs in this immune suppression are not completely understood. In a few studies, high presence of Foxp3⁺ cells is noted in the lamina propria of the involved parts of the colon of IBD patients. [4], [5] In addition, the population of Foxp3⁺ T cells in intestinal biopsies from patients with either Crohn's disease or ulcerative colitis is highly associated with the histological grade of local inflammation. [4] In order to explore Tregs inhibitory activity, it is mentioned that in patients with IBD, Tregs from the bowel are equally suppressive as those from healthy controls. [6], [7]

Because of the factors mentioned above, which are still quite obscure, the human immune system fails to fully control inflammation in the case of IBDs. How the presence of an infectious factor could influence immune response in similar patients with IBD? We attempted to study whether coexistence of CMV in patients colon promote or restrict Tregs from mediating tissue immune defense.

Cytomegalovirus is a member of the family of Herpesviridae, a double-stranded DNA virus that affects from 40% up to nearly 100% of general population. This is possible to happen either as a result of acute infection due to CMV or after

reactivation of a preceding infection. CMV infects immunodeficient patients much more often than immunocompetent patients. In the life cycle of CMV, the virus induces a latent infection to macrophages and cells of the bone marrow mainly and to some other tissues. The fusion of infected cells leads to the formation of multinucleated giant cells and hence the virus was named Cytomegalovirus. [8] In 1961, CMV was associated with IBD for the first time when the virus was detected in a patient with ulcerative colitis. Since then, additional studies proved that CMV infection may constitute an activating factor in recrudescence of IBD. Therefore, CMV is accused of causing immunosuppression or aggravation of the symptoms of IBD. In most cases, CMV infection was a reactivation of a latent virus due to immunosuppressive medication that patients, while acute infections were rare. [9] According to bibliographic data, Tregs function after CMV infection remains immunosuppressive in an attempt to suppress local tissue inflammation. [3]

The present study aims to investigate T-regulatory cells' presence and role in samples of intestinal tissue from patients with inflammatory bowel disease when an infectious factor, such as CMV, further complicates the already existent local inflammation. We applied immunohistochemical methods (polyclonal antibodies against forkhead box protein 3-Foxp3- which claim to be the basic transcriptional factor for T regulatory cells) in 61 bowel tissue samples with inflammatory bowel disease, thirty of which concern patients with IBD complicated by CMV infection. Research results were aimed to be combined with data from the already existent relevant bibliography in an attempt to investigate Tregs role in immunodeficient patients with or without CMV infection.

Materials and Methods

A retrospective analysis of 61 formalin fixed, paraffin-embedded samples obtained from intestinal biopsies (mainly colonic) during diagnostic endoscopic examination or follow-up examination of already diagnosed patients derived from 61 patients bearing the diagnosis of Inflammatory bowel disease (either ulcerative colitis or Crohn's disease) was performed. In 31 of the collected cases no infectious factor had been detected, while in the rest 30 cases CMV's presence was detected. From every case paraffin sections with 4µm thickness were obtained. Hematoxylin-Eosin stained slides as well as immunohistochemically stained slides with CMV antibodies were evaluated. The samples were collected in a period of five years (2012-2016) in the Department of Pathology at General Hospital of Athens "G.Gennimatas" as well as the 1st Department of Pathology, Medical School, National and Kapodistrian University of Athens.

For immunostaining, the polyclonal rabbit antibody Tinto Foxp3 (supplied by Bio SB, USA) was used. It was diluted in buffer containing BSA and sodium azide as a preservative at pH 7.5. Serial sections of 4µm thickness were cut and subjected to 56°C. Afterwards, deparaffinization, hydration and antigenic position opening was performed at pH 9.0. After heating and washing with buffer, the slides were incubated with primary antibody and in the next stage with the secondary antibody binded to peroxidase. After washing with TBST, chromogen DAB was infused and hematoxylin was used as a counterstain just before xylene and alcohol were added.

In order to estimate the level of expression, the number of Foxp3+ cells was measured in ten optical fields per slide and hence an average score was calculated for every sample. The results were statistically analysed in order to

correlate Tregs levels with CMV presence, disease activity and the quantity of eosinophils.

Statistical analysis was performed with R3.5.0. Statistical significance was set at 5% ($\alpha=0,05$). The relation between the mean value of positive cells per optical field and other parameters were examined with Student's independent sample t-test. The relation between CMV presence, IBD activity and eosinophils were examined with Pearson's χ^2 test.

Results

The study included 61 cases of inflammatory bowel disease (ulcerative colitis or Crohn's disease). In 30 of them CMV coexisted with IBD, while in the rest 31 cases no infectious factor was detected. As mentioned above, tissue samples were obtained from intestinal biopsies (mainly colonic) during diagnostic endoscopic examination or follow-up examination of already diagnosed patients and were studied immunohistochemically (polyclonal antibodies against Foxp3) in an effort to evaluate the quantity of regulatory T cells at the examined tissues.

Regarding IBD activation in patients, in 41 of the samples, signs of active inflammatory bowel disease were detected, whereas in the rest 20 cases the findings were suggestive of disease remission. Increased numbers of eosinophils were present in 42 patients, while in 19 cases no remarkable increase of the number of eosinophils was noticed. Tissue sample characteristics are listed in Table 1.

Statistical analysis reached the result that Foxp3 expression and, as a consequence, the number of regulatory T cells correlates with CMV presence in the examined samples. More specifically, Tregs were detected in larger numbers in patient tissues where IBD coexists with CMV ($p=0,041$). On

average, 85,25 regulatory T cells per ten optical fields were counted in tissues with IBD and CMV versus 59,86 Tregs in tissues from patients only with IBD. (**Figure 1:** slide with high expression of Foxp3 and increased number of Tregs, **Figure 2:** low Foxp3 expression and lower Treg number)

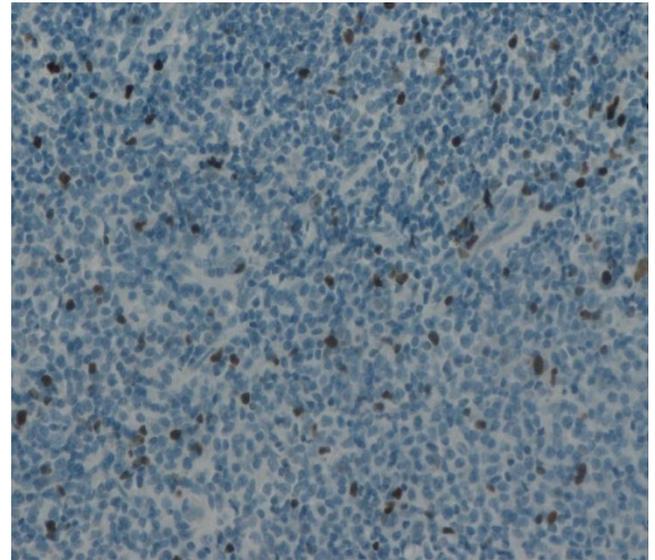


Figure 1. High expression of Foxp3 and increased number of Tregs (Immunohistochemistry, x200)

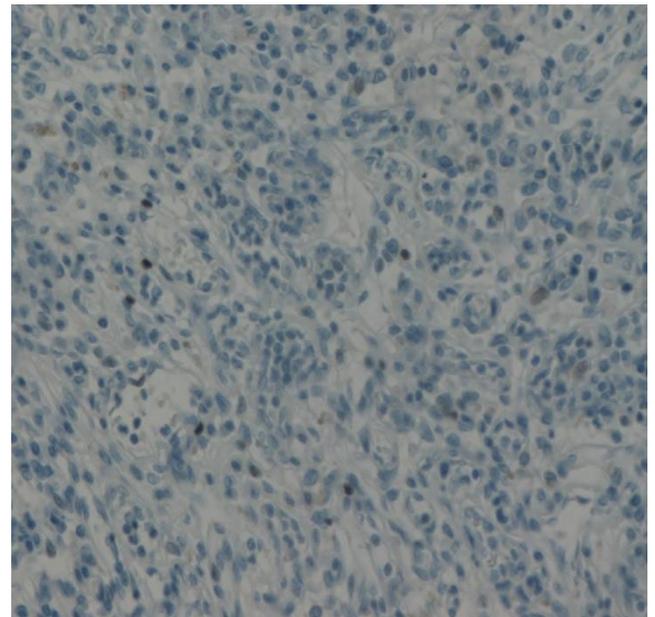


Figure 2. Low Foxp3 expression and decreased Treg number (Immunohistochemistry, x200)

The effect of other parameters, such as IBD activity and eosinophilia, on the Tregs number was also studied. Increased Tregs numbers were detected in patients with histological findings of active IBD. In addition, tissue samples with eosinophilia demonstrated high Tregs numbers,

in contrast to those that were not characterised by an abundance of eosiphils.

Table 1. CMV presence and tissue samples characteristics

Characteristics		Immunohistochemistry cases		
		CMV present	No CMV detection	Total
CMV presence		30 (49,2%)	31 (50,8%)	61 (100,0%)
IBD activation	Active IBD	21 (51,2%)	20 (48,8%)	41 (100,0%)
	Inactive IBD	9 (45,0%)	11 (55,0%)	20 (100,0%)
	Total	30 (49,2%)	31 (50,8%)	61 (100,0%)
Eosinophilia	Increased number of eosinophils	15 (35,7%)	27 (64,3%)	42 (100,0%)
	Not increased eosinophils number	15 (78,9%)	4 (21,1%)	19 (100,0%)
	Total	30 (49,2%)	31 (50,8%)	61 (100,0%)

Results processed with statistical analysis disclosing correlation of evaluated parameters are collected in Table 2 with P values performance.

Table 2. Statistical analysis of factors associating with Foxp3 expression

Factors	P value (Foxp3 expression in colon biopsies)
CMV	0.041
IBD activity	0.014
Ssdd Eosinophilia	0.037

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Discussion

In the majority of cases, cytomegalovirus infection causes no symptoms at all. After primary infection, CMV usually remains lifelong in a latent state. However, immunocompromised patients, such as patients with Crohn’s disease or ulcerative colitis, are in a vulnerable position in regards to cytomegalovirus infection, in contrast to immunocompetent individuals. Patients with a past silent CMV infection may show symptoms of activation of this infection after getting immunocompromised due to an IBD or its treatment. [10] More recent experimental studies in murines also revealed a triggering role of CMV in the development of Crohn’s disease and ulcerative colitis. [11]

It has been suggested that Inflammatory bowel diseases may practically be the clinical expression of diminished immunotolerance to commensal bacteria. Treg cells are critical for the immune tolerance in the intestine. Many studies have so far indicated that Tregs play a protective role in IBD. [12] Treg phenotype shows no alterations in patients with IBD despite the mucosal immune dysregulation. If wanted to characterize Treg phenotype concerning activation and inhibitory molecule, it may be characterized as a fully regulatory phenotype. [13]

Experimental data have revealed that T cell immune reactivity against antigens can be

controlled by immunoregulatory T cells. However, if requested to examine these cells and functions, a specific marker for Treg identification is required. Recent researches concerning Foxp3 protein revealed that it can be used for the quantification and enumeration of T cells with regulatory potency. [14]

In our research, immunohistochemical staining with a polyclonal antibody against Foxp3 was used in order to identify Tregs. With the method described above we were enabled to evaluate Treg population at the colon tissue and compare its presence in IBD patients with and without CMV colon infection.

The results of our study showed that Tregs were detected in larger numbers in the samples from patients with IBD that coexisted with CMV. The number of Foxp3+ Tregs was significantly higher in the mucosa of IBD patients with CMV infection in comparison to IBD patients without any intestinal infection. This finding suggests a remarkable increase of Treg cell pool in the infected colon mucosa due to causes that remain to be further investigated and clarified.

It is of great interest the fact that according to several articles increased Treg numbers are present in CMV colitis and similar mucosal infections. Recent studies suggest that such infiltrates can be the outcome of local inflammation caused by viral or bacterial infections. [14] Immunohistochemical staining for

nuclear Foxp3 gives the advantage to identify and enumerate even very low Treg numbers. Given the fact that Tregs maintain regulatory phenotype in IBD patients, a suppressive activity on behalf of Tregs is expected in the inflamed mucosa accordingly to the grade of inflammation. [4] In similar investigation patterns many studies demonstrated that the depletion of Foxp3+ T cells in local mucosal tissues launched the development of GvHD-like reactions in target organs. [15], [16]

Further results with regard to Foxp3+ T cells and their correlation with IBD activity grade may be easily explained considering that in IBD the immune system makes an effort to transcend the slight immune deficiency that led to IBD outbreak and to get protected against it. [13] As a result, elevated numbers of Tregs are found in the intestinal mucosa and circulation of patients with IBD, when compared with healthy controls. [17]

Eosinophilia is generally considered a sign of chronic inflammation. In our research, increased presence of eosinophils was associated with higher Treg numbers. Inflammatory bowel diseases are undoubtedly medical conditions with a long clinical course. Therefore, a continuous chronic local inflammation is expected to derange mucosal Treg number. Some studies correlate

chronic inflammatory diseases with Treg deficiency in peripheral blood. [14] However, Tregs quantity in peripheral blood does not reflect accurately their presence in target tissue. The mucosal sequestration of Tregs may offer a presumable explanation of their paucity in blood circulation. [3], [14], [18], [19]

Conclusion

In conclusion, the findings of our study demonstrate that Treg infiltration in inflamed mucosal tissues of IBD patients show a significant increase when CMV infection coexists in comparison with IBD patients without any intestinal infectious disease. Treg cells play a crucial role in suppressing local inflammation induced by viral intestinal infections and their regulatory potential may improve the state of intestinal mucosa by providing an active defensive mechanism against CMV, even in patients with partial immune deficiency, like IBD patients. The influence of Tregs over viral colon infection of patients with IBD should be further studied retrospectively and prospectively in order to determine whether these Foxp3+ T cells can be of value in a therapeutic context in order to control ongoing inflammation

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