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CD20cy and CD45RO immunoexpression in early rheumatoid arthritis synovium

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Abstract

In this paper, we studied 15 cases of early rheumatoid arthritis presenting with inflammatory lesions in different degrees of evolution. We want to highlight B- and T-lymphocytes in synovial tissue collected from patients diagnosed with early rheumatoid arthritis, to establish the pattern of their distribution, possibly in relation to local neovascularisation to determine the role played by these types of cells. The pathological samples were represented by synovial membrane biopsy fragments, which were examined by histopathological and immunohistochemical methods. We noticed a perivascular distribution of lymphocyte infiltrate, up to formation of lymphoid follicles with germinal centers. There is a close interdependence between B- and T-lymphocytes in these lesions, and their presence in the synovial membrane in relation to newly formed blood vessels facilitates their action and their chemical mediators. Studying the interdependence of different types of lymphocytes and their connection with blood vessels may generate new therapeutic targets.

Keywords: synovium, B-lymphocyte, CD20, T-lymphocyte, CD45RO.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with autoimmune nature. This condition raises serious problems of morbidity and mortality. In 2002, were diagnosed in the USA alone over one million of patients [1]. A study conducted on 208 patients over a period of 25 years has revealed that rheumatoid arthritis shortens life by about seven years in men and by three years in women [2].

The initiation and self-sustaining of rheumatic inflammation is a complex phenomena in which we find cell proliferation, angiogenesis, tissue modelling and activetion of proinflammatory factors [3].

One of the key elements of the pathogenesis in rheumatoid arthritis is the inflammation [4, 5]. T-cells are one of the most dominant cell population in rheumatoid synovium, majority of them are CD4+, with an important role in pathogenical process. More new evidences now suggests that the T-cell population is systemically abnormal in RA, having an excessive proliferative history; the result is a contracted T-cell receptor repertoire and an immunosenescent phenotype [6], such premature ageing of the immune system appears to predate the onset of clinical RA.

The therapeutic effect of rituximab, an anti-CD20 agent, in rheumatoid arthritis has confirmed the importance of B-cells in disease pathogenesis [7]. Ectopic germinal-centre-like structures in the synovia of some patients create a spatially organized microenvironment ideally suited to humoral immune responses. B-cells can process and present antigenic peptides to CD4+ T-cells, resulting in classical adaptive humoral responses, and similar cognate interactions with B-cells may prime naive T-cells in some cases [7]. The rheumatoid synovium appears to provide a context in which B-cell tolerance may be broken, autoantibody production enhanced, and an aberrant immune response upheld [8].

B- and T-lymphocyte cells are basic components of the inflammatory infiltrate and play key roles in disease pathogenesis [5, 9].

The purpose of this study is to highlight the presence and distribution of B- and T-lymphocytes in the inflamed synovial tissue and to establish the role of these cells in the context of inflammation.

Material and Methods

The study included 15 cases diagnosed with early rheumatoid arthritis, within six months from the onset of disease, in the Laboratory of Pathological Anatomy of the Emergency County Hospital Craiova in 2008.

Biological material was represented by fragments of synovial biopsy, collected by ultrasound-guided synovial biopsy, specimens that were processed using formalin-fixed, paraffin-embedded usual histopathological technique, followed by Hematoxylin–Eosin stain.

The immunohistochemical processing was made on serial sections from each paraffin-embedded block using Envision/HRP polymer amplification system (Dako,

Redox, Romania). As antigen retrieval, we used citrate solution pH 6 and sections were boiled for 15 minutes at microwave. Incubation with primary antibodies mouse anti-human CD20cy (clone L26, IgG2a kappa, dilution 1/1000, Dako, Redox, Romania) and mouse anti-human CD45RO (clone UCHL 1, dilution 1/200, Dako, Redox, Romania) achieved at room temperature for two hours. Signal detection was performed by incubation with Envision™ + Dual Link System–HRP for one hour at room temperature. To visualize the reaction we used diaminobenzidine-tetrahydrochloride (DAB), followed by counterstain with Hematoxylin. It was used a positive external control tissue represented by tonsil tissue for CD20, respectively spleen tissue for CD45RO and negative internal control by omitting the primary antibody.

We quantify the degree of the inflammatory infiltrate both histopathological and immunohistochemical, respectively B- and T-lymphocyte population in terms of its density and distribution pattern. Classification was done in accordance with the literature data [10] (Table 1).

Table 1 – ***Quantifying immunoexpression of CD20cy in the inflammatory infiltrate cell population***

The presence of rare lymphocytes diffusely distributed	Perivascular moderate arrangement	Perivascular massive clusters with formation of lymphoid follicles	Diffuse massive disposure and formation of lymphoid follicles with germinal centers
Grade 1	Grade 2	Grade 3	Grade 4

Sections were examined and the images were captured from 20× and 40× objective with Nikon Eclipse 90i microscope (Nikon, Apidrag, Bucharest) equipped with a 5 megapixels CCD camera. The acquisition itself was made with the software Nikon NIS-Elements by three observers (CP, SA, CM). Images captured at 40× objective were analyzed in terms of number of labeled cells per microscopic field.

Results

Histopathological analysis showed the presence of inflammatory infiltrate in all cases. In two cases, it had a diffuse distribution with rare lymphocytes. In 10 cases, the arrangement was moderate or massive perivascular and in three cases were identified follicular clusters of lymphocytes. The massive and diffuse disposal of inflammatory infiltrate and lymphoid follicles with germinal centers were not identified in the sections studied (Figure 1).

Tonsil CD20cy control sections showed B-lymphocytes from the germinal centers, from the mantle zone of lymphoid follicles and also B-lymphocytes dispersed in the interfollicular space. As regards spleen control sections for marking with CD45RO, their immunostain signal was diffuse in the T-lymphocytes areas of the spleen or clustered in the paracortical areas of normal and reactive lymphoid follicles.

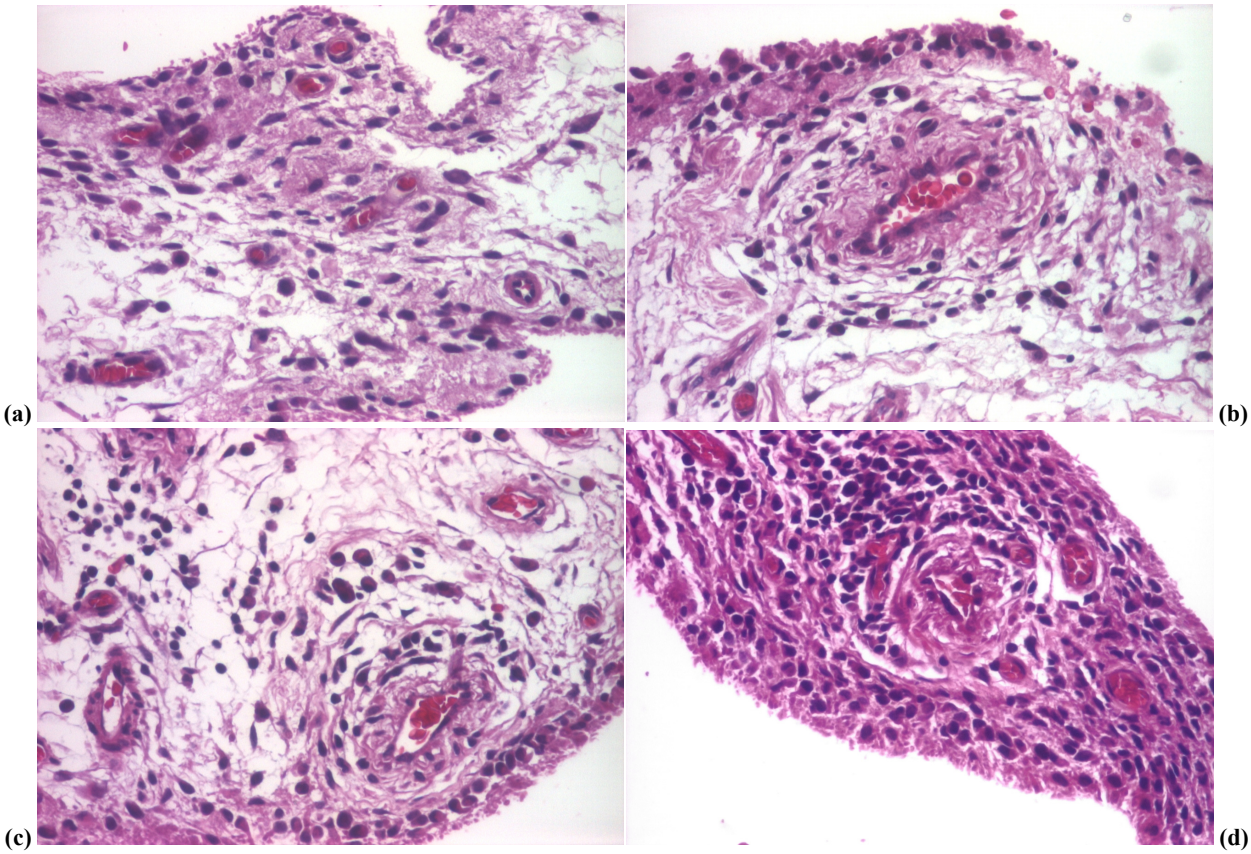


Figure 1 – ***(a) Rare lymphocytes diffusely distributed (HE stain, ×200). (b) Moderate lymphocyte numbers with perivascular distribution (HE stain, ×200). (c) Moderate inflammatory cells diffuse and perivascular (HE stain, ×200). (d) Follicular distribution of lymphocytes close to a blood vessel (HE stain, ×200).***

CD20cy immunoreaction was present in all cases included in the study and the immunostain was membranary and apical cytoplasmic. B-lymphocytes had a diffuse distribution in four cases, labeled cells were rare (grade 1). In eight cases, the lymphocytes were disposed perivascular, being present a moderate number of marked cells (grade 2). In three cases, B-lymphocytes were present in large numbers, perivascular disposed, forming follicular shaped structures (grade 3). In these cases, were also present grade 1 and 2 immunological marked areas. The transition between areas with different marking degrees was gradually and even with the presence of areas in which lymphocytes were absent (Figure 2).

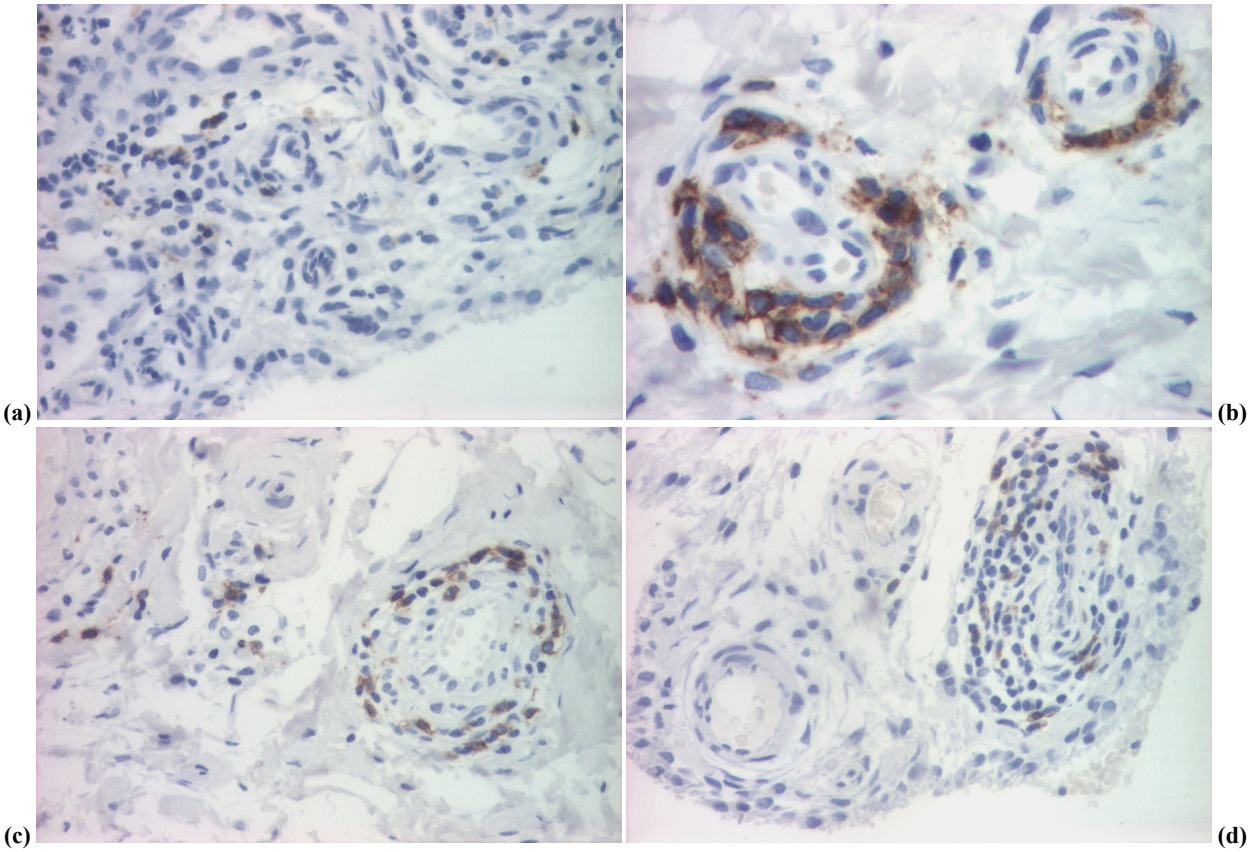


Figure 2 – ***(a) CD20cy immunostaining, rare lymphocytes with diffuse disposure, ×200. (b) CD20cy immunostaining, perivascular lymphocytes (grade 2), ×400. (c) CD20cy immunostaining, diffuse perivascular lymphocytes (grade 2), ×200. (d) CD20cy immunostaining, follicular disposed lymphocytes (grade 3), ×200.***

Immunohistochemical reaction for CD45RO was positive in all cases, with a membranar and apical cytoplasmic T-lymphocytes stain. In two cases, marked cells were rare and diffusely arranged without relation to the blood vessels (grade 1). In other cases, T-lymphocytes were perivascular disposed in a moderate number (10 cases, grade 2) or massively with a follicular aspect (three cases, grade 3). In two cases, the appearance of follicular distribution was not in relationship with closed blood vessels (Figure 3).

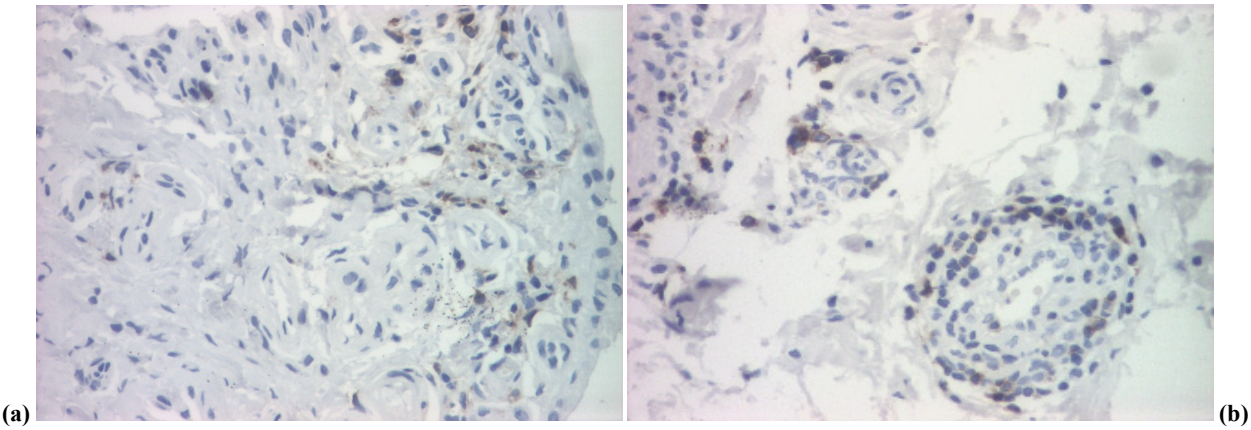
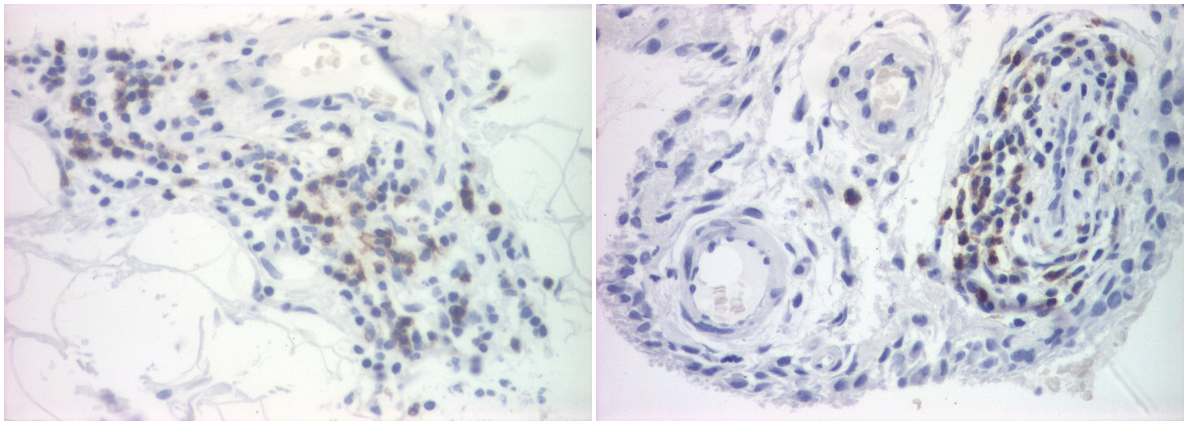


Figure 3 – ***(a) CD45RO immunostaining, rare lymphocytes diffuse disposure, ×200. (b) CD45RO immunostaining, diffuse perivascular disposure of T-lymphocytes (grade 2), ×200.***



(c) **(d)**

Figure 3 – ***(c) CD45RO immunostaining, (grade 3), close to a blood vessel, ×200. (d) CD45RO immunostaining, T-lymphocytes massive overcrowding, with follicular aspect, ×200.***

Perivascular distribution for both markers was done in relation to small and medium size blood vessels. Only a few blood vessels presented B- or T-lymphocytes around them, especially when they were located near areas of fibrinoid necrosis or underlying areas of epithelium with increased proliferation.

Following the counting of immunostained lymphocytes, we found lymphocytes CD45RO-positive predominance, B-/T-lymphocytes ratio being 1/1.6 in the lymphoid clusters and 1/2 in areas that had a diffuse inflammatory infiltrate.

Besides, in areas with diffuse distribution of lymphoid infiltrate, T-cells were preferentially presented to the synovial membrane surface, in conjunction with synoviocytes layers. T-cells prevailed in the external areas of lymphoid areas in these areas being present fewer marked CD20-cells. In most cases, B-lymphocytes were in direct relationship with neoformation blood vessels.

Discussion

B- and T-lymphocytes, macrophages and dendritic cells are invading the synovial membrane, forming micro-structural complexes that are producing inflammatory or tissue destructive lesions. The discovery of autoantibodies generated by B-lymphocytes on patients with rheumatoid arthritis provided the first evidence that rheumatoid arthritis would be an immune-mediated disease [11].

B-cells have many potential key roles: they can act as antigen presenting cells, secrete pro-inflammatory cytokines, produce rheumatoid factor (RF) and other auto-antibodies and auto-activate T-cells. B-cells are acting as antigen presenting cells by peptidic antigen processing and antigen presentations to T-cells that become activated, proliferate and execute pro-inflammatory activities [12].

CD20+ B-lymphocytes are a predominant cell population in rheumatoid synovial tissue and they are present in most patients with rheumatoid arthritis. Our study revealed that B-lymphocytes were present perivascular in different distribution patterns depending on the different stages of the disease. Other authors indicate that they may have a distinct follicular form that may resemble to a functional unit of secondary lymphoid tissues-germinal centers [11].

In 2001, Takemura S *et al.* noted that the three days administration of anti-CD20 antibodies leads to destruction of follicular structures of B-lymphocytes and also a dramatic level decrease of interleukin-1β (IL-1β) and interferon-γ (IFN-γ), suggesting that B-lymphocytes influence the function of T-lymphocytes and macrophages that are generating these proinflammatory cytokines [13]. In 2004, Edwards JC *et al.* tested on 161 patients the targeted therapy with Rituximab (a monoclonal antibody pointed against CD20) compared with the methotrexate therapy. The 24 weeks analysis showed significantly better effects ($p \leq 0.025$) on patients treated with Rituximab [14]. In 2009, after conducting experiments with injection of immune cells taken from the joints of patients with rheumatoid arthritis, on mice's skin with immune depression, Constantino Pitzalis said that "lymphocytes B are making their way to the affected joints where they are settling structures called germinal centers. These lymphocytes act as antibodies factories, producing antibodies that later attack the joint tissue" [15].

CD4+ and CD8+ T-cells can be differentiated according to their surface expression of CD45RA or CD45RO molecules. T CD45R0-lymphocytes indicate a cell differentiation and are associated with a memory phenotype. This indicates that those cells were previously stimulated by an antigen receptor. T-cells are activated by the linking to the trimolecular complex consisting of antigen peptide, HLA-DR4, and T-lymphocyte's receptor. This leads to a cascade of events that includes IL-2 production that further stimulates clone expansion of T-lymphocytes [12].

In synovial biopsy fragments studied, we found large numbers of T-cells present in synovial subintimal layer. Following T-cell population in terms of distribution in relation to vasculature and cell density, we observed their location under synovial surface or perivascular, ranging in some cases to formation of lymphoid follicles with germinal centers.

There are two basic patterns of infiltration with T-cells [16]. First, there might be perivascular lymphocyte aggregates containing, in particular, CD4+ lymphocytes in combination with B-lymphocytes, few CD8+

lymphocytes and dendritic cells. The second pattern is diffuse infiltration of T-lymphocytes scattered all over synovial membrane.

In some studies, it is shown that a population of T-lymphocytes represents a majority in the inflammatory infiltrate in rheumatoid arthritis [17]. In this study, the ratio between B-lymphocytes/T-lymphocytes was subunit both in areas with diffuse distribution and in those with follicular architecture of inflammatory factors. Also, the strong correlation with the surface synoviocytes can show involvement in the activation of T-lymphocytes and their proliferation. In 2005, Tran CN *et al.* showed that the surface T-cells and the synoviocytes maintain their mutual proinflammatory activity. The same author evidentiates the close relationship between B- and T-lymphocytes in the follicular areas where the two-lymphocyte populations appear to interact [18].

In 1997, Klimiuk PA *et al.* notes that in some patients, granulomatous necrobiosis areas are obvious. These areas are characterized by regions of fibrinoid necrosis surrounded by rim of epithelioid histiocytes and granulation tissue [19].

The role of T-lymphocytes in the pathogenesis of rheumatoid arthritis is well established, while the contribution of B-lymphocytes is less clarified. T-lymphocytes activation by B-lymphocytes leads to the production of IL-2 that stimulates further clone expansion of T-lymphocytes, and to express several surface molecules – CD69, TNFα (α factor tumor necrosis) and RANKL (RANK-ligand) which activates macrophages, synovial fibroblasts and osteoclasts respectively. T-cells produce IL-17 and IFN-γ [20], which stimulates further release of other cytokines. In 2000, McInnes IB *et al.* shows how T-cells activated in the synovial membrane and synovial fluid of patients with rheumatoid arthritis can directly activate macrophages, synoviocytes and osteoclasts by intercellular interactions [21].

A possible biological effect at synovial level, of the perivascular activated T-lymphocytes is the activation of the migrating macrophage populations through direct cell contact. This mechanism is known as stimulating *in vitro* production of cytokines and MMP from macrophages [22]. It was shown that a human serum factor, identified as apolipoprotein A-I (apo A-I), would inhibit contact-mediated stimulation of monocytes by in vitro activated T-lymphocytes [23]. It has been speculated that apo A-1 may play an important role in modulating the effects mediated by T-lymphocytes in the acute and chronic form of inflammation. Furthermore, many T-cells in synovial tissue are in a state of hiporeactivity [16, 24]. Interdigital dendritic cells, which are powerful antigen presenter cells, are located near the CD4+ T-lymphocytes in the lymphocyte aggregates and close to the intimate layer [25–27].

Conclusions

Our study has shown the involvement in the pathogenesis of rheumatoid arthritis of both types of lymphocytes, the developed lesions being a consequence of activation and cooperation of the two cell types. The ratio of the two cell populations and the close relationship of T-lymphocytes with the synoviocytes above, underlines their crucial role in disease development. The close distribution of B- and T-lymphocytes in the inflammatory infiltrate proves interdependent pathogenic mechanisms, and the direct relationship between the B-lymphocytes and newly formed blood vessels may be a valuable therapeutic target.

Acknowledgements

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