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The Role of CD8+ CD40L+ T Cells in the Formation of Germinal Centers in Rheumatoid Synovitis

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In rheumatoid synovitis, lymphocytes can be arranged in follicular structures resembling secondary lymphoid follicles. To understand the organizing principles of this ectopic lymphoid tissue, the cellular components contributing to synovial follicles were examined. In 9 of 24 synovial tissue biopsies, lymphoid aggregates were found consisting of CD4+ T cells and CD20+ B cells. In four of the nine patients, the follicular centers were occupied by CD23+CD21+ cellular networks representing follicular dendritic cells involved in germinal center reactions. In five patients, CD23+ cells were absent from the centers of the aggregates, suggesting that fully developed germinal centers are generated in only a subset of patients. To identify factors involved in the regulation of the synovial microarchitecture, cell populations contributing to the follicles were quantified by digital image analysis of immunostained tissue and by flow cytometry of tissue-derived lymphocytes. Proportions of CD4+, CD20+, and CD68+ cell subsets were surprisingly invariant, irrespective of the presence or absence of CD23+ follicular dendritic cells. Instead, tissue biopsies with CD23+ germinal center-like regions could be distinguished from those with CD23- T-cell-B-cell aggregates by a fourfold increase in the frequency of tissue-infiltrating CD8+ T cells, a fraction of which expressed CD40 ligand (CD40L). The data suggest a previously unsuspected role of CD8+ lymphocytes in modulating germinal center formation and raise the possibility that CD8+ CD40L+ T cells are involved in aggravating pathologic immune responses in rheumatoid synovitis. The Journal of Immunology, 1998, 161: 6390–6397.

The follicular formations adopted by T cells and B cells in rheumatoid synovium have microscopic characteristics reminiscent of germinal centers (GCs) (4, 5). GCs are specialized microanatomical structures that are required for the generation of high-affinity Abs and the selection of memory B cells in response to protein Ags (6–8). Immunization with Ag induces activation of T cells and B cells in the T-cell-rich areas of secondary lymphoid tissues with cognate- and costimulation-dependent expansion of Ag-specific lymphocytes (9). Activated T cells and B cells from these populations migrate into adjacent B cell zones to form GCs (10). GCs arise when B cells accumulate among the processes of follicular dendritic cells (FDCs) and undergo intense proliferation, apoptosis, and V(D)J gene hypermutation. The current paradigm holds that somatically mutated cell surface Igs are probed on Ags bound to FDCs and that B cells producing high-affinity Abs are selected to survive, while B cells that fail to recognize Ag die by apoptotic programmed cell death (6, 11). Only 10% of GC cells are CD3+ T cells, virtually all of which express the CD4 marker (12). CD8+ T cells account for <3% of the T cells. Although they represent a minority of GC cells, CD8+ T cells are requisite for GC formation. In murine studies, CD4+ T cells migrating into the GC bear αβ TCRs reactive to the Ag driving the GC reaction (13, 14).

Molecular mechanisms involved in GC formation are beginning to be understood. Patients with X-linked immunodeficiency causing hyper-IgM production do not generate secondary Ab responses, lack memory B cells, and possess lymph nodes with primary follicles lacking GC reactions (15). The molecular defect underlying this syndrome has been localized to the CD40 ligand (CD40L) gene (16, 17). According to the current model, CD40-CD40L interactions are critical for the recruitment of GC precursors, with CD40L+ T cells and interdigitating dendritic cells providing signals to B cell precursors in extrafollicular areas (18–20). B cell memory generation appears to be regulated by the interaction of CD40+ centrocytes with CD40L+ intrafollicular T cells.
Other molecules implicated in GC reactions include the integrin, VLA-4, on lymphocytes interacting with VCAM-1 on FDCs, possibly facilitating the adherence of B cells to the FDC reticulum (23). Additionally, mice deficient in either lymphotixin α (24), TNF-α (25), or the type-I TNFR (26) fail to develop secondary lymphoid follicles and GCs. In mice lacking expression of either CD28 or its ligands CD80 and CD86, B cells accumulate in the lymphoid follicle following antigenic challenge (27, 28). However, these cells fail to undergo proliferative expansion, do not initiate GCs, and do not acquire somatic mutations, emphasizing the importance of CD28-CD80/CD86 interactions in GC reactions.

The goal of the current study was to examine the follicular structures formed by tissue-infiltrating lymphocytes in rheumatoid synovitis, with special emphasis on the cellular components required for GC generation. Immunohistochemical studies combined with digital image analysis demonstrated that in only one-half of the patients with follicular synovitis did T cell-B cell follicles contain all of the cellular components of classical GCs. In the remaining patients, CD23+ FDCs were lacking in T cell-B cell aggregates that otherwise had normal proportions of intrafollicular CD4+ T cells and CD20+ B cells, suggesting an aborted GC reaction. Surprisingly, patients with and without typical GCs could be distinguished based on the presence of CD8+ T cells. GC formation was associated with increased recruitment of CD8+ T cells to both the border of T cell-B cell follicles as well as to interfollicular areas. Classical GC reactions occurred in patients who had an expansion of CD40L-expressing CD8+ T cells. Because CD40L expression is critically involved in GC reactions, CD8+ CD40L+ T cells may be able to support the generation of pathologic immune responses in rheumatoid synovium.

Materials and Methods

Patients

Synovial tissue specimens were obtained from patients who underwent synovectomy or joint replacement surgery. Patients enrolled into this study fulfilled the diagnostic criteria for RA and had active disease at the time of tissue biopsy (Table I). Tissue sections were stained with hematoxylin and eosin, and specimens with follicular lymphoid aggregates were selected for further studies.

Immunohistochemistry

Synovial tissue was embedded and frozen in O.C.T. compound (Miles, Elkhart, IN) and was cut into 5-μm sections and mounted onto gelatin-coated slides. Slides were air dried, fixed in acetone, and stored at −80°C. Sections were fixed in 1% paraformaldehyde, blocked with 5% normal goat serum (Life Technologies, Grand Island, NY), and incubated for 30 min at room temperature with the following Abs: anti-CD4 (Leu-3a, dilution 1:100, Becton Dickinson, San Jose, CA); anti-CD8 (Leu-2a, 1:5, Becton Dickinson); anti-CD20 (L26, 1:50, Dako, Carpenteria, CA); anti-CD21 (1F8, 1:200, Dako); anti-CD23 (MHM6, 1:50, Dako); anti-CD68 (KP1, 1:250, Dako); anti-MIB-1 (KI-67, 1:60, Coulter Immunotech, Westbrook, ME); and anti-IgD (A093, 1:1000, Dako). Anti-CD40L (anti-gp39, Ancell, Bayport, MN) was used at a dilution of 1:500, and slides were incubated overnight at 4°C. Secondary species-specific Abs were applied for 30 min at room temperature followed by detection with the streptavidin-biotin complex immunoperoxidase or alkaline phosphatase technique. For light microscopy, 3- amino-9-ethylcarbazole (AEC, Sigma, St. Louis, MO) or 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) substrate solutions were used. For digital fluorescence imaging, the Vectastain ABC kit and alkaline phosphatase substrate kit 1 (Vector Red, Vector Laboratories, Burlingame, CA) were used for detection. For two-color immunohistochemistry, slides were washed for 10 min in 1% Triton X-100 in PBS and blocked with 5% normal goat serum before adding the second Ab. Sections were counterstained with hematoxylin and permanently mounted in Cytoseal-280 (Stephens Scientific, Riverdale, NJ).

Digital imaging

Fluorescence-stained tissue sections from serial sections were scanned using a confocal microscope (LSM310, Carl Zeiss, Oberkochen, Germany) with an argon-krypton laser, excitation 568 nm. Fluorescence signals for each Ab were translated into pseudocolors. For analysis of spatial relationships, images from the immunohistochemical analysis for CD68, CD4, CD8, CD20, and CD23 expression were overlaid. Correct positioning of the images was ensured by placing the digitized scans on a light microscope background that showed tissue landmarks and cellular infiltrates. The area stained with each Ab was calculated by an image analysis program (KS 400, Kontron Elektronik, Munich, Germany) as a percentage of the total area of the lymphoid aggregate.

Tissue digestion and flow cytometry

Fresh synovial tissue was cut into 2- to 4-mm³ pieces and washed once in complete medium (RPMI 1640, 10% FCS, 200 μM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Then, 1 cm³ of tissue was incubated in 10 ml digestion solution (0.05 M HEPES buffer, 3 mg/ml type I A collagenase, 1 mg/ml hyaluronidase, and 0.1 mg/ml type IV deoxyribonuclease I in RPMI 1640) at 37°C for 30 to 45 min. Lymphocytes were isolated using a Ficoll density-gradient and incubated with FITC-labeled anti-CD40L (Ancell) and phycoerythin-conjugated anti-CD4 or anti-CD8 Abs (Becton Dickinson) for 30 min at 4°C in the dark.

Statistical analysis

All statistical analyses were performed using SigmaStat software (Jandel, San Rafael, CA). Student’s t test and the nonparametric Mann-Whitney test were used where appropriate.

Results

Heterogeneity of follicular structures in rheumatoid synovium

To identify factors involved in regulating the microarchitecture of rheumatoid synovitis, synovial tissue biopsies from 24 consecutive patients with unequivocal RA and active synovitis at the time of collection were screened by standard light microscopy. Nine tissues had prominent lymphoid follicles in the sublining stroma and were selected for further analysis. Besides having follicular organization, all of these samples also had diffuse mononuclear infiltrates and collections of small lymphocytes surrounding the capillaries. Scattered lymphocytes were found in the interfollicular zones. Clinical characteristics of the nine patients enrolled into the study are given in Table I. The group included six females and three males. Seven of the nine patients produced rheumatoid factor. Disease duration ranged from 1–38 yr. Specimens were harvested from the shoulder, elbow, wrist, hip, or knee.

Immunohistochemistry analysis demonstrated that the lymphoid aggregates in the synovium were composed of CD20+ B cells intermingled with CD3+ CD4+ CD45RO+ T cells. In some tissue sections, a ring of CD4+ T cells was arranged around the CD20+ B cells. Often CD4+ T cells and CD20+ B cells were not spatially separated but formed a mixed population (Fig. 1). CD8+ T cells were a minor population. Anti-CD23 Abs reacted with cells in the center of T cell-B cell aggregates and less frequently with cells in the mantle zone of some of the T cell-B cell clusters. However, not
all of the follicular structures analyzed in this study contained centrally located CD23-expressing cells. As shown in Fig. 2, essentially two different types of T cell-B cell aggregates were found; in one, anti-CD23-specific Abs revealed networks of centrally located CD23-expressing cells, consistent with the presence of FDCs; in the other, the centralized CD23 staining pattern was distinctly absent.

To investigate whether tissues without CD23+ cells in the center of the follicles expressed an unusual FDC phenotype, adjacent tissue sections were stained with anti-CD21, anti-MIB-1, and anti-IgD. Representative results are shown in Fig. 2. These experiments revealed that lymphoid aggregates in rheumatoid synovium can be categorized into two subsets. Aggregates resembling classical GCs had central CD23+ networks. MIB-1+ cells were frequent, occupying the center of the cell clusters with only scattered cells throughout the remainder of the follicle. IgD was expressed on the majority of B cells, but was absent from the most centrally localized B cells, consistent with phenotypic changes that result as these cells participate in a GC reaction. In contrast, five of the nine synovial biopsies contained prominent and demarcated T cell-B cell aggregates that characteristically lacked central CD23+ and CD21+ cells. In these tissues, irregularly distributed CD23+ cells were occasionally seen in the outer layers of the follicular structures. To assure that the absence of centrally localized CD23+ CD21+ cells was not a sampling artifact, serial sections of the regions of interest were examined. These studies failed to reveal evidence for FDCs in the center of the T cell-B cell clusters. As shown in Fig. 2, the absence of CD23+ FDC networks was associated with a low frequency of MIB-1+ cells in the T cell-B cell aggregates. Also, IgD+ B cells were dispersed throughout the follicles without the central clearing observed in the CD23+ follicles.

In summary, these studies provided evidence that follicular organizations in the rheumatoid synovium can involve all of the cellular components and possess the microanatomical arrangement typical of classical GCs. However, only a subset of T cell-B cell aggregates fulfilled the criteria for GCs, whereas the others were characterized by a lack of central FDCs and a lack of B cell proliferation and differentiation. These two forms of follicular structures were not encountered side-by-side within the same tissue but were, instead, mutually exclusive. Individual patients either had fully developed GCs or atypical follicles, suggesting a critical role of host factors in the formation of ectopic lymphoid tissue in the synovium.
Formation of GCs in the synovium is associated with the recruitment of high numbers of CD8⁺ T cells

To identify potential factors influencing the formation of either typical GCs or CD23⁺ T cell-B cell aggregates, the representation of different cell populations in tissues of both categories was quantified by digital image analysis. Compound images were constructed of consecutive tissue sections analyzed by confocal microscopy. These images allowed for detailed inspection of the spatial relationship of different cell populations. Representative examples of a follicle with centrally located CD23⁺ FDCs and a T-B aggregate lacking CD23⁺ cells are shown in Fig. 3. The area stained with each of the specific Abs was calculated and expressed as a percentage of the total area occupied by the follicular structure. The data from the four patients with CD23⁺ follicles and the five patients with CD23⁺ T cell-B cell aggregates are summarized in Table II.

This quantitative approach demonstrated that the contribution of each cell type to the follicular structures was surprisingly invariable. In CD23-containing GCs, the follicle consisted of 32.8% CD4⁺ T cells and 30.9% CD20⁺ B cells by area. CD23-expressing...
cells contributed 8.1% and CD68^+ macrophages accounted for 10.8% of the area. Unexpectedly, CD8^+ T cells were frequent in these centers. They were found predominantly at the outer edge of the demarcated follicle and accounted for 17.5% of the follicular area. Lymphoid aggregates lacking centrally localized FDCs were composed of 43.4% CD4^+ T cells and 41.7% CD20^+ B cells. CD68^+ macrophages were again a minor population (mean area of 8.4%). CD8^+ T cells only represented 5.5% of the total area. The ratio of the two major cell populations in the follicles, CD4^+ T cells and CD20^+ B cells, was well-maintained among the patients and was essentially identical when classical GCs and CD23 aggregates were compared. The contribution of CD8^+ T cells to the follicles clearly distinguished classical GCs and CD23 aggregates (p < 0.04).

So far, CD8^+ T cells have not been considered a cell population of relevance in GC formation. Therefore, it was surprising to find a large number of CD8^+ T cells at the outer edge of typical GCs.

To further examine the possibility that variations in the frequency of synovial CD8^+ T cells could be correlated with tissue organization, the total number of CD8^+ T cells in the perifollicular regions, the interfollicular zones, and the perivascular cell accumulations was counted. As shown in Fig. 4, synovial tissue from patients with GCs contained an average of 68 CD8^+ T cells per high-power field. In patients without GCs, CD8^+ tissue-infiltrating T cells were much less frequent (16 cells per high-power field, p < 0.001). These data indicated that the representation of CD8^+ T cells was variable among patients and was closely associated with the emergence of GCs.

**Tissue-infiltrating CD8^+ T cells in patients with GC formation express CD40L**

To further examine the role of synovial CD4^+ and CD8^+ T cells, mononuclear cells were isolated from freshly harvested synovial tissue biopsies. Tissues from three patients with GC formation and three patients with CD23^+ T cell-B cell aggregates were analyzed by flow cytometry. As expected, the majority of CD3^+ T cell network in the center of A, the distribution of CD20^-, CD4^-, CD8^-, and CD68^+ cells is similar in the CD23^+ aggregate (A) compared with the CD23 aggregate (B).
However, a difference emerged for the percentage of CD8+ T cells with up-regulated CD40L expression (Fig. 5). In synovium with CD23-lymphoid aggregates, CD8+CD40L+ T cells accounted for only 1.2% of T lymphocytes. The CD8+CD40L+ subset was expanded three- to ninefold in specimens with typical GCs. All three patients had a significant proportion of CD40L-expressing CD8+ cells (3.8–10.8% of T lymphocytes, p < 0.001).

Two-color immunohistochemistry with anti-CD8- and anti-CD40L-specific Abs was used to localize CD8+ cells with up-regulated CD40L expression. In specimens from patients with CD23+ T cell-B cell follicles, CD8+CD40L+ cells were distinctly infrequent and multiple tissue sections had to be searched to identify an occasional cell with this phenotype. In patients with fully developed GCs, CD8+CD40L+ cells were found in a perifollicular distribution, whereas CD8+ T cells in the interfollicular zones and in the perivascular regions remained negative for CD40L (Fig. 6).

**Discussion**

The formation of follicle-like structures in rheumatoid synovitis has been cited as evidence for the involvement of a T cell-B cell response in disease pathogenesis (4). Lymphocytes are a component of most inflammatory infiltrates, and their presence does not prove a fundamental role in pathology. The synovial membrane is primarily not a lymphoid tissue and the generation of ectopic lymphoid architecture is indeed a highly significant finding. The data presented here confirm that the conditions in rheumatoid synovitis are unique and that the synovial microenvironment is able to support the formation of follicles that have all of the characteristics of functional GCs. The more important finding of our study relates to the distinction between patients who have classical GCs in the synovium and those who generate T cell-B cell aggregates that lack the typical features of B cell proliferation and down-regulation of IgD cell surface expression. This suggests that not all of the signals required for GC organization are present in some individuals and that the search for specific mediators of such differences could provide important insight into synovial immune responses.
Lymphoid aggregates without GC reaction lacked networks of CD23+ CD21+ FDCs. More surprisingly, we found that the number of tissue-infiltrating CD8+ T cells was a predictor for the absence or presence of fully developed GCs. As CD8+ T cells have not been implicated in contributing to GC formation, it would be expected that patients with a low representation of CD8+ T cells in the infiltrates would be particularly successful in establishing synovium GCs. The opposite was the case, suggesting an hitherto unsuspected contribution of CD8+ T cells to rheumatoid synovitis.

Ab responses to protein Ags begin in the T cell-rich zones of secondary lymphoid tissues where interrogating dendritic cells, T cells, and B cells make contact. However, this encounter is insufficient for the maturation of humoral immune responses and for the development of memory B cells. T cell-B cell interaction in GCs must provide unique signals that allow for B cell proliferation, positive selection based on affinity of the expressed Ig, and emergence of memory B cells. Therefore, the formation of these highly specialized centers in the synovial membrane cannot be overemphasized, and it stresses the contribution of T cells, B cells, and specific Ag to the pathologic events defining this syndrome.

Deficiency of GC formation is a hallmark of patients with severe X-linked immunodeficiency with hyper-IgM syndrome (29). Careful studies of these patients have revealed the crucial role of CD40 signaling in GC formation and T cell-dependent Ag responses (30, 31). Mutations of the CD40L gene not only produce the clinical syndrome, which is characterized by increased occurrence of opportunistic infections and severe neutropenia, but is also associated with failure to produce GC reactions (29). Comparison of synovial tissue CD23+ T cell-B cell aggregates with lymphoid follicles in CD40L-deficient patients raised the possibility of shared features. Lymph node biopsies from patients with mutated CD40L lacked GCs or expressed scant GC reactions while the architecture and cellular distribution in the paracortical areas was normal. Interestingly, these patients displayed quantitative and qualitative abnormalities of FDCs, most importantly, poor expression of CD21 and CD23. It has been suggested that the loss of FDCs and their phenotypic abnormalities in these patients might result in poor Ag trapping and inefficient rescue of B cells from apoptosis. Alterations in the function of FDCs or differences in the recruitment of this specialized cell type to the synovial tissue environment could therefore explain why some individuals do not develop complete GC reactions in this ectopic site.

A role for CD40L in rheumatoid synovitis was suggested by the intriguing finding that the frequencies of synovial CD8+ CD40L+ cells correlated with the formation of GC-like follicular structures. CD40L+ T cells have recently attracted attention because several reports have associated increased and prolonged expression of CD40L with systemic lupus erythematosus (32). Because production of a multitude of autoantibodies is a characteristic feature of patients with systemic lupus erythematosus, defects in mechanisms underlying T cell-dependent B cell activation are obvious candidates for disease-risk factors. CD40L expression has been reported to occur on synovial tissue T cells in RA (33). While it could be easily imagined that up-regulation of CD40L has a place in chronically persistent CD4 T cell-B cell responses, a contribution of CD8+ T cells has so far not been described.

CD8+ T cells were fourfold more frequent in the tissue of patients with GCs. In these patients, CD8+ T cells accounted for a significant proportion of the cells in T cell-B cell follicles. Often, they accumulated at the outer edge of the cell clusters. CD8+ T cells were not only represented more frequently in direct association with the follicles, they were also more numerous in the interfollicular areas. Two models could explain this unexpected finding: 1) CD8+ T cells migrate into the synovial membrane in an attempt to down-regulate the GC reactions; or 2) CD8+ T cells assume helper cell functions in the synovium. In the first model, CD4+ CD40L T cells would be the primary initiating cells of GC formation and the recruitment of CD8 T cells would be a secondary event. Although this interpretation cannot be excluded, a primarily inhibitory function of the CD8 T cells is difficult to reconcile with the expression of CD40L. Also, if CD8+ T cells, through a yet unknown mechanism, could shut down GC reactions, burned-out GCs with pale cells arranged in an onionskin fashion should be found. Such structures were not encountered in the CD8+ T cell-rich tissues. Rather, the frequency of tissue-infiltrating CD8+ T cells was an excellent predictor for typically structured GCs. In support of the second model, Cronin et al. have reported that IL-4-producing CD8+ T cells can provide B cell help (34). We have no direct evidence to support this interpretation, but the expression of CD40L would be highly suggestive of a possible helper function for this cell subset. This issue will have to be addressed by isolating these CD8+ T cells and assessing their functional profile in T cell-B cell interactions. The quantification of cell populations contributing to the GCs indicated that CD4+ T cells were less abundant in typical CD23+ follicles than in CD23 aggregates. This again would support the interpretation that CD8+ T cells might be able to assume helper cell functions under special circumstances.

In summary, our studies on the composition of follicular structures formed in rheumatoid synovial tissue revealed a critical role for CD8+ T cells in regulating the organization of ectopic lymphoid tissue. Formation of synovial tissue GCs coincided with the recruitment of CD8+ T cells and the up-regulation of CD40L on these cells. Evidence is emerging that RA is not a single disease entity but includes several distinct variants of polyarthritis. The restriction of GC formation to a subset of RA patients raises the possibility that variations in CD8+ T cell function represent disease-risk factors and contribute to the heterogeneity of the rheumatoid disease process. Investigating the regulation and function of synovial tissue CD8+ T cells could eventually provide clues on factors critically involved in establishing a specialized microenvironment for chronic immune responses and in modulating inflammatory pathways in the synovial membrane.

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