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Tissue Cytokine Patterns Distinguish Variants of Rheumatoid Synovitis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease with primary manifestations in the synovial membrane. Tissue infiltrates are composed of T cells, B cells, and macrophages, but histopathological appearances vary widely and are rarely pathognomonic. Mechanisms underlying the phenotypic heterogeneity of rheumatoid synovitis are not known. To explore whether a correlation exists between the microscopic patterns of rheumatoid synovitis and *in situ* production of cytokines, tissue samples from 21 consecutive patients with clinically active RA were examined. Based upon the organization of the lymphocyte infiltrate, the synovial biopsies were categorized into three distinct subsets. Ten samples were characterized by diffuse lymphoid infiltrates without further microarrangement. In seven samples, lymphoid follicles with germinal center formation were detected, and in four specimens, granuloma formation was identified. In all specimens, cytokine transcription of interferon (IFN)-γ, interleukin (IL)-4, IL-1β, tumor necrosis factor (TNF)-α, IL-10, and transforming growth factor-β1 was semiquantified with polymerase chain reaction and liquid phase hybridization. Each of the morphologically defined variants of synovitis displayed a unique cytokine profile. Low-level transcription of IFN-γ, IL-4, IL-1β, and TNF-α was typical of diffuse synovitis. In follicular synovitis, IFN-γ was the dominant cytokine, IL-4 was virtually undetectable, and IL-10 was abundant. Granulomatous synovitis demonstrated high transcription of IFN-γ, IL-4, IL-1β, and TNF-α and could be clearly distinguished from the other phenotypes. To investigate whether differences in the synovial lesions were related to host factors, patients were compared for clinical parameters. Diffuse synovitis was seen in most of the patients with seronegative RA, the mildest form of the disease. In contrast, extra-articular spreading of RA with nodule formation was typically associated with granulomatous synovitis. In summary, RA patients display reproducible patterns in the organization and activity of synovial infiltrates. The correlation of microanatomy with tissue cytokine production suggests that several pathomechanisms can modulate the expression of the immune response in the synovial membrane. *(Am J Pathol 1997, 151:1311–1319)*

Rheumatoid arthritis (RA) is a systemic inflammatory disease that primarily manifests itself as synovial inflammation of diarthrodial joints. The typical histopathological changes include dense infiltration of the synovial membrane by mononuclear cells, neoangiogenesis, and hypertrophy and hyperplasia of the synovial lining.[1,2] The etiopathogenesis of the syndrome is not understood. Several lines of evidence support a central role of T lymphocytes in the disease-specific pathogenic events.[3–5] An alternative hypothesis, namely, that macrophages are the pivotal cell type in rheumatoid synovitis, has also been proposed.[6,7] Whether only T cells or only macrophages or both are the causative elements in RA remains a matter of controversy.[8,9]

RA is primarily a clinical diagnosis. Symmetrical joint involvement, dominant manifestations in peripheral joints, rheumatoid factor production, and the formation of rheumatoid nodules are considered when the diagnosis is made.[10] The histological appearance of the synovium varies quite extensively and the pathological findings are usually not helpful in distinguishing RA from other inflammatory arthropathies.[2] Many patients with RA present with diffuse infiltrates of the synovial membrane without the formation of pathognomonic structures. One possible exception is the formation of T-B cell aggregates, which often form germinal-center-like structures and are considered to be rather characteristic of RA.[11] No information is available on the mechanisms underlying the topographical arrangement of the inflammatory infiltrate in the rheumatoid synovium. The variability of the pathological changes suggest that multiple mechanisms regulate the synovial inflammation and that the contribution of T cells and macrophages may be different in individual patients. These distinct mechanisms may correlate with different disease manifestations as well as outcomes.

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In this study, we explored the hypothesis that the pattern of cytokines produced in the rheumatoid synovium is predictive of the morphological appearance of the disease. The ultimate goal of this project was to use the accumulated knowledge on the functional profile of individual cytokines to attempt to understand how inflammatory cells in the rheumatoid synovium communicate with each other, why they are arranged in defined patterns, how they interact with resident cells, and through what mechanisms they may damage tissues. Here we report that rheumatoid synovitis is a heterogeneous entity with three distinct histologically defined phenotypes. The phenotypic heterogeneity is correlated to a specific combination of T-cell- and macrophage-derived cytokines, raising the possibility that several pathomechanisms may cause an RA-like syndrome.

Materials and Methods

Study Population

Fresh synovial tissue was obtained from 21 consecutive patients with active RA who fulfilled the American College of Rheumatology 1987 revised criteria for RA[10] and who underwent joint surgery.

Histopathological Evaluations

Hematoxylin and eosin sections of the tissue samples were analyzed for the organizational structure of the inflammatory infiltrate with particular attention to the topographical arrangement of T cells, B cells, and macrophages as well as the degree of angiogenesis and the relationship of the mononuclear infiltrate to the subsynovial lining. All tissue samples were reviewed by one pathologist (J. Björnsson) who was unaware of any clinical, serological, or immunohistological findings. To control for patchiness of the inflammation and intra-specimen variation, multiple independent specimens were included from 10 patients with the pathologist blinded to the identity of the specimens. There was concordance for the identification of follicles and granulomatous lesions for all of the independently graded specimens.

Immunohistochemistry

Frozen tissues embedded in OCT (Miles, Elkhart, IN) were cut into 5-μm sections, mounted on gel-coated slides (Superfrost/Plus, Fisher Scientific, Pittsburgh, PA), and dried in a 37°C desiccator. Slides were stored at −70°C. Before staining, slides were fixed in acetone for 10 minutes, air dried, and fixed in 1% paraformaldehyde/EDTA, pH 7.2, for 3 minutes. Endogenous peroxidase was blocked with 0.3% H_2O_2 in 0.1% sodium azide. Nonspecific binding was blocked with 5% rabbit serum (Life Technologies, Grand Island, NY) for 15 minutes. Sections were stained with monoclonal mouse anti-interferon (IFN)-γ Ab, 1:100 (Genzyme Diagnostics, Cambridge, MA) for 60 minutes or monoclonal mouse anti-CD20 Ab, 1:40 (Dako, Carpinteria, CA) for 30 minutes at room temperature. After incubation with biotinylated rabbit anti-mouse antibody, 1:300 (Dako), the slides were developed with streptavidin-peroxidase, 1:250 (Dako) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO).

Slides stained with anti-IFN-γ were washed in 0.5% Triton X-100 in PBS for 10 minutes. Nonspecific binding was blocked for 15 minutes with 5% normal goat serum (Life Technologies). Sections were stained with a monoclonal mouse anti-CD45RO Ab, 1:60 (Dako), for 30 minutes at room temperature. After incubation with a biotinylated rabbit anti-mouse antibody, 1:300 (Dako), the slides were developed with a Vectastain ABC-AP kit and alkaline phosphatase substrate kit 1 (Vector Laboratories, Burlingame, CA). Negative controls without primary Ab were processed in parallel. Sections were counterstained with hematoxylin and permanently mounted in Cytoseal (Stephens Scientific, Riverdale, NJ).

Cytokine Measurement

Total RNA was extracted from synovial tissue by using a commercially available kit (Trizol, Life Technologies). cDNA from synovial tissue specimens was adjusted to contain equal numbers of β-actin transcripts. Adjusted cDNA was amplified under nonsaturating conditions with cytokine-specific primers (Table 1) by polymerase chain reaction (PCR) in parallel with a standard with a known number of cytokine sequences as described.[12] Primers were chosen to specifically amplify cDNA and not DNA. To achieve this goal, several primers had been designed to span an intron.[13] Amplified products were labeled with digoxygenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) and then semiquantified in a liquid hybridization assay with biotinylated internal probes (Table 1) using a commercially available PCR ELISA kit (Boehringer Mannheim). In this assay, the labeled PCR products were hybridized with 200 ng/ml probe at 42°C for β-actin, interleukin (IL)-4, IFN-γ, transforming growth factor (TGF)-β1, and tumor necrosis factor (TNF)-α and at 55°C for IL-1β and IL-10 for 2 hours. Hybrids were immobilized on streptavidin-coated microtiter plates and, after washing, were detected with a peroxidase-labeled anti-digoxigenin antibody. Plates were developed by a color reaction using ABTS (2,2′-azino-di[3-ethylbenzthiazoline sulfonate (6)] diammonium salt) substrate and quantitated using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The number of cytokine-specific sequences was determined by interpolation with a standard curve and was expressed as the number of cytokine sequences per 2×10^6 β-actin sequences.

Statistical Analysis

The clinical presentations of the histopathologically defined patient subsets were compared by using a Fisher's exact test. *In situ* cytokine production was compared using a nonparametric Kruskal-Wallis test.

Table 1. Nucleotide Sequences of PCR Primers and Biotinylated Probes

		Oligonucleotide sequence
β-Actin	5′ Primer	ATG GCC ACG GCT GCT TCC AGC
	3′ Primer	CAT GGT GGT GCC GCC AGA CAG
	Probe	TAC AGG TCT TTG CGG ATG TC
IL-1β	5′ Primer	GAC ACA TGG GAT AAC GAG GC
	3′ Primer	GGG ATC TAC ACT CTC CAG CTG
	Probe	AGC TTT TTT GCT GTG AGT CCC GGA G
IL-4	5′ Primer	CTT CCC CCT CTG TTC TTC CT
	3′ Primer	TTC CTG TCG AGC CGT TTC AG
	Probe	AGA GCA GAA GAC TCT GTG CAC CGA G
IL-10	5′ Primer	CAG TTT TAC CTG GAG GAG
	3′ Primer	CAA TAA GGT TTC TCA AGG GGC TGG GTC
	Probe	CTA CGG CGC TGT CAT CGA TTT CTT
IFN-γ	5′ Primer	ACC TTA AGA AAT ATT TTA ATG C
	3′ Primer	ACC GAA TAA TTA GTC AGC TT
	Probe	ATT TGG CTC TGC ATT ATT TTT CTG T
TGF-β1	5′ Primer	AAG TGG ACA TCA ACG GGT TCA CTA
	3′ Primer	GCT GCA CTT GCA GGA GCG CAC
	Probe	ATC TGC AAA GCT CCC GGC AC
TNF-α	5′ Primer	TAG CCC ATG TTG TAG CAA ACC C
	3′ Primer	TCG GCA AAG TCG AGA TAG TC
	Probe	AAT GGC GTG GAG CTG AGA GAT AAC

Results

Three Distinct Histopathological Patterns in Rheumatoid Synovitis

Microscopic evaluation of rheumatoid synovium demonstrates fibrin exudation, hyperplasia of synovial lining cells, often assuming a villous configuration, stromal fibrosis, capillary angiogenesis, and diffuse stromal inflammation. Tissue-infiltrating cells include T cells, macrophages, and B cells. Giant cells are variably present. To identify pathological patterns of the synovial inflammation, a series of 21 tissues from patients undergoing joint surgery was analyzed. To exclude features due to long-standing and burned out disease, only patients with clinically active synovitis were enrolled.

Upon analysis by conventional histology, three patterns emerged (Figure 1). Ten tissue samples were categorized as diffuse synovitis (Table 2). These tissues were characterized by a diffuse infiltrate of lymphocytes and macrophages without any additional microanatomical arrangements. The infiltrate tended to be sparse and was accompanied by moderate edema and delicate and diffuse fibrosis. A second pattern manifested as demarcated lymphocytic aggregates with sparing of the intervening stroma. In some patients, indistinct germinal center formation with central clearing of the aggregates was apparent. Immunohistochemical analysis showed that follicular structures displayed a central accumulation of B cells surrounded by T cells. Such pseudo-follicular organizations were detected in seven tissues and were classified as follicular synovitis. Four patients displayed necrobiotic granulomas. In this pattern, a fibrinoid necrotic center was lined by a collar of epithelioid histocytes, with or without giant cells. External to this palisade, or garland, of histiocytes was a zone of granulation tissue with angiogenesis and a mixed inflammatory infiltrate composed of lymphocytes, histiocytes (macrophages), and plasma cells. No specimens displayed both follicular synovitis and granulomatous necrobiosis. We further evaluated each specimen with respect to fibrinous exudates, capillary neovascularization, and linear subsynovial inflammatory arrays. These features were variable and were not correlated with each other or any of the three main inflammatory patterns (data not shown).

T-Cell-Derived Cytokines in Distinct Types of Rheumatoid Synovitis

T cells are a consistent component of tissue infiltrates. Previous studies have emphasized that the production of T cell mediators such as IL-2 and IFN-γ is low in rheumatoid synovium.[6] However, it has become increasingly clear that the fraction of activated T cells producing inflammatory mediators in autoimmune disorders often represents a minority. We used a semiquantitative PCR/liquid hybridization assay to detect low concentrations of cytokines in tissue extracts. To directly address the question of whether a correlation exists between the tissue organization of inflammatory cells and lymphokine production, the three histomorphologically defined types of synovitis were compared for *in situ* transcription of the IFN-γ and IL-4 genes. To correct for variations in the amount of tissue used for RNA extraction, all cDNAs were adjusted to a concentration equivalent to 2×10^6 copies of the β-actin gene product. Results are summarized in Figure 2. IFN-γ mRNA production was a characteristic finding for granulomatous synovitis. Tissues with follicular synovitis contained variable concentrations of IFN-γ mRNA copies (median of 114 copies) whereas the lowest levels were found in extracts derived from material with diffuse synovitis (median of 55 copies). As demonstrated by two-color immunohistochemistry, the major IFN-γ-producing cells in all three forms of synovitis were T cells. The high rate of IFN-γ transcription distinguished patients with granuloma formation from the diffuse subtype ($P = 0.007$).

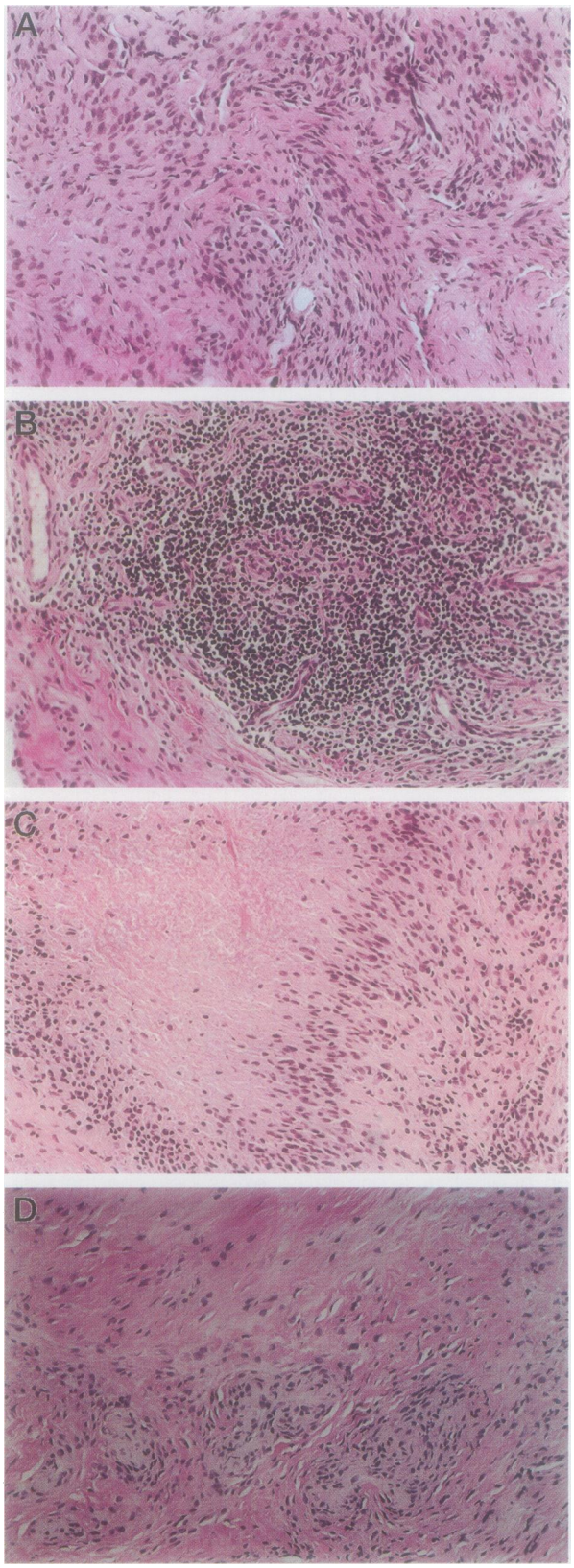

Figure 1. Histopathological patterns of rheumatoid synovitis. All tissue samples were evaluated for characteristic features of inflammatory infiltrate. Representative examples are shown for the three patterns that emerged. Original magnification, × 200. **A:** Diffuse inflammatory infiltrate without organizational structure; **B:** follicular aggregates of T and B cells; **C:** granulomatous synovitis; and **D:** angiogenesis adjacent to granuloma.

Table 2. Histomorphological Characteristics of Rheumatoid Synovium

Patient	Diffuse infiltrate	Follicle formation	Granuloma formation
RA 1	+	−	−
RA 2	+	−	−
RA 3	+	−	−
RA 4	+	−	−
RA 12	+	−	−
RA 13	+	−	−
RA 14	+	−	−
RA 15	+	−	−
RA 18	+	−	−
RA 21	+	−	−
RA 7	+	+	−
RA 8	+	+	−
RA 10	+	+	−
RA 16	+	+	−
RA 17	+	+	−
RA 19	+	+	−
RA 20	+	+	−
RA 5	+	−	+
RA 6	+	−	+
RA 9	+	−	+
RA 11	+	−	+

A different pattern of tissue distribution was seen for IL-4 mRNA. IFN-γ and IL-4 are both derived from T cells, but they are usually secreted by distinct subsets of T helper cells. T cells with a commitment to the TH1 pathway release IFN-γ and IL-2. Conversely, IL-4 is the typical product of a TH2 cell. IFN-γ and IL-4 can be considered as antagonistic effectors with IFN-γ driving inflammatory responses and IL-4 acting as an anti-inflammatory mediator. The highest concentration of IL-4 mRNA was determined in samples with granulomatous synovitis (median of 116 copies). IL-4 transcripts were essentially absent in biopsies from patients with follicular synovitis and were detectable only at low levels in diffuse synovitis.

Taken together, the studies on T-cell-derived cytokines indicated that diffuse synovitis correlated with low concentrations of IFN-γ and IL-4 mRNA. This pattern might indicate a low degree of activation of predominantly noncommitted TH0 cells. Follicular synovitis followed the paradigm for germinal centers in that IFN-γ was the dominant T cell product and IL-4 was virtually absent. This type of RA would be best described as a TH1-mediated response. The constellation of high levels of IFN-γ combined with high transcription of IL-4 mRNA found in granulomatous synovitis did not fit the current paradigm. The presence of IL-4 mRNA was surprising and raised the question of how these two antagonistic cytokines can co-exist in the lesions.

Differences in Macrophage Activation Distinguish the Three Variants of Rheumatoid Synovitis

To address the question of whether the different organizational forms of rheumatoid synovitis are associated with differences in the activation of synovial macrophages, the number of IL-1β and TNF-α transcripts was determined in

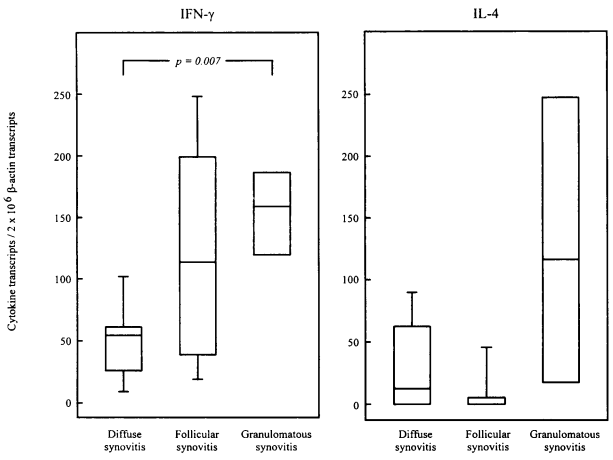

Figure 2. *In situ* transcription of T-cell-derived cytokines in rheumatoid synovitis. In all tissue samples, the number of IFN-γ and IL-4 transcripts were semiquantified by PCR and liquid oligonucleotide hybridization in a PCR ELISA. Tissues were stratified according to their histopathological appearance. Results of the cytokine measurements are shown as box plots with medians, 25th and 75th percentiles, and whiskers representing the 10th and 90th percentiles. Diffuse synovitis was characterized by low production of IFN-γ and IL-4. In the follicular synovitis, the production of IFN-γ dominated, whereas in the granulomatous synovitis, increased copy numbers of both IFN-γ and IL-4 were found.

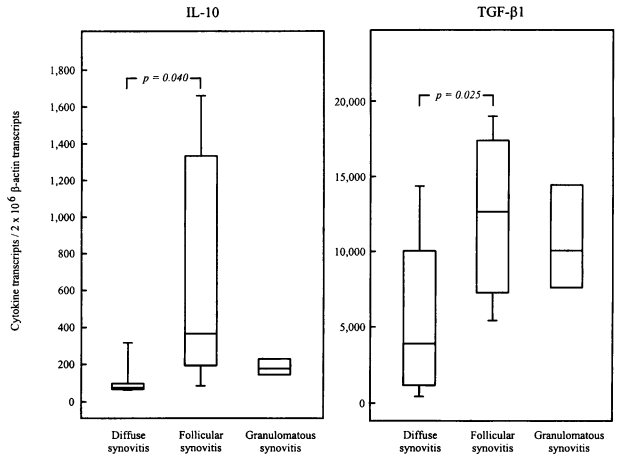

Figure 4. *In situ* transcription of IL-10 and TGF-β1. The three different histologically defined types of synovitis were analyzed for the *in situ* production of IL-10 and TGF-β1 mRNA by semiquantitative PCR as described in Figure 2. IL-10 and TGF-β1 transcription was low in diffuse synovitis, suggesting that the immunosuppressive function of these two cytokines did not contribute to the subdued immune responses in these tissues. IL-10 production was high in follicular synovitis, suggesting a role of this cytokine in follicle formation.

the tissue extracts. IL-1β and TNF-α-specific sequences were detected in all samples. As shown in Figure 3, concentrations of *in situ* transcribed IL-1β varied extensively, ranging from 103 to 17,400 copies per 2 × 10^6 β-actin sequences. Low copy numbers were frequently found in specimens with diffuse synovitis (median of 270 copies), and intermediate levels of IL-1β transcription were typical for follicular synovitis (median of 2038 copies). This difference was statistically significant (P = 0.019). The highest rate of IL-1β mRNA synthesis, assigned to the tissue sections with granuloma formation, was a median number of 10,044 copies and could be clearly distinguished from the low-level transcription in tissues with diffuse infiltrates (P = 0.005). The results for TNF-α paralleled the findings for IL-1β (r^2 = 0.860; P < 0.001; Figure 3). A low copy number (median of 660 copies) was found in diffusely infiltrated tissue compared with an intermediate level of TNF-α transcription in follicular tissue (median of 1799 copies; P = 0.04) and high concentrations in granulomatous synovitis (median of 9568 copies; P = 0.005).

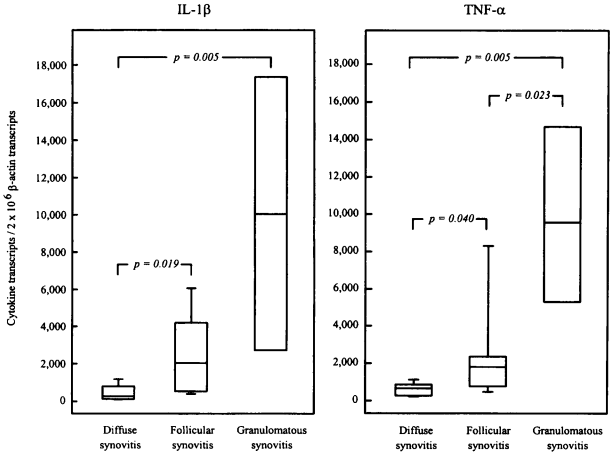

Figure 3. *In situ* transcription of macrophage-derived cytokines in rheumatoid synovitis. Tissue samples were stratified and *in situ* transcription of IL-1β and TNF-α was determined as described in Figure 2. Again, the results are shown as box plots. Only low levels of IL-1β and TNF-α were found in the tissues with diffuse synovitis. Follicular synovitis was characterized by intermediate production of both monokines whereas the highest tissue concentrations were found in granulomatous synovitis.

Low, intermediate, and high IL-1β and TNF-α production correlated with IFN-γ transcription in all three disease variants (r^2 = 0.674 and P < 0.001 for IL-1β and r^2 = 0.607 and P < 0.001 for TNF-α compared with IFN-γ). This relationship is compatible with a regulatory role of the T cell product, IFN-γ, in macrophage activation. In this model, rheumatoid granulomas could be regarded as sites of marked T cell and macrophage stimulation. The histiocyte formation characteristic of the granulomas would be consistent with this hypothesis.

Anti-Inflammatory Cytokines in Distinct Variants of Rheumatoid Synovitis

It is now well established that cytokines function in networks with positive and negative feedback loops acting through pro- and anti-inflammatory mediators. IFN-γ, IL-1β, and TNF-α have been classified as pro-inflammatory cytokines. IL-10 and TGF-β1 have been demonstrated to suppress rather than stimulate the activation of TH1 cells and macrophages. To test the hypothesis that the low level of IFN-γ and IL-1β transcription in diffuse synovitis resulted from the action of anti-inflammatory cytokines, the concentrations of IL-10 and TGF-β1 mRNA in the tissues were semiquantified.

IL-10 transcripts were consistently found (Figure 4). The lowest tissue concentrations of IL-10 mRNA correlated with the presence of diffuse synovitis. In these patients, the median copy number of cytokine per 2 ×

Table 3. Characteristics of Patient Populations Defined by Histopathological Patterns

	Sex (female/male)	Median age in years (range)	Median disease duration in years (range)	Rheumatoid factor positive*	Rheumatoid nodules†
Diffuse synovitis	5/5	57 (52–73)	16.5 (1–51)	50%	10%
Follicular synovitis	6/1	57 (34–67)	10 (4–50)	100%	0%
Granulomatous synovitis	3/1	61.5 (24–68)	8 (5–32)	75%	100%

*$P = 0.04$ for diffuse *versus* follicular synovitis.
†$P = 0.005$ for diffuse *versus* granulomatous and $P = 0.003$ for follicular *versus* granulomatous synovitis.
All other comparisons were nonsignificant.

10^6 β-actin sequences was 76. In contrast, abundance of IL-10 transcripts was a characteristic feature of follicular synovitis. Patients with follicular aggregates synthesized a median of 331 copies of IL-10-specific sequences ($P = 0.04$). Despite the marked stimulation of macrophages in the granulomatous synovitis, IL-10 was present at only low levels (median of 181 copies). Although macrophages are the main producer of IL-10 in the inflamed synovium, macrophage stimulation as indicated by IL-1β production can thus be differentiated from IL-10 production.

TGF-β1 is understood as a powerful suppressive cytokine. To explore whether the subdued production of T cell and macrophage products in the diffuse synovitis was correlated with a dominance of TGF-β1 mRNA, this cytokine was analyzed in all three variants of rheumatoid synovitis. TGF-β1 sequences could be easily detected in all but one patient. Transcript concentrations ranged from 0 to 19,020 copies (Figure 4). Low levels of TGF-β1 transcription was a common denominator among patients with diffuse synovitis. They synthesized a median of 3901 copies, a finding that distinguished them from patients with follicular disease (median of 12,636 copies; $P = 0.025$).

Therefore, neither IL-10 nor TGF-β1 could account for the aborted cytokine response in diffuse synovitis. IL-10, so far recognized as a suppressive cytokine, was transcribed in tissue samples with follicular T-B aggregates and was distinctly low in granulomatous synovitis whereas TGF-β1 mRNA was found in all three forms of synovitis.

Host Factors Correlate with the Organization and Function of Inflammatory Cells in the Rheumatoid Synovium

Results on tissue cytokine pattern suggested that more than one type of rheumatoid synovitis exists with distinct pathways of inflammation. Phenotypic heterogeneity of RA could be attributed to differences in host factors. To address this issue, we compared the three patient subsets for demographic features and for similarities in clinical presentation. Also, it could be argued that differences in the histopathological appearance and functional profiles of accumulated cells could be influenced by therapeutic intervention. Treatment given in the last few weeks before harvesting of the tissue could be particularly important in affecting the disease process in the synovial membrane.

The demographic characteristics of the patient cohorts stratified according to the histomorphology of the synovitis are presented in Table 3. Sex, age, and disease duration did not predict which variant of synovitis the patient had developed. All patients had long-standing disease, but no differences were seen that correlated with a specific organizational and functional pattern in the tissue. Patients lacking rheumatoid factor production accumulated among the cohort of individuals found to have diffuse synovitis. The group of patients with diffuse synovitis included 5 of 10 patients who were seronegative, whereas all patients who had developed follicular synovitis secreted rheumatoid factor ($P = 0.04$). The most intriguing finding was that the generation of rheumatoid nodules was characteristic for individuals with synovial granuloma formation. All patients with granulomatous synovitis, but none of the patients with follicular synovitis and only 1 of 10 patients with diffuse synovitis, had rheumatoid nodules of the skin. The accumulation of patients with nodular disease in the category of granulomatous synovitis was statistically significant ($P = 0.005$ *versus* diffuse synovitis; $P = 0.003$ *versus* follicular synovitis). Pertinent treatment information is summarized in Table 4. All individuals with granulomatous synovitis had been treated with disease-modifying agents (DMARDs) such as hydroxychloroquine, steroids, gold, sulphasalazine, azothioprine, and methotrexate. None of these patients

Table 4. Past and Current Treatment in the Different Patient Categories

	Diffuse synovitis (A)	Follicular synovitis (B)	Granulomatous synovitis (C)	*p*		
				A *vs* B	A *vs* C	B *vs* C
NSAID only (last 3 months before surgery)	4/10	1/7	0/4	NS	NS	NS
DMARD (last 3 months before surgery)	6/10	6/7	4/4	NS	NS	NS
Methotrexate (last 3 months before surgery)	3/10	2/7	4/4	NS	0.07	0.06
DMARD (total)	7/10	6/7	4/4	NS	NS	NS

NSAID, nonsteroidal anti-inflammatory drugs; DMARD, disease-modifying anti-rheumatic drugs; NS, not significant.

had been managed with nonsteroidal anti-inflammatory drugs (NSAIDs) alone. The group of patients with diffuse synovitis appeared to have been treated less aggressively. One-third of these patients was managed with NSAIDs only. All patients with granuloma formation in the synovia, but only 30 and 29%, respectively, of patients in the diffuse and follicular categories, were on methotrexate. Despite the low number of individuals analyzed, these differences showed a trend toward significance ($P = 0.07$ and $P = 0.06$, respectively). This analysis suggested that host factors and/or treatment may contribute to the microanatomy in the joint, or *vice versa*, that a correlation exists between systemic manifestations and certain types of rheumatoid synovitis.

Discussion

RA is a chronic inflammatory disease with profound phenotypic variability.[1] The pattern of involvement, the course and destructive potential of the disease, and the frequency of extra-articular manifestations vary significantly. Reasons for the phenotypic heterogeneity are not completely understood but may include variable combinations of disease risk genes.[14,15] Here we report that heterogeneity of the disease process includes the synovial lesion and that patients display considerable differences in the organization and the functional commitment of the inflammatory infiltrates. The microanatomy of the inflamed synovium showed a correlation with profiles of tissue cytokines, supporting the model that different mechanisms are functional in regulating rheumatoid synovitis. Tissue destruction and possibly other aspects of the disease process are related to cell-cell interactions in the infiltrates. Thus, understanding the rules underlying the emergence of a defined microanatomical structure in the synovial membrane promises to shed light on fundamentally important pathological events leading up to RA.

The current study suggests that the topographical arrangement of the mononuclear infiltrate can be used to define three variants of rheumatoid synovitis. These three patterns correlated with the combination and the amount of cytokines produced in the tissue. In general, the level of transcription of T-cell-derived cytokines is low in synovial tissue, and the study therefore had to employ a semiquantitative PCR approach. This technique can have limitations that need to be considered when interpreting the data. As it is a semiquantitative method, differences have to be large to be distinguished. Also, the level of cytokine mRNA may not necessarily reflect the amount of functionally active protein, particularly in the case of TGF-β1 and IL-1β. Cytokine production was confirmed by immunohistochemistry, which, however, is not a suitable technique to determine quantitative differences. The finding that 21 samples were sufficient to accomplish a dissection of the three variants emphasizes that the differences in cytokine production were pronounced.

The first variant of RA emerging from the study is a disease phenotype characterized by diffuse infiltrates in the synovia, a lower probability of rheumatoid factor production, and a clinically milder disease that is responsive to nonaggressive treatment. The low transcription of pro-inflammatory mediators of a TH0 pattern suggested that the T cell response was not highly differentiated. Consistent with the interpretation is the absence of a microanatomical organization of the mononuclear infiltrate. The reason for this subdued activation of inflammatory cells is unclear. Production of the anti-inflammatory cytokines IL-10 and TGF-β were generally low, and no evidence was found for an activate suppressive mechanism.

The second variant of RA distinguished in this study represents the histomorphological pattern that is typically associated with RA, the formation of follicular structures composed of T and B cells. Recent molecular characterization of B cells isolated from RA tissues with follicular centers have confirmed that these T-B cell aggregates functionally resemble germinal centers.[16,17] Germinal centers are the site of B cell differentiation, somatic mutation, and affinity maturation, all of which are T-cell-dependent processes.[18–20] Berek et al[16] were able to provide evidence for somatic mutation in follicles isolated from synovial tissue.

The tissue cytokine profile that emerged for this category included intermediate levels of IFN-γ with essentially absent IL-4 transcription. This pattern would suggest a TH1 deviation of the immune response.[21] The predominance of a TH1 pattern in patients with follicular synovitis is in line with current knowledge on cytokine production in germinal centers.[20] Although IL-4-deficient mice generated by gene targeting are not able to form lymphoid follicles,[22] T cells accumulating in germinal centers typically do not produce IL-4 but IFN-γ. Also, the production of IL-10 found in the tissues with follicular aggregates may relate to germinal center formation.[23] IL-10 can be produced by a variety of cells, including TH1- and TH2-type T cells, macrophages, and B cells.[24] It acts by inhibiting the production of numerous pro-inflammatory monokines and by attenuating TH1-mediated immune responses. In contrast to this immunosuppressive effect, IL-10 has marked stimulatory effects on B cells and supports B cell proliferation and differentiation. In particular, the production of rheumatoid factor appears to be IL-10 dependent.[25] Our data would suggest that the production of IL-10 in follicular synovitis relates to providing a microenvironment for B cell proliferation and not for suppressing macrophage activation.

The third variant of RA identified in this study was the least frequent and was characterized by the most abundant production of T-cell- and macrophage-derived cytokines. From a clinical perspective, granulomatous synovitis occurred in patients with the most serious presentation of the disease, extra-articular RA. The morphological structures encountered in the synovia resembled rheumatoid nodules, which are usually found in the skin. The presence of granuloma formation in the synovium was not surprising. It was, however, unexpected that granulomatous and follicular synovitis did not co-occur. On the other hand, follicular structures are distinctly absent from rheumatoid nodules. Although the number of patients analyzed may be too small to address whether these two disease expressions are mutually exclusive in the synovia, the cytokine profiles correlating

with the two histomorphologies were distinct. Granulomatous disease was characterized by high production of IFN-γ, IL-4, IL-1β, and TNF-α whereas the follicular disease resembled a classical TH1 response with the virtual absence of IL-4.

The co-production of IFN-γ and IL-4 is unusual for a granulomatous reaction. Participation of cytokines in granuloma formation has been studied under various experimental conditions. In general, hypersensitivity granuloma (in contrast to the nonimmune foreign-body-type lesions) represents a chronic inflammatory infiltrate of macrophages, in particular, epithelioid macrophages, multinucleated giant cells, and T cells.[26] The two most widely used experimental systems are mycobacteria- and schistosoma-egg-induced granuloma formation. In these two systems, granuloma formation has been associated with quite distinct patterns of tissue cytokines. The mycobacteria-induced lesion is a characteristic TH1 response, with predominantly IFN-γ at the site of inflammation.[27] In addition to IFN-γ, TNF-α has been found to be critical in the granuloma formation during bacille Calmette Guerin (BCG) infection.[28] Because IFN-γ is known to augment TNF production, it is likely to be a critical mediator in granuloma formation. A similar cytokine pattern is seen in the chronic inflammation of giant cell arteritis, which is also characterized by granuloma formation.[29] In contrast, the schistosoma-egg-induced granuloma is characterized by a TH2-type response with the prominent production of IL-4, IL-5, and IL-10.[30,31] How TH2-derived cytokines, in particular IL-4, participate in this type of granuloma formation is not entirely clear. In particular, it is not understood what the macrophage-activating agent in this type of lesion is. Nevertheless, TH1- as well as TH2-associated cytokines can apparently participate in granuloma formation.

The granuloma formation in the rheumatoid synovium was different from these experimental models in that IFN-γ and IL-4 were co-produced. Whether and how these two cytokines synergize in granuloma formation needs to be explored. Previous results have emphasized their antagonistic effects.[32–36] Neutralization of IFN-γ production enhances granuloma formation in the schistosoma model although it abrogates the inflammation in the microbacterial model.[37] However, it is possible that these findings are restricted to the early stages of granuloma formation and may not apply to chronic inflammation, as is the case in the rheumatoid synovium. Indeed, a temporal participation of TH1 as well TH2 cells has been described for the mycobacterial infection as well as for the schistosoma-egg-induced granuloma.[38–40]

Data presented here provide insights into the contribution of different cytokines in controlling the microanatomy of the synovial inflammation and also emphasize the necessity to re-evaluate the role of immune pathways in the pathogenesis of RA. Immune deviation is now widely accepted as a concept in explaining the pathogenesis of autoimmune diseases. This model implies that chronic inflammation is a consequence of the aberrant commitment to an immune pathway in response to a given antigen.[41] Studies in diseases such as infection with leishmania major and mycobacterium leprae have fueled the hypothesis that immune reactions to a given antigen can take very different paths, and accordingly, a certain type of disease may develop.[42,43] Tuberculoid leprosy and lepratomatous leprosy have been distinguished as two disease phenotypes, the distinction being mainly attributed to the involvement of different cytokine networks. Following this model, RA has been considered a consequence of an immune deviation toward a TH1 response, and the use of IL-4 and IL-10 have been proposed as therapeutic interventions.[44–47] Our finding that RA encompasses different cytokine patterns suggests that the choices of the host in terms of cytokine recruitment do not determine whether or not the individual develops the disease but can influence severity and organ involvement.

Alternatively, the three disease phenotypes may reflect distinct pathomechanisms including different disease initiators. As the initial events in RA are not understood, both models remain feasible. However, searches into the instigators of RA could benefit from the realization that there exist several variants of the disease. It might be misleading to search for common denominators in a cohort of RA patients if multiple distinct disease variants are represented. Focusing on a single entity of RA may enhance the identification of the shared pathomechanisms, genetic risk factors, and antigens driving rheumatoid synovitis. Equally important, phenotypic variants of RA should be considered in the design of treatment trials and in the application of therapeutic agents in individual patients.

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