B Lymphocyte Autoimmunity in Rheumatoid Synovitis Is Independent of Ectopic Lymphoid Neogenesis[1]

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B lymphocyte autoimmunity plays a crucial role in the pathogenesis of rheumatoid arthritis. The local production of autoantibodies and the presence of ectopic lymphoid neogenesis in the rheumatoid synovium suggest that these dedicated microenvironments resembling canonical lymphoid follicles may regulate the initiation and maturation of B cell autoimmunity. In this study, we assessed experimentally the relevance of ectopic lymphoid neogenesis for B cell autoimmunity by a detailed structural, molecular, and serological analysis of seropositive and seronegative human synovitis. We demonstrate that synovial lymphoid neogenesis is a reversible process associated with inflammation which is neither restricted to nor preferentially associated with autoantibody positive rheumatic conditions. Despite the abundant expression of key chemokines and cytokines required for full differentiation toward germinal center reactions, synovial lymphoid neogenesis in rheumatoid arthritis only occasionally progresses toward fully differentiated follicles. In agreement with that observation, we could not detect Ag-driven clonal expansion and affinity maturation of B lymphocytes. Furthermore, ectopic lymphoid neogenesis is not directly associated with local production of anti-citrullinated protein Abs and rheumatoid factor in the rheumatoid joint. Therefore, we conclude that synovial lymphoid neogenesis is not a major determinant of these rheumatoid arthritis-specific autoantibody responses. *The Journal of Immunology,* 2008, 181: 785–794.

A utoimmunity of B lymphocytes plays a central role in the pathogenesis of many autoimmune disorders, including rheumatoid arthritis (RA).[3] Despite experimental models recapitulating specific pathogenic events, it remains unclear to which extend humoral autoimmunity is a primary driver of tissue inflammation or, on the contrary, results from the local inflammatory events that may in turn help to break self-tolerance. A prototype example is the RA-specific anti-citrullinated protein Ab (ACPA) response directed toward peptide epitopes, which have been posttranslationally modified by arginine deimination (1). ACPA can contribute to the enhancement and progression of subclinical experimental arthritis (2), which fits in a model of preexisting Abs driving out of control a local, non-specific inflammation (3). In contrast, ACPA are also produced locally in the inflamed tissues containing citrullinated Ags (4–7), which suggests that local inflammation may promote the adaptive autoimmune response. However, the presence of citrullinated Ags in an inflammatory context is not sufficient to break B cell tolerance and trigger ACPA responses, even in the appropriate HLA background (8, 9). This may, at least in part, relate to the regulation of peripheral tolerance by the immunological microenvironment.

Lymphoid structures, which are the primary anatomical site of maturation of T cell dependent humoral immune responses, can also be found ectopically in a variety of inflamed tissues, such as the synovial membrane. One third of the RA tissues display follicular synovitis (10), with up to 20% containing lymphoid aggregates with germinal centers (GC) (11–13). Together with the demonstration that B cells can proliferate and accumulate somatic mutations in inflamed synovial tissue (14), these observations raised the possibility that ectopic lymphoid neogenesis promotes the maturation of humoral autoimmune responses. However, these synovial structures displayed important structural differences with secondary lymphoid organs (SLO), including the lack of clear T/B cell segregation (12), the dependency on CD8+ lymphocytes (11), and the lack of follicular dendritic cells (FDC) in lymphoid aggregates without GC (15).

Despite the detailed structural characterization in RA and other autoimmune conditions, the functional relevance of ectopic lymphoid neogenesis remains unknown. More specifically, it remains unknown whether peripheral B cell tolerance and autoantibody

[3] Abbreviations used in this paper: RA, rheumatoid arthritis; ACPA, anti-citrullinated protein antibodies; anti-CCP, anti-cyclic citrullinated peptide; APRIL, A proliferation-inducing ligand; BAFF, B cell activation factor of the tumor necrosis factor family; FDC, follicular dendritic cells; GC, germinal center; HEV, high endothelial venules; LTb, lymphotoxin β; OA, osteoarthritis; PNAd, peripheral node addressin; RF, rheumatoid factor; SpA, spondyloarthritis; SLO, secondary lymphoid organs; TACI, transmembrane activator and calcium modulator and cyclophilin ligand-interactor; SF, synovial fluid.

Table I. *Demographic and clinical features of the patient cohorts*

	RA (n = 112)	SpA (n = 74)	OA (n = 18)	Crystal-Induced Arthritis (n = 35)	SpA TNFα Blockade (n = 18)
Age (years)	56.5[a] (47.5–66)	42 (32–52)	65 (56–71)	52 (44–66)	43 (35–57.5)
Gender (male/female)	30/82	44/30	7/11	27/8	14/4
Disease duration (years)	8[b] (2–18.5)	4.25 (1–12)	5 (1–15)	2 (0.1–9)	8 (2.5–17)
Swollen joint count	7 (3–12)	2 (1–3)	1 (1–2)	2 (1–2)	3.5 (2–10.5)
C-reactive protein (g/L)	37 (16–98)	9 (2–26)	3 (2–12)	36 (9–90)	23 (13–72)
Erythrocyte sedimentation rate (mm/h)	42 (28–64)	17 (5–29)	13 (3–32)	36 (18–65)	25 (13.5–56)
Therapy					
DMARD[c]	65	28	1	3	0
Corticosteroids	45	4	0	1	0
NSAID[d]	105	71	14	26	18

[a] Median value (interquartile range).
[b] Absolute numbers.
[c] Disease-modifying antirheumatic drugs.
[d] Nonsteroidal anti-inflammatory drugs.

production in rheumatoid synovitis is regulated by these structures. We assessed this issue by addressing the following questions: is synovial lymphoid neogenesis committed to autoantigen-specific B cell responses rather than driven by inflammation? Does synovial lymphoid neogenesis structurally resemble SLO? Are humoral autoimmune responses in RA qualitatively or quantitatively modulated by synovial lymphoid neogenesis?

Materials and Methods

Patients and samples

Synovial tissue biopsies and paired synovial fluid (SF) were obtained in 250 arthritis patients by needle arthroscopy from affected knee joints (16, 17). The population consisted of 112 patients fulfilling the American College of Rheumatology criteria for RA (18) and 74 patients fulfilling the European Spondyloarthropathy Study Group criteria for spondyloarthritis (SpA) (19). SpA is the second most frequent form of chronic inflammatory arthritis but, in contrast to RA, is not associated with known autoantibodies. The RA cohort consisted of 47 patients with early RA without DMARD (median disease duration 7 mo) and 65 patients with persistent active RA despite DMARD treatment (median disease duration 56 mo). Disease activity was similar in both groups (data not shown). Additional controls included 18 patients with inflammatory osteoarthritis (OA) and 35 patients with crystal-induced arthritis. All patients had active disease characterized by inflammation of at least one knee joint and none of the patients was treated with biological therapy. To study specifically the effect of TNFα blockade, biopsies were obtained in an additional cohort of 18 SpA patients (20, 21) at baseline and after 12 wk of treatment with infliximab (5 mg/kg i.v. at week 0, 2, and 6) (n = 11) or etanercept (25 mg subcutaneously twice a week) (n = 7). All patients gave written informed consent before inclusion as approved by the local Ethics Committees. The demographic and clinical data are shown in Table I. For all analysis (histology, real-time PCR (RT-PCR), and BCR sequencing) 6–8 pooled biopsies were used to avoid biases related to patchy distribution of aggregates.

Synovial histology

Synovial lymphoid neogenesis, defined as microstructural organization of lymphoid infiltrates, has been assessed by different criteria, including the presence of lymphoid aggregates (10), the size of these aggregates (22, 23), and the presence of additional features of SLO, such as FDC and GC (15). These different criteria reflect the fact that tertiary lymphoid organs are under constant development and change (24) and do not always display the prototype characteristics of activated follicles in SLO such as a dark and light zone, T/B cell segregation, and FDC (15, 25). In this study, we defined synovial lymphoid neogenesis as the presence of organized aggregates of lymphoid cells with a diameter of ≥12 cells as smaller synovial lymphoid aggregates do not display features of lymphoid organogenesis and aggregates of this size are always composed of B and T cells (23, 26). This definition includes both the aggregate and GC-like infiltrates (defined by the presence of FDC) described by others (15) as one of the aims of the study was to assess the degree of differentiation toward GC-reactions rather than to assume that the distinction between aggregates and GC-like infil-

trates has a biological or clinical relevance. The presence or absence of lymphoid neogenesis was assessed on H&E stained sections. Two to three different levels of a tissue block consisting of ≥6 synovial biopsies were examined by two independent observers. Global synovial infiltration was assessed as previously described (17, 20).

Quantitative RT-PCR

RNA was extracted from 6 to 8 synovial tissue biopsies of 13 SpA samples without lymphoid neogenesis, 11 SpA samples with lymphoid neogenesis, and 14 RA samples with lymphoid neogenesis. cDNA was synthesized with the RevertAid H minus First strand cDNA synthesis kit (MBI Fermentas). Primers were designed using Primer Express software (PE Applied Biosystems) (Table II). Quantitative RT-PCR was performed on an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems). A standard curve was constructed for each gene to correct for different primer efficiencies. Gene expression levels were expressed relative to GAPDH (27).

Immunohistochemistry

Synovial tissue biopsies were processed as described previously (17). 6–8 biopsies of each patient were pooled and evaluated. Infiltrating lymphocytic cells were phenotyped on frozen sections by immunohistochemistry with Abs directed toward CD3 (clone UCHT1; DakoCytomation), CD20 (clone L26; DakoCytomation), and CD138 (clone CBL455; Chemicon).

Synovial lymphoid neogenesis in 16 RA samples (of which 9 ACPA and RF positive) and 19 SpA samples was further characterized on paraffin embedded sections using following markers: CD3 (clone SP7, LabVision), CD20

Table II. *Forward and reverse primers used for quantitative real-time PCR*

|	Sequence
GAPDH F	GCCCAGGATGCCCTTGA
GAPDH R	ACCTGACCTGCCGTCTAGAAAA
CXCL13 F	CTCTGCTTCTCATGCTGCTGG
CXCL13 R	TGAATTCGATCAATGAAGCGTCT
CCL19 F	TGTCTGTGACCCAGAAACCCA
CCL19 R	TGAACACTACAGCAGGCACCC
CCL21 F	CTGCTCCATCCCAGCTATCCT
CCL21 R	GGTGTCTTGTCCAGATGCTGC
LTβ F	CACTTCTCTGGTGACCTTGTTGC
LTβ R	CTCCGTTACCAGTCCTCCCTG
CD21L F	TCCTGAAATACCAGTTTGTGAAAAAG
CD21L R	GTGTAGTCTACAGTCATCCCAGAGACA
APRIL F	AAGGATGACTCCGATGTGACAGA
APRIL R	TCCTGGATTCGGACACCATATC
TAC1 F	GCGGAGTGGGAGAAGTTGAAAAC
TAC1 R	GGAGAGCTGGACTTGCTTCTGA
RAG1 F	GGCATGAGATCCCAAGACCTT
RAG1 R	CTCACTCACGTCTCCCATTCC
BAFF F	TCTGGTGACTTTGTTTCGATGTATTC
BAFF R	GTTTTGCAATGCCAGCTGAA
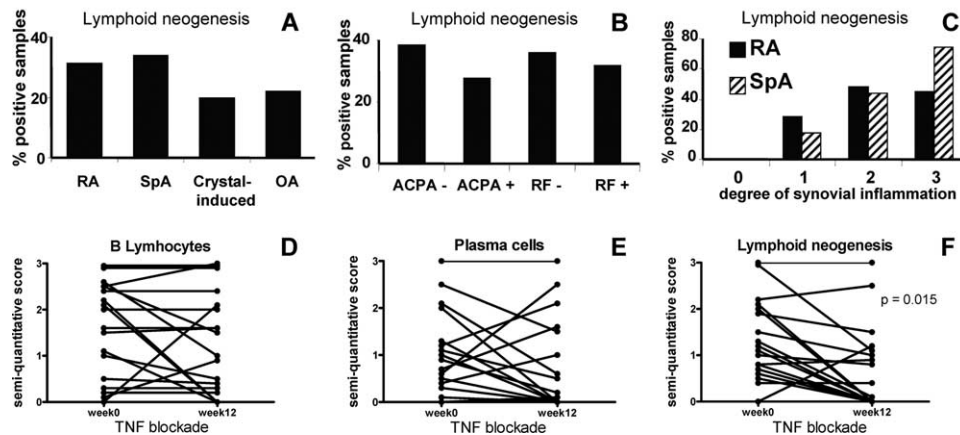
FIGURE 1. Synovial lymphoid neogenesis is related to the degree rather than the type of synovial inflammation. *A*, Percentage of synovial samples with lymphoid neogenesis in rheumatoid arthritis (RA) (*n* = 112), spondyloarthritis (SpA) (*n* = 74), crystal-induced arthritis (*n* = 35), and osteoarthritis (OA) (*n* = 18). *B*, Percentage of RA synovial samples with synovial lymphoid neogenesis in anti-citrullinated protein Ab (ACPA)-negative and -positive disease and in rheumatoid factor (RF)-negative and -positive disease. *C*, Percentage of RA and SpA with synovial lymphoid neogenesis in relation to the degree of synovial inflammation as scored by histology on a 0–3 semiquantitative scale. *D*, Before and after plot of synovial B cell infiltration in 18 SpA patients at baseline and after 12 wk of TNF blockade. *E*, Before and after plot of synovial plasma cell infiltration in 18 SpA patients at baseline and after 12 wk of TNF blockade. *F*, Before and after plot of synovial lymphoid neogenesis in 18 SpA patients at baseline and after 12 wk of TNF blockade.

(clone L26; DakoCytomation), CD23 (clone 1B12; Monosan), CD27 (clone 137B4, Novocastra), CD79α (clone JCB117; DakoCytomation), CD138 (clone MI15; DakoCytomation), IgD (clone IgD26; Dako Cytomation), IgG (clone A57H; DakoCytomation), FDC (clone CNA.42; DakoCytomation), PNAd (clone MECA-79; BD Biosciences), and Ki67 (clone MIB-1; DakoCytomation). Sections were blocked with 10% swine serum for 30 min, and incubated with primary Ab for 30 min. After blocking endogenous peroxidase with 1% H_2O_2, stainings were developed with the LSAB$^+$ kit and AEC substrate (DakoCytomation), except for the PNAd staining which was visualized with a HRP-labeled goat-anti-rat secondary Ab (Southern Biotechnology). Concentration- and isotype-mached control Abs were included as negative control and tonsil sections were included as positive control. Sections were scored on a semiquantitative four-point scale by two independent observers blinded for diagnosis and clinical data (17).

For double immunofluorscence, slides were incubated with anti-CD3 Ab (clone SP7; LabVision) and subsequently with goat anti-rabbit Alexa555 (Invitrogen), followed by anti-CD20 (clone L26; DakoCytomation) Ab and goat anti-mouse Alexa486 (Invitrogen).

Immunglobulin variable H chain gene sequencing

We performed Ig variable H chain (Ig V_H) gene analysis of 4 RA samples with synovial lymphoid neogenesis and 4 RA samples without synovial lymphoid neogenesis as described previously (28). Total RNA was extracted from RA synovial biopsies (6 biopsies per patient) using an RNAeasy kit (Qiagen). Ig V_H and VDJ genes were amplified by PCR and subsequently ligated into the pGEM T vector (Promega). *Escherichia coli* DH5a cells were transformed with the ligation mixture (29). For each sample, 8–18 clones were picked at random and grown overnight in Luria-Bertani medium. Extracted DNA from the colonies containing V_H gene inserts was analyzed by *Eco*RI digestion and sequenced using a high efficiency DNA sequencer (MCLab). A mucosal B cell lymphoma and peripheral blood cells from a healthy individual were used as positive and negative control. Using exactly the same approach on OA synovial tissue, we recently demonstrated that the number of analyzed sequences is sufficient to evaluate clonal expansion and affinity maturation (28).

Autoantibody measurement in SF and serum

Autoantibodies in serum and SF were measured in the total RA cohort (*n* = 112). ACPA were determined by the CCP-2 ELISA kit (Eurodiagnostica) (cutoff 25 U/ml in serum). Rheumatoid factor (RF) IgM was determined with the RF IgM ELISA (cutoff 12.5 U/ml in serum) (Sanquin) and RF IgG with the Quanta Lite RF IgG ELISA (cutoff 6 U/ml in serum) (Inova Diagnostics). Total IgG and IgM levels were determined by nephelometry using the latex IgG and IgM test (Dade Behring). All analyses were performed in duplo using an optimal dilution according to the standard curve.

Statistical analysis

Because the obtained data were not normally distributed, the results were expressed as median (interquartile range). The Mann-Whitney *U* test and the paired Wilcoxon test were used for comparison of unpaired and paired data. Correlations were calculated with the Spearman's ρ test. Values of $p < 0.05$ were considered to be statistically significant. For the B cell clonality analysis, the positive control showed identical B cell clones in >80% of the randomly selected sequences and the negative control in 0%. Because in this situation analysis of 7 sequences provides 85% power to detect clonal expansions (28), we analyzed 8–18 sequences for each sample to avoid false negative results.

Results

Synovial lymphoid neogenesis is not restricted to RA

As ectopic lymphoid neogenesis is observed in 30–50% of end-stage RA synovial samples obtained during joint replacement surgery (10) but also in chronic inflammatory disorders not associated with humoral autoimmunity (30), we investigated whether synovial lymphoid neogenesis was preferentially observed in RA compared with seronegative forms of arthritis, with SpA as prototype. In agreement with previous studies (10), large organized lymphoid structures were observed in 35/112 (31%) RA samples (Fig. 1*A*). There was no difference between early RA and treatment resistant RA (16/47 vs 19/65). Synovial lymphoid neogenesis was also seen in 25 of 74 (34%) tissue samples obtained in SpA, (Fig. 1*A*). Similar lymphoid structures were seen in 4 of 18 (19%) samples in OA, a chronic degenerative joint disorder with secondary synovial inflammation, and in 7 of 35 (20%) samples obtained during acute synovitis in crystal-induced arthritis (Fig. 1*A*).

Analyzing the RA cohort in relation to autoantibody status, synovial lymphoid neogenesis was equally observed in the RF-positive (32%) and -negative (36%) samples (Fig. 1*B*). Moreover, synovial lymphoid neogenesis was slightly albeit not significantly less frequent in ACPA-positive (28%) than in ACPA-negative (38%) RA patients (Fig. 1*B*), independently of potentially confounding factors such as the MHC background (HLA-DR shared epitope) and the presence of intracellular citrullinated proteins (data not shown) (31, 32). These data confirm and extend our previous observations (26, 28) showing that ectopic lymphoid neogenesis in the synovial membrane
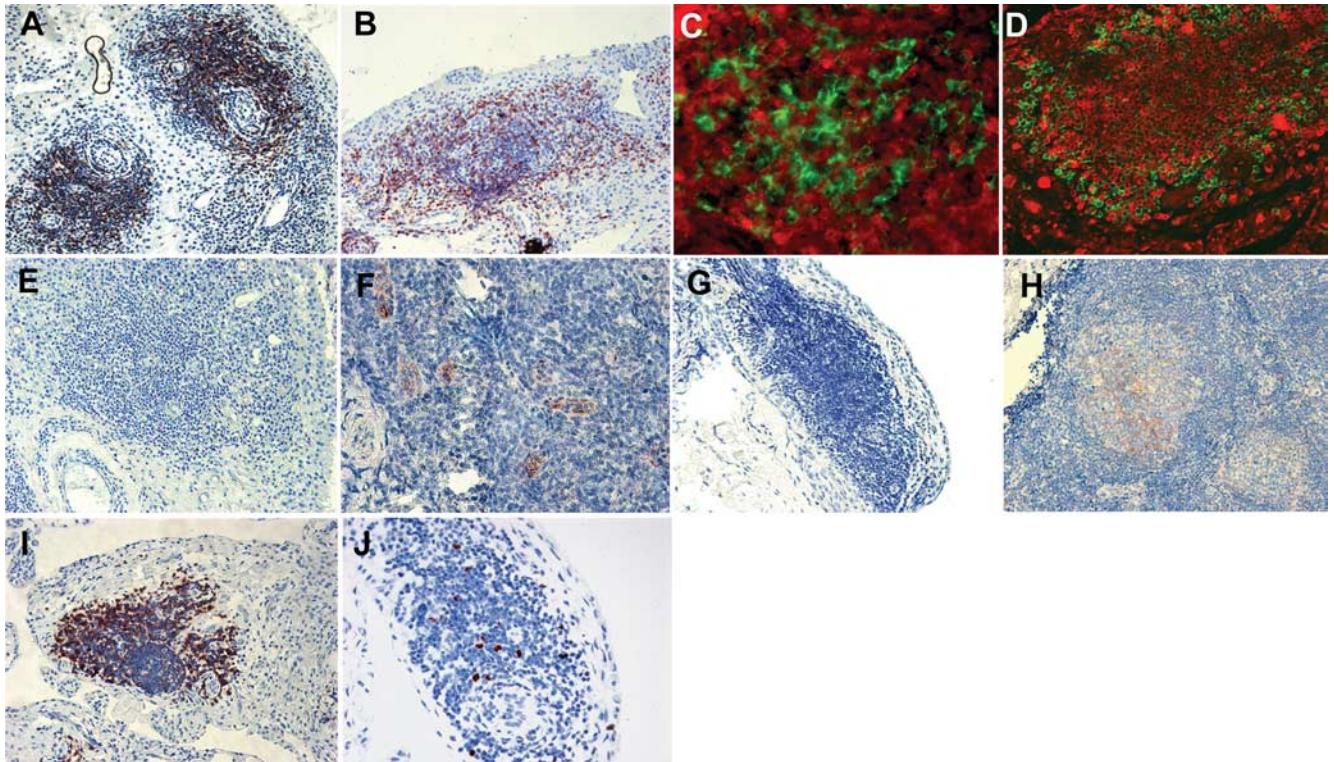
FIGURE 2. Synovial lymphoid neogenesis only occasionally progresses to germinal center reactions. 6–8 biopsies of 16 RA samples (of which 9 ACPA and RF positive) and 19 SpA samples were evaluated. *A*, CD20$^+$ B cells are almost exclusively found within lymphoid aggregates. *B*, CD3$^+$ T cells were found both inside the aggregates and dispersed throughout the synovial tissue. *C*, In almost all cases of lymphoid neogenesis, CD3 (green) and CD20 (red)-positive cells are interspersed throughout the lymphoid structures without clear T-B cell segregation. *D*, In one single case, CD3$^+$ T cells (green) show a distinct spatial segregation from CD20$^+$ B cells (red) in the lymphoid structure. *E*, PNAd$^+$ HEV were not detected in synovial lymphoid neogenesis, although sections of a tonsil used as positive control were clearly positive (*F*). *G*, Follicular dendritic cells were not detected in synovial lymphoid neogenesis, although sections of a tonsil used as positive control were clearly positive (*H*). *I*, The dark and light zone classically observed in activated follicles of SLO was seen only in one single case of synovial lymphoid neogenesis, which was the same sample depicting clear T-B cell segregation (red: CD3$^+$ cells; see also *D*). *J*, Ki67 staining, indicating proliferating cells, does not stain a dark zone of proliferating centroblasts but stains occasionally single cells both within and outside the synovial lymphoid structures.

is neither restricted to nor preferentially associated with autoantibody positive rheumatic conditions.

Synovial lymphoid neogenesis is associated with the degree of synovial inflammation

Because synovial lymphoid neogenesis occurs in different arthritic conditions, we investigated whether this phenomenon is related to the degree rather than the type of synovial inflammation. Synovial lymphoid neogenesis was associated with the degree of synovial inflammatory infiltration in RA (Fig. 1*C*). The presence of CD20$^+$ B lymphocytes ($p = 0.050$) and CD138$^+$ plasma cells ($p = 0.011$) was higher in RA synovium with lymphoid neogenesis than in diffuse synovitis, with a similar trend for CD3$^+$ T lymphocytes. The association between the degree of synovial inflammatory infiltration and lymphoid neogenesis is not disease-specific because it was even more pronounced in SpA (Fig. 1*C*). The number of CD3$^+$ T lymphocytes ($p < 0.001$), CD20$^+$ B lymphocytes ($p < 0.001$), and CD138$^+$ plasma cells ($p < 0.001$) was significantly higher in SpA tissues with lymphoid neogenesis than in diffuse synovitis. Similar results were obtained in OA and crystal-induced arthritis (data not shown).

To confirm the associaton between inflammation and lymphoid neogenesis, we assessed the effect of down modulation of synovial inflammation by TNFα blockade on lymphoid neogenesis in 18 SpA patients. Synovial inflammation was strongly reduced after 12 wk of treatment ($p = 0.004$) (20, 21). Despite the persistent pres-

ence of infiltrating B lymphocytes ($p = 0.194$) and plasma cells ($p = 0.376$), down modulation of synovial inflammation is paralleled by the disorganization of large lymphoid aggregates ($p = 0.015$): synovial lymphoid neogenesis decreased in 12 of 18 samples and even completely disappeared in 9 samples (Fig. 1, *D–F*). This was observed in both the infliximab and etanercept treated patients. Moreover this phenomenon was related neither to the type of arthritis nor to the specific targeting of TNFα because synovial lymphoid neogenesis also decreased in 4 of 6 RA patients treated for 2 wk with high dose prednisolon and remained stable in the 2 others (data not shown). These data indicate that synovial lymphoid neogenesis is a reversible process associated with the degree rather than the type of joint inflammation.

Synovial lymphoid neogenesis only occasionally progresses to GC reactions

As lymphoid neogenesis is a dynamic process with progressive maturation toward T/B cell segregation, development of high endothelial venules (HEV) and FDC networks, and GC-reactions, we investigated the lymphoid architecture by detailed immunohistochemical stainings to assess whether there were qualitative differences between RA and SpA synovitis. The cellular composition of the lymphoid microstructures was similar in RA and SpA with CD20$^+$ B lymphocytes being almost exclusively found inside these aggregates (Fig. 2*A*), indicating that these cells have a tendency to aggregate when they infiltrate the synovial compartment.
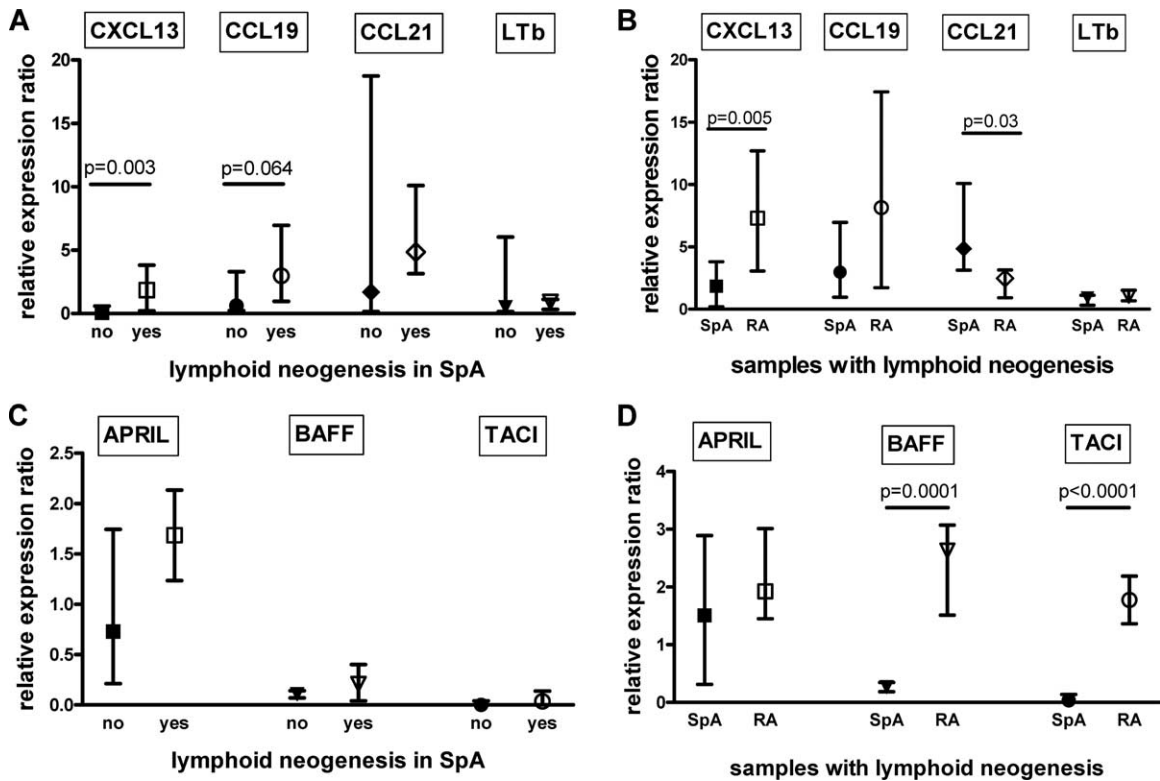
FIGURE 3. Differential expression of lymphoid chemokines in synovial lymphoid neogenesis in spondyloarthritis (SpA) and rheumatoid arthritis (RA). mRNA levels of chemokines, cytokines, and growth factors in synovial tissue were assessed by quantitative real-time PCR and expressed relative to GAPDH (median-interquartile range). *A*, Comparison of the expression levels of CXCL13, CCL19, CCL21, and LTb between synovial SpA samples without ($n = 13$) and with lymphoid neogenesis ($n = 11$). *B*, Comparison of the expression levels of CXCL13, CCL19, CCL21, and LTb between SpA ($n = 11$) and RA ($n = 14$) samples with synovial lymphoid neogenesis. *C*, Comparison of the expression levels of APRIL, BAFF, and TACI between synovial SpA samples without ($n = 11$) and with lymphoid neogenesis ($n = 13$). *D*, Comparison of the expression levels of APRIL, BAFF, and TACI between SpA ($n = 11$) and RA ($n = 14$) samples with synovial lymphoid neogenesis.

In contrast, $CD3^+$ T lymphocytes were found both inside the organized aggregates and dispersed throughout the inflamed synovium (Fig. 2*B*). In the aggregates, B and T cells were scattered throughout the lymphoid structure and did not display compartmentalization (Fig. 2*C*), with exception of one single RA sample with clear T/B cell segregation (Fig. 2*D*). In sharp contrast to follicles in SLO, $PNAd^+$ HEV were found in only 2 SpA samples and none of the RA tissues (Fig. 2, *E–F*). FDC were found in 2 of 35 synovial samples with lymphoid neogenesis (1 RA and 1 SpA) (Fig. 2*G*), in contrast with the positive staining in tonsilar follicles (Fig. 2*H*). This scarcity was confirmed by quantitative RT-PCR experiments which detected FDC-specific CD21L mRNA (33) in only 2 of 17 synovial samples with lymphoid neogenesis. The prototypic dark and light zone of an activated lymphoid follicle was seen only in the single RA sample with clear T/B cell segregation (Fig. 2*I*). Accordingly, Ki67 staining showed proliferating cells in the organized lymphoid aggregate of the same RA tissue but not in the 34 other synovia with lymphoid neogenesis (Fig. 2*J*). For all these analyses, there was no difference between seropositive and seronegative RA. This detailed structural analysis indicates that synovial lymphoid structures are similar in RA and SpA and are distinct from canonical follicles in SLO (13). Even in RA, lymphoid neogenesis only occasionally progresses toward fully differentiated follicles with T/B segregation, HEV, FDC networks, and GC reactions.

Differential chemokine expression profile of synovial lymphoid neogenesis in RA and SpA

Chemokines (CXCL13, CCL19, and CCL21) and cytokines (lyphotoxin β, LTb) play a central role in the development, orga-

nization, and maintenance of lymphoid architecture (34) and are produced locally during RA synovitis (15, 23, 35). To understand why synovial lymphoid neogenesis only occasionally progresses toward differentiated GC-reactions, we assessed the synovial expression of these factors by quantitative RT-PCR. Analyzing SpA, the expression of CXCL13, a major B cell attractant which drives the formation of organized lymphoid aggregates (36), was higher in synovitis with lymphoid neogenesis than in the samples without organized lymphoid aggregates ($p = 0.003$) (Fig. 3*A*) and correlated with the number of infiltrating B lymphocytes (r = 0.46; $p = 0.031$). As to the CCR7 ligands which regulate T cell movement, a similar trend toward increased expression in synovia with lymphoid neogenesis was seen for CCL19 ($p = 0.064$) but not for CCL21 (Fig. 3*A*). In contrast, LT β, which supports the establishment of FDC networks (37, 38), was not increased in follicular SpA synovitis (Fig. 3*A*). Because the low expression of this key mediator may explain why synovial lymphoid neogenesis does not progress toward full maturation in SpA, we assessed this issue in RA by comparing synovial tissue samples with lymphoid neogenesis between SpA and RA. Although the level of synovial inflammation assessed by histology was similar in these SpA and RA samples, there was a further 6- to 25-fold increase of the transcript levels of CXCL13 ($p = 0.005$) in the latter, with a similar trend for CCL19 ($p = 0.09$) (Fig. 3*B*). In contrast, CCL21 was higher in SpA than RA ($p = 0.03$). Levels of LTb were not different. Thus, RA and SpA synovitis have distinct expression of the key chemokines and cytokines required for full maturation of lymphoid neogenesis.

Increased BAFF and TACI expression in RA synovitis

The B cell growth and survival factors BAFF (B cell activation factor of the TNF family) and APRIL (A proliferation-inducing ligand) also contribute to the establishment of FDC networks and GC-reactions in SLO as well as in RA synovitis (39, 40). One of their receptors, TACI (soluble transmembrane activator and calcium modulator and cyclophilin ligand-interactor), negatively regulates this process (41, 42). Investigating why synovial lymphoid neogenesis in RA only occasionally progresses toward differentiated follicles despite high expression of CXCL13 and CCL19, we assessed these factors by quantitative RT-PCR. Whereas in SpA synovitis BAFF and TACI mRNA levels were very low in all samples, APRIL mRNA was clearly expressed and slightly albeit not significantly higher in samples with lymphoid neogenesis compared with diffuse synovitis (Fig. 3*C*). APRIL levels were similar in RA and SpA samples with lymphoid neogenesis (Fig. 3*D*). BAFF levels, in contrast, were increased in RA synovial tissue ($p = 0.0001$). However, the expression of the inhibitory receptor TACI was also increased in RA compared with SpA synovial samples with lymphoid neogenesis ($p < 0.0001$) (Fig. 3*D*). The overexpression of TACI in RA synovium with lymphoid neogenesis may be one of the factors preventing these structures from evolving to GC-reactions.

Synovial lymphoid neogenesis does not support Ag-driven clonal expansion and affinity maturation

Because ectopic lymphoid neogenesis in inflamed synovium only partially resembles fully differentiated follicles in SLO, we questioned their function in terms of Ag-driven clonal expansion and affinity maturation of B cells. In agreement with the absence of HEV, naive CD23, or IgD-positive cells were almost absent from the inflamed synovium. B cells identified in the organized lymphoid aggregates by the expression of CD20 and CD79a, appeared to be Ag-experienced with expression of the CD27 marker, which is expressed on memory B cell, plasma cells and GC cells (Fig. 4, *A* and *B*). CD138-positive, class-switched IgG plasma cells surrounded these lymphoid structures but were also sometimes found in the diffuse infiltrate (Fig. 4, *C–F*). Together with the previously mentioned absence of Ki67 staining and dark zones, these data indicate that these organized lymphoid aggregates are mainly composed of non-dividing, preactivated memory cells.

To assess whether these B cells originate from local Ag-driven clonal expansions, we sequenced the IgV$_H$ gene in RA synovial samples with and without lymphoid neogenesis with an identical method as performed in OA synovial tissue (28). Using this approach, we recently demonstrated clonally related sequences and clustered somatic mutations in the CDR (28). As shown in Table III, oligoclonal B cell populations were found in all but one sample with synovial lymphoid neogenesis: two samples showed a single B cell expansion with 3/10 and 3/12 sequences with identical rearrangements, and one samples showed two different clones with 6/17 and 4/17 sequences with identical rearrangements (supplemental material, Fig. 1, *A* and *B*). From a total of 47 sequences analyzed from four samples, 16 showed identical rearrangements. Similar oligoclonal populations were found in all four cases of diffuse synovitis with in total 25 of 75 sequences with identical rearrangements. Somatic mutations as observed during intraclonal diversification were found in only one sample with diffuse synovitis and in none of the cases with lymphoid neogenesis. The five offsprings of clone V$_H$3–11-01 in this RA sample without lymphoid neogenesis were characterized by high ratios of replacement to silent mutation in the CDR but not FWR regions, indicating Ag-driven affinity maturation (supplemental material, Fig. 1, *C* and
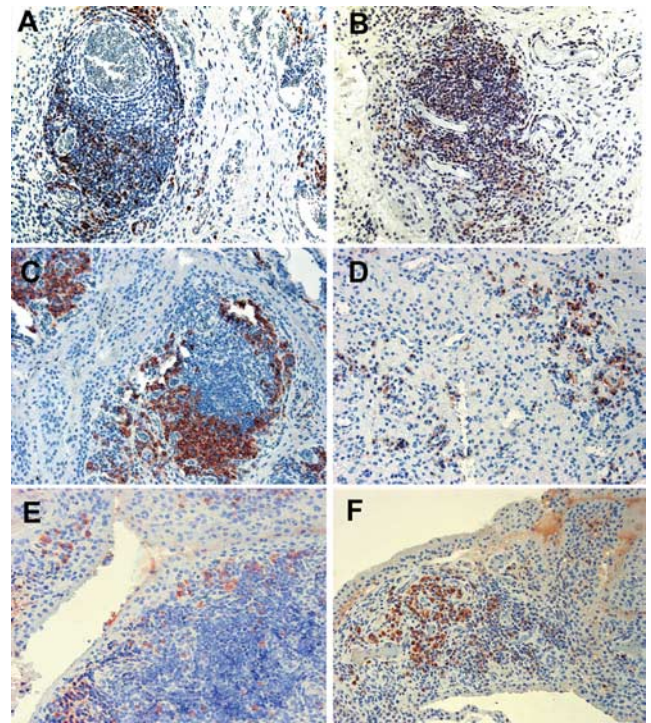

FIGURE 4. B lymphocytes in synovial lymphoid neogenesis are mainly preactivated, memory B cells. Synovial tissue samples with pronounced lymphoid neogenesis were obtained from rheumatoid arthritis ($n = 16$) and spondyloarthritis ($n = 19$) patients and stained for a variety of B cell phenotypic markers. *A*, CD79a staining demonstrates the abundant presence of B cells in the large lymphoid aggregates, but these cells did not express IgD or CD23 as markers of naive B cells. *B*, CD27 staining, which stains memory B cells, plasma cells and GC cells, indicates that almost all lymphocytes present in the large, organized lymphoid aggregates are Ag-experienced cells. *C*, CD138$^+$ plasma cells are not present inside the aggregates but rather surround these structures. *D*, In contrast with CD20$^+$, CD79a$^+$ B cells which are almost exclusively found in lymphoid aggregates, CD138$^+$ plasma cells are also found dispersed throughout the synovial tissue. *E*, IgG staining indicates that the plasma cells surrounding large lymphoid aggregates have already undergone class switching. *F*, Also plasma cells not associated with lymphoid structures are mainly IgG positive.

D). Taken together, these data suggest that RA synovitis is frequently characterized by oligoclonality of infiltrating B cells (14). This could result from local clonal expansion wihout further diversification, which may be due to the lack of supporting, fully matured lymphoid structures. Alternatively, however, it may results from the migration of prediverified, clonally related cells, being it B cells or plasmablasts. Indeed, it needs to be emphasized that the technical approach of this analysis does not allow the

Table III. *Analysis of B cell clonality by IgV$_H$ sequencing*

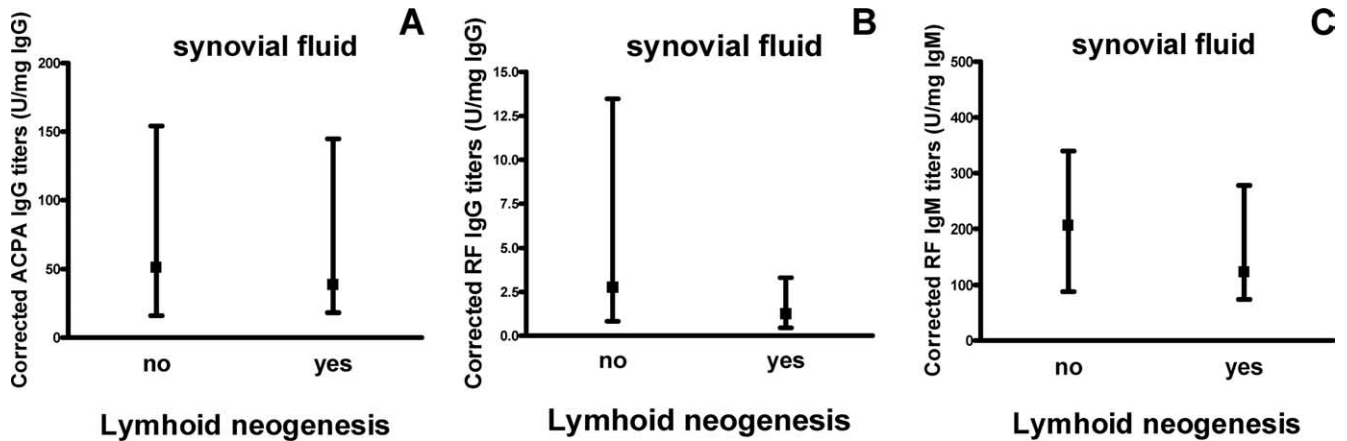| Lymphoid Neogenesis	Sample	Number of Sequences Analyzed	Number of Clonal Expansions	Number of Identical Sequences	Intraclonal Offspring Mutations
No	RA1	15	2	4/15 and 3/15	No
No	RA2	17	1	4/17	No
No	RA3	18	1	5/18	Yes
No	RA4	15	2	4/15 and 5/15	No
Yes	RA5	17	2	6/17 and 4/17	No
Yes	RA6	12	1	3/12	No
Yes	RA7	8	0	NA	NA
Yes	RA8	10	1	3/10	No

FIGURE 5. Synovial lymphoid neogenesis does not support local autoantibody production in rheumatoid arthritis (RA) ($n = 112$). Levels of autoantibodies were measured in SF and corrected for the total amount of SF IgG or IgM as appropriate. Data are presented as median and interquartile range. *A*, In ACPA-positive patients (cutoff: 25 U/ml in serum, $n = 73$), relative titers of SF ACPA are not higher in RA with vs without synovial lymphoid neogenesis. *B*, In RF IgG-positive patients (cutoff: 6 U/ml in serum, $n = 65$), relative titers of SF IgG RF are not higher in RA with vs without synovial lymphoid neogenesis, with even a trend toward higher levels in the latter group ($p = 0.064$). *C*, In RF IgM-positive patients (cutoff: 12.5 U/ml in serum, $n = 68$), relative titers of SF IgM RF are not higher in RA with vs without synovial lymphoid neogenesis.

discrimination between B cells and plasma cells and thus the additional experiments are warranted to discriminate between these different possibilities. Independently of the underlying mechanism, of importance for the present research question is the fact that this process is not directly related to ectopic lymphoid neogenesis.

Synovial lymphoid neogenesis does not support autoantibody production in RA

Even in the absence of naive B cells and Ag-driven maturation of B cell responses, synovial lymphoid neogenesis could still contribute to humoral autoimmunity by amplifying and/or perpetuating an ongoing response that was originally generated in canonical SLO. As indicated previously, synovial lymphoid neogenesis occurs as frequently in seropositive as seronegative RA (Fig. 1*B*) but it may specifically enhance the local production of ACPA and RF as prototype autoimmune responses in RA. Assessing this issue in ACPA-positive RA as defined by serum anti-CCP Abs ($n = 73$), local ACPA levels in SF were not different in samples with vs without synovial lymphoid neogenesis (Fig. 5*A*). Other factors contributing to the ACPA response such as the presence of synovial citrullinated proteins, the specific antigenic targets of this autoimmune response, and HLA-DR shared epitope were similar in both groups (data not shown). These findings are not restricted to the ACPA response as they were confirmed by similar analyses for IgM and IgG RF (only in RF IgM ($n = 68$)- and RF IgG ($n = 65$)-positive patients for serum values, respectively), with even a clear trend toward a 2-fold lower rather than higher IgG RF production in those joints characterized by synovial lymphoid neogenesis (2.7 (0.8–13.1) vs 1.3 (0.5–3.3) U/mg IgG; $p = 0.063$) (Fig. 5, *B* and *C*). Also after correction for the relative frequencies of plasma cells, which do correlate with total IgG levels in SF, there was no difference in ACPA and RF SF levels between samples with and without lymphoid neogenesis (data not shown). These data indicate that synovial lymphoid neogenesis is not a major determinant of the local production of these prototype RA autoantibodies.

Discussion

In SLO, Ag-specific activation of B lymphocytes leads to GC-reactions. Similar mechanisms may operate in ectopic lymphoid neogenesis as suggested by the binding of target Ags to these lymphoid structures in thyroid autoimmune disease and Sjögren's syn-

drome (43, 44). As the presence of developed lymphoid structures in non-autoimmune diseases such as idiopathic long fibrosis (30) and osteoarthritis (28, 45) questions the commitment of ectopic lymphoid neogenesis to specific B cell autoantigens, we investigated whether synovial lymphoid neogenesis occurs preferentially in seropositive rheumatic diseases. Using a unique collection of human samples we demonstrate that lymphoid neogenesis occurs as frequently in RA as in seronegative chronic joint diseases and, within RA, in RF and ACPA-positive vs -negative RA. The association between the degree of synovial inflammation and lymphoid neogenesis suggests that the latter is rather related to the severity than the type of inflammation. Although the description of associations rather than causal relationships is an intrinsic limitation of human studies, this concept was further supported by the fact that down modulation of synovial inflammation by either TNF blockers or prednisolone is associated with a reversal of synovial lymphoid neogenesis (20, 46, 47). Moreover, we recently demonstrated that B cell depletion by Rituximab decreased synovial tissue infiltration as well as lymphoid neogenesis after 16 wk of treatment (48). Taken together with the coexistence of different degrees of lymphoid organization in a single sample (23, 26), these data indicate that synovial lymphoid neogenesis is a dynamic process which could be related to inflammation rather than a fixed pathological feature committed to specific B cell autoantigens.

In SLO, Ag-driven B cell responses require the full maturation of lymphoid structures toward GC-reactions, a complex process orchestrated by lymphoid chemokines and cytokines (49, 50) Defective expression of these mediators leads to abortive follicle formation and impaired B cell responses (49, 51). The overexpression of these cytokines in RA synovial lymphoid neogenesis suggests that they could have a similar function in synovial tissue and thus that full maturation toward GC-reactions may be more relevant to B cell autoimmune responses than the mere presence of large lymphoid aggregates (15, 23). Testing this hypothesis in SpA vs RA, we confirm that the lymphoid chemokines CXCL13 and CCL19 are significantly up-regulated in synovial tissues with lymphoid neogenesis (35, 39) and demonstrate that they are even further up-regulated in RA vs SpA follicular synovitis. CCL21 transcripts are not increased compared with tissues without synovial lymphoid neogenesis, which is in line with CCL21 being also expressed in nonorganized clusters and minor aggregational stages in

RA synovium (23). Although both CCL19 and CCL21 recruit CCR7-positive T lymphocytes and dendritic cells, their functions are not redundant as only CCL21 over expression leads to organized lymphoid structures (52). Therefore, the lack of up-regulation of CCL21, especially in RA synovitis, is of interest in relation to the observed poor T/B cell segregation in synovial lymphoid neogenesis (12). A second factor of interest is the low expression of LTb, even in the presence of lymphoid neogenesis. LTb controls HEV development and function in SLO (24, 53) and predicts FDC recruitment in inflamed synovium (15). The low LTb expression is consistent with our observation that these tissues do no contain HEVs and FDCs. The third and major finding is that RA lymphoid neogenesis rarely depicts the prototypic features of GC formation despite high expression of CXCL13, CCL19, and BAFF. Moreover, this is independent of the seropositivity for ACPA and RF. One factor which may control and restrict the maturation of synovial lymphoid neogenesis in RA is TACI, as TACI-Fc treatment counteracts synovial GC formation in the SCID model (39). However, no causality has been demonstrated between TACI overexpression in the RA-synovium with inhibition of evolution to GC reactions.

Whereas the exact interplay between the different chemokines, cytokines, and growth factors remains difficult to decipher in human pathology, our data confirm molecular and structural similarities but also important differences between SLO and synovial lymphoid neogenesis (15, 54). Although synovial lymphoid organization can progress to full maturation in single cases, our data contradict previous reports that RA synovial lymphoid neogenesis display genuine GC-reactions in half of the cases (13). As our data are in agreement with the presence of FDC in 6% of the synovia in an independent analysis of >100 RA samples (55), these earlier studies may have been biased by the selection of severe, end-stage synovial tissue and the use of non-specific markers such as CD21 and CD23 for FDC (15, 23). The major conclusion of this molecular and structural analysis is that synovial lymphoid neogenesis is generally limited to the organized accumulation of T and B lymphocytes without development of HEV, T/B cell segregation, and FDC networks, and that not only the mere presence but also the degree of organization of synovial lymphoid neogenesis is similar between seropositive and seronegative disease.

These findings question whether synovial lymphoid neogenesis shares functional properties with SLO in terms of induction and/or maturation of B cell responses. This process includes the influx of naive B cells through HEV, priming of these cells trough Ag presentation by FDC, and subsequent clonal proliferation and affinity maturation. In inflamed synovial tissue, the presence of activated endothelial cells and numerous APCs, especially RF^+ B cells, could bypass the need for HEV and FDC (38). This hypothesis is not supported by our histological analysis demonstrating the absence of naive B lymphocytes and the dark zone of proliferating centroblasts in synovial lymphoid neogenesis. To assess Ag-driven B cell maturation at the molecular level, we sequenced the IgV_H of 112 B cell clones obtained from RA synovium with and without lymphoid neogenesis. Confirming previous results (14), our data demonstrate multiple clonally related sequences in most RA samples. However, intraclonal mutations suggestive of local Ag driven B cell maturation, as observed previously in OA synovium (28) are found only in 1 sample with diffuse synovits. In all other samples, clonal accumulation could result either from infiltration of clonally related cells (B cells or plasmablasts) or from local clonal expansions without any further diversification. Importantly, this occurred independently of synovial lymphoid neogenesis. This is not only relevant to the humoral autoimmune responses generated by priming and maturation of germline autoreactive naive B cells, but

also to the acquisition of self-reactivity by somatic hypermutation of B lymphocytes with other reactivities (56, 57). We failed to detect mRNA for recombination-activating genes (data not shown), indicating that these structures do not contribute to the generation of new and potentially autoreactive specificities by receptor revision (43, 58). Taken together, we could not demonstrate a qualitative impact of synovial lymphoid neogenesis on B cell responses in RA.

Synovial lymphoid neogenesis, even when involving memory rather than naive B cell, could have a quantitative impact on humoral autoimmunity in RA by amplifying and perpetuating an ongoing autoimmune response that was generated in SLO. In this scenario, the plasma cells surrounding the synovial lymphoid follicles should be clonally related to the B cells within these follicles, which was demonstrated not to be the case (59). Our extensive analysis failed to provide evidence that synovial lymphoid neogenesis contributes quantitatively to the local production of ACPA, RF IgG, and RF IgM in the affected joint. Albeit depicting a wide variability between individual samples, all three investigated Abs showed even a trend toward a 2-fold decreased local production in samples with vs without synovial lymphoid neogenesis. Although any causal interpretation of this finding remains speculative, we emphasize that local ACPA and RF production was measured in function of total Ig production in the joint. The relative decrease of these autoantibodies may thus relate to an increase in other Ab specificities. Alternatively, these data may prompt us to reconsider the assumption that lymphoid neogenesis would per definition be detrimental in autoimmune disease (60). In contrast, there is increasing evidence that peripheral tolerance checkpoints in GC control autoimmunity (61–65). The instalment of similar checkpoints in the inflamed tissue may help to restrict the induction of humoral immune responses to the multiple autoantigens that are released during chronic inflammation. Finally, one should consider that even without production of autoantibodies, the accumulation of autoreactive B cells within lymphoid structures could still promote autoimmunity and inflammation by activating pathogenic self-reactive T cells (15, 66, 67). Confirmation of the new data provided by our translational study and further analysis of the exact immunological role of ectopic lymphoid structures would obviously require additional functional studies in vitro and/or in experimental animal models. However, as of today we have no good ACPA-related animal models of arthritis to address this question mechanistically.

In conclusion, our data indicate that the ectopic lymphoid microstructures in the synovial target organ are not restricted to seropositive arthritis but rather may be related to the degree of inflammation, do only occasionally mature toward the full-blown structure of SLO, and does not appear to influence the induction, perpetuation, or amplification of the prototypic RA autoantibody responses. Therefore, the development of synovial lymphoid neogenesis is not a key determinant of humoral autoimmunity in RA.

Disclosures

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