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T Cell Activation in Rheumatoid Synovium Is B Cell Dependent¹

Seisuke Takemura, Piotr A. Klimiuk, Andrea Braun, Jörg J. Goronzy, and Cornelia M. Weyand²

Rheumatoid arthritis results from a T cell-driven inflammation in the synovial membrane that is frequently associated with the formation of tertiary lymphoid structures. The significance of this extranodal lymphoid neogenesis is unknown. Microdissection was used to isolate CD4 T cells residing in synovial tissue T cell/B cell follicles. CD4 T cells with identical TCR sequences were represented in independent, nonadjacent follicles, suggesting recognition of the same Ag in different germinal centers. When adoptively transferred into rheumatoid arthritis synovium-SCID mouse chimeras, these CD4 T cell clones enhanced the production of IFN- γ , IL-1 β , and TNF- α . In vivo activity of adoptively transferred CD4 T cells required matching of *HLA-DRB1* alleles and also the presence of T cell/B cell follicles. *HLA-DRB1*-matched synovial tissues that were infiltrated by T cells, macrophages, and dendritic cells, but that lacked B cells, did not support the activation of adoptively transferred CD4 T cell clones, raising the possibility that B cells provided a critical function in T cell activation or harbored the relevant Ag. Dependence of T cell activation on B cells was confirmed in B cell depletion studies. Treatment of chimeric mice with anti-CD20 mAb inhibited the production of IFN- γ and IL-1 β , indicating that APCs other than B cells could not substitute in maintaining T cell activation. The central role of B cells in synovial inflammation identifies them as excellent targets for immunosuppressive therapy. *The Journal of Immunology*, 2001, 167: 4710–4718.

Rheumatoid arthritis (RA)³ is a chronic inflammatory disease that causes irreversible destruction of cartilage, tendons, and bones, and shortens the life expectancy of patients by affecting major organ systems (1, 2). The rheumatoid synovial lesion is a highly vascularized and invasive neotissue infiltrated by lymphocytes and macrophages (3). Numerous lines of evidence support the model that the ultimate cause of synovitis is the recognition of arthritogenic Ag by T lymphocytes (4–6). The Ag causing RA have not been identified, nor is it known whether Ag recognized in the joints of different patients are identical or diverse. Important information on the nature of the Ag could come from identifying the APC and the site of Ag recognition.

In most autoimmune syndromes, chronic inflammatory lesions are arranged as diffuse infiltrates of mixed cell populations. This is not the case in RA, in which cells invading the synovial membrane organize themselves into sophisticated microstructures. Most interesting is a subset of patients with RA in whom T cells and B cells form aggregates and eventually develop germinal centers (GCs) (7). As in lymph nodes, T cells and B cells are arranged around a network of follicular dendritic cells (8, 9). Mechanisms regulating this process of lymphoid neogenesis (10, 11) in RA synovium are not understood, but the topographical arrangement as well as the overall number and ratio of T cells and B cells are

strictly controlled (12). GCs in RA synovitis are functionally competent, allowing for affinity maturation via IgG hypermutation (7). Analogous to normal secondary lymphoid tissue, extranodal GCs with surrounding T cell populations should be ideal sites for Ag capture. Ag presentation is most likely accomplished by interdigitating dendritic cells that activate Ag-specific T cells in the T cell-rich zones, followed by migration of these T cells into the GCs (8, 9, 13–16).

In the current study, CD4⁺ T cells in synovial GCs were isolated by microdissection. Activation requirements for these follicle-derived CD4 T cells were analyzed in adoptive transfer experiments. Distinct GCs from the same patient contained identical CD4 T cell clones that, upon transfer into heterologous synovial tissues, were able to increase the production of proinflammatory mediators. Two factors were critical in determining the functional activity of follicular CD4 T cells, matching with the MHC class II polymorphism of the implanted synovium and the presence of B lymphocytes in the tissue. Several possible mechanisms could underlie the critical role of B cells in regulating the activation of tissue-invading CD4 T cells. Given the ability of B cells to specifically capture Ag with their Ig receptors and present it to T cells (17, 18), B cells may be uniquely situated to stimulate proinflammatory T cells in rheumatoid synovitis.

Materials and Methods

Patients

Synovial tissue specimens were obtained from patients with RA with active synovitis who were undergoing synovial biopsy, synovectomy, or total joint replacement surgery. All patients had unequivocal destructive RA, were seropositive for rheumatoid factor (RF), and fulfilled the American College of Rheumatology (Atlanta, GA) criteria for the diagnosis of RA (19). Clinical and demographic data are given in Table I. Matching blood samples were collected before the surgical procedure. The protocol was approved by the Mayo Clinic Internal Review Board, and all patients provided written informed consent.

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; BV, TCR β -chain variable region; GC, germinal center; NOD, nonobese diabetic; RF, rheumatoid factor.

Table I. Patient characteristics

Patient	Sex ^a	Age (years)	Disease Duration (years)	RF	HLA-DRB1	GC
RA-1	F	57	12	+	401/404	+
RA-2	F	63	4	+	404/08	+
RA-3	F	74	17	+	01/404	+
RA-4	F	69	15	+	401/403	+
RA-5	F	38	20	+	01/401	+
RA-6	F	56	3	+	401/403	+
RA-7	F	78	42	+	15/15	+
RA-8	F	62	26	+	01/401	+
RA-9	F	39	8	+	401/08	+
RA-10	F	33	20	+	01/03	+
RA-11	F	70	42	+	401/403	—
RA-12	F	55	41	+	401/07	—
RA-13	M	59	18	+	401/07	—
RA-14	F	66	24	+	401/07	—
RA-15	F	48	4	+	03/401	—

^a F, Female; M, male.

Generation of human synovium-SCID mouse chimeras

NOD.CB17-Prkdc^{scid}/J mice (nonobese diabetic (NOD)-SCID) were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at age 6–8 wk. For tissue implantation, mice were anesthetized with 50 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL) i.p. and methoxyflurane (Medical Developments Australia, Springvale, Victoria, Australia) inhalation. Pieces of human synovial tissue with inflammatory infiltrates were placed into a s.c. pocket on the upper dorsal midline. In this model, complete engraftment is reached within 1 wk (20). At the completion of the experiment, the mice were sacrificed and the synovial tissue was harvested and embedded in OCT (Tissue-Tek; Sakura Finetek, Torrance, CA) for histological studies or was shock frozen in liquid nitrogen for RNA extraction.

Treatment with anti-CD20 mAb

Ab treatment was initiated 12 days after tissue implantation. Human synovium-SCID mouse chimeras were injected i.p. three times daily with anti-CD20 mAb (Rituxan; IDEC Pharmaceuticals, San Diego, CA) for 3 consecutive days. Mice received either 100 or 200 μ g per injection (300 or 600 μ g/day, respectively). Tissue grafts were explanted on day 20 and embedded in OCT for immunohistochemistry or shock frozen in liquid nitrogen for subsequent analysis of in situ cytokine transcription by PCR-ELISA.

Microdissection of GCs and TCR sequencing

Fractions of lymphoid aggregates were collected from 8- μ m cryosections with a 50- μ m microcapillary under an inverted microscope. cDNA from each pick was amplified by PCR with TCR β -chain variable region (BV) (21)- and nested β -chain constant region (BC)-specific primers (5'-CTGTGCACCTCCTTCCCATTC-3' and 5'-GTGGGAGATCTCTGCTTCTG-3'), cloned with the TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA), and sequenced. To select BV elements used by follicular CD4 T cells, immunohistochemical staining with BV-specific mAb was performed on serial tissue sections directly adjacent to the sections used for microdissection. The panel of anti-BV mAb was specific against BV3S1, BV5S1, BV5S2/S3, BV6S7, and BV8 (T Cell Diagnostics/Endogen, Cambridge, MA). From each patient, material from two to three independent follicular picks was subjected to sequence analysis. Subsequently, 50–70 follicular picks were screened for specific TCR sequences in hybridization experiments. In these experiments, cDNA was amplified with the appropriate primers and hybridized with biotinylated oligonucleotides representing the N-D-N region of TCR sequences that were expanded in the follicles, using the hybridization protocol described below for the cytokine semiquantification. Specificity and sensitivity of the assay system have been described (22). A signal was considered positive if it exceeded a nonspecific binding signal by at least 10% of the positive control.

T cell cloning and adoptive transfer experiments

T cell clones were generated from synovial tissue by limiting dilution cloning (21) and maintained by weekly polyclonal restimulation and 20 IU/ml human rIL-2 (Proleukin; Chiron, Emeryville, CA). Three T cell clones

were identified that expressed a TCR β -chain nucleotide sequence also found in microdissected GCs from the same patient. All three clones preferentially produced IFN- γ after in vitro stimulation. Autologous control clones were generated from anti-CD3-activated PBMC and maintained in parallel. Because tissue-derived clones had a Th1 profile of cytokine production, control clones were selected to also preferentially produce IFN- γ . For adoptive transfer experiments, synovium-SCID mouse chimeras were generated, and 12–14 days after tissue implantation, 5×10^6 T cell clones were slowly injected into the tail vein. Synovial grafts were explanted on day 20–22 and embedded in OCT for immunohistochemistry or shock frozen in liquid nitrogen for tissue cytokine measurements.

Histopathological evaluation and immunohistochemistry

Hematoxylin-stained sections of the synovial tissue samples were examined for the organizational structure of the inflammatory infiltrate, paying particular attention to the topographical arrangement of T cells, B cells, and macrophages; the degree of angiogenesis and synovial hyperplasia; and the presence of plasma cells. GC reactions, diagnosed by histomorphology, were confirmed by immunohistochemical staining for CD23⁺ networks (12). Tissues were classified as having diffuse synovitis if neither granulomas nor T cell/B cell clusters could be detected in serial sections from multiple independent sites of the specimen.

Cell populations in the synovial samples were identified by immunohistochemical staining of frozen sections. Tissues embedded in OCT were cut into 5- μ m sections and mounted on slides (SuperFrost/Plus; Fisher Scientific, Pittsburgh, PA). Before staining, the slides were fixed in acetone for 10 min, air dried, and fixed in 1% paraformaldehyde/EDTA (pH 7.2) for 3 min. Endogenous peroxidase was blocked with 0.3% H₂O₂ in 0.1% sodium azide. Nonspecific binding was blocked with 5% normal goat serum (Invitrogen Life Technologies) for 15 min. Sections were stained with anti-human CD3 (1:100; BD Biosciences, San Jose, CA), anti-human CD4 (1:100), anti-human CD20 (1:100), or anti-human CD23 (1:100) mAb (all from DAKO, Carpinteria, CA) for 60 min at room temperature. After incubation with biotinylated rabbit anti-mouse Ig Ab (1:300; DAKO), the slides were developed with diaminobenzidine tetrahydrochloride substrate for 3 min, counterstained with hematoxylin for 5 s, and mounted in Cytoseal-60 (Stephens Scientific, Riverdale, NJ). Negative controls were stained in parallel without the primary Ab.

Cytokine semiquantification

Total RNA was extracted from synovial tissue specimens using TRIzol reagent (Invitrogen Life Technologies). cDNA samples were analyzed for β -actin transcripts by semiquantitative PCR-ELISA and were adjusted to contain equal numbers of β -actin transcripts (20, 23). The adjusted cDNA was amplified by PCR for 30 cycles under nonsaturating conditions with cytokine-specific primers. Standard curves were generated by amplifying serial dilutions of known concentrations of cytokine-specific sequences. Detailed procedures and primer sequences for IFN- γ , IL-1 β , and TNF- α have been reported (23). Each PCR amplification cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min, with 10-min initial denaturation at 94°C and a final 10-min extension at 72°C. Amplified products were labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals-Boehringer Mannheim, Indianapolis, IN) and semiquantified in a liquid hybridization assay with biotinylated internal probes (23) using a commercially available PCR-ELISA kit (Roche Molecular Biochemicals-Boehringer Mannheim). In this assay, the labeled PCR products were hybridized with 200 ng/ml probe at 55°C for 2.5 h. Hybrids were immobilized on streptavidin-coated microtiter plates and, after washing, were detected with a peroxidase-labeled anti-digoxigenin Ab. Plates were developed by a color reaction using ABTS substrate and quantitated using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The number of cytokine-specific sequences was determined by interpolation on a standard curve, and was expressed as the number of cytokine sequences per 2×10^6 β -actin sequences. Data are given as mean \pm SD of triplicate PCR.

Results

Follicular CD4 T cell clones stimulate synovial inflammation

The population of CD4 T cells in synovial lesions is diverse (24). Not knowing which Ag are critical in the disease process, it has not been possible to isolate T cells relevant for synovitis. To overcome this, we used synovial tissues with GCs. We reasoned that CD4 T cells participating in these microstructures would be specific for disease-relevant Ag. Tissues with follicles and CD23⁺ follicular dendritic networks from five patients bearing HLA-DRB1*04 were

selected for analysis. Synovial follicles were isolated by microdissection. cDNA was generated from the dissected material and was amplified with TCR *BV*- and *BC*-specific primers, cloned, and sequenced. From all five patients, sequences derived from the follicular structures had limited diversity, with sets of 25 sequences consistently containing two to three sequences present in multiple copies. More importantly, identical TCR sequences were demonstrated in distinct follicles from the same patient. All of these shared sequences were identical at the nucleotide and not only at the protein level, suggesting that they derive from the same founder cell. TCR sequences expressed in at least three independent follicles were considered to have derived from T cells specifically activated in the lesions and were selected. Parallel samples of synovial tissues from the same patients were used to establish T cell clones that were screened for these relevant TCR sequences. Three *BV6S7*⁺ T cell clones that expressed nucleotide sequences identical to sequences in the follicular picks (*BV6S7-TTATTCGGGACAGACACAGAT-BJ2S3*; *BV6S7-TTAGGGGTGCTAGCGCGGGAG-BJ2S3*; *BV6S7-CCAACAGGGGTGCGC GCAGAT-BJ2S5*) were retrieved from an *HLA-DRB1*0401/0403*⁺ patient, RA-4. These three T cell clones were phenotyped as *CD4*⁺*CD8*^{null}*CD28*⁺. Their distribution in multiple follicles of the donor tissue is described in Table II.

The biological activity of these *CD4*⁺ T cell clones was examined in adoptive transfer experiments. Autologous synovial tissue fragments from RA-4 were implanted into SCID mice, and 5×10^6 tissue-derived T cell clones were injected i.v. 10 days later. *CD4*⁺*CD8*^{null}*CD28*⁺ T cell clone isolated from the peripheral blood served as controls. To examine the effect of *CD4* T cell clones on synovial inflammation, we monitored the production of IFN- γ , TNF- α , and IL-1 β cytokines, all previously implicated in the disease process (1). The transfer of the control clone did not affect baseline IFN- γ transcription in the engrafted synovium. In contrast, infusion of follicle-derived T cell clones resulted in a 3- to 4-fold increase in tissue IFN- γ production (Fig. 1). Similar increases were obtained in two additional experiments using the same synovial T cell clones and two different control clones. Also, the number of IL-1 β - and TNF- α -specific transcripts increased 2- to 3-fold over baseline following transfer of autologous follicular

Table II. Clonotype distribution among synovial tissue follicles

Tissue Source	TCR CDR3 Sequence (<i>BV-BJ</i>)		
	LFGTDTD (<i>BV6S7-BJ2S3</i>)	LGVLARE (<i>BV6S7-BJ2S5</i>)	PTGVGAD (<i>BV6S7-BJ2S3</i>)
Follicle 1	—	—	—
Follicle 2	—	—	—
Follicle 3	+	+	—
Follicle 4	—	—	—
Follicle 5	—	—	—
Follicle 6	—	—	—
Follicle 7	+	+	—
Follicle 8	—	—	—
Follicle 9	+	+	—
Follicle 10	+	—	+
Follicle 11	—	—	—
Follicle 12	—	+	—
Follicle 13	—	—	—
Follicle 14	—	—	+
Follicle 15	—	—	+
Follicle 16	+	—	+
Follicle 17	+	+	—
Follicle 18	—	—	+
Follicle 19	+	—	—
Follicle 20	+	—	—

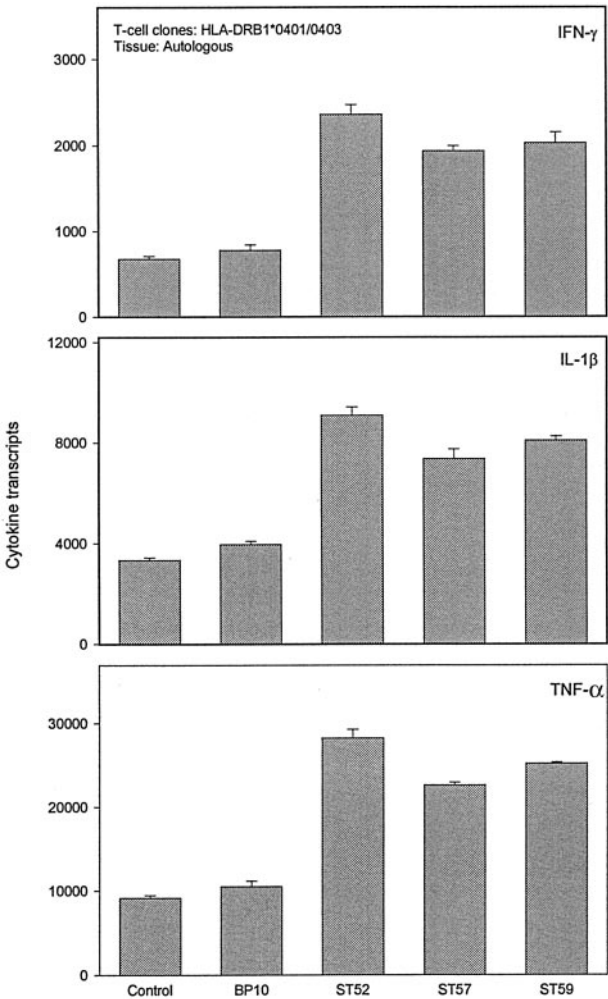


FIGURE 1. Proinflammatory function of adoptively transferred follicular *CD4* T cell clones. TCR sequences of follicular *CD4* T cells represented in several independent synovial follicles were identified by microdissection of fresh tissue (Table II). Corresponding *CD4* T cells were isolated by limiting dilution. A total of 5×10^6 T cell clones was adoptively transferred by i.v. injection into the tail vein of synovium-SCID mouse chimeras engrafted with autologous synovial tissue. Eight days following the adoptive transfer, synovial grafts were retrieved and tissue cytokines were quantified by PCR-ELISA. Control grafts were recovered from chimeras injected with saline only or a control *CD4* T cell clone isolated from the peripheral blood. Results are the means of triplicate measurements from one of three adoptive transfer experiments. All three follicular *CD4* T cell clones were able to increase IFN- γ , IL-1 β , and TNF- α transcripts in the synovial lesions.

CD4 T cell clones, but remained unaffected when the control clones were injected. The selective activation of follicular T cells is most consistent with the model that these cells recognize a local Ag, although additional differences between control and follicular T cells (such as differences in homing receptors) may contribute. The T cell clones did not produce TNF- α or IL-1 in vitro. Therefore, the increased production of these cytokines must be considered a downstream event, documenting that T cell activation and increases in IFN- γ transcripts were of functional importance. There was a trend for T cell clone ST52 to boost synovial cytokines most efficiently, but all three synovial T cell clones had strong proinflammatory properties. These data suggested that *CD4* T cell clones isolated from follicular centers are effective regulatory cells in rheumatoid inflammation.

Follicular CD4 T cell clones are activated in tissues from distinct patients and are HLA-DRB1 restricted

To investigate whether the activation of adoptively transferred CD4 T cells was restricted by HLA class II molecules, adoptive transfer experiments were performed with SCID mouse chimeras carrying *HLA-DRB1*-matched and -mismatched tissues. Donor-recipient combinations included matches for both *HLA-DRB1* alleles (RA-6), matches for one *HLA-DRB1* allele (RA-8 and RA-9), and mismatching for both (RA-7 and RA-10). Results of representative experiments are shown in Fig. 2. All three T cell clones, ST52, ST57, and ST59, which had been shown to function in autologous tissue grafts, were able to act as proinflammatory cells in heterologous tissue. Transfer into human synovium-SCID mouse chimeras implanted with tissue matched for both *HLA-DRB1* alleles resulted in a 3- to 4-fold increase in the transcription of IFN- γ , IL-1 β , and TNF- α (Fig. 2, upper panel). Similar results were obtained when the T cell clones and the recipient tissue were matched for only one *HLA-DRB1* allele, *HLA-DRB1**0401 (Fig. 2, middle panel). Mismatch of both *HLA-DRB1* alleles resulted in the loss of T cell clone function, with none of the inflammatory cytokines responding to the transfer of cloned follicular T cells. These experiments demonstrated that the necessary components to trigger the activity of follicle-derived CD4 T cell clones are present in

heterologous synovial tissues, suggesting that Ags driving the inflammation are shared by different patients.

Follicular CD4 T cells function only in the presence of B cells

Experiments shown in Fig. 2 were performed with tissues containing GCs. To address the question as to whether T cell activation is dependent upon the presence of these microstructures, we made use of the observation that not all patients with rheumatoid synovitis form classical GCs. In a subset of patients with RA, no T cell/B cell aggregates were found in the synovial membrane. Instead, T cells were dispersed throughout the tissue and CD20⁺ B cells were essentially absent (Fig. 3). In both follicular and diffuse synovitis, CD83⁺ interdigitating dendritic cells were present as possible APC (data not shown). The pattern of synovitis was consistent within patients, and synovial tissues from distinct joints showed the same type of inflammatory lesion. Also, longitudinal studies have demonstrated that diffuse synovitis does not convert into follicular synovitis or vice versa (data not shown).

Tissue fragments that had mononuclear infiltrates, but very few or no infiltrating B cells, were obtained from patients expressing one or both *HLA-DRB1* alleles identical to those of the tissue-derived CD4 T cell clones and were selected for implantation. Results from adoptive transfer experiments are shown in Fig. 4.

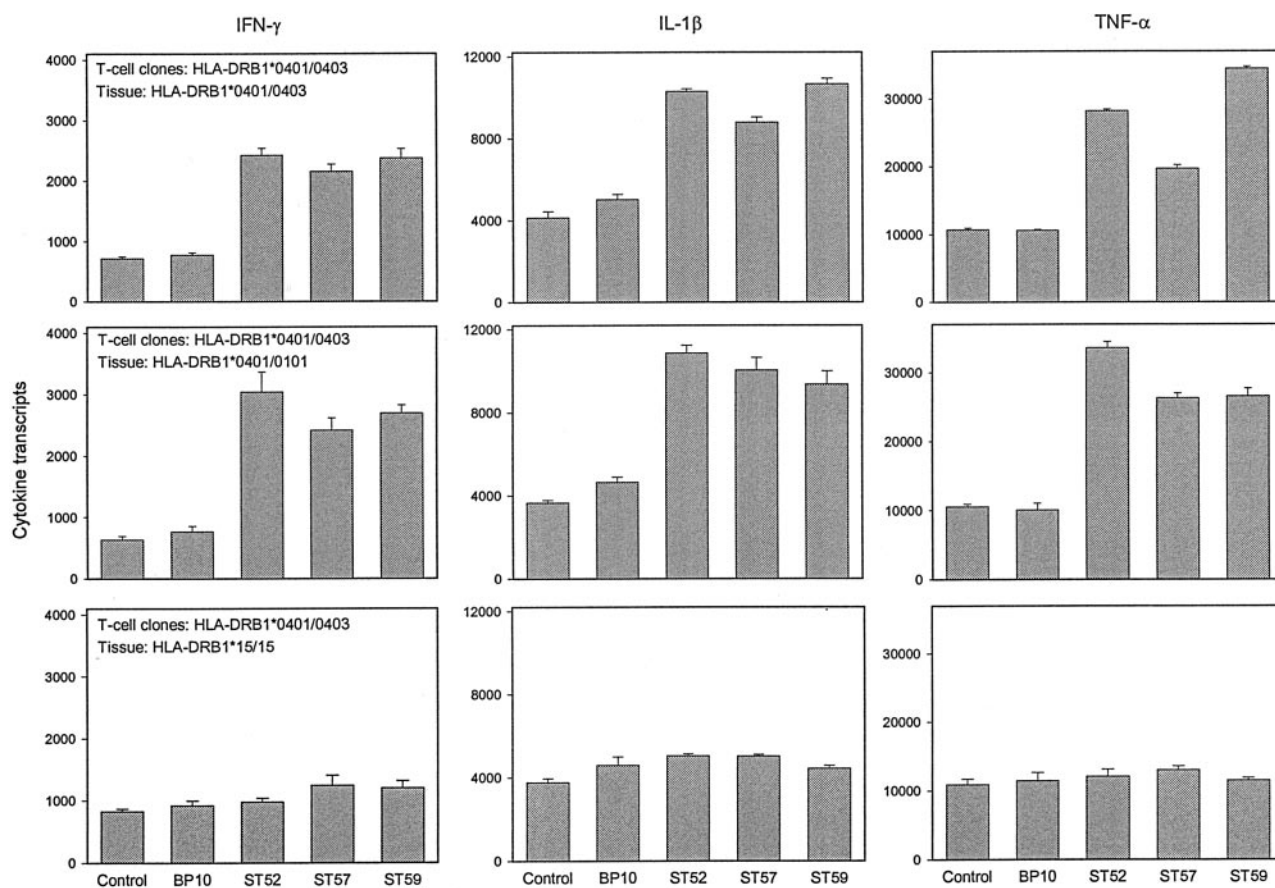


FIGURE 2. Adoptively transferred follicular CD4 T cell clones can be activated in heterologous HLA-DRB1-matched synovial tissue. Follicular CD4 T cell clones were identified, isolated, and adoptively transferred, as described in Fig. 1. Human synovium-SCID mouse chimeras were created by implanting synovial tissue from patients matched for two (upper panel), one (middle panel), or none (lower panel) of the *HLA-DRB1* alleles of the T cell clones. Control grafts were harvested from chimeras injected with saline only or a control clone, BP10. Eight days following cell infusion, the synovial grafts were explanted and tissue cytokine transcription was quantified by PCR-ELISA. Results for tissue production of IFN- γ , IL-1 β , and TNF- α transcripts are given as the means of triplicate analysis. The follicular CD4 T cell clones, ST52, ST57, and ST59, could be activated in heterologous tissue as long as they were matched for the *HLA-DRB1**0401 allele. No functional activity of any of the three follicular CD4 T cell clones was detected when transferred into *HLA-DRB1*-mismatched tissue (lower panel).

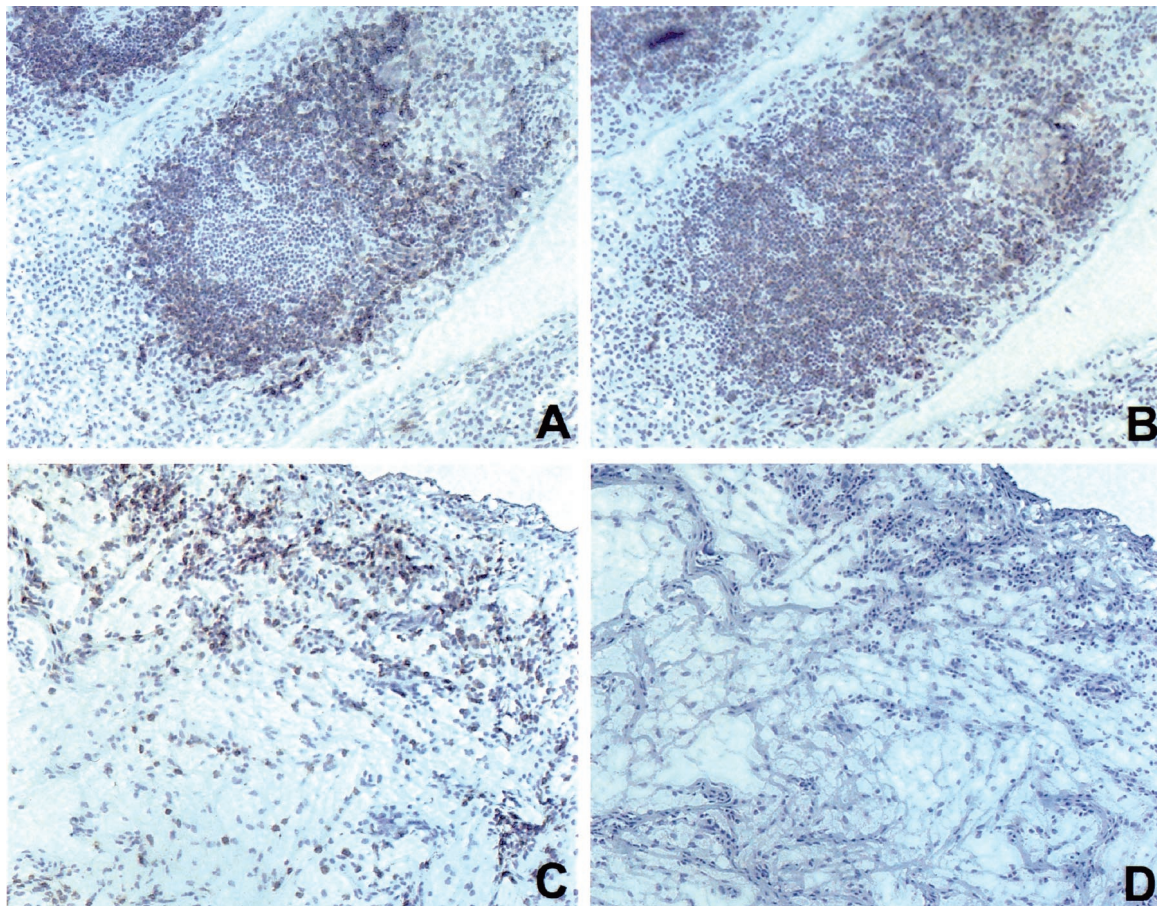


FIGURE 3. Different lymphoid microstructures in rheumatoid synovitis. Lymphocytes infiltrating the synovial membrane acquire different topographical arrangements. Each patient develops a pattern that is maintained over time. Serial sections of OCT-embedded tissues from two patients with RA were stained with either anti-CD4 (A and C) or anti-CD20 (B and D) mAb. Immunoperoxidase staining and 3,3'-diaminobenzidine tetrahydrochloride substrate were used. A and B, An example of follicular synovitis with CD4⁺ T cells and CD20⁺ B cells arranged in a tertiary follicle with GC reaction. C and D, An example of diffuse synovitis lacking tissue-residing B cells is shown. Original magnification, $\times 100$.

Synovial tissues lacking B cells were not able to support the activation of any of the three follicle-derived CD4 T cell clones. Levels of tissue IFN- γ transcripts were indistinguishable between control mice, mice injected with a PBMC-derived CD4 T cell clone, and mice injected with the follicular CD4 T cell clones. Data shown in the *upper panel* of Fig. 4 are derived from experiments using recipient tissue that typed *HLA-DRB1*0401/0403* (RA-11), and thus, was matched for both alleles. Despite this perfect match, the transferred CD4 T cell clones remained nonfunctional. Tissue concentrations for TNF- α and IL-1 β transcripts also remained unaltered. These experiments suggested that B cells were critical in inducing activation of selected CD4 T cells.

B cell depletion abrogates activation of synovial T cells

To examine the role of B cells in the activation of tissue-residing T cells, NOD-SCID mice were implanted with synovial tissue containing GCs and were then injected with anti-CD20 mAb for 3 consecutive days. Tissues from eight different patients, four with GCs and four with only diffuse infiltrates, were used. Grafts were harvested and analyzed for tissue histomorphology and cytokine production. The cell diversity of the infiltrate was semiquantified by counting CD4 T cells in 20 high powered fields per tissue. As shown in Fig. 5, treatment with anti-CD20 mAb resulted in the dissociation of the follicular structures. Not only were the follicles dissipated, the overall density of the infiltrates was decreased with a concomitant loss of CD4⁺ T cells. Upon treatment, the average

number of CD3⁺ T cells per high powered field declined to $\sim 25\%$ in controls. Functional studies demonstrated that the production of IFN- γ in the tissue with GC decreased markedly (Fig. 6). In grafts retrieved from chimeras treated with 300 μg anti-CD20 mAb per day, the concentration of IFN- γ mRNA in the tissue was reduced by 60–80%. In synovial tissues explanted from chimeras injected with a daily dose of 600 μg anti-CD20 mAb, IFN- γ transcription essentially ceased. In parallel to the suppression of T cell activation and IFN- γ production, the transcription of the proinflammatory monokine IL-1 β decreased by 80% in comparison with the controls (Fig. 6).

Discussion

This study links T cell activation in rheumatoid synovitis to the presence of B cells, thus assigning a novel function to B cells in the disease process. Adoptively transferred CD4 T cell clones derived from synovial follicles functioned selectively in tissues with CD20⁺ B cell clusters, but could not be triggered in tissues lacking B cell infiltrates; and elimination of B cells from the synovial tissue disrupted T cell activation and the production of proinflammatory monokines. Our data target the search for arthritogenic Ags toward the B cell and encourage the therapeutic use of B cell-depleting agents in RA.

Several mechanisms could account for the critical position of B cells in supporting the activation of synovial CD4 T cells. A direct involvement of secreted Ab is unlikely. B cells, however, are

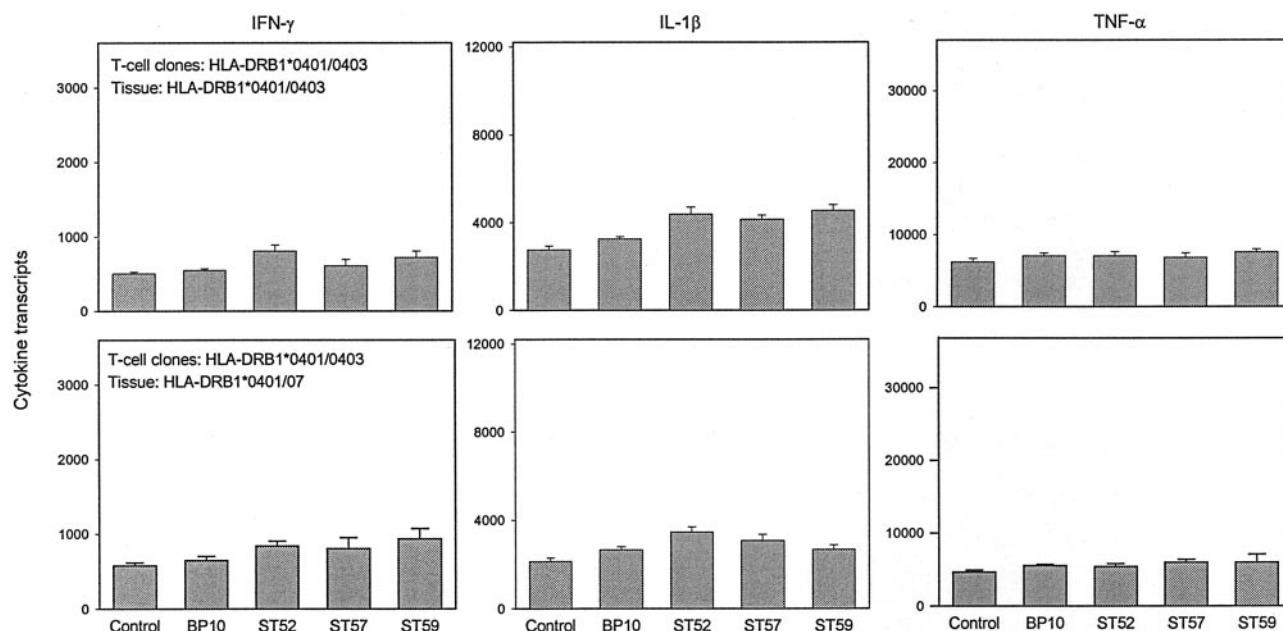


FIGURE 4. Synovial tissues lacking B cells do not sustain activation of follicular CD4 T cell clones. Synovial tissues lacking tissue-infiltrating B cells were implanted into NOD-SCID mice, and follicular CD4 T cell clones were adoptively transferred, as described in Figs. 1 and 2. B cell-poor synovial tissues were *HLA-DRB1* matched with the adoptively transferred T cell clones for at least one allele, as indicated. Control animals received saline only or the CD4 T cell clone BP10, cloned from the peripheral blood. Eight days after the cell infusion, the synovial grafts were harvested and analyzed for cytokine transcripts by PCR-ELISA. None of the three follicular CD4 T cell clones, ST52, ST57, or ST59, was activated in heterologous *HLA-DRB1*-matched tissues lacking T cell/B cell follicles.

highly efficient in Ag presentation because they accumulate and incorporate Ag by specifically capturing them with their Ig receptors. This mechanism is particularly important for B cells with RF reactivity because RF-positive B cells bind immune complexes and process them for Ag presentation (18, 25). They could, therefore, function as APCs in a cognate interaction. Alternatively, B cells could regulate T cell homing and survival and, thereby, facilitate T cell activity. Recent studies have clearly identified B cells as critical mediators of lymphoid organogenesis, in part through their ability to express lymphotoxin- β on their cell surface (11, 14). Finally, B cells could be the source of the relevant Ag, requiring their presence for effective T cell activation.

Direct relevance of B cells in disease pathogenesis has been shown for several experimental models of autoimmunity, including murine models of lupus erythematosus and diabetes mellitus (26–30). Persistence of lupus nephritis in mice with B cells deficient in secreting Ig has excluded the simple explanation that B cells influence the disease process through the production of autoantibodies (31). In MRL/*lpr* mice, B cells have been implicated in inducing a highly activated phenotype of CD4 and CD8 T cells (26). Taken together, B cells influence autoimmunity through multiple pathways, thereby representing important therapeutic targets in autoimmune diseases.

The precise role of B cells in RA is not well understood. RA was originally considered an Ab-driven disease (32, 33) with immune complex-mediated tissue injury (34–36). The failure to correlate disease activity to the levels of autoantibodies and the realization that RA exists in individuals not expressing RF focused interest on T lymphocytes and T cell-dependent effector mechanisms. The current paradigm proposes that T cell-derived cytokines, released upon T cell recognition of Ag in the synovial membrane, induce activation of macrophages and synovial fibroblasts, causing the formation of tissue-invasive pannus. Indirect evidence for an important contribution of B cells in rheumatoid synovitis could be inferred from the finding that the synovial membrane can be a site

of lymphoid neogenesis. Synovial GCs have the morphological and functional characteristics of GCs in lymph nodes, with the exception that T cells account for a higher proportion of the cells contributing to the core (12). The formation of extranodal GCs is uncommon in Ag-driven immune responses and has only been described for a very few nonmalignant syndromes (37). In RA, ~20% of all patients have the ability to generate these complex microstructures in the joint (38). The current study provides direct evidence that in this subset with GCs, macrophages, synovial fibroblasts, or interdigitating dendritic cells are not sufficient for sustaining T cell activation, but that B cells are critical. The adoptive transfer data are based on experiments with T cell clones from only one patient. However, similar results were found in chimeric mice implanted with tissues from different donors, and the conclusion is further supported by the B cell depletion experiments with tissues from different patients. Recent data in a TCR transgenic mouse model have emphasized that Ab produced by autoreactive B cells can have a direct role in inducing synovial inflammation, indicated by the transfer of joint inflammation from affected to unaffected animals by serum (39). In contrast, B cell functions distinct from the release of Ab must be underlying the critical involvement of B cells in human rheumatoid synovitis. The property of B cells in controlling stimulation of CD4 T cells in the lesion cannot be explained by autoantibody production. Rather, it indicates a different contribution of B cells to the RA process.

An interesting finding of the current study relates to the distribution of CD4 T cells able to boost synovial inflammation. Based on the reasoning that CD4 T cells participating in the GC reaction would almost certainly represent Ag-reactive T cells undergoing in situ activation, a microdissection approach was developed. TCR sequences isolated from the GC were restricted in diversity. More importantly, T cells with identical TCRs were isolated from different, nonadjacent T cell/B cell follicles. This finding supports the notion that the same Ag is driving the GC reaction in different follicles. TCR sequences isolated from microdissected follicular

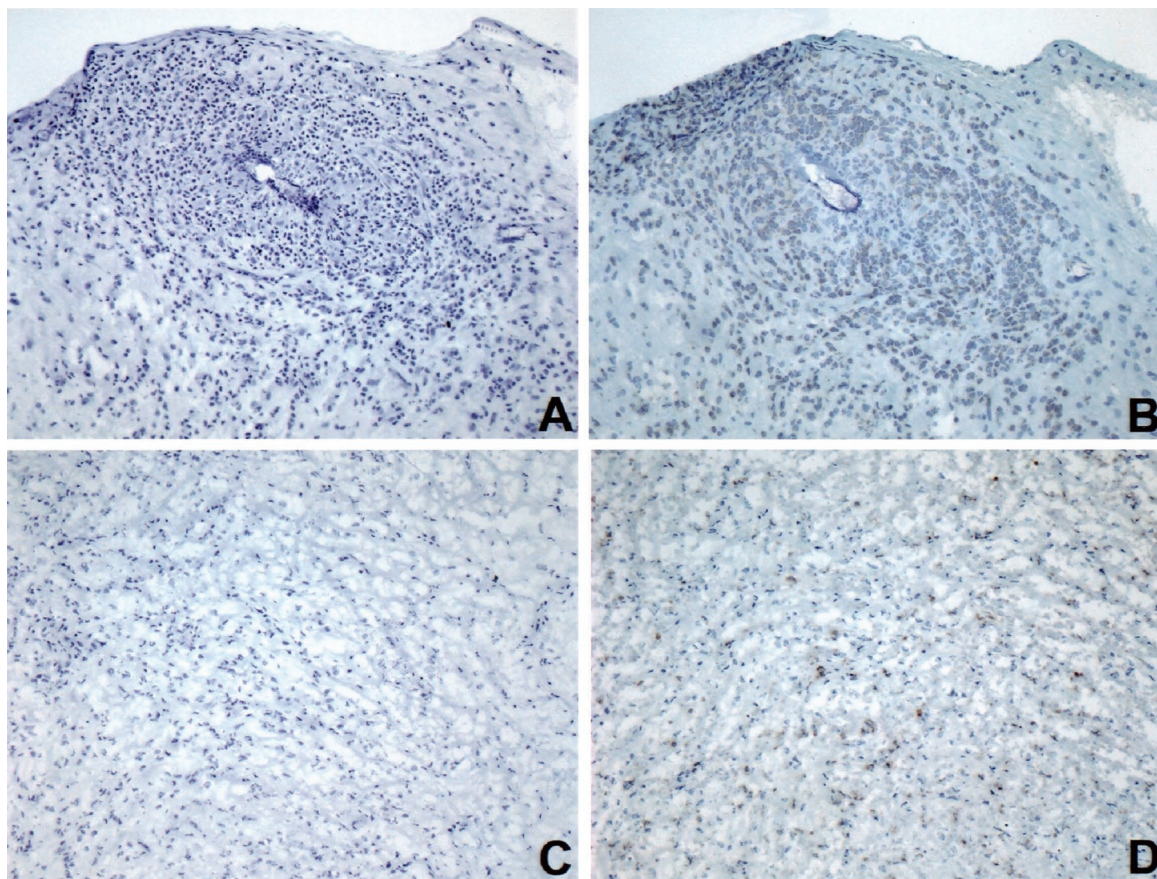


FIGURE 5. Architecture of synovial infiltrates after depletion of CD20⁺ B cells. Human synovium-SCID mouse chimeras implanted with synovial tissue were treated with either buffer or Rituxan (anti-CD20 mAb) given as three daily injections of 100 or 200 μ g for 3 consecutive days. Tissue grafts were explanted and analyzed by standard histomorphology and immunohistochemistry to identify the cellular components of the tissue-infiltrating cells. Tissues treated with control buffer contained prominent and demarcated lymphoid aggregates with CD3⁺ T cells accumulating in the outer follicles (A, hematoxylin; B, anti-CD3). In the tissues treated with anti-CD20 mAb, no follicle-like structures could be identified. In addition, overall cellularity was decreased, and only a small number of CD3⁺ T cells remained in the infiltrates. Representative areas are shown (C, hematoxylin; D, anti-CD3). Original magnification, $\times 100$.

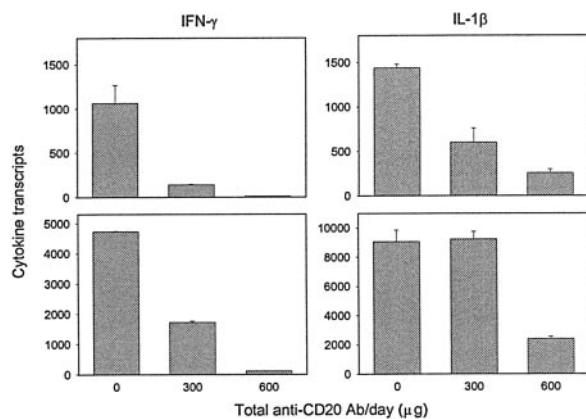


FIGURE 6. Effect of B cell depletion on synovial IFN- γ and IL-1 β production. Human synovium-SCID mouse chimeras were treated with anti-CD20 mAb for 3 consecutive days with the daily doses indicated. Synovial grafts were retrieved 6 days after Ab injection, and tissue cytokines were quantified by PCR-ELISA. Results from two independent experiments using tissues from two different patients with follicular synovitis are shown. Cytokine quantities are shown as means of triplicate measurement. Ab treatment resulted in a dose-dependent reduction of IFN- γ transcripts with almost complete abrogation of IFN- γ transcription at high Ab doses (A and C). In parallel to the reduction of tissue IFN- γ production, the activity of macrophages/synoviocytes also decreased, as indicated by the decline in IL-1 β transcription (B and D).

structures are only infrequently found outside of the clusters (data not shown). The restriction of the TCR and the sharing of T cells among different GCs strongly support a role for few Ag driving the formation of tertiary lymphoid tissue and subsequent tissue destruction in the rheumatoid joint. These Ag are obviously shared among patients because the follicular CD4 T cell clones underwent stimulation in the autologous as well as heterologous tissues. The restriction to *HLA-DRB1* molecules is in line with the recognition of a classical peptide Ag. The possibility has to be considered that the failure of follicular CD4 T cells to function in B cell-poor tissues is a reflection of a lack of the relevant Ag. In this model, follicular and diffuse synovitis would be different diseases. The Ag recognized in the synovium would be shared between different patients with follicular synovitis, but would be different in diffuse synovitis. It was proposed that CD8 T cells in the joints of patients with RA are triggered by EBV-related Ag (40, 41), but subsequent studies did not confirm a pathogenic role of EBV infection in RA (42–44). The CD4 T cell clones used in the current study did not respond to autologous EBV-transformed lymphoblasts (data not shown). Synovial B cells could also harbor other Ag and, thus, hold a critical position in the synovial immune response.

Although this interpretation cannot be excluded, a B cell-derived Ag presented by B cells as well as other APCs would not explain the rapid decline in CD4 T cell activity after the elimination of CD20⁺ B cells. The mAb treatment had profound effects.

Not only did the follicles disappear, but the frequency of tissue-infiltrating T cells and macrophages decreased markedly, to the extent of abrogating synovial inflammation. This observation supported a direct contribution of B cells in maintaining stimulation of proinflammatory T cells. Synovial B cells could have specialized Ag-presenting function, superior to all other accessory cells in the inflamed tissue. Several cell populations in the inflamed synovial membrane have been suspected to serve as APCs, including dendritic cells, synoviocytes, and macrophages (45–49). It is unlikely that primed T cells would be exclusively restricted to recognize Ag on B cells, especially when considering that GC T cells are thought to make their first Ag contact by interacting with dendritic cells in the T cell-rich zones. Anti-CD20 mAb treatment spared dendritic cells, but their presence was insufficient to sustain synovitis. Also, synovial tissues with diffuse infiltrates contain CD83⁺ dendritic cells and are able to promote T cell activation (20). However, in both experimental systems, the adoptive transfer experiments in follicular and diffuse synovitis and in the B cell depletion experiments, B cells proved to be critical for the functional activity of proinflammatory CD4 T cells. B cells may be superior to dendritic cells in capturing Ag, in particular, if Ag concentrations are limiting. B cells expressing Ig with RF activity could take up immune complexes presented by the follicular dendritic cells and thereby enrich for an infrequent Ag included in these complexes (18, 25).

Finally, it should be considered that the dependence of T cell stimulation on CD20⁺ B cells is a reflection of the complexity of the microenvironment. In situ activation of tissue-infiltrating CD4 T cells may not only depend on the presence of Ag and APCs, but it may be critically modulated by the spatial arrangement of the cells, local gradients of cytokines and mediators, and the competition of cells for space and resources. This model would predict that T cell triggering in the synovial lesions is optimized in follicular centers, and that the disease process in diffuse tissues is low grade and smoldering. Evidence has been provided that small concentrations of Ag are more effectively recognized when presented in the specialized microenvironment of lymphoid tissue (50). Indeed, previous studies have shown higher IFN- γ , IL-1 β , and TNF- α production in RA tissues with follicular synovitis than in tissues with diffuse synovitis (23).

The data reported in this work have potential clinical applications. Based on the finding in the human synovium-SCID mouse chimera model that T cell activation and its downstream effects, such as production of the proinflammatory monokines, TNF- α and IL-1 β , were suppressed by depleting CD20⁺ B cells, elimination of B cells in patients could be developed into a potent immunosuppressive therapy. Anti-B cell reagents have been generated for treatment of patients with lymphoproliferative disorders and have been shown to be relatively safe (51–53). Edwards and Cambridge (54) have recently reported five RA patients who responded to anti-CD20-mediated B cell depletion in combination with cyclosporin and prednisone. Three of the five patients had sustained improvement for more than 1 year. Because this study used a combination therapy, the relative contribution of B cell depletion to the treatment success is unclear. It is known how many of these five patients had synovial GCs. Our study suggests that B cell-depleting Abs or reagents interfering with B cell stimulation pathways, such as BLS, may directly influence the synovial inflammation in the subset of RA patients that generate tertiary lymphoid microstructures in the synovium.

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