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Autoantibodies from Synovial Lesions in Chronic, Antibiotic Treatment-Resistant Lyme Arthritis Bind Cytokeratin-10

Srimoyee Ghosh,* Robert Seward,‡ Catherine E. Costello,‡ B. David Stollar,† and Brigitte T. Huber²*

Although the causative agent of Lyme disease is definitively known to be the tick-borne spirochete, Borrelia burgdorferi, the etiology of chronic joint inflammation that ensues in a subset of patients remains less well understood. Persistence of arthritis after apparent eradication of the spirochete suggests an autoimmune reaction downstream of the original bacterial infection. We have generated recombinant Ab probes from synovial lesions within affected arthritic joints in an attempt to recapitulate disease-relevant Ag-binding specificities at the site of injury. Using this panel of intra-articular probes, as well as Ab fragments derived from patient peripheral blood, we have identified cytokeratin 10, present in synovial microvascular endothelium, as a target ligand and a putative autoantigen in chronic, antibiotic treatment-resistant Lyme arthritis. Furthermore, there is cross-reactivity between cytokeratin 10 and a prominent B. burgdorferi Ag, outer surface protein A. Release of the self protein in the context of inflammation-induced tissue injury and the resulting in situ response to it could set in motion a feed-forward loop, which amplifies the inflammatory process, thereby rendering it chronic and self-perpetuating, even in the absence of the inciting pathogen. The Journal of Immunology, 2006, 177: 2486–2494.

Lyme disease, caused by the tick-borne spirochete Borrelia burgdorferi (Bb), continues to be a major public health concern, with an average of >14,000 cases reported each year (1–3). Arthritis is a late-stage manifestation of this multiphasic, multisystem illness and resolves in most cases by the administration of antibiotics (1). Treatment-resistant Lyme arthritis is a chronic, debilitating condition that continues unabated for several months to years in a subset of patients, despite standard antibiotic treatment regimens that typically eliminate the causative agent, Bb (4). Amelioration of symptoms during this phase entails the usage of either nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic agents, or arthrosopic synovectomy (5). Spirochetal DNA, readily detected in patient synovial samples by PCR before antibiotic treatment, is absent from both synovial fluid and tissue of these patients, suggesting successful clearance of the pathogen and pointing toward a role for postinfectious autoimmune sequelae in the propagation of chronic joint inflammation (5, 6).

Previous work has focused on the role of T cell-mediated autoimmunity in chronic Lyme arthritis, Ab autoactivity during this phase remaining a largely unexplored area (7–11). Early studies of the humoral response have ruled out a role for anti-nuclear autoantibodies or rheumatoid factors in mediating either severity or duration of joint inflammation (12, 13). As the arthritis becomes more chronic, circulating immune complexes, measured by complement fixation assays, disappear from the serum of patients and progressively accumulate in the synovial fluid, their levels being proportional to the number of neutrophils there (14, 15). This points toward a role for immune complexes, some of which may be locally generated, in mediating leukotactic and phagocytic stimuli that contribute to pathophysiology.

Arthritic lesions, present in synovial sections from patient biopsies, contain ectopic germinal center (GC)-like structures with intermixed aggregates of T and B cells, follicular dendritic cells, and plasma cell clusters (5). Such structures, actively maintained in the apparent absence of Bb, could conceivably be sampling self Ags, resulting in the secretion of Abs with progressively higher binding affinities into the closed joint space. Based on the rationale that B cells within the ectopic follicles are undergoing a GC reaction and differentiating into plasma cells, an analysis of IgGs synthesized by the latter can provide insights into the nature of the Ab response that develops within the diseased joint, in the context of chronic inflammation, as well as its antigenic targets. Our previous study of Ig sequences expressed within synovial lesions of antibiotic treatment-resistant Lyme arthritis patients reveals the hallmarks of an affinity-matured response occurring in situ, with a degree of restriction of the Ig repertoire, expansion and diversification of specific clones, somatic hypermutation, Ag-mediated selection of Ig V domains, and class switching to the traditionally more pathogenic IgG isotype (16).

Given the evidence for a sustained in situ Ig response, in this study we sought to determine the identity of Ag(s) that is responsible for its perpetuation. Intratissue lymphoid follicles in other autoimmune diseases, such as rheumatoid arthritis (RA) and Hashimoto’s thyroiditis, have been associated with the production of Abs that recognize local, disease-relevant Ags, underlining the pathologic significance of the locally generated Ig response (17, 18). Using Ig V domains selected from our ensemble of sequences,
single-chain V region fragments (scFvs) were synthesized and used to generate affinity matrices. We screened human synovial tissue lysates for the isolation of cognate ligands that bind Lyme-derived scFvs, leading to the identification of cytokeratin (CK)-10, a scaffolding protein present in the intima of synovial blood capillaries (19, 20), as a candidate autoantigen in chronic, antibiotic treatment-resistant Lyme arthritis. Additionally, scFvs specific for outer surface protein A (OspA), an abundant Bb Ag, also bound CK10. This cross-reactivity suggests a degree of antigenic mimicry that might contribute to setting the stage for autoimmunity, sustaining the response originally elicited by Bb, long after elimination of the spirochete.

**Materials and Methods**

**Clinical samples**

Synovial tissue samples came from an archival collection, obtained from Lyme arthritis patients who had persistent arthritis after antibiotic treatment and had undergone synovectomy. Synovial tissue from two patients, A and B, were selected for detailed analysis, based on a previous report documenting marked inflammatory and lymphocytic infiltration in the synovium (11). Duration of arthritis before antibiotic therapy was 1 mo for patient A and 6 mo for patient B. Both patients were treated with oral doxycycline for a period of 30 days, as well as oral amoxicillin (patient A) and I.V. ceftriaxone (patient B). After antibiotic therapy, synovial samples from A and B were negative in PCRs done to detect Bb DNA. Duration of arthritis posttreatment and before synovectomy was ≈4 mo in both of the cases. A frozen aliquot (stored in liquid nitrogen) of PBMCs from another treatment-resistant Lyme arthritis patient, C, who was seropositive for OspA by Western blot, was used to isolate OspA-specific B cells from circulation. Serum samples for assaying levels of anti-Ck10 IgG came from a panel comprising 15 treatment-resistant Lyme arthritis patients, 5 treatment-responsive Lyme arthritis patients, 5 RA patients (synovial fluid obtained from 4 other RA patients (courtesy of J. Sicat, Tufts University, Boston, MA) were also included in the panel), and 16 healthy control subjects. All synovial tissue and blood samples from treatment-resistant Lyme arthritis patients were contributed by A. Steere (Harvard Medical School, Boston, MA). All human studies have been reviewed and approved by the Institutional Review Board at Tufts/New England Medical Center.

**Isolation of synovial plasma cells, Ig V region RT-PCR, and sequence analysis**

These are described in detail elsewhere (16, 21).

**OspA-specific B cells and associated V regions**

**Identification of OspA-specific B cells from peripheral blood.** OspA was labeled with Alexa 488, as per the manufacturer’s protocol (Molecular Probes). A vial of frozen PBMCs from patient C was rapidly thawed and washed three times in FBS (Atlanta Biologicals). Cells were stained with CyChrome-conjugated anti-human CD19 (BD Biosciences) and OspA: Alexa 488 in FACS buffer, comprising 20% FBS in 1× sterile Dulbecco’s PBS (Invitrogen Life Technologies). PBMCs from an RA patient, RA1, were processed in parallel. Samples were incubated in the dark on ice and washed three times in FACS buffer, followed by single-cell sorting by FACS.

**In vitro production of Ig in the EL4-B5 system.** The EL4-B5 thymoma line (22, 23) (courtesy of R. Zuber, University Hospital, Geneva, Switzerland, and M. Corazza, Medaurex, Milpitas, CA) was cultured in standard tissue culture medium: RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM 2-ME. Cell density was not allowed to exceed 2 × 10^7 cells/ml to maintain the Th function of the thymoma line. For the in vitro assay, a requisite number of EL4-B5 cells was irradiated at 5000 rad, and 5 × 10^6 irradiated cells in 100 μl of medium were added per well to 96-well tissue culture plates (Corning Glass). A total of 100 μl of activated T cell supernatant (T-SN), at a concentration of 10%, was also added to each well. The activated TSN, containing a mixture of T-derived, activation-induced cytokines, was prepared as follows: nylon wool columns (Polysciences) were washed in medium and preincubated for 1 h at 37°C. Freshly isolated human PBMCs were applied onto prewashed columns and incubated for 1–1.5 h at 37°C to allow for binding of B cells to the matrix. Following this, the columns were washed and T cells were collected in the flow-through and washed (purity of >96%, as tested by FACS). The T cells thus obtained were activated at 1 × 10^6 cells/ml, using PHA at 5 μg/ml and PMA at 10 ng/ml for 36–40 h. Following this period, T cells were spun down and the activated T-SN was passed through 0.22-μm filters, aliquoted, and stored at −80°C. OspA CD19+ cells were singly sorted into 96-well plates containing the EL4-B5 cells and activated T-SN, followed by −10 days in culture at 37°C. Supernatants from these cultures were tested in parallel for the production of IgG and OspA-specific IgG by the use of specific ELISAs.

**IgG and OspA-IgG ELISA.** Nunc-Immuno MaxiSorp ELISA plates (Nunc) were coated overnight at 4°C with goat anti-human IgG (Southern Biotechnology Associates) or OspA at a concentration of 2 μg/ml in binding buffer (0.1 M NaHPO4 (pH 9)). The plates were washed in PBS/0.05% Tween 20 (PBS/T) and blocked in 2% BSA (Sigma-Aldrich) for 2 h. Culture supernatants were added in duplicate to the ELISA plates and incubated at room temperature for 2 h. Alkaline phosphatase (AP)-conjugated goat anti-human IgG (Southern Biotechnology Associates) at a 1/2000 dilution for the detection. The ELISA was developed using the AP substrate, paranitrophenyl phosphate (Pierce), in 0.2% diethanolamine substrate buffer (Pierce) for 15 min, and plates were read at 405 nm.

**Ig RT-PCR and sequencing.** RNA was isolated from clones that yielded positive results in the OspA-IgG ELISA, using the RNAeasy kit (Invitrogen Life Technologies). Amplification of expressed Ig Vg4 and Vh regions was conducted, followed by cloning of the PCR products into TOPO-TA vectors (Invitrogen Life Technologies) and sequencing of plasmid mini-preps, as described previously (16).

**Production of scFv**

Ig Vg4 and Vh regions were cloned into the pg20 vector, the VH domain upstream of the corresponding VH domain, separated by a flexible (GGGGS) linker sequence and under the transcriptional control of a T7 RNA polymerase promoter (24). A 5′-XhoI restriction site (CCCCGGG) and a 3′-XbaI restriction site (TCTAGA) were incorporated into the Vh product by an additional PCR, while primers for the second PCR amplification for the VH regions contained 5′-BglII (AGATCT) and 3′-NcoI (CCATGG) sites. Vg4 and Vh regions were sequentially incorporated into the pg20 vector by digesting with each pair of restriction enzymes, followed by ligation with T4 DNA ligase (USB). A 5′ secretory bacterial AP leader peptide (phoA) enabled secretion of the recombinant protein, and a 3′ protein A tag allowed for purification using IgG-Sepharose (24). Plasmids containing the recombinant scFv constructs were used to transform BL21(DE3)E. coli (Invitrogen Life Technologies), which were then grown on Luria-Bertani-agar plates containing ampicillin (200 μg/ml) and chloramphenicol (20 μg/ml). Isopropyl-β-D-thiogalactopyranoside-sensitive colonies were identified by replica plating, followed by inoculation of selected colonies into liquid culture and incubation with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37°C. Bacterial cultures were spun at 5000 × g for 20 min at 4°C in an ultracentrifuge and supernatants were collected. Large-scale protein preparations were conducted at the Center for Gastroenterology Research on Absorptive and Secretory Processes, Tufts University (courtesy of A. Kane). IgG-Sepharose beads (Amersham Biosciences) were activated by alternating washes in 50 mM Tris/150 mM saline/0.05% Tween 20 (pH 7.6) and 0.5 M acetic acid (pH 3.4), as per the manufacturer’s instructions. Supernatants were filtered and applied onto the matrix, followed by extensive washing in 50 mM Tris/150 mM saline/0.05% Tween 20, a short wash in 5 mM ammonium acetate (pH 5), and elutions at a low pH, using 0.5 M acetic acid. Each elution was neutralized to pH 7–80°C. OspA by Western blot, was used to isolate OspA-specific B cells from

**Preparation of tissue lysates**

Snap-frozen samples of normal, cadaveric synovium were obtained from the Cooperative Human Tissue Network (University of Pennsylvania). Frozen tissue was dipped in liquid nitrogen and ground in a prechilled mortar and pestle. Powdered tissue fragments were suspended in chilled lysis buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl2, 2 mM EDTA, 1% Nonidet P-40, protease inhibitor mixture (Sigma-Aldrich), 5 mM sodium fluoride, and 2 mM sodium vanadate). The suspension was incubated for 30–45 min at 37°C, followed by centrifugation at 10,000 g for 25 min. The tissue supernatant was collected, aliquoted, and stored at −80°C. Protein in the tissue extract was estimated using the bichinonic acid kit (Pierce).
Generation and operation of affinity orientation columns

cScFv affinity orientation columns were generated using a modified version of the Seize X Immunoprecipitation kits (Pierce), using IgG-Sepharose as the column matrix, instead of the provided protein A beads. Approximately 60–500 μg of each scFv in 1× PBS was added to 260 μl of IgG-Sepharose beads in a miniSeize X spin column (provided in the kit) and incubated overnight at 4°C with end-over shaking. The column was then washed three times in PBS, and 25 μl of the cross-linking agent, disuccinimidyl suberate (DSS) dissolved in dimethyl formamide, was added to the beads, followed by end-over shaking of the column for 1 h at room temperature. The reaction was extensively quenched by 5× washes in the provided elution buffer, containing primary amines at pH 2.8, followed by 3× washes in PBS to restore neutrality. The prepared columns were sealed with parafilm and stored at 4°C until further use. Approximately 250 μg of normal synovial extract, in a volume of 400 μl, was added to each column and incubated overnight at 4°C, with end-over shaking. The column was washed five times in PBS and eluted in the elution buffer (pH 2.8). Three elution fractions of 150 μl each were collected and neutralized to pH ~7, using 1 M Tris buffer. Wash and elution fractions were run on SDS-PAGE and stained using a silver-staining kit (Amersham Biosciences) to visualize bound proteins.

Preparation of samples for mass spectroscopy (MS)

For the analysis of proteins by MS, gels were stained using a MS-compatible silver-staining kit (Invitrogen Life Technologies), which omits the formaldehyde fixation step common to most silver-staining protocols. Selected gel bands were excised with clean scalpels, destained, and stored in Eppendorf tubes at 4°C until further processing. Gel bands were cut into 1-mm3 pieces and washed twice for 15 min with 50% acetonitrile (ACN)/water. Gel pieces were reduced for 1 h at 56°C with 10 mM DTT in 100 mM ammonium bicarbonate (pH 8) and alkylated for 45 min at room temperature (protected from light) with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (pH 8). Gel pieces were washed twice for 15 min with 50% ACN, dried in a centrifugal concentrator and resuspended with 15–20 μl of 30 mM ammonium bicarbonate (pH 8)) on ice, and incubated overnight at 37°C. Peptides were collected by removing the solution from the gel pieces to a clean tube, followed by three extractions of 30 min each with 0.1% trifluoroacetic acid (TFA) in water, 25% ACN/0.1% TFA, and 50% ACN/0.1% TFA at room temperature. Pooled extracts were dried in a centrifugal concentrator and resuspended with 15–20 μl of 50% ACN/0.1% TFA. Peptides were analyzed by MALDI-TOF MS on a Bruker Reflex IV (Bruker) operated in positive ion mode using α-cyano matrix. Peptide mass fingerprint analysis was performed using the Mascot search engine (Matrix Science).

CK ELISAs

Recombinant CKs, CK10 (CK10 and rabbit polyclonal CK10 antiserum; courtesy of T. Foster and E. Walsh, Trinity College, Dublin, Ireland), CK8, and CK18 (Research Diagnostics), were used to coat ELISA plates overnight at 4°C at a concentration of 5 μg/ml in binding buffer. Plates were also coated with 5 μg/ml BSA, as a negative control protein. The plates were washed in PBS/T and blocked in 2% BSA/PBS/T for 2 h. For the detection of scFv binding, plates were incubated for 1.5 h with each scFv in duplicate, diluted in blocking buffer. HRP-conjugated chicken anti-CK ELISAs (Research Diagnostics) were used at a dilution of 1/1000 for detection of the protein A tag on the scFvs. The plates were developed using the HRP substrate, tetramethylbenzidine (Research Diagnostics), for 5–10 min. The enzymatic reaction was stopped using 2 N sulfuric acid, and plates were read at 405 nm. For the serum ELISAs, 1/100 diluted serum samples were incubated in duplicate on CK10-coated plates for 1.5 h. AP-conjugated goat anti-human IgG (Southern Biotechnology Associates) was used at a 1/2000 dilution for detection. The ELISA was developed for ~15 min using paranitrophenyl phosphate, and plates were read at 405 nm.

Statistics

ELISA data from the clinical serum samples were analyzed using a cutoff value equal to mean of healthy controls plus 3 SDs (OD units = 0.33) to distinguish positive from low/negative samples. Results were used to generate a 2 x 2 contingency table in a commercially available statistical

FIGURE 1. Generation of scFvs. a, Summary of the source and techniques used in the isolation of Ig sequences from patients, to be cloned as scFvs; indirect immunofluorescence laser-capture microdissection (IF-LCM). b, Table of scFvs generated from antibiotic treatment-resistant Lyme arthritis patients; syn, synovium; pb, peripheral blood. c, Depiction of the plg20 plasmid and scFv construct, under transcriptional control of the T7 promoter; phoA, secretory peptide from bacterial AP.
software package (GraphPad Prism). Numbers of different patients in specific subgroups were analyzed from contingency tables using Fisher’s exact test. Statistical significance was assumed for $p$ values ≤0.05.

**Results**

**Generation of scFvs**

**Synovial Ig probes.** Synovial plasma cells were identified by indirect immunofluorescence for syndecan-1 (CD138) on frozen tissue sections, obtained from two treatment-resistant Lyme arthritis patients, A and B (Materials and Methods). Targeted analysis of plasma cells within focal lesions and the availability of only archival tissue sections necessitated a microdissection approach. Syndecan-positive cells were isolated using laser-capture microdissection, followed by RT-PCR amplification of their expressed IgGs and detailed sequence analysis, as described previously (16, 21, 25). Ig V domains were selected for synthesis as scFvs based on the following criteria: overrepresentation within the synovial repertoire, affinity maturation, evidence for clonal expansion, and/or intraclonal diversification within the synovium (Fig. 1, a and b) (16).

**OspA-specific Ig probes.** Borrelial OspA has been implicated in the pathogenesis of treatment-resistant Lyme arthritis, increased titers of Abs to OspA marking the transition from the initial, acute arthritis to chronic joint disease (26, 27); hence, the anti-OspA IgG response was also of interest to us. We generated OspA-specific scFvs from PBMCs of a treatment-resistant patient (patient C; Materials and Methods), using a well-characterized system: first, Ag-specific B cells were identified with labeled OspA (OspA-Alexa 488) and singly sorted by FACS; second, isolated B cells were expanded in vitro with the help of a specialized thymoma line (EL4-B5), which, in the presence of a mixture of activated T-SN, drives the differentiation of B cells to the plasma cell stage (22, 23); and finally, supernatants from the monoclonal plasma cell cultures thus obtained were tested in an ELISA for the production of OspA-specific IgG (data not shown). This allowed for two specificity screens: one at the level of surface Ig binding to the labeled Ag, visualized by FACS, and the other at the level of actual production of OspA-specific IgGs, as assessed by ELISA. Next, we isolated RNA from OspA-IgG-positive wells and prepared OspA-specific V regions using Ig RT-PCR amplification, as performed on the synovial plasma cells (16) (Fig. 1c).

$V_H$ and $V_L$ were cloned into the plg20 vector, the $V_H$ domain upstream of the corresponding $V_L$ domain, connected through a flexible linker sequence (24) (Fig. 1c). Given the infrequent incidence of glycosylations in Ig V regions, an *E. coli* system was used for the production of our joint-derived and PBMC-derived scFvs. A C-terminal protein A tag allowed for both the purification of
recombinant proteins, as well as their detection/manipulation in subsequent biochemical assays. We generated a collection of seven such monoclonal scFvs: three from patient A synovium (2-1, 2-2, and DC7), two from patient B synovium (5-1g4 and 5-1g5), and two OspA-specific scFvs from patient C PBMCs (3-24 and 8-10) (Fig. 1c). The topological arrangement of V_{H}V_{L} domains in an scFv represents the Ag binding site of the corresponding Ab (28). Although synovial scFvs recapitulate the ligand-binding specificities of potentially pathogenic Abs produced at the site of injury in chronic Lyme arthritis patients, the anti-OspA scFvs are important as probes for putative molecular mimics of the bacterial protein.

Identification of CK10 as an affinity ligand

As an initial screen, using both ELISAs and Western blots, we tested and ruled out reactivity of our Ab fragments with some common autoantigens, such as collagen (29), glucose-phosphate isomerase (30), and deiminated fibrinogen (31), which have been implicated in other chronic arthritides such as RA (data not shown). LFA-1 has been previously proposed as a candidate T cell autoantigen in antibiotic treatment-resistant Lyme arthritis, with a role for molecular mimicry between an immunodominant OspA epitope and a homologous LFA-1/H9251 peptide (8). Although direct binding to the purified LFA-1/H9251 chain (CD11a) was not assessed, we immunoblotted activated T cell lysates with our panel of scFvs for the identification of specific protein bands, LFA-1 being an adhesion molecule that is prominently up-regulated on activated T cells (32). One of the Lyme-derived scFvs selectively and reproducibly bound two protein bands, running at 36 and 38 kDa, present primarily in activated T cells (compared with resting T cells or activated B cells) (data not shown). Although the identity of these ligands is of interest, limited amounts of protein, affinity purified using the cognate scFv, have to date precluded their identification by MS. Human LFA-1α runs at ~180 kDa on SDS-PAGE; the absence of specific protein bands in this molecular mass range suggests that LFA-1 is unlikely to be an Ag recognized by our current collection of Ab fragments.

![Graphs showing specific binding of scFvs to CK10 and OspA](http://www.jimmunol.org/Download/graphics)
Next, we generated affinity matrices with the panel of recombinant probes, using the C-terminal protein A tag to tether and cross-link scFvs to IgG Sepharose beads via a protein cross-linker (DSS), leaving the Ag-binding domain exposed to solvent (Fig. 2a). Human joint tissue lysates were run on scFv columns, to screen for ligands present within the synovial microenvironment. An affinity column comprising an scFv derived from patient A (2-2; Fig. 1c), when loaded with extract from normal human synovial tissue, reproducibly yielded a specific band at $\approx 100$ kDa in the first elution fraction, compared with 3-24, an OspA-specific scFv (Fig. 2b). Upon MALDI-TOF MS and peptide mass fingerprint analysis, CK10 and CK1, respectively, were identified with the most significant Mascot scores from the $\approx 100$-kDa band (Fig. 2, b and c; data not shown). No significant CK scores were obtained from the other six bands that were excised alongside the $\approx 100$-kDa band. Because CK10 runs at $\approx 56$ kDa on SDS-PAGE and CK1 at $\approx 65$ kDa, we hypothesized that the $\approx 100$-kDa band could possibly be an incompletely reduced dimer of the two forms. Next, the gel was rerun, and a $\approx 50$-kDa band from the same elution fraction was excised and analyzed, based on the premise that this could be the CK10 monomeric form (Fig. 2b). MALDI-TOF MS analysis indeed yielded CK10 as the most significant score for this band. Additionally, the elutions were immunoblotted with rabbit polyclonal antiserum generated against CK10, to confirm the identity of the affinity ligand (Fig. 2d). Somewhat unexpectedly, 3-24 also bound CK10, and 2-2 appeared to be a relatively weak binder. Unlike the other scFvs, in which the affinity column contained $60-100$ g of the immobilized probe, the 2-2 affinity column contained a large excess, $500$ g, of the scFv. Possibly the large amount of the scFv probe allowed for the capture of detectable amounts of CK10, despite a weak interaction.

For an unambiguous assessment of binding interactions with the protein, we tested our panel of scFvs for direct binding to rCK10 in an ELISA. The 2-1 was the strongest binder, while the others bound the protein to varying degrees, compared with an irrelevant, non-Lyme control, C22 (Fig. 3a). The scFvs did not bind at equivalent levels to two other rCKs that were tested, CK8 and CK18, which share 28 and 40% sequence homology, respectively, as assessed by

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Clinical serum samples were tested for CK10 binding. A panel of serum samples from three separate patient groups and healthy controls was tested at a 1/100 dilution for CK10 binding in an ELISA. Goat anti-human IgG conjugated to AP was used for detection. A cutoff value of 3 SDs above the mean of the healthy controls (OD = 0.33) yields a frequency of 3 of 15 treatment-resistant Lyme arthritis patients (20%) that are positive for anti-CK10 IgG, vs none of the three control groups (0%). Net ODs, i.e., OD obtained with CK10 minus OD obtained with BSA for a given serum sample, have been graphed.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** OspA Vs CK10 binding by clonally related OspA-specific scFvs from patient C peripheral blood. **a**, Specific binding of OspA by supernatants of the 3-24 and 8-10 in vitro EL4-B5 plasma cell cultures. Specific binding refers to the OspA binding units per ng of IgG in the culture supernatant, as assessed by an OspA-IgG and total IgG ELISA, respectively. OspA binding was arbitrarily standardized against an OspA-specific mouse mAb (LA2), which was run in parallel, and detected using a mouse-specific secondary Ab. One binding unit is equal to the OD given by 100 ng of LA2 in the same OspA-IgG ELISA. **b**, OspA- and CK10-specific binding in corresponding ELISAs by purified scFvs 3-24 and 8-10. Inset, Ratio of ODs in an OspA-specific or CK10-specific ELISA, given by scFvs 3-24 and 8-10. **c**, Pairwise BLAST alignment of OspA and CK10; *, denotes conservation of chemical nature for the corresponding amino acid residues.
Proposed model depicting a feed-forward loop in the production of CK10 Abs in the inflamed synovial milieu. E, endothelial cell; B, B cell; Mφ, macrophage; Nφ, neutrophil; P, plasma cell; T, T cell; and bc, blood capillary.

**FIGURE 6.** Proposed model depicting a feed-forward loop in the production of CK10 Abs in the inflamed synovial milieu. E, endothelial cell; B, B cell; Mφ, macrophage; Nφ, neutrophil; P, plasma cell; T, T cell; and bc, blood capillary.

**Discussion**

Lyme arthritis begins with intermittent episodes of joint swelling and pain, typically affecting the large joints, especially the knee (5, 34). *Bb* lipoproteins like the Osps (including OspA) are potent inducers of proinflammatory cytokines, chemokines, proteolytic enzymes, and free radicals; the affected joint thus develops into a site of intense inflammatory activity (3, 35). It has become increasingly clear that this initial, acute phase constitutes a necessary first step toward progression to chronic arthritis, the strong anti-*Bb* response being attributed an adjuvant effect that could set the stage for the eventual development of persistent pathology (35, 36). Our previous analysis of synovial Ig sequences points toward a dynamic modulation of the Ab response within artitic lesions, with a focusing of the repertoire, clonal diversification, and Ag-driven affinity maturation (16). The local response at the site of injury suggests a role for Abs in driving chronic joint inflammation, via the recognition of putative self Ags that are exposed or up-regulated in the prevailing inflammatory milieu. In this study, we have generated scFvs, representative of synovial IgGs, to screen human joint tissue lysates and identified CK10 as an antigentic target of this in situ response.
CKs are members of the intermediate filament family, a diverse group of cytoskeletal proteins that maintain the intracellular, structural scaffolding in eukaryotic cells (37). Abs to cytoskeletal elements in general, and CKs in particular (although not CK10), have been observed in several human diseases such as RA, systemic lupus erythematosus, and psoriatic arthritis (33, 38–40). Although CK autoantibodies are clearly not unique to Lyme arthritis, CK10 appears to be a relatively specific autoantigen, given its cross-reactive potential with Bb OspA and the observed CK10-selective binding by our panel of scFvs (Fig. 3). Although there are no obvious structural similarities between CK10 and OspA, alignment of their primary sequences reveals discontinuous stretches of amino acid homology, which could constitute shared, conformational epitopes. Interestingly, a pairwise BLAST with CK10 assigned the highest homology scores to a C-terminal fragment of OspA, encompassing residues 189–273 (Fig. 5c). IgG responses elicited by epitopes in this region, mainly OspA168–273, have been shown to correlate directly with both severity and duration of chronic Lyme arthritis (27).

CK10 (with its dimeric partner, CK1) is reportedly present in the squamous cells that constitute the endothelial layer of synovial blood capillaries, the neovascularity observed in chronic Lyme arthritis probably enhancing its presence within the inflamed joint (19, 20, 41, 42). The initial, acute arthritis, triggered by Bb, with the accompanying release of numerous proinflammatory mediators, most likely causes endothelial cell damage, and hence, release of CK10. Although recognition of the self protein by our OspA-specific scFvs suggests a role for antigen mimicry in initiating the anti-CK10 response, bystander mechanisms could also come into play, given that Bb lipoproteins such as OspAs A and B are capable of stimulating B cells nonspecifically (43). Tolerogenic checkpoints in B cell development appear to be less efficient at eliminating autoantibodies recognizing cytosolic components, attributable possibly to the relative inaccessibility of these Ags; hence, a subset of mature B cells continues to encode low-affinity, anticytosolic Igs (44). Polyclonal stimulation can result in the expansion of hitherto quiescent, self-reactive B cells; these can bind their cognate Ag(s) from among the array of self proteins released in the context of inflammation-induced cell death and undergo the GC reaction, resulting in the generation of high affinity, self-reactive Abs with greater pathogenic potential. Indeed, a majority of our synovial V domains and the synthesized scFvs were highly mutated, and scFv 2-1, the strongest CK10 binder (Fig. 3), came from a plasma cell cluster that underwent intracellular diversification in patient A synovium (16). We envisage a feed-forward loop, wherein a local response to the protein further amplifies the ensuing inflammatory process, which thus becomes self-perpetuating and persists in the absence of the pathogen, the latter being largely eliminated either as a result of intense inflammation or antibiotics or both (Fig. 6).

In conclusion, CK10 is an autoantigenic target of Abs that are expressed within synovial disease lesions associated with chronic, antibiotic treatment-resistant Lyme arthritis. There is evidence for a role of other Ags as well, such as LFA-1, and as yet unidentified proteins in activated T cell lysates (data not shown). Development of persistent joint inflammation following Bb infection is most likely to arise through a complex interplay of multiple factors, such as the host genetic background, period of spirochetalemia before antibiotic treatment, the innate and adaptive responses to Bb, and involvement of a subset of self Ags, rather than a single, dominant protein (4, 26). Further screening using an expanded collection of scFvs, generated from our synovial Ig sequences, could shed more light on additional disease-relevant Ags in chronic Lyme arthritis. Because it has a known inciting agent, a dissection of molecular processes that sustain pathology could contribute to a better understanding of autoimmune mechanisms in general, both Ag specific and nonspecific, that drive pathogenesis in other chronic arthropathies and autoimmune diseases.

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Disclosures

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