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Clonal Diversification in OspA-specific Antibodies from Peripheral Circulation of a Chronic Lyme Arthritis Patient

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Abstract

Chronic, antibiotic treatment-resistant Lyme arthritis develops in a subset of patients following infection with the tick-borne spirochete *Borrelia burgdorferi* and persists after apparent microbial clearance. IgG responses to Outer Surface Protein (Osp) A, an abundant spirochetal lipoprotein, correlate with both severity and duration of joint inflammation. Characterization of this OspA-directed antibody response is, therefore, important for understanding some of the mechanisms that sustain persistent pathology. Such analyses in Lyme arthritis patients have been previously hampered by relatively small amounts of clinical blood samples, as well as the general intractability and low success rates of B-cell immortalization procedures. Here we describe a robust method for generation of OspA-specific monoclonal antibody fragments from archival cell samples employing a three-step procedure – isolation of single OspA-specific B-cells, their *ex vivo* clonal expansion and production of expressed immunoglobulins as single chain variable region fragments (scFvs). Interestingly, two of three scFvs generated from a single patient were of a common clonal origin, additional somatic mutations in the downstream member resulting in a concomitant modulation of antigen binding affinity. Computational docking of OspA into corresponding Fv domains, generated by molecular modeling, reveals subtle binding site differences which could account for the observed alteration in ligand binding. Besides their utility as standards in routine diagnostic assays, being the first described OspA-specific human monoclonal reagents, these scFvs are useful tools for analysis of the anti-OspA repertoire in patients and for identification of putative human mimics of the bacterial protein.

Keywords

Anti-OspA; single B-cell; human monoclonals; chronic Lyme arthritis

Introduction

Lyme disease is a complex, multifactorial illness caused by *Borrelia burgdorferi* (*Bb*), a spirochete transmitted through the bite of infected *Ixodes* ticks (Burgdorfer et al., 1982; Steere, 2001; Steere et al., 2004). *Bb* disseminates rapidly via its numerous adhesins and affects multiple organ systems (Steere et al., 2004). Infection of the joints results in Lyme arthritis, a major late-stage manifestation of the disease (Steere, 2001; Steere et al., 2001). In a majority of patients, arthritic symptoms resolve following elimination of the pathogen through oral and/or intravenous antibiotic therapy (Steere et al., 1994). However, in ~10% of affected cases,

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joint inflammation persists despite antibiotic treatment regimens and apparent absence of infection, pointing towards self-sustaining autoimmune phenomena downstream of microbial containment (Gross et al., 1998;Gross et al., 2001;Steere et al., 2001).

Development of chronic arthritic sequelae has long been associated with the induction of cellular and humoral immune responses to *Bb* OspA, a prominent lipoprotein on the spirochetal outer envelope (Gross et al., 1998;Meyer et al., 2000). In about 70% of Lyme arthritis patients, IgG responses to OspA develop near the beginning of prolonged periods of arthritis and correlate with both severity and duration of inflammation, no such correlation being reported with IgM levels (Kalish et al., 1993;Akin et al., 1999); IgG titers elicited by the C-terminal fragment of the protein (OspA₁₆₈₋₂₇₃) have the strongest association, suggesting a causal role for the humoral response in sustaining chronic inflammation (Akin et al., 1999). The anti-OspA response has, thus, been under considerable investigation for its potential role in the pathophysiology of chronic, antibiotic treatment-resistant Lyme arthritis (Klempner and Huber, 1999;Guerau-de-Arellano and Huber, 2002).

Mouse models exist for the initial subacute arthritis observed in humans (Weis, 2002); however, the current lack of a murine model for the chronic phase necessitates its study exclusively through human clinical samples, most of which are archival, having been collected at discrete time points during the course of disease. Traditionally, immunorepertoires have been studied using (i) classical hybridoma technology, with associated issues of laborious screening, subcloning and intrinsic genetic instability of hybridoma fusions; (ii) transformation of B-cells with Epstein Barr Virus (EBV) to generate lymphoblastoid cell lines (LCLs), the disadvantage of this technique being preferential immortalization of select B-cell subsets, resulting in biased sampling; and (iii) combinatorial phage display libraries, which enable quick and efficient selection of antibody fragments recognizing a wide variety of antigens, but with the obvious drawback of being unable to pinpoint original, *in vivo* Ig heavy (H) and light (L) chain pairings (Cole et al., 1984;Niedbala and Stott, 1998;Little et al., 2000;Kretzschmar and von Ruden, 2002). More recently, some of these limitations have been bypassed by the EL4-B5 system, which has been used in a number of disease scenarios, including rheumatoid arthritis (RA) (Rudolphi et al., 1997), systemic lupus erythematosus (SLE) (de Wildt et al., 1997) and systemic sclerosis (Weber et al., 2003). EL4-B5 cells, representing a mutant subclone of the mouse EL4 thymoma line, drive the *in vitro* differentiation of murine and human B-cells to the plasma cell stage, allowing for secretion of Igs in culture (Grimaitre et al., 1997). This process requires the presence of activated T-cell derived cytokines and is mediated largely by CD40-CD40L binding, although other cognate interactions are also believed to play a role (Wen et al., 1987;Grimaitre et al., 1997).

Here we use this system to focus on single, unmanipulated OspA-specific B-cells in the peripheral circulation of a treatment-resistant Lyme arthritis patient, breaking down the polyclonal anti-OspA serum IgG pool into some of its monoclonal constituents. Specifically, our approach encompasses the following steps - (a) identification and isolation of single OspA-specific B-cells by FACS, (b) clonal expansion of sorted cells in the EL4-B5 system, followed by screening of secreted Igs for OspA-reactivity, (c) RT-PCR amplification of expressed Ig H and L variable (V) regions (VH & VL) and (d) *in vitro* production of VH and VL domains as single chain variable region fragments (scFvs), using a bacterial system. Recombinant scFvs thus generated bound OspA, both in its purified state, as well as on the surface of permeabilized *Bb*. Remarkably, molecular analysis of Ig V-region sequences reveals a high degree of clonal relatedness between two of the scFvs produced. Intracлонаl diversification appears to have led to corresponding changes in OspA binding, illustrative of a process of affinity maturation impacting this clone.

Materials and methods

Clinical samples

Frozen peripheral blood mononuclear cells (PBMCs) from treatment-resistant Lyme arthritis patient, C (courtesy of Dr. Allen Steere, Harvard Medical School) and samples #0613 and #4190 from a Food and Drug Administration (FDA) OspA vaccine study (courtesy of Dr. Abbie Meyer, Tufts University School of Medicine) were used in this report. Patient C and sample #0613 were seropositive for OspA by western blotting; sample #4190 and PBMCs freshly isolated from blood donated by a rheumatoid arthritis patient (RA1) were seronegative for OspA. This study was approved by the Institutional Review Board of Tufts-New England Medical Center, Tufts University Health Sciences (Protocol #4074).

Isolation of OspA-specific B-cells and in vitro Ig production

OspA, produced as a Glutathione-S-Transferase (GST)-fusion protein, was purified using glutathione-Sepharose (Pharmacia Amersham Biosciences), the GST tag cleaved off by thrombin and recombinant OspA labeled with Alexa 488 (Molecular Probes, OR). Frozen PBMCs were rapidly thawed, washed 3X in fetal bovine serum (FBS) (Atlanta Biologicals, GA) and stained with cychrome-conjugated anti-human CD19 (or anti-mouse B220 for murine splenocytes) (BD Biosciences Pharmingen, NJ) and OspA:Alexa 488 in FACS buffer, comprising 20% FBS in 1X sterile Dulbecco's phosphate buffered saline (DPBS, Gibco, Invitrogen, CA). Samples were incubated in the dark on ice, washed 3X in FACS buffer, followed by single-cell sorting by FACS. Single cells were cultured with 5×10^4 irradiated (5000 rads) EL4-B5 thymomas (courtesy of Dr. Rudolf Zubler, University Hospital, Geneva and Dr. Marco Coccia, Medarex Inc, CA) and a cocktail of activated T-cell cytokines, as per the recommended protocol (Wen et al., 1987; Grimaitre et al., 1997). After ~10 days, culture supernatants were added in duplicate to either OspA- or anti-human IgG-coated (at 2 µg/ml in 0.1M Na₂HPO₄, pH=9) ELISA plates (Nunc, Rochester, NY) which had been blocked using 2% Bovine Serum Albumin (BSA) in PBS/0.05% Tween 20. Alkaline phosphatase conjugated goat anti-human IgG (Southern Biotech, Birmingham, AL) was used at a 1:2000 dilution for detection. Plates were developed using para-nitrophenyl phosphate (PNPP) (Pierce, Rockford, IL) in 0.2% diethanolamine substrate buffer (Pierce, Rockford, IL) for 15' and read at 405 nm.

Ig RT-PCR, sequencing and scFv production (Described in detail elsewhere) (Polymenis and Stollar, 1995; Wang and Stollar, 2000; Ghosh et al., 2005; Ghosh et al., 2006)

Briefly, RNA was isolated from OspA⁺ IgG⁺ clones using the RNeasy kit (Invitrogen, CA). Synthesis of cDNA, followed by 2 rounds of nested PCRs (using primer sets complementary to sequences in Ig constant regions and VH or VL family leaders for the 1st PCR and VH or VL family FWR1 for the second PCR), allowed for amplification of expressed VH and VL regions. PCR products were cloned into TOPO-TA vectors (Invitrogen, CA) followed by sequencing of plasmid minipreps (Tufts University Core Facility) and detailed sequence analysis. For scFv production, VH domains were cloned into the pIg20 vector, upstream of VL domains and connected through a flexible (GGGGS)₃ linker sequence (Polymenis and Stollar, 1995). The construct, under transcriptional control of a T7 RNA polymerase promoter, was used to transfect BL21(DE3)pLysE *E. Coli* (Invitrogen, CA). Isopropyl-β-D-thiogalactopyranoside (IPTG)-induction allowed for production of recombinant protein, most of which was secreted owing to a 5' phoA tag. ScFvs, thus generated also contained a 3' protein A tag, enabling their purification using IgG-Sepharose columns (Pharmacia Amersham Biosciences).

Bb immunofluorescence

Overnight *Bb* cultures in Barbour-Stoenner-Kelly (BSK) medium, supplemented with 6% rabbit serum, were spun down at 4000xg for 20' at 4°C, briefly washed and resuspended in 1X PBS. 20 µl aliquots were spotted onto Superfrost glass slides (Fisher) and air-dried for 1h in a 34°C incubator. Spots were fixed by immersion in methanol for 10', dried for 10–15', followed by two washes in 1X PBS for 2' each. Each *Bb* spot was incubated with individual scFvs at a concentration of 3 µg/ml, 100 µl per spot, for ~1h at 34°C, followed by two 5' washes in PBS, and then incubation in rabbit IgG:Alexa 488 (Molecular Probes) at a 1:500 dilution for 1h in the dark. Slides were washed thrice in PBS, mounted, and visualized.

Analysis of Ig sequences and mutations

For identification of the closest germline matches, sequences were aligned against Ig genes in the International ImMunoGeneTics (IMGT) database (Giudicelli et al., 2004; Lefranc, 2004) using the V-QUEST program (<http://imgt.cines.fr/textes/vquest/>). All sequences were deposited with GenBank under the accession numbers EF028212 through EF028224. CDRs and FWRs were defined based on the Kabat numbering system. (Johnson and Wu, 2001). The first eight codons in FWR1, being primer-encoded, were excluded from analysis. Mutations were calculated for CDRs 1, 2 and 3 for V_L sequences, and CDRs 1 and 2 for V_H sequences. For multinomial analysis, the germline gene sequence corresponding to each Ig sequence was imported into the JAVA applet at <http://www-stat.stanford.edu/immunoglobulin> (Lossos et al., 2000) along with the associated set of R and S mutation values for FWRs and CDRs.

Molecular modeling of Fv domains

Three dimensional structural models of Fv domains were generated using the Web Antibody Modeling (WAM) program (<http://antibody.bath.ac.uk/index.html>) (Whitelegg and Rees, 2000). H and L FWRs were built upon the most sequence-homologous FWR structures; CDR loops were identified as belonging to specific canonical classes and modeled on those with maximum sequence homology, within the corresponding class. Non-canonical and H3 loops were built using the CAMAL (Combined Antibody Modeling Algorithm) method, consisting of a combined database/conformational search (Martin et al., 1989). Canonical non-conserved and non-canonical residue side chains were modeled using dead-end elimination. Final energy minimizations on CAMAL-generated conformations were performed by employing the accessibility profile screen for Fvs 3–24 and 8–10, and the VFF screen for 7–15 (Whitelegg and Rees, 2000).

Analysis of all-atom contacts and geometry of WAM-generated models was assessed using the MolProbity software (<http://molprobity.biochem.duke.edu/>) (Davis et al., 2004). In Ramachandran plots for 3–24 and 8–10, 207 and 206 residues, respectively, (>94%) were in favored ϕ, ψ conformations while 10 and 11 residues, respectively, (4–5%) were in allowed regions. There were 2 outliers (<1%) in either case, both in the V_H domain, pro in FWR2 and leu in H3. Rotamer analysis for 3–24 and 8–10 identified 13 (6.7%) and 8 (5%) side chains, respectively, with unusual torsion angles that did not fall into any of the known canonical classes. MolProbity was also applied to 4 randomly selected examples of actual X-ray crystallized unliganded Fvs and yielded values ranging from 0–0.88% for Ramachandran outliers and 3–22% for non-canonical rotamer outliers.

Computational docking of OspA

Protein Databank (PDB) files of OspA (PDB identifier, 1fj1E) and each modeled Fv were uploaded onto the ClusPro server (<http://nrc.bu.edu/cluster/>) (Comeau et al., 2004). ClusPro has a relatively high predictive value of ~70%, performing consistently well in a blind, web-based experiment for accurate prediction of binding modes, called CAPRI (Critical Assessment

of Prediction of Interactions) (Comeau et al., 2005). The DOT (Daughter of Turnip) algorithm, based on Fast Fourier Transform (FFT) correlation techniques, was used to perform rigid body docking for each OspA-Fv pair using default grid parameters. Docked complexes were filtered based on shape complementarity (an approximation of van der Waals contact energy), as well as desolvation and electrostatic free energies, followed by clustering based on a pairwise binding site root mean squared deviation criterion. The top cluster centers were CHARMM minimized and concatenated complexes in PDB NMR format were received via email. Individual docked models were then parsed using the `split_models` command in Perl script, available for download on the ClusPro website. All structures were visualized using the RasMol program (<http://www.umass.edu/microbio/rasmol/>) (Sayle and Milner-White, 1995).

Results

Isolation of OspA-specific B-cells

Recombinant OspA was labeled with Alexa 488, and detection of antigen-specific B-cells by the labeled protein, presumably via engagement of cognate surface Igs, was verified by staining splenocytes from an immunized mouse (Fig 1a& b). OspA-Alexa 488, in conjunction with a pan B-cell marker like CD19, allowed for identification of OspA-specific B-cells in human PBMCs, yielding a significantly higher percentage of OspA⁺, CD19⁺ cells in seropositive individuals relative to controls ($p=0.031$, Wilcoxon matched pairs test; data not shown).

OspA-specific B-cells thus identified were singly sorted by FACS from frozen PBMC samples of an OspA vaccine recipient (Lymerix®; Sample #0613) in order to initially establish the system, and from a chronic, treatment-resistant Lyme arthritis patient (patient C) (Fig 1c & Table 1). PBMCs from a seronegative individual (Sample #4190) and from a rheumatoid arthritis patient (RA1) served as negative controls for the respective sorts. Single cells were deposited and co-cultured with irradiated EL4-B5 thymomas and a cytokine supernatant derived from activated T-cells for a period of ~10 days (Grimaitre et al., 1997). Given that high titers of IgG antibodies to OspA mark the transition from episodic to chronic arthritis (Akin et al., 1999), we were primarily interested in OspA-specific antibodies of this traditionally more pathogenic isotype. IgG secreted by individual cultures were tested for OspA binding by an antigen-specific ELISA. Hence, anti-OspA Igs were obtained through a dual screen - surface binding by antigen to B-cell receptors, visualized by FACS, followed by elimination of potential false positives by testing for actual production of antigen-specific Igs in culture, downstream of the sort.

A relatively high frequency of OspA-IgG⁺ cells were recovered from the immunized donor - 7 out of a total of 300 seeded wells (2.3%) (Table 1). This number was predictably lower in patient PBMCs (3 out of a total of 472; 0.64%), most likely attributable to this patient not having been vaccinated with purified protein, as well as possible B-cell recovery losses during thawing of the archival sample after prolonged storage in liquid nitrogen (Table 1). Only three of 35 IgG producers from patient C bound OspA in an ELISA. One possibility is that the remaining 32 IgG clones were weak binders, and owing to steric constraints imposed by a plate-bound ELISA, were unable to bind OspA with sufficient affinity to register a positive signal. Alternatively, these B-cells might represent false positives that bound OspA-Alexa 488 non-specifically during the sorting step. The negative control (RA1) also contained a small percentage of double positive-staining cells (0.03% compared to 0.1% in patient C) (Fig 1c). Although freshly isolated PBMCs were used for this sample, applying the same gates as in patient C, we were able to sort out only 120 single cells, of which one was an IgG producer that did not bind OspA (Table 1). Hence, non-specific B-cells appear to be largely excluded from this gate, and the double positive population in RA1 (Fig 1c) possibly represents debris or dead cells that retain dye indiscriminately. The antigenic targets of the OspA non-binders from patient C remain unclear.

Amplification of Ig V-regions, scFv generation and OspA binding

RNA was isolated from the 3 OspA-IgG⁺ wells from patient C (3–24, 7–15 & 8–10), as well as from 4 of the non-binders, and also from the 7 OspA-IgG⁺ wells from the vaccine recipient. Expressed Ig VH and VL regions were amplified using a nested PCR scheme (Wang and Stollar, 2000), yielding PCR products at ~400 bp, as visualized on a 1% agarose gel (Fig 1d). Since the main focus of this study was isolation and characterization of potentially pathogenic anti-OspA Igs from a chronic, treatment-resistant Lyme arthritis patient, the OspA-IgG clones from the immunized subject, principally employed to set up the system, were not pursued further. VH and VL sequences from 3–24, 7–15 and 8–10 were cloned as scFvs, the VH domain upstream of the VL domain with an intervening, flexible glycine linker sequence (Polymenis and Stollar, 1995).

As is apparent from the OspA-IgG ELISA performed using the sorted cell culture supernatants, 3–24 was the strongest binder, 7–15 and 8–10 binding OspA at comparable levels (Fig 2a, b & c). Specific binding to OspA was further confirmed by running lysates from either wild type or OspA-deficient *Bb* on SDS-PAGE and immunoblotting with scFv 3–24, yielding the ~31 kD OspA band in all lanes except for the OspA null mutant (Fig 2d). ScFvs 3–24, 7–15 and 8–10 could also detect the native protein on the surface of permeabilized spirochetes by indirect immunofluorescence, relative to non-specific Fv controls (Fig 3).

Sequence analysis

Analyses of OspA-specific V-region sequences enabled identification of the closest germline matches from which H or L chains were derived, while calculation of replacement to silent mutation ratios (R/S) in complementarity determining regions (CDRs) or framework regions (FWRs) and application of the multinomial model allowed for assessment of antigen-selective pressure on individual V-domains (Lossos et al., 2000). Both 3–24 and 8–10 contained the same H-L pair (VH1-2/JH3), 8–10 being more mutated compared to 3–24 (Table 2 & Fig 4). Strikingly, an analysis of somatic mutations in these V-regions reveals a hierarchy in their acquisition, a pattern of step-wise accumulation indicating a common clonal lineage. The L chain of 8–10 clearly appears to be derived from that of 3–24, inheriting some mutations from the latter and accumulating *de novo* replacements of its own. Similarly, the 8–10 H chain shares some mutations with its counterpart in 3–24, acquires some unique mutations, but also appears to retain germline residues at still other positions (at three sites located in CDR1, FWR2 and CDR2) (Fig 4). 7–15, the other OspA-IgG clone contained an unrelated H-L pair, although the H chain used the same JH segment (JH3) as the other two (Table 2).

Given that they were obtained as a result of a fairly specific FACS screen, Ig sequences from the OspA non-binders were also analyzed (Table 3). Three out of 4 clones examined contained germline H and L chains, possibly representing naïve B-cells encoding Igs with intrinsic OspA affinity, but, in the absence of hypermutation, being unable to bind the protein under the more stringent conditions of an ELISA, relative to flow cytometry. The fourth (1-1) contained a mutated λ chain (Table 3), but did not yield a H product following PCR amplification. All H chains isolated belonged to the commonly used VH3 family (Table 3). We also noted that clones 4–10 and 4–35 contained closely related VH and VL domains. The VH sequences were generated by identical VDJ recombination events (VH3-73/D6-6/JH6), but with slight differences in the patterns of N-nucleotide acquisition, resulting in homologous, but non-identical junctions (data not shown); VL domains contained the same V κ (V κ 3–20) segment recombined with J κ 4 in 4–35 and the more downstream J κ 5 segment in 4–10 (Table 3).

Somatic hypermutation in OspA-specific V-regions

Typically, a good correlate of antigen selective pressure operating on hypermutating B-centrocytes is a preferential clustering of replacement mutations within CDRs, which bind

antigen, as opposed to silent mutations in FWRs, which maintain antibody structure. The multinomial analysis further verifies whether the relative abundance of R mutations in CDRs and S mutations in FWRs is statistically significant, accumulating at a rate higher than that predicted by random chance alone, based on codon composition of the parent germline sequence (Lossos et al., 2000). OspA has been previously associated with B-cell mitogenic potential (Ma and Weis, 1993); however, all 3 anti-OspA IgGs isolated from patient C appear to contain true, somatically mutated, antigen-selected V-regions. Both H and L chains of the 3 anti-OspA V-regions had significantly higher R/S ratios in CDRs, relative to FWRs (R mutations as a fraction of total mutations were compared by Student's T-test; $p=0.033$ and 0.028 for H and L chains, respectively). The 3–24 H chain and 7–15 L chain both had a significant excess of R mutations in their CDRs by the multinomial model, ($p_{\text{cdr}} < 0.05$; Table 2), while H and L sequences of all 3 V-regions had a significant paucity of R mutations in the FWRs ($p_{\text{fwr}} < 0.05$; Table 2), indicative of antigen-imposed constraints. Despite being the upstream member of a clonal diversification pathway, 3–24 appears to be more “antigen-selected” by the multinomial model (especially the VH domain with both p_{cdr} and $p_{\text{fwr}} < 0.05$; Table 2), statistical predictions being borne out by its enhanced *in vitro* binding of OspA, compared to 8–10 (Fig 2).

Molecular modeling of Fv domains

To examine the structural impact of hypermutation on this pair of intraclonal variants, we next sought a molecular basis for the perceived alteration in OspA binding between 3–24 and 8–10, a set of point mutations distinguishing one V-region from the other. Lacking crystallographic structures for our Fvs, we studied their putative paratopes and postulated their binding modes with antigen, using a “template-based” approach to first generate structural models, followed by computational docking of OspA, whose crystal structure has been previously determined (Li et al., 1997b). The 3–24 and 8–10 V-region sequences were approximated to a large database of similar templates with known crystal structures, using the online modeling tool, WAM (Web Antibody Modeling), which uses mainly homology-based and also *ab initio* methods for model building (Whitelegg and Rees, 2000). CDR loops L1, L2, H1 and H2 were classified as belonging to Chothia canonical classes 2, 1, 2, 1, respectively, while L3 was deemed non-canonical (WAMPredict). In both cases, H3 was predicted to have a “kinked”, instead of “extended”, conformation, owing to the absence of an asp residue at position 101. Further, structural models were validated using the MolProbity software, generating Ramachandran plots and performing rotamer analyses, in order to check the peptide backbone and amino acid side chains for adherence to known geometric constraints (Davis et al., 2004). In general, both Fv models exhibited good compliance with some of the standard parameters for structural integrity and, as visualized in RasMol, contained the characteristic Ig fold with antiparallel β -barrels, enclosing a deep central cavity flanked by VH and VL CDRs, presumably accommodating the antigen binding site (Fig 5a).

OspA was docked into each Fv domain using an automated, web-based program, ClusPro, wherein docking algorithms evaluate billions of possible Fv-OspA complexes, retaining a preset number of energetically favorable solutions, which are then scored based on the assumption that the native or near-native complex resides at a global free energy minimum (Comeau et al., 2004). The majority of interatomic contacts between OspA and both Fv domains in the docked complexes appear to be mediated by residues in the two barrel sheets of OspA (Fig 5b) – for 3–24, the most proximal side chains reside predominantly in barrel sheet 2, while for 8–10, the OspA molecule appears to have swiveled forward slightly, allowing for more contacts with strands and turns in barrel sheet 1 as well (Fig 5c & e). Like, 8–10, the 7–15 Fv domain also interacts with residues in barrel sheets 1 and 2 (data not shown). Notably, both barrel sheets constitute a major portion of OspA_{168–273}, the fragment most strongly linked to arthritis severity and chronicity (Akin et al., 1999).

In terms of specific substitutions, we focused on those that appear to be the most antigen-proximal in the docked complexes, with side chains impinging upon the binding cavity. By this metric, the most significant changes from 3–24 to 8–10 were (i) thr → gly at position 50 (L2), (ii) ser → thr at position 93 (L3) and (iii) ile → val at position 104 (H3). For the 3–24 Fv domain, lys231 in barrel sheet 2 of OspA is the closest contact residue for thr50, which would allow for H-bonding between the γ -oxygen of thr and a H atom contributed by the lys side chain amino group. Ser93 is within H-bonding distance of thr253 of OspA, while ile104 is most proximal to a buried ala208 side chain in barrel sheet 1, contact likely mediated by hydrophobic interactions between the two (Fig 5d). In the OspA:8–10 complex, lys231 appears to have swung away, possibly due to electrostatic repulsive effects of the imidazole ring of cationic his, which replaces neutral asn at position 34 in L1 (not shown in figure). Hydrophilic thr50 is substituted here by a smaller-sized and completely neutral gly residue, closest to ala215 in OspA; at position 93, thr replaces ser and appears to contact ala208 in OspA, rendering the binding environment at these positions more hydrophobic in the 8–10 Fv domain, relative to 3–24 (Fig 5f). Ile at position 104 in H3 is replaced by val – the shorter aliphatic side chain renders the latter out of range for mediating hydrophobic contacts with OspA in the docked complex, thereby diminishing antigen binding interactions contributed by this position.

To summarize, we have followed OspA-specific antibodies from patient peripheral blood, through their isolation on corresponding B-cells, their *in vitro* generation as recombinant scFvs, and finally, characterized a pair of clonally diversified variants via sequence analysis and computational investigation of their respective binding modes with antigen, in the context of binding data. While exact elucidation of binding site geometry is clearly outside their scope, we have used our *in silico* models to glean clues as to possible alterations in molecular interactions between OspA and each of the clonally diversified Fvs, replacements from 3–24 to 8–10 appearing to abate antigen binding interactions at the substituted positions. Given that affinity maturation results in Ig receptors with enhanced antigenic affinity, reduced OspA binding by the more mutated 8–10, compared to 3–24, appears somewhat paradoxical. Conceivably, downstream of the original microbial challenge, additional antigens skew the response towards themselves and away from OspA, as discussed below.

Discussion

OspA is possibly the most intensely investigated of all *Borrelial* proteins, given its essential role in the natural enzootic *Bb* lifecycle, its strong inflammatory potential and its elicitation of a cellular and humoral immune response that appears to be central to the development of chronic arthritic pathology in the human host (Weis et al., 1994; Gross and Huber, 2000; Yang et al., 2004). OspA is not essential for the early events of mammalian infection, its surface expression diminishing upon *Bb* entry into the host; it gets upregulated in response to inflammatory cues by host-adapted *Bb* later on in the course of disease, its function during this stage being largely uncharacterized (de Silva et al., 1996; Schwan and Piesman, 2000; Crowley and Huber, 2003). Correspondingly, in longitudinal studies of serial serum samples from untreated patients (collected prior to the use of antibiotic therapy for Lyme disease treatment), development of IgG reactivity to OspA and its operonic partner OspB marks the terminal event in natural evolution of the antibody response to *Bb* proteins (Kalish et al., 1993; Akin et al., 1999). IgG titers to OspA (and to a lesser extent, OspB) correlate directly with severity of chronic Lyme arthritis, while IgG titers to the C-terminal third of OspA (OspA_{168–273}) correlate with both severity and duration, suggesting the strongest causal link with epitopes contained within this fragment (Akin et al., 1999).

Most traditional methods for generation of monoclonal antibodies (mAbs) involve dedicated, time-intensive protocols. In this case, characterization of the anti-OspA IgG response necessitated revival of corresponding B-cells from archival clinical samples and their activation

at sufficient levels for subsequent mAb production, which posed an even greater challenge. We addressed this by using the EL4-B5 system, which allowed for recovery of single, OspA-specific B-cells from a small volume of frozen PBMCs by providing robust T-cell help post-sorting, both via cognate and non-cognate interactions (Wen et al., 1987; Grimaire et al., 1997). Testing the *ex vivo* culture supernatants in anti-OspA IgG ELISAs constituted an additional screen for antigenic specificity, the first being surface staining of Ig receptors by labeled OspA. Ig RT-PCR amplification and production of recombinant scFvs in an *E. coli* system provided a rapid and efficient means for procuring OspA-directed IgGs expressed in the patient, in the context of chronic Lyme arthritis. We noted a small population of CD19⁻, OspA⁺ cells in patient C (Fig 1c) - OspA is known to interact with toll-like receptor 2 (TLR2) on monocytes and neutrophils in peripheral blood (Hirschfeld et al., 1999), which theoretically should have been excluded from the lymphocyte gate; this population was absent in the RA sample and not characterized further.

Remarkably, given that antigen-specific B-cells had been randomly sorted, 2 out of the 3 anti-OspA IgGs obtained from patient C were clonally related. Despite being the more downstream member of an affinity maturation pathway, 8–10 exhibited weaker OspA binding compared to the less mutated 3–24. One possibility could be that 8–10 arose downstream of 3–24 during the hypermutation process, albeit without additional beneficial replacements, but perhaps retained sufficiently strong OspA affinity over threshold, allowing for selection of the corresponding centrocyte in the germinal center. Alternatively, the selective influence of additional antigen(s) could be involved in further diversification of this clone, post-selection on *Bb* OspA. It has been previously shown that both 3–24 and 8–10, along with Fvs derived from chronic Lyme arthritic synovium, bind cytokeratin 10 (CK10), a self-protein reportedly expressed in the joints under both arthritic and normal conditions, mainly in the endothelial layer of tissue microvasculature (Ghosh et al., 2006). A pairwise BLAST alignment of OspA with CK10 assigned the highest homology score to a 71 amino acid stretch of the latter protein with residues 189–273 of OspA, contained within the potentially arthritogenic OspA_{168–273} fragment (Ghosh et al., 2006). The joint compartment is conceivably accessed by both locally-generated and peripheral blood Igs. In this case, the affinities are reversed, 8–10 being the stronger CK10 binder, relative to 3–24 (Ghosh et al., 2006). 8–10 could be envisaged as being on the cusp of an affinity shift, the primary microbial antigen OspA being progressively supplanted by self-antigen, increasing amounts of the latter being released in the context of chronic tissue damage.

We used bioinformatics and computational tools for modeling OspA-Fv binding, to probe the nature of epitope-paratope interactions on a molecular level. The current unavailability of 3D structural data on CK10 precludes similar analyses of its binding interactions here. Both 3–24 and 8–10 appear to bind epitopes in the OspA barrel sheets, located in the OspA_{168–273} fragment. As evidenced in epitope mapping studies, this region appears to be particularly immunogenic, being also bound by murine anti-OspA monoclonals, LA2 and 336, observed in their crystal structures, in complex with OspA (Ding et al., 2000). The OspA N-terminal tethers it to the *Bb* membrane via lipid moieties; hence, topologically, the C-terminal region is the most distal from the spirochetal surface, and thus, likely to be the most accessible (Ding et al., 2000). Putative contact residues for substituted positions in both Fvs (ala208, ala215, lys231 and thr253) are contained in three of the most polymorphic regions of OspA, which otherwise exhibits low sequence variability (loops 1, 2 and 3 encompassing residues 203 – 220, 224 – 233 and 246 – 257, respectively) (Ding et al., 2000). Interestingly, ala residues at positions 208 and 215 are unique to the more arthritogenic *Bb sensu stricto* strains, being replaced by structurally non-conservative side chains in the two other pathogenic *Borrelia* genospecies, *B. garinii* and *B. afzelii*, more commonly associated with neurologic and cutaneous manifestations of Lyme disease (van Dam et al., 1993; Ding et al., 2000).

In conclusion, chronic Lyme arthritis is a debilitating, antibiotic-refractory condition, which develops downstream of *Bb* infection, the immune response to OspA being linked to the development of persistent pathology (Steere and Glickstein, 2004). In the current study, we describe the generation of OspA-specific monoclonal scFvs from peripheral circulation of a chronic, treatment-resistant Lyme arthritis patient from the time of active disease, using an alternative molecular approach based on isolation of single, antigen-specific B-cells, their *ex vivo* expansion, Ig RT-PCR and cloning, circumventing some of the disadvantages associated with more traditional methods of mAb generation. Further, we focus on a pair of intracloal V-domain variants, using computational models to provide a rationale for their differential binding of OspA. Ultimately, characterization of OspA-specific monoclonal antibodies from patient samples and delineation of putative epitopes bound by them can yield insights into maturation and diversification of this potentially pathogenic anti-OspA IgG response in the prevailing inflammatory milieu, as well as mechanisms that contribute to its self-perpetuation in the apparent absence of the inciting agent.

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Abbreviations

Bb	<i>Borrelia burgdorferi</i>
OspA	Outer Surface Protein A
Ig	Immunoglobulin
scFv	single chain variable region fragment
H	heavy

L	light
V	variable
PBMC	peripheral blood mononuclear cell
R	replacement
S	silent
CDR	complementarity determining region
FWR	framework region
mAb	monoclonal antibody

Fig 1. Isolation of OspA-specific B-cells from peripheral circulation and Ig RT-PCR

(a) Alexa 488 labeled OspA was run on a non-denaturing gel and visualized with an UV filter. Labeled IgG was run in parallel as a positive control. (b) Specific binding by the labeled protein was tested by FACS, using OspA-Alexa 488 and B220-PE to identify OspA-specific B-cells in the splenocytes of an immunized mouse. Splenocytes from a non-immunized mouse were used as negative control. (c) OspA⁺CD19⁺ cells were identified by FACS in PBMCs from Lyme patient C. Cells from a RA patient, RA1, were processed in parallel, as negative control. Double positive cells were singly sorted into irradiated EL4-B5 thymoma cells and a cocktail of activated T-cell cytokines. (d) Products of γ κ or λ -specific Ig RT-PCRs performed on RNA isolated from anti-OspA IgG clones from patient C; NT, no template control.

Fig 2. OspA binding

(a) by supernatants from individual wells of singly sorted B-cells from patient C cultured with irradiated EL4-B5 thymomas (wells were numbered as plate number-well number; 1-1, 2-46, 4-10 and 4-35 were IgG⁺, OspA non-binders from this patient; OspA binding units were standardized against murine anti-OspA monoclonal antibody, LA2 such that 1 binding unit yields an equivalent OD as 100 ng of LA2 in the same ELISA) and (b & c) scFvs 3-24, 7-15 and 8-10 (DS1 and DS2 represent non-Lyme scFv controls, derived from neonatal cord blood). (d) Recombinant OspA, sonicated *Bb* and lysates from wild type or OspA⁻*Bb* immunoblotted with scFv 3-24. Rabbit polyclonal IgG was used for detection of the protein A tag. Goat anti-rabbit IgG: HRP was used as a secondary antibody.

Fig 3. *Bb* immunofluorescence visualized using anti-OspA scFvs

Aliquots from two day old *Bb* cultures were fixed with methanol onto positively charged glass slides and probed with each scFv at 3 μ g/ml. Rabbit IgG conjugated to Alexa 488 (green) was used for detection of the protein A tag. (a) Wild type or OspA⁻ *Bb* were probed with anti-OspA scFv (3–24), an OspA-specific mouse monoclonal antibody (LA2) and a flagellin-specific monoclonal antibody (CB1); images at 100 $\times$ magnification. (b) Wild type *Bb* probed with anti-OspA scFvs, 3–24, 7–15 and 8–10. Synovial scFv DC7 and neonatal cord blood derived scFv DS1, which do not bind OspA were used as negative controls; sec, staining with secondary antibody alone; images at 40 $\times$ magnification.

Fig 4. Clonally related OspA-specific scFvs from patient C
Amino acid sequence alignments of (a) VH and (b) VL domains of OspA-specific scFvs, 3–24 and 8–10, compared to germline Ig sequences (denoted as G). The closest germline matches for the H and L chains were VH1-2 and Vκ1D-39, respectively (Table 2). CDRs 1, 2 and 3 have been indicated. Dashes signify sequence identity. Amino acid replacements have been indicated at the corresponding positions.

Fig 5. Computational docking of OspA into Lyme-derived scFvs

(a) Central cavity in the Fv domain, flanked by VH and VL CDRs 1 (blue), 2 (purple) and 3 (green). The dotted circle denotes the putative antigen binding site. (b) Crystal structure of OspA (Li et al., 1997a) visualized in RasMol, depicting secondary structural elements; N-terminal β -sheet (gray), central β -sheet (yellow), barrel sheet 1 (cyan), barrel sheet 2 (magenta) and C-terminal helix (orange). (c & e) Docking orientation of OspA with Fvs 3-24 and 8-10, respectively; CDRs are depicted in red; bs1, barrel sheet 1, bs2, barrel sheet 2. (d & f) Amino acid replacements in Fv CDRs (labeled in red) and OspA contacts (labeled in gray), as visualized in docked complexes, generated using the ClusPro server (Materials and methods). Aliphatic residues are shown in yellow; oxygen (red), nitrogen (blue).

Table 1PBMC samples used for sorting single OspA⁺ CD19⁺ cells

Sample ID	Description	OspA seroreactivity	Total cells sorted	Number IgG + (%)	Number OspA + (%)	Period in liquid nitrogen
0613 ¹	OspA-immunized	yes	300	n.d. ²	7 (2.3)	8 mo
Patient C	Treatment-resistant Lyme arthritis patient	yes	472	35 (7.41)	3 (0.64)	14 y
RA1	Rheumatoid arthritis patient	no	120	1 (0.083)	0 (0)	-

¹ PBMCs from a seronegative individual (sample # 4190), served as negative control to set up gates for the sort; cells were not sorted from this control sample.

² n.d.; not done.

Table 2

Evidence of antigenic selection in OspA-IgG clones from patient C

Clone	Isotype	VH				pCDR	VL				pCDR
		VDJ	R/S FWR	R/S CDR	pFWR		VJ	R/S FWR	R/S CDR	pFWR	
3-24	IgG	VH1-2/JH3	5/5	9/1	0.0017	0.0033	Vκ1D-39/Jκ3	2/5	4/1	< 0.0001	0.16
8-10	IgG	VH1-2/JH3	11/10	6/3	0.01	0.39	Vκ1D-39/Jκ3	5/9	6/1	< 0.0001	0.2
7-15	IgG	VH4-30-4/JH3	2/3	4/1	< 0.0001	0.06	Vκ3-20/Jκ2	0/0	2/0	na	0.02

Multinomial pFWR or pCDR values <0.05 have been indicated in boldface; na, not applicable.

Table 3

OspA non-binders, as detected by ELISA, isolated from patient C

Clone	IgG (ng/ml)	VH	VL	Mutational status - VH	Mutational status - VL
1-1	3.7	-	V λ 5-45/J λ 3	-	m
2-46	11.7	VH3-15/D6-13/JH3	V λ 3-1/J λ 2	g	g
4-10	3.6	VH3-73/D6-6/JH6	V κ 3-20/J κ 5	g	g
4-35	47.6	VH3-73/D6-6/JH6	V κ 3-20/J κ 4	g	g

g, germline; m, mutated.