

Supplemental Materials

Enhanced protective immunogenicity of homodimeric *Borrelia burgdorferi* Outer Surface Protein C

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Methods

Construction of recombinant vectors encoding for proteins from Borrelia.

Total DNA from *B. burgdorferi* strains B31, 297, and N40 was used as the template for PCR amplification of OspC coding sequences (UNIPROT U01894, E4QRR2, E4QHI7) and BmpA_{B31} (GenBank # X81515) coding sequences using the primer combinations provided in Table S1. PCR generated coding fragments for the OspC variants OspC(C130S)_{B31} (designated as D_{B31}), OspC(C19G, C130S)_{B31} (M_{B31}), OspC(C131S)₂₉₇ (D₂₉₇), OspC(C19G, C131S)₂₉₇ (M₂₉₇), OspC(C129S)_{N40} (D_{N40}), and OspC(C19G, C129S)_{N40} (M_{N40}), respectively. Some OspC variants contain internal cysteine residues and we found that those variants tend to create large multimeric complexes (data not shown). To ensure that only homodimers were formed we mutated the internal cysteine codon of those variants to a serine codon by overlap extension PCR. Subsequently, the PCR products were digested with the appropriate restriction enzymes and integrated into the prokaryotic expression vector pET24d-N as described for OspC_{VS461} and OspC₂₀₀₄₇ variants (5) thereby adding N-terminal hexahistidine-tags to the recombinant proteins. All vector constructs were controlled by DNA sequencing (MWG Biotech).

Expression and purification of recombinant proteins

Recombinant proteins were expressed in *E. coli* Rosetta (DE3) pLacI (Novagen, Darmstadt, Germany) as described for another recombinant protein (6). Bacteria were harvested by centrifugation at 1,800 x g for 30 minutes and sediments were resuspended in 10 mmol/L

Tris-HCl pH 8.0, 300 mmol/L sodium chloride, 10 mmol/L imidazole. To release the recombinant proteins, the cell suspension was sonicated 3 times for 20 seconds (50% Output, level 7, micro-tip, Branson Sonifier, Dietzenbach, Germany) on ice. Lysed bacteria were centrifuged again at 15,000 x g for 20 minutes to sediment insoluble proteins. Recombinant proteins were further purified from the resulting supernatant by immobilized metal affinity chromatography (IMAC) using Ni-NTA sepharose (Qiagen, Hilden, Germany) and 10 mmol/L Tris-HCl pH 8.0, 300 mmol/L sodium chloride, 150 mmol/L imidazole as eluent. OspCs eluted from IMAC were dialysed extensively against 25 mmol/L HEPES pH 7.0 and then applied onto a cation exchange resin (POROS HS, Applied Biosystems, Darmstadt, Germany) equilibrated with 25 mmol/L HEPES pH 7.0. After thorough washing, the recombinant protein was eluted from the column by a linear gradient of sodium chloride in 25 mmol/L HEPES pH 7.0 with a final concentration of 1 mol/L. BmpA eluted from IMAC was dialysed extensively against 25 mmol/L Tris-HCl pH 8.5 and then applied onto an anion exchange resin (POROS HQ, Applied Biosystems) equilibrated with 25 mmol/L Tris-HCl pH 8.5. After thorough washing, the column was eluted by a linear gradient of sodium chloride in 25 mmol/L Tris-HCl pH 8.5 with a final concentration of 1 mol/L. Pooled fractions containing recombinant proteins were stored in aliquots at -20 °C until use. All recombinant proteins were prepared with this standardized protocol in a quality-managed environment to guarantee homogenous quality.

Characterization of OspC in solution by Dynamic Light Scattering (DLS)

For DLS, proteins were brought to a final concentration of 1 µg/mL in 20 mM Tris-HCl pH 7.4, 150 mM sodium chloride. Reducing conditions were generated by addition of 10 mM DTT. The final samples were filtered with 0.2 µm membrane filters to remove particles, transferred to the 12 µL quartz cuvette and analyzed with a laser light scattering instrument

(DynaPro MS/X, Wyatt Technology Europe GmbH, Dernbach, Germany) at 25 °C, 15 repetitions with 20 second intervals. The resulting data were analyzed with the DynaPro Dynamics 6.7.3 software package assuming a globular shape with parameters set to viscosity (20°C) = 1,019 cP and hydrodynamic radius = 3.978 nm.

Pepscan analysis

For the determination of antibodies to individual peptide epitopes in OspC_{B31}, representative murine sera were analyzed with slides on which peptides spanning the complete sequence (length: 12 amino acids, overlap: 11 amino acid) had been synthesized in duplicate using a laser printing technique (PEPperPRINT, Heidelberg, Germany) (1). Flag-tag peptides were included as reference. Slides were blocked for 60 minutes with modified sample buffer (0.5% (w/v) bovine serum albumin, 0.05% (w/v) Tween-20 in PBS), washed three times with washing buffer, and incubated with serum diluted 1:100 in sample buffer supplemented with a murine monoclonal antibody against Flag M2 (Sigma-Aldrich, 1:200) for 60 minutes. After washing three times, bound antibodies were detected by incubation with anti-mouse IgG-Cy2 conjugate (Jackson Research, UK) diluted 1:200 in sample buffer for 30 minutes. Slides were subsequently analyzed with a BioAnalyzer 4F (LaVision Biotec, Germany). For reuse, slides were stripped with 1% (w/v) sodium dodecyl sulphate, 20 mmol/L dithiotreitol in PBS overnight followed by 3 rinses with deionized water and drying. All steps were carried out at room temperature.

Miscellaneous

If not stated otherwise all reagents were purchased from Merck (Germany) at their highest purities. NuPAGE, Blue Native-PAGE (Thermo-Fisher Scientific) and Mini-Protean TGX were run according to manufacturers' instructions. Gels were either stained with Coomassie R250 (Sigma-Aldrich), silver (2) or trichloroethanol (4) and analyzed with a Bio-Rad XR+

equipped with Image Lab 5.0 (Bio-Rad, Germany). Protein concentrations were determined by bicinchoninic acid assay (Sigma-Adrich). MALDI-TOF fingerprinting after SDS-PAGE and tryptic cleavage was conducted as described by Koy et al. (3).

Supplemental Tables

Table S1. Sequences of primers used for the generation of PCR products from total DNA of *B. burgdorferi* B31, 297, and N40. F, forward primer; R, reverse primer. OspC coding DNAs were amplified without regions coding for the signal peptides; C19G, point mutation leading to a conversion Cys→Gly at position 19 of the native sequence; C129/130/131S, point mutation leading to a conversion Cys→Ser at the designated positions of the native sequence.

Protein	Restriction sites	Primer sequences (5'→3')
D-OspC _{B31} (C130S)	Esp3I	F: ATATCGTCTCCCATGTGCAATAATTCAGGGAAAGATGGGAATAC
	Esp3I	R: ATACGTCTCTAGATTTCTTAGCCGCATCAATTTTTTCC
	Esp3I	F: GCGGCGTCTCAATCTTCTGAAACATTTACTAATAAATTAAG
	XhoI	R: GCAGAGTGCCTCGAGTTAAGGTTTTTTTTGGACTTTCTGCCAC
M-OspC _{B31} (C19G, C130S)	NcoI	F: CGCACCATGGGTAATAATTCAGGGAAAGATGGGAATAC
	Esp3I	R: ATACGTCTCTAGATTTCTTAGCCGCATCAATTTTTTCC
	Esp3I	F: GCGGCGTCTCAATCTTCTGAAACATTTACTAATAAATTAAG
	XhoI	R: GCAGAGTGCCTCGAGTTAAGGTTTTTTTTGGACTTTCTGCCAC
D-OspC ₂₉₇ (C131S)	BsmBI	F: ATACGTCTCCCATGTGTAATAATTCAGGAAAAGATGG
	BsmBI	R: TATCGTCTCTCAGAGCTTTTCTTAGCATTTTC
	BsmBI	F: ATACGTCTCCTCTGAAGATTTTACTA
	BsmBI	R: TATCGTCTCCTCGATTAAGGGTTTTTTGGAC
M-OspC ₂₉₇ (C19G, C131S)	BsmBI	F: ATACGTCTCCCATGGGCAATAATTCAGGAAAAGATGG
	BsmBI	R: TATCGTCTCTCAGAGCTTTTCTTAGCATTTTC
	BsmBI	F: ATACGTCTCCTCTGAAGATTTTACTA
	BsmBI	R: TATCGTCTCCTCGATTAAGGGTTTTTTGGAC
D-OspC _{N40} (C129S)	BsmBI	F: ATACGTCTCCCATGTGTAATAATTCAGG
	BsmBI	R: TATCGTCTCGTAGAGCTTTGCTTAGCTGTATCAATC
	BsmBI	F: ATACGTCTCGTCTACAGAATTTACTAATAAAC
	BsmBI	R: TATCGTCTCGTCTCGATTAAGGTTTTTTTTGGACTTTCTGCC
M-OspC _{N40} (C19G, C129S)	BsmBI	F: ATACGTCTCCCATGGGCAATAATTCAGGAAAAGATGG
	BsmBI	R: TATCGTCTCGTAGAGCTTTGCTTAGCTGTATCAATC
	BsmBI	F: ATACGTCTCGTCTACAGAATTTACTAATAAAC
	BsmBI	R: TATCGTCTCGTCTCGATTAAGGTTTTTTTTGGACTTTCTGCC
BmpA _{B31}	NcoI	F: ATACCATGGGTAGTGGTAAAGGTAGTCTTGGG
	XhoI	R: ATACTCGAGAATAAATTCCTTAAGAACTTCTC

Table S2. Number of tick recovered/positive from tick-challenged mice.

Day 7 post	Mouse ID	Recovered Ticks	Positive Ticks
Control	1	2	2
	2	2	2
	3	3	3
	4	1	1
	5	2	2
	6	2	2
Monomer	7	3	3
	8	0	0
	9	2	2
	10	2	2
	11	0	0
	12	1	1
Dimer	13	1	1
	14	3	3
	15	2	2
	16	0	0
	17	1	1
	18	3	3

Table S3. Reactivity of pre- and post-challenge sera from mice immunized with D-OspC_{B31} (Dimer) or M-OspC₃₁ (Monomer) to *B. burgdorferi* antigens.

Condition ^a	Cultures Positive/ Total	Immunoblot	ELISA					
		VisE, p39, p18 reactivity	Mean Absorbance _{post-challenge} / Mean Absorbance _{pre-challenge}					
			His-OspC _{B31} (dimer)		FTNK15	KELT15	His-BmpA _{B31}	
			pan-IgG	IgG3	IgG3	IgG3	pan-IgG	IgG3
Alum only	6/6	6/6	64.4	180.4	90.8	11.9	30.7	76.4
0.1 µg Dimer	1/6	1/6	1.8	6.8	8.5	1.1	1.0	2.5
1 µg Dimer	0/6	0/6	1.2	0.8	0.9	0.8	1.0	0.9
10 µg Dimer	1/6	1/6	1.3	6.3	2.9	12.1	1.0	2.0
0.1 µg Monomer	3/3	3/3	5.3	80.1	7.3	10.9	18.3	65.5
1 µg Monomer	3/3	3/3	1.6	65.6	4.6	8.8	3.6	37.9
10 µg Monomer	2/3	2/3	1.5	25.1	2.0	14.2	12.3	57.9
100 µg Monomer	4/6	4/6	1.2	17.2	1.3	9.1	9.4	27.4

^a All immunizations in this study utilized alum as the adjuvant.

Table S4. Reactivity of monoclonal antibodies, as determined by enzyme immunoassay and immunoblot analysis.

Antigen used in ELISA or immunoblot	Reactivity of Monoclonal Antibody with Antigen			
	1-5-A10 (IgG1)	1-8-B7 (IgG1)	2-2-H8 (IgG1)	2-8-C10 (IgG1)
D-OspC _{B31}	strong	strong	strong	strong
M-OspC _{B31}	weak	weak	strong	medium
FTNK15	ND ^a	ND	strong	ND
KELT15	ND	ND	ND	ND

^a ND = not detected

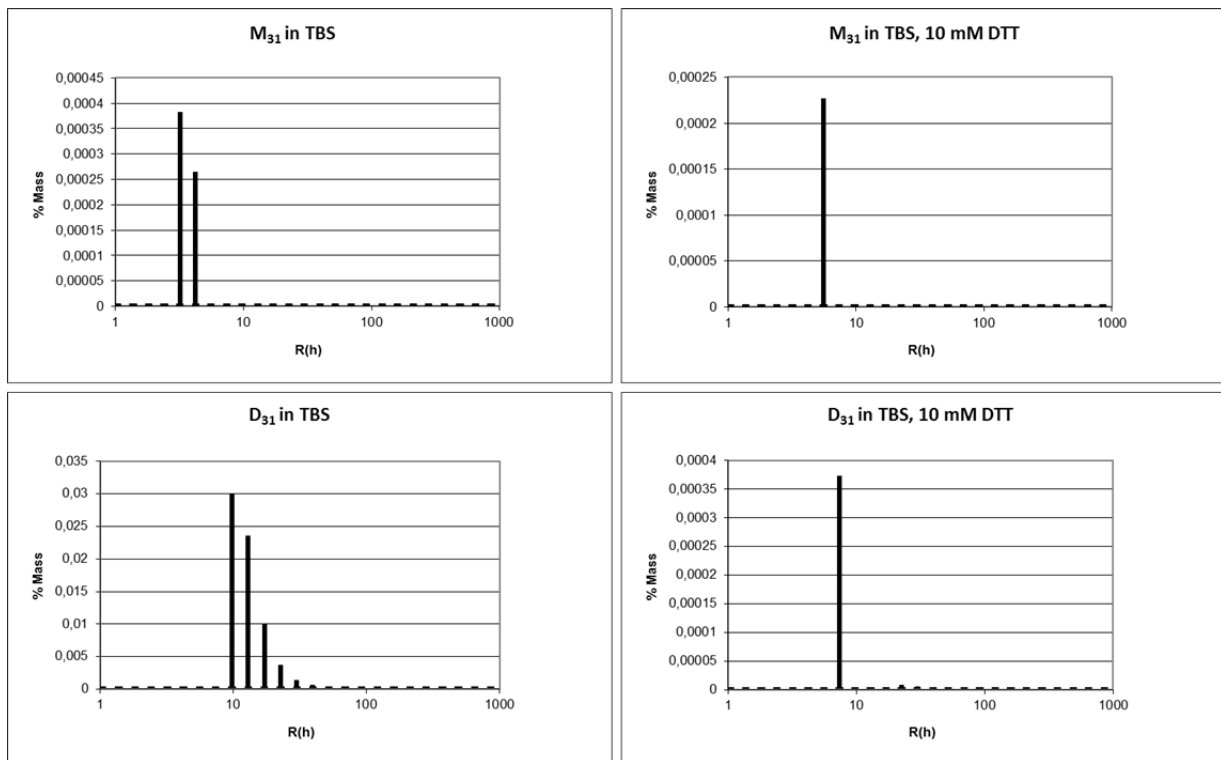


Figure S1. Dynamic light scattering analysis of purified OspC- M_{B31} and OspC- D_{B31} . The proteins were diluted to a final concentration of 1 mg/mL in TBS with or without 10 mM dithiothreitol (DTT).

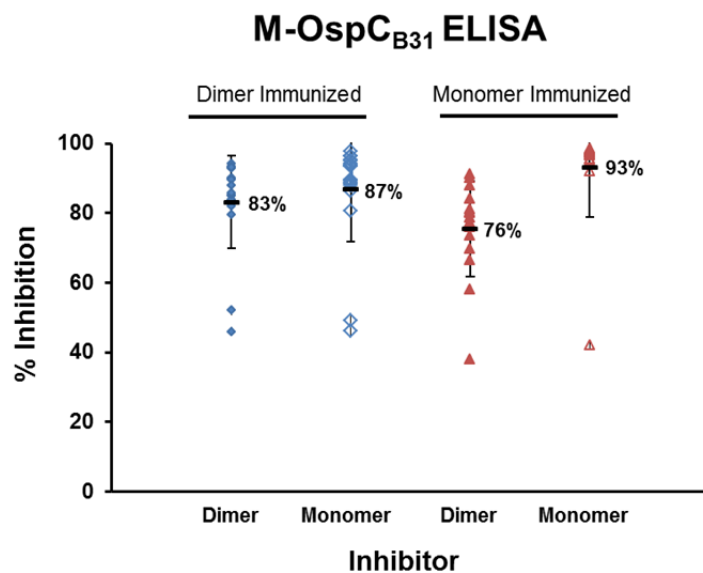
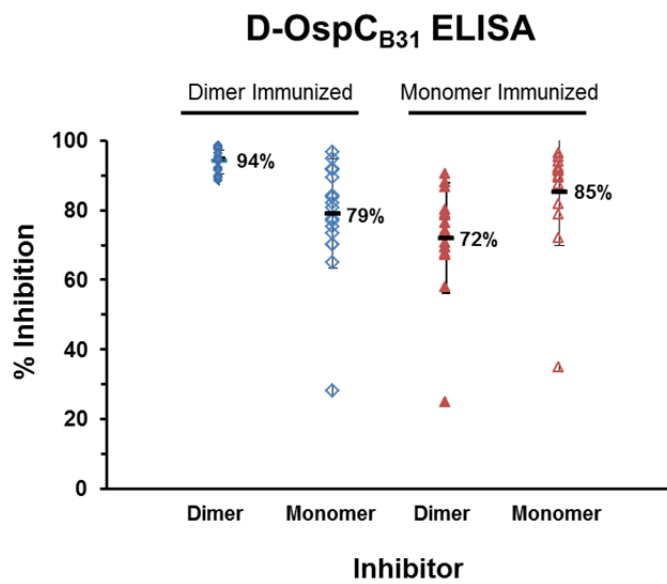


Fig. S2. Inhibition of binding of D-OspC_{B31} (Dimer) and M-OspC_{B31} (Monomer) induced antibodies by soluble Dimer and Monomer proteins. Diluted sera were incubated with 10 µg/ml of the indicated protein 30 minutes prior to addition to ELISA plates coated with A) D-OspC_{B31} or B) M-OspC_{B31}. Data points represent results obtained with pre-challenge sera from individual mice in Experiment 2 (see Table S1). Mean \pm SD of inhibition relative to replicate wells without added soluble inhibitor.

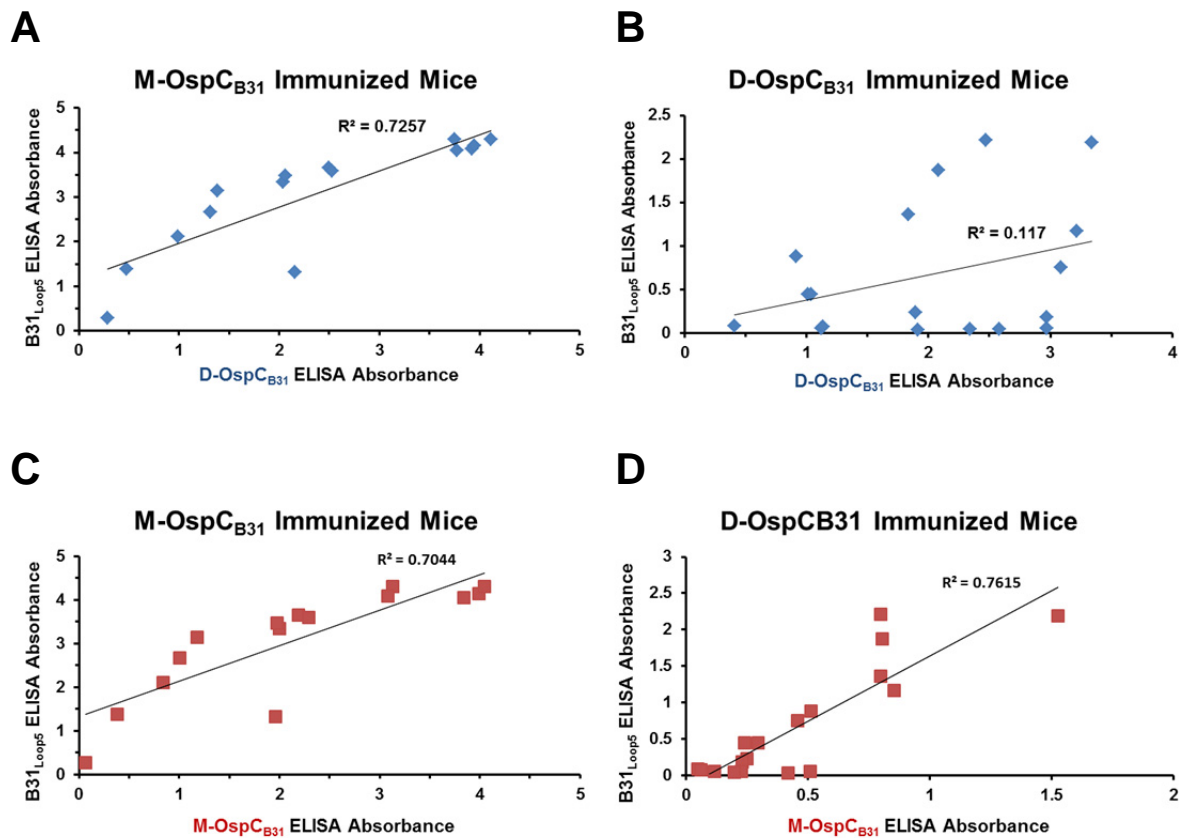


Fig. S3. Correlation of antibody reactivity with monomeric and dimeric OspC_{B31} proteins and peptide B31_{Loop5}. Mice were immunized with either M-OspC_{B31} or D-OspC_{B31} as described in Materials and Methods, and prechallenge sera were tested for IgG1 antibodies reactive with the two forms of OspC_{B31} (X-axis) and B31_{Loop5} (Y-axis) by ELISA. A) High correlation of D-OspC_{B31} and B31_{Loop5} reactivity in mice immunized with M-OspC_{B31} ($R^2 = 0.7257$). B) Low correlation of reactivity in mice immunized with D-OspC_{B31} ($R^2 = 0.117$). Correlation of reactivity in B31_{loop} and M-OspC_{B31} ELISAs was high for sera from both M-OspC_{B31}- and D-OspC_{B31}-immunized mice (panels C and D, respectively). Data are from Experiment 2 (Table S2) and include all antigen dosages.

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