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Deciphering the interface between a CD40 receptor and borrelial ligand OspA

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ABSTRACT

Neuroborreliosis is serious sequelae of Lyme borreliosis. Neuroinvasion is largely relied on successful translocation of *Borrelia* across the blood–brain barrier. Adherence of *Borrelia* to brain microvascular endothelial cell (BMEC) seems to be critical for translocation. Here we unfold the interface between OspA and CD40 molecules, major ligand and receptor, that are involved in adhesion of *Borrelia* to BMECs. We found that a region between Asn127 and Asp205 of OspA forms the CD40-receptor binding site. This region encompasses human umbilical vein endothelial cell (HUVEC) binding domain and contains a potential ligand-binding pocket lined by three amino acid residues: Arg139, Glu160 and Lys189. Disruption of this pocket (by truncation of the HUVEC binding domain) caused complete abrogation of its ability to bind CD40. To identify the amino acid residues within the HUVEC binding domain involved in the CD40 binding, site-directed mutagenesis and binding assays were performed. Results showed that Asp149, Phe165, Ala172, Val186 and Leu192 might form interface with CD40 molecule. Other side of the interface was also identified with the help of a ligand-binding assay with OspA and truncated CD40 fragments. Results exposed that cysteine rich domain 2 (CRD2) of CD40 might be the site for OspA binding. Precise knowledge of the molecular basis of the ligand–receptor interactions is essential in order to understand mechanisms of pathogenesis and could help in the development of novel therapeutics and vaccines.

1. Introduction

Crossing of the blood–brain barrier (BBB) by *Borrelia* is a crucial step in the development of neuroborreliosis, however, its precise molecular mechanism is still unknown. Multistage process of borrelial dissemination from peripheral vasculature was described earlier, which includes transient tethering-type associations, short-term dragging interactions, and a stationary adhesion (Moriarty et al., 2008). Stationary adhesion of *Borrelia* is commonly observed at a site of intercellular junctions of peripheral endothelial cells (Moriarty et al., 2008). Various interactions of spirochetes with endothelial cells, such as adhesion, passage through intercellular space or exploitation of host-derived proteolytic enzymes (such as plasmin and matrix metalloproteinases – MMPs) to disrupt intercellular junctions are essential for crossing of the endothelial barriers (Hu et al., 1995; Coleman and Benach, 2003; Zhao et al., 2007; Moriarty et al., 2008).

Previously it was demonstrated that OspA mediates adhesion of *Borrelia* to the peripheral blood endothelial cells (Comstock et al., 1993). Recently it was shown that OspA of neuroinvasive *Borrelia* and CD40 on the brain microvascular endothelial cells (BMEC) take part in the transient association (primary adhesion) during the translocation of spirochete across BBB (Pulzova et al., 2011). It was also shown that OspA of SKT-7.1 (neuroinvasive strain of *Borrelia garinii*, recently renamed as *Borrelia bavariensis*), but not of SKT-2 (non-neuroinvasive strain of *Borrelia burgdorferi* s.s.), is able to interact with CD40 (Pulzova et al., 2011). This differential binding ability of OspA to the CD40 was due to the sequence variations in

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0944-5013/

the coding region of OspA of neuroinvasive and non-neuroinvasive strains. The formation of OspA–CD40 complex results in the production of proinflammatory cytokines, increased expression of integrins and proteolytic enzymes (Pulzova et al., 2011) that may facilitate paracellular translocation of *Borrelia* across BBB. Hence a role of OspA and CD40 molecules in the initial transient association is apparent, which triggers off multiple signaling events necessary for stationary adhesion of *Borrelia* on the endothelial cells and its subsequent extravasation.

OspA is the multifunctional protein that interacts with various host receptors and plays a significant role in the establishment of chronic infection, neuroinvasion and survival of *Borrelia* in CNS (Hsieh et al., 2007). Expression of OspA is down-regulated almost immediately after a tick blood meal (Schwan et al., 1995), however, OspA expression *in vivo* can be significantly induced if the spirochetes are kept in an inflammatory environment (Crowley and Huber, 2003). Interestingly, OspA was found in the unique environment of the cerebrospinal fluid (CSF), but not in the serum (Schutzer et al., 1997). Moreover, all neuronal cell-adherent borreliae were OspA positive and OspC negative (Rupprecht et al., 2006). This finding supports the hypothesis that OspA plays an important role in binding to neuronal cells and indicates that OspA must be upregulated during the CNS invasion.

In this study, interface between OspA and CD40 was exposed. A region from Asn127 to Asp205 of OspA was found as the only site involved in the interaction with CD40, while Asp149, Phe165, Ala172, Val186 and Leu192 of OspA were found as functional residues that are responsible for the formation of interface. On the other side, cysteine-rich domain 2 (CRD2) seems to be involved in binding of OspA. Unfolding the OspA–CD40 interface might be helpful in the development of engineered vaccine, designed to abolish transient adhesion of *Borrelia* to BMECs and thus to block the translocation of *Borrelia* across BBB.

2. Materials and methods

2.1. Cultivation of Borrelia

Neuroinvasive strain SKT-7.1 (*B. garinii* serotype 4, recently designated as *B. bavariensis*) was cultured as described earlier (Pulzova et al., 2011).

2.2. Expression of truncated form of OspA

To map the domain/s of OspA interacting with CD40, four truncated forms of OspA were prepared. The first fragment contained tick gut endothelium (TGE) binding domains 1–2 (18–132 aa), the second encompassed human umbilical vein endothelial cells (HUVEC) binding domain (127–205 aa), the third contained TGE binding domain 3 (204–261 aa) while the fourth fragment ($OspA_{18–249}$) consisted all TGE and HUVEC binding domains (numbering based on GenBank accession number GU906888). Briefly, four regions were amplified from genomic DNA of SKT–7.1 by PCR using primers depicted in Table 2. Purified PCR products were digested with restriction enzymes (Thermo Scientific, Bratislava, Slovakia), ligated into previously digested pQE-30 UA (Qiagen, Hilden, Germany) or pQE-30 UA-GFP (in house modified vector, which inserts GFP fusion at C-terminus of insert) and proteins were overexpressed in *Escherichia coli* M15 or SG13009 (Qiagen).

For TGE and HUVEC binding domains pQE-30 UA vector was used, while for $OspA_{18–249}$ expression pQE-30 UA-GFP was used. GFP fusion of $OspA_{18–249}$ was essential for discrimination of OspA from its interacting partner (CD40) in subsequent pull down assay as both proteins have similar molecular weights. Recombinant clones were selected on LB agar plate containing 50 μg/ml carbenicilin and 25 μg/ml kanamycin. The integration of the OspA fragments in the vector in transformants was examined by PCR using vector-specific primers (Table 2) and sequencing. Expression of proteins was induced by adding isopropyl β-D-1-thiogalactopyranoside (1 mM). Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole) and sonicated on ice. The recombinant proteins were purified from the cleared cell lysate with the help of metal affinity chromatography according to manufacturers' instructions (Clontech, Mountain View, USA)

2.3. Cultivation of rat BMECs and extraction of membrane proteins

BMECs were cultivated exactly according to the procedures described before (Pulzova et al., 2011). Proteins were extracted with ProteoJET™ Membrane Protein Extraction Kit (Thermo Scientific) and protein concentration was measured with Bradford assay (Thermo Scientific).

2.4. Assessment of binding affinity of truncated OspA fragments to CD40 by western blotting

Membrane proteins of BMECs were fractionated by SDS-PAGE (8 cm well width/total protein load 400 μg) and electrotransferred onto nitrocellulose membrane. Membranes were cut into 3 mm vertical strips for further assays. Strips were blocked for 45 min in TTBS (TBS [25 mM Tris, 150 mM NaCl, pH 7.2] with 0.05% Tween 20) containing 2% BSA and incubated overnight at 4 °C with purified truncated OspA proteins. As a negative control one strip was incubated only in TTBS, while as a positive control one strip was incubated with purified $OspA_{18–249}$. After washing with TTBS, strips were incubated with HisProbe-HRP conjugate (Thermo Scientific) and the protein interaction was visualized on X-ray film using the ECL substrate (Thermo Scientific). Experiments were repeated three times to confirm the results.

2.5. Assessment of binding affinity of truncated OspA fragments to CD40 by pull-down assay

Truncated OspA fragments were bound to metal affinity beads (Talon, Clontech, Mountain View, USA) under the native condition as per manufacturer's instructions. After two washings with native wash buffer the beads were incubated with membrane proteins of BMECs. Unbound proteins were washed out and the protein complex was eluted. Proteins were separated by SDS-PAGE as well as detected with the help of MALDI-TOF. Detailed protocol of pull-down assay is described in our recent publication (Mlynarcik et al., 2012). Identification of the protein observed on the gel was performed with peptide mass fingerprinting as described earlier (Bhide et al., 2009). Experiment was repeated three times.

2.6. Further truncation of HUVEC binding domain and affinity of truncated fragments to CD40

Further truncation of HUVEC binding domain was performed as follows: $HUVEC_{127–155}$, $HUVEC_{154–205}$, $HUVEC_{127–171}$ and $HUVEC_{127–185}$. Amplification of gene fragments (primers depicted in Table 2), their ligation into pQE-30 UA-GFP vector, protein expression, cell lysis and metal affinity chromatography were performed as described above. Interaction between fragments of HUVEC binding domain and CD40 was assessed by western blotting as described above. As a positive control BMEC proteins were incubated with HUVEC binding domain (127–205 aa). As a negative

Table 1
Amino acid variations in OspA sequence of SKT-7.1 and SKT-2 strains.

Amino acid variation	SKT-2	SKT-7.1	Amino acid variation	SKT-2	SKT-7.1
Glu37Gly	37 Glu (−3.5)	37 Gly (−0.4)	**Val179Lys**	**179 Val (4.2)**	**179 Lys (−3.9)**
Lys39Thr	39 Lys (−3.9)	39 Thr (−0.7)	**Lys181Thr**	**181 Lys (−3.9)**	**181 Thr (−0.7)**
Asn47Asp	47 Asn (−3.5)	47 Asp (−3.5)	**Thr186Val**	**186 Thr (−0.7)**	**186 Val (4.2)**
Asp53Ser	53 Asp (−3.5)	53 Ser (−0.8)	**Ser192Leu**	**192 Ser (−0.8)**	**192 Leu (3.8)**
Ile55Glu	55 Ile (4.5)	55 Glu (−3.5)	**Val197Ile**	**197 Val (4.2)**	**197 Ile (4.5)**
Val75Thr	75 Val (4.2)	75 Thr (−0.7)	**Ser198Thr**	**198 Ser (−0.8)**	**198 Thr (−0.7)**
Val79Glu	79 Val (4.2)	79 Glu (−3.5)	**Glu200Aal**	**200 Glu (−3.5)**	**200 Ala (1.8)**
Ala81Thr	81 Ala (1.8)	81 Thr (−0.7)	**Asn202Asp**	**202 Asn (−3.5)**	**202 Asp (−3.5)**
Ser91Ala	91 Ser (−0.8)	91 Ala (1.8)	**Thr204Ser**	**204 Thr (−0.7)**	**204 Ser (−0.8)**
Gly95Ser	95 Gly (−0.4)	95 Ser (−0.8)	Ser206Thr	206 Ser (−0.8)	206 Thr (−0.7)
Thr98Lys	98 Thr (−0.7)	98 Lys (−3.9)	Ser207Thr	207 Ser (−0.8)	207 Thr (−0.7)
Leu99Phe	99 Leu (3.8)	99 Phe (2.8)	Ala208Gln	208 Ala (1.8)	208 Gln (−3.5)
Val101Ile	101 Val (4.2)	101 Ile (4.5)	Ala214Gly	214 Ala (1.8)	214 Gly (−0.4)
Ser116Leu	116 Ser (−0.8)	116 Leu (3.8)	Ala215Lys	215 Ala (1.8)	215 Lys (−3.9)
Val132Thr	**132 Val (4.2)**	**132 Thr (−0.7)**	Asn217Asp	217 Asn (−3.5)	217 Asp (−3.5)
Ile136Thr	**136 Ile (4.5)**	**136 Thr (−0.7)**	Gly219KLys	219 Gly (−0.4)	219 Lys (−3.9)
Thr138Val	**138 Thr (−0.7)**	**138 Val (4.2)**	Thr226Ser	226 Thr (−0.7)	226 Ser (−0.8)
Asp141Asn	**141 Asp (−3.5)**	**141 Asn (−3.5)**	Lys230Gln	230 Lys (−3.9)	230 Gln (−3.5)
Gly149Asp	**149 Gly (−0.4)**	**149 Asp (−3.5)**	Asp234Asn	234 Asp (−3.5)	234 Asn (−3.5)
Gly164Asp	**164 Gly (−0.4)**	**164 Asp (−3.5)**	Asn241Asp	241 Asn (−3.5)	241 Asp (−3.5)
Tyr165Phe	**165 Tyr (−1.3)**	**165 Phe (2.8)**	Gln247Lys	247 Gln (−3.5)	247 Lys (−3.9)
Val166Thr	**166 Val (4.2)**	**166 Thr (−0.7)**	Asn251Ala	251 Asn (−3.5)	251 Ala (1.8)
Thr172Ala	**172 Thr (−0.7)**	**172 Ala (1.8)**	Ile255Leu	255 Ile (4.5)	255 Leu (3.8)
Glu174Asp	**174 Glu (−3.5)**	**174 Asp (−3.5)**	Ser258Lys	258 Ser (−0.8)	258 Lys (−3.9)
–175Gly	**175 –**	**175 Gly (−0.4)**			

Numbers in the parenthesis indicate Kyte and Doolittle hydropathy profile. Amino acid changes depicted in the bold letters are located in the HUVEC binding domain.

control none of the HUVEC fragment was included in the western blotting. Experiment was repeated three times.

2.7. Site-directed mutagenesis and mapping of amino acid residues within HUVEC binding domain responsible for binding to CD40

In our previous study, we have shown that OspA of neuroinvasive strain SKT-7.1, but not of non-neuroinvasive strain SKT-2, interacted with CD40 (Pulzova et al., 2011). This suggested that difference in the binding ability to CD40 might be due to amino acid variations in HUVEC binding domain of OspA. This fact can be used effectively to map functional amino acids. In short, amino acid sequences of OspA from both strains were compared (SKT-7.1, accession number GU906888; SKT-2, accession number AY597037; Geneious pro software, Biomatters, San Francisco, USA), wherein 20 amino acids variations were found in HUVEC binding domain. These variable residues were subjected to site-directed mutagenesis (Fig. 1). Primers for mutagenesis were designed using software (https://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15) (Table 3). pQE-30UA-GFP carrying OspA with desired mutation was constructed using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Kirkland, USA). All steps in site-directed mutagenesis, like amplification of the vector, digestion of PCR product with *DpnI*, transformation of mutated vector in XL-10 gold *E. coli*, clonal selection of transformants and plasmid isolation, were performed exactly as described in the kit. Plasmids carrying mutation were transformed into M15 or SG13009 *E. coli*, proteins were overexpressed and purified with metal affinity chromatography as described above. Proteins were dialyzed (MWCO: 6000) against TBS and purity of His-tagged protein was confirmed by SDS-PAGE. Nucleotide sequences of mutants were submitted to GenBank: JX889248 to JX889267.

Table 2
Primers used for amplification of OspA fragments.

Primer name	Sequence
$OspA_{18–249}$	*Sense* GT**GATATC**GAAGCAAAATGTTAGCAGCCTT
	Antisense ATA**GTCGAC**TCTCCCTTCTATGTCGTATTTTTGTAC
TGE binding domains 1,2	*Sense* AAA**GGATCC**AAGCAAAATGTTAGCAGCCTT
	Antisense ATA**GTCGAC**TGTTTCACCCTTTTCGTTGAA
HUVEC binding domain	*Sense* AACGAAAAGGGTGAAACA
	Antisense GTCAGAGTCATCAAGTGC
TGE 3 binding domain	*Sense* AAA**GGATCC**TCTGACACTACTCAGGCT
	Antisense ATA**GTCGAC**TTCGACTGCTTTGCCTTC
$HUVEC_{127–155}$	*Sense* CCA**GGATCC**AACGAAAAGGGTGAAACATCT
	Antisense ATA**GTCGAC**TCTCCCTTCTATTCCATCGCTTTTTATGTCTG
$HUVEC_{154–205}$	*Sense* AG**GATATC**TCCGGAAAAGCTAAAGAAGTT
	Antisense ATA**GTCGAC**TCTCCCTTCTATGTCAGAGTCATCAAGTGC
$HUVEC_{127–171}$	*Sense* AG**GATATC**TAACGAAAAGGGTGAAACA
	Antisense TAC**GTCGAC**TAGAGTTCCTTCAAGAGTAAA
$HUVEC_{127–185}$	*Sense* AG**GATATC**TAACGAAAAGGGTGAAACA
	Antisense ATA**GTCGAC**AACAGTGCCTTCTGTAAC
Confirmation of proper ligation of insert in vector	
Vector specific primers	*Sense* CGCATCACCATCACCATCACG
	Antisense ACCAAAATTGGGACAACACCAGTG

Restriction enzyme site is indicated in bold

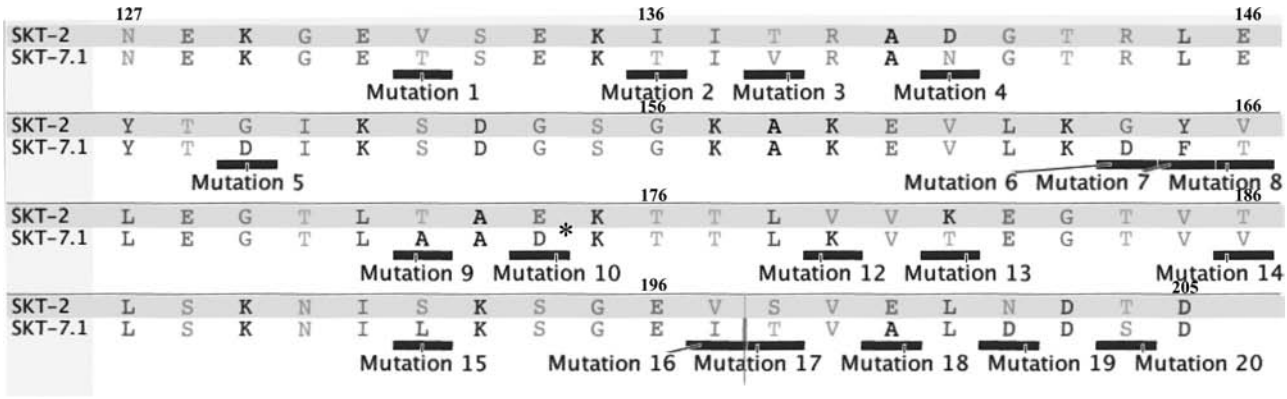

Fig. 1. Comparative sequence analysis of HUVEC binding domain of OspA of SKT-7.1 and SKT-2. * indicates glycine insertion observed in OspA of SKT-7.1 opposed to SKT-2. Amino acids changed with the help of site-directed mutagenesis are indicated as Mutation 1, 2 and so on. Glycine insertion (*) is mutation # 11.

Table 3
Primers used for site-directed mutagenesis of OspA.

Primer name is the same as mutations	Sequence
Thr132Val	*Sense* CAACAGAAGAAAAATTCAACGAAAAGGGTGAAGTATCTGAAAAAACAATAGTAAGAG
Thr136Ile	*Sense* ACGAAAAGGGTGAAACATCTGAAAAAATAATAGTAAGAGCAAATGGA
Val138Thr	*Sense* ACGAAAAGGGTGAAACATCTGAAAAAACAATAACAAGAGCAAATGGAACCA
Asn141Asp	*Sense* CATCTGAAAAAACAATAGTAAGAGCAGACGGAACCAGACTTGAATACACAGA
Asp149Gly	*Sense* GAACCAGACTTGAATACACAGGAATAAAAAGCGATGGATCCGGA
Asp164Gly	*Sense* GAAAAGCTAAAGAAGTTTTAAAAGGCTTTACTCTTGAAGGAACTCTAGC
Phe165Tyr	*Sense* AGCTAAAGAAGTTTTAAAAGACTATACTCTTGAAGGAACTCTAGCTG
Thr166Val	*Sense* TCCGGAAAAGCTAAAGAAGTTTTAAAAGACTTTGTTCTTGAAGGAACTCTAG
Ala172Thr	*Sense* CTTTACTCTTGAAGGAACTCTAACTGCTGACGGCA
Asp174Glu	*Sense* TGAAGGAACTCTAGCTGCTGAAGGCAAAACAACATTG
Gly175Ala	*Sense* AAGGAACTCTAGCTGCTGACGCTAAAACAACATTGAAAGTTACAG
Lys179Val	*Sense* CTGACGGCAAAACAACATTGGTGGTTACAGAAGGCACTGTTGTTTTAAG
Thr181Lys	*Sense* CTGACGGCAAAACAACATTGAAAGTTAAAGAAGGCACTGTTG
Val186Thr	*Sense* CATTGAAAGTTACAGAAGGCACTGTTACTTTAAGCAAGAACATTTTAAAATCCG
Leu192Ser	*Sense* AGGCACTGTTGTTTTAAGCAAGAACATTTCAAAATCCGGAGAAATAAC
Ile197Val	*Sense* CAAGAACATTTTAAAATCCGGAGAAGTTACAGTTGCACTTGATGACTCTG
Thr198Ser	*Sense* AACATTTTAAAATCCGGAGAAATATCAGTTGCACTTGATGACTCTG
Ala200Glu	*Sense* CCGGAGAAATAACAGTTGAACTTGATGACTCTGACAC
Asp202Asn	*Sense* TCCGGAGAAATAACAGTTGCACTTAATGACTCTGACAC
Ser204Thr	*Sense* GAAATAACAGTTGCACTTGATGACACTGACACTACTCAGGCT

For site-directed mutagenesis pairs of primers were used where the antisense primers (not shown here) are the reverse complement of the given sense primers. Mutation point is underlined.

2.8. Expression of recombinant CD40 (r-CD40)

An expression vector pLEXSY_IE-blecherry3 (Jena Bioscience) was extensively modified by the addition of: (1) factor Xa site (between target protein and GFP tag), (2) coding sequence for GFP (at C terminus to factor Xa site), (3) Myc-tag (at C terminus to GFP). Generated vector, pLEXSY_I-eGFP-Myc-Ble3, was used further for the expression of CD40 fusion proteins.

Coding region encompassing amino acids 28–192 (numbering based on GenBank accession number BC097949) of CD40 was amplified by PCR using primers presented in Table 4. Amplicons were digested with restriction enzymes *XbaI* and *AflI* (Thermo Scientific), column purified, ligated into the digested pLEXSY_I-eGFP-Myc-Ble3 vector and transfected into *E. coli* strain DH5α. Transformed colonies were selected on LB agar plates containing 50 μg/ml carbenicillin. Recombinant plasmid DNA

Table 4
Primers used to amplify CD40 fragments.

Primer name	Sequence
Expression of CD40 in *L. tarentolae* system	
r-CD40	*Sense* CCA**TCTAGA**GGACAAACAGTACCTCCAAGGT
	Antisense ATATA**CTTAAG**CATCCGGGGCTGGAAACC
$CD40_{D1}$	*Sense* GCTGGCGCCTC**TCTAGA**CACAGACAAACAGTACCTCCAAGGT
	Antisense GCTTCTCCCTTCTATGGTACC**CTTAAG**GTCGCACGGTTGGCATTGGGT
$CD40_{D2}$	*Sense* GCTGGCGCCTC**TCTAGA**CACAGAGAAGACCCAATGCCAACCG
	Antisense GCTTCTCCCTTCTATGGTACC**CTTAAG**GCAGTGCTGCCCTTCCTTGCA
$CD40_{D3}$	*Sense* GCTGGCGCCTC**TCTAGA**CACAACCGCGGTTTCAGACACT
	Antisense GCTTCTCCCTTCTATGGTACC**CTTAAG**GAAGAATCCGACCGGGCA
$CD40_{D4}$	*Sense* GCTGGCGCCTC**TCTAGA**CACAGATACTGTCTGCCAACCCTGC
	Antisense GCTTCTCCCTTCTATGGTACC**CTTAAG**CATCCGGGGCTGGAAACC

Restriction enzyme site is indicated in bold.

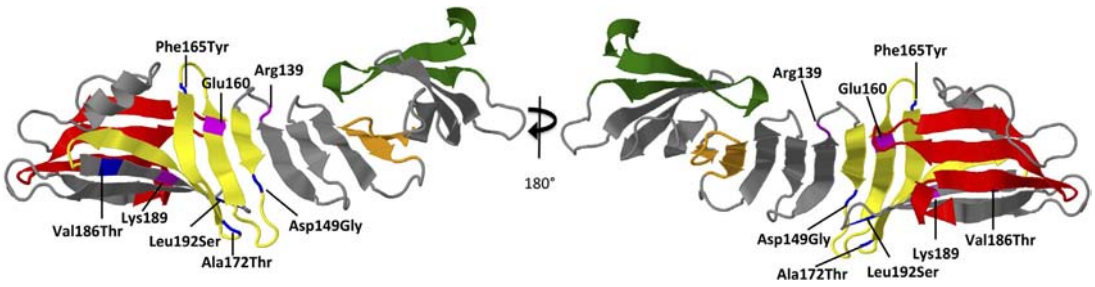

Fig. 2. Stereo view of binding domains and putative ligand binding pocket in OspA. TGE binding domain 1 (green), TGE binding domain 2 (orange), HUVEC binding domain (yellow) and TGE binding domain 3 (red). Figure also depicts three residues (Arg139, Glu160 and Lys189, in magenta) that form a potential binding pocket for ligands (predicted by Li et al., 1997). Residues with blue color present mutations that abolished binding ability of OspA to CD40.

was isolated from *E. coli* transformants, 5 μg of plasmid was digested with *Swa*I to linearize the cassette and electroporated into *Leishmania tarentolae* cells (LEXSY T7-TR, Jena Bioscience). Stable transformants were selected in BHI medium containing bleomycin (100 μg/ml) for 5 days at 26 °C. Protein expression was induced with tetracycline (10 μg/ml, Duchefa, Haarlem, The Netherlands). Overexpressed protein secreted in the BHI medium was purified according to manufacturers instructions (http://www.jenabioscience.com/images/ae3a4f50f1/EGE-1410.pdf). Protein was resolved by SDS-PAGE, transferred onto nitrocellulose membrane and presence of r-CD40 protein was verified by western blot analysis using anti-Myc-tag HRP antibody (Sigma).

2.9. Detection of interaction between r-CD40 and OspA mutants by western blotting and ELISA

In the western blotting membranes with immobilized r-CD40 were incubated with purified His-tagged OspA mutants. Protein–protein interaction was detected using HisProbe-HRP conjugate, as described above. Experiments were repeated three times.

Interaction between CD40 and OspA mutants was also confirmed by ELISA. In short, r-CD40 (10 μg/ml) diluted in carbonate buffer (50 mM sodium carbonate, pH 9.6) was coated in the microtiter wells for 2 h at room temperature, unbound r-CD40 was washed with TTBS and non-specific binding sites were blocked with 1% skimmed milk in TTBS. One hundred microliter of OspA mutants (100 μg diluted in TTBS with 1% skimmed milk) was added in the wells and incubated for 1 h at room temperature with constant shaking. Wells were washed three times with TTBS and incubated with 100 μl of HisProbe-HRP conjugate (diluted 1:1000 in TTBS) for 1 h. After incubation wells were washed three times with TTBS and 100 μl of ABTS enzyme substrate (Sigma, Bratislava, Slovakia; 160 mg of ABTS in 400 ml of citric phosphate buffer pH 4.3 and 1% of 1:40 H_2O_2) was added. Absorbance was measured at 405 nm and 0.1 was set as a cut-off value. Absorbance above cut-off was considered positive. Experiment was repeated three times.

2.10. Expression of truncated fragments of CD40

CD40 consists of four CRDs. Gene fragments encompassing each CRD were amplified from rat cDNA with PCR (primers in Table 4) and subcloned into pLEXSY_I-eGFP-Myc-Ble3. Truncated proteins were overexpressed in *Leishmania tarentolae* expression system and purified as described above. Four truncated proteins were designated as: $CD40_{D1}$ (28–62 aa, encompassing CRD1), $CD40_{D2}$ (55–111 aa, encompassing CRD2), $CD40_{D3}$ (96–151 aa, encompassing CRD3) and $CD40_{D4}$ (140–192 aa, encompassing CRD4). Presence of protein was checked with anti-Myc-tag HRP antibody as described above.

2.11. Mapping of OspA binding domain of CD40

Truncated CD40 fragments and r-CD40 (positive control) were spotted (10 μg) on nitrocellulose membrane and the non-specific binding sites were blocked with 2% BSA in TTBS. Membranes were incubated with purified recombinant $OspA_{18–249}$ (80 μg total protein resuspended in TTBS) or only with TTBS containing 1% BSA

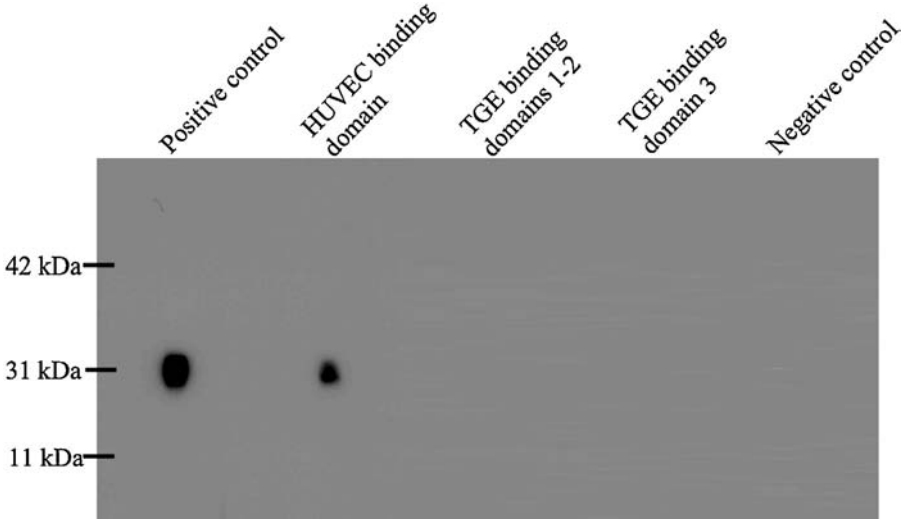

Fig. 3. Interaction between OspA domains and rat BMEC receptor CD40. Positive control – BMEC proteins incubated with $OspA_{18–249}$. Negative control – BMEC proteins incubated only with TTBS.

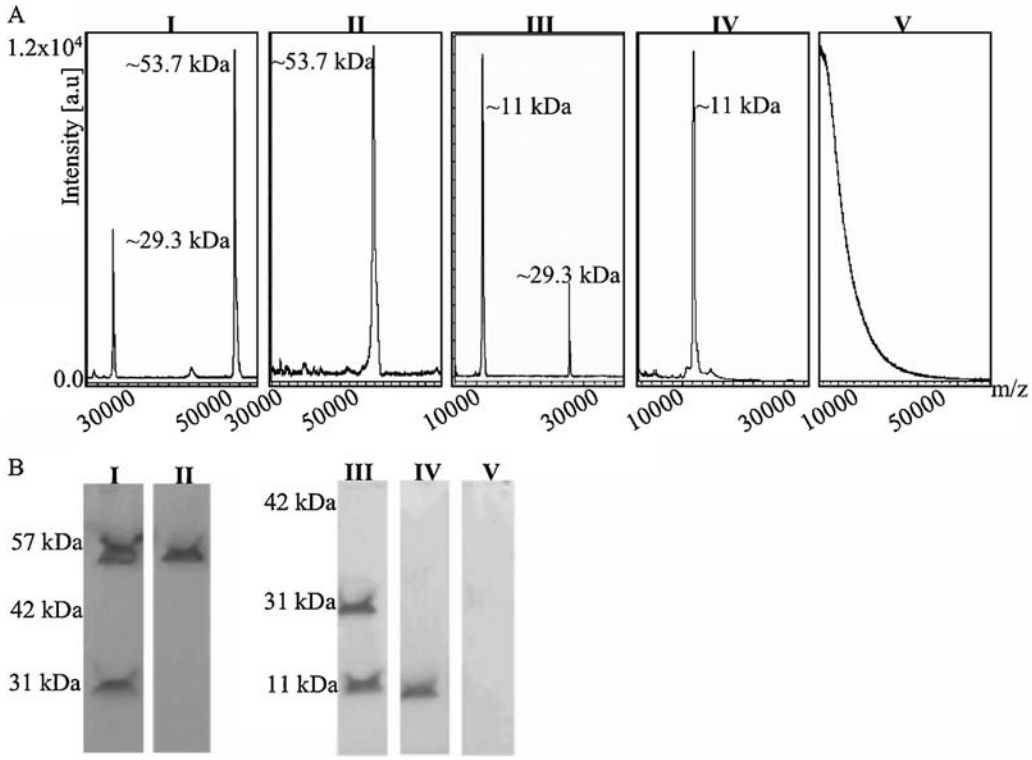

Fig. 4. Binding ability of truncated OspA fragments to CD40. Pull down assay. Proteins in eluate were detected with mass spectrometry (A) and SDS-PAGE (B). In both (A) and (B) – (I) immobilized $OspA_{18–249}$ (~53.7 kDa) was incubated with cell lysate of rat BMECs; ~29.3 kDa protein corresponds to the molecular weight of CD40. (II) Input control – only $OspA_{18–249}$ was captured on affinity beads and eluted. (III) Immobilized HUVEC binding domain (~11 kDa) incubated with cell lysate of rat BMECs; ~29.3 kDa protein corresponds to molecular weight of CD40. (IV) Input control – HUVEC binding domain was captured on affinity beads and eluted. (V) Negative control – only lysate of BMECs was incubated with affinity beads.

(negative control). Membrane was incubated with HisProbe-HRP conjugate (Thermo Scientific) and signals were developed with ECL chemiluminescence substrate. All experiments were repeated three times. Interaction was also confirmed with ELISA as described above.

3. Results

Central region of OspA is polymorphic while the C-terminal region is relatively conserved. Amino acid variations in the central polymorphic region may alter the binding ability of OspA to various cell receptors like CD40. To map functional sites in the OspA amino acid sequence, data from protein databases (Uniprot, EMBL-EBI, Pfam, etc.) and previous studies were retrieved (Schubach et al., 1991; McGrath et al., 1995; Pal et al., 2000; Nagasawa et al., 2006). Fig. 2 depicts three tick gut endothelium binding domains (TGE binding domain) and a human umbilical vein endothelial cells binding domain (HUVEC binding domain).

3.1. Residues within the region of Asn127–Asp205 of OspA (HUVEC binding domain) interact with rat CD40 receptor

To identify the region of OspA interacting with CD40, three truncated forms of the OspA were prepared. The truncation was based on OspA functional domains depicted in Fig. 2. Purity of the truncated fragments was confirmed by the SDS-PAGE in which no or very slight protein degradation was found (Supplemental Fig. 1).

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In the western blotting, immobilized rat BMEC proteins were incubated with truncated OspA fragments coding the each functional domain, wherein we found that only $OspA_{18–249}$ (encompassed all functional domains, served as a positive control) and HUVEC binding domain were able to bind CD40 (Fig. 3). Binding ability of $OspA_{18–249}$ and HUVEC binding domain to CD40 was further assessed by pull-down assay in which truncated OspA fragments were stably linked to metal affinity beads and incubated with cell extract of rat BMECs. Protein complex was eluted from affinity beads and subjected to MALDI-MS or separated on PAGE gel. In both cases, $OspA_{18–249}$ (53.7 kDa) and HUVEC binding domain (11 kDa) were accompanied with a 29.3 kDa protein (Fig. 4). This 29.3 kDa protein, interacting with OspA, was identified as CD40 with peptide mass fingerprinting.

3.2. Further truncation of HUVEC binding domain abolishes CD40 binding ability of OspA

Previously, Li and colleagues described that three charged residues Arg139, Glu160 and Lys189 in the β-strand 10, 12 and 15 of OspA form a potential binding pocket for an unknown ligand (Li et al., 1997). Based on this knowledge, further truncation of HUVEC binding domain was performed: $HUVEC_{127–155}$ fragment containing Arg139 and $HUVEC_{154–205}$ containing remaining two amino acids. After confirmation of the purity of the truncated HUVEC fragments (Supplemental Fig. 1), they were incubated with immobilized proteins of rat BMECs. This western blotting revealed that none of the HUVEC binding domain fragments was able to bind CD40 (Fig. 5). Based on this observation, another two fragments ($HUVEC_{127–171}$ and $HUVEC_{127–185}$) of HUVEC binding domain were constructed, which encompassed Arg139 and Glu160, but not Lys189 (Supplemental Fig. 1). None of these two fragments showed binding ability to CD40 (Fig. 5). These results suggest that all three amino acid residues (Arg139, Glu160 and Lys189) might be essential to maintain the affinity of OspA to CD40. Results also suggested

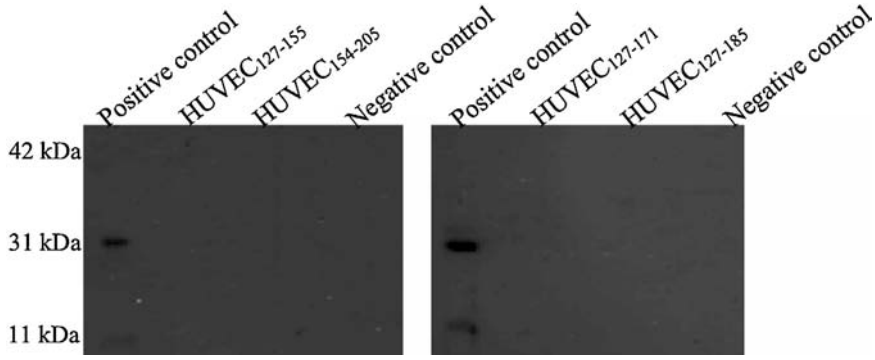

Fig. 5. Interaction of fragment of HUVEC binding domain with rat BMEC receptor CD40. Interaction of truncated fragments of HUVEC binding domains with ~30 kDa protein (CD40) of rat BMECs. Positive control – recombinant HUVEC binding domain (127–205 aa) incubated with CD40 on membrane (detected with HisProbe-HRP). Negative control – membrane was incubated only with TTBS.

that fragmentation of the binding pocket abrogates interface formation between OspA and CD40.

3.3. Asp149, Phe165, Ala172, Val186 and Leu192 of OspA are involved in OspA–CD40 interface

Previously it was shown that OspA of neuroinvasive *Borrelia* (strain SKT-7.1, *B. bavariensis*) possess ability to bind CD40, however the OspA of non-neuroinvasive *Borrelia* (SKT-2, *B. burgdorferi* sensu stricto) do not show binding ability to this receptor. In the sequence alignment of HUVEC binding domain of OspA of SKT-2 and SKT-7.1 a total of twenty amino acid variations were observed (Table 1, Fig. 1). To prove experimentally the importance of variable residues in CD40 binding, individual amino acid residues from OspA of neuroinvasive strain were changed to residues that are present in non-neuroinvasive stain. Site-directed mutagenesis was employed to construct twenty mutant OspA proteins with His tag at N terminus and GFP tag at C terminus: (Thr132Val, Thr136Ile, Val138Thr, Asn141Asp, Asp149Gly, Asp164Gly, Phe165Tyr, Thr166Val, Ala172Thr, Asp174Glu, Gly175Ala, Lys179Val, Thr181Lys, Val186Thr, Leu192Ser, Ile197Val, Thr198Ser, Ala200Glu, Asp202Asn and Ser204Thr. The purity of each mutant protein was confirmed on SDS-PAGE (Supplemental Fig. 2). Simultaneously, Myc-tagged GFP-CD40 fusion protein was overexpressed in *L. tarentolae* expression system. Both recombinant candidates (OspA mutants and r-CD40) were used in western blotting and ELISA, in which r-CD40 was immobilized and incubated with mutant OspA proteins. Results of both assays showed that amino acid variations at Asp149Gly (mutation # 5), Phe165Tyr (mutation # 7), Ala172Thr (mutation

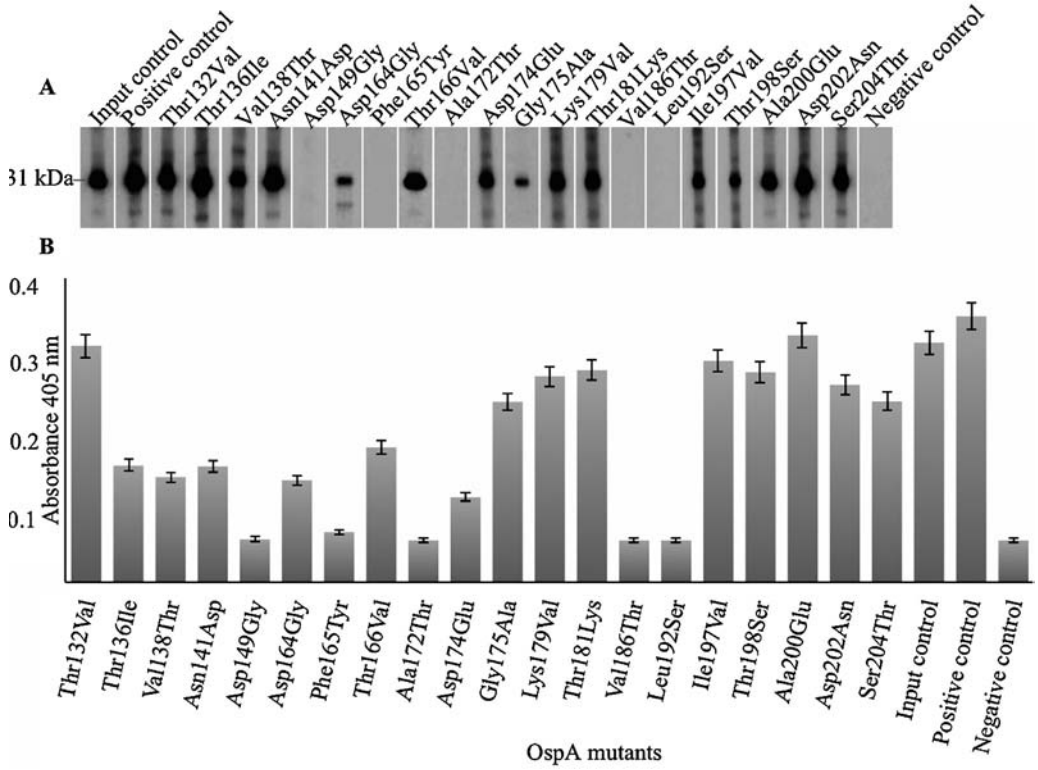

Fig. 6. Mapping of the amino acid residues involved in the binding of OspA to CD40. Western blotting (A) and ELISA (B). Input control – r-CD40 immobilized on membrane or in the microtiter well was detected with anti-Myc-HRP antibody. Positive control – $OspA_{18–249}$ was incubated with r-CD40 and bound OspA was detected with HisProbe-HRP. Negative control – any non-specific binding of the HisProbe-HRP conjugate to r-CD40 in the western blotting or ELISA was ruled out by incubating the r-CD40 only with HisProbe-HRP.

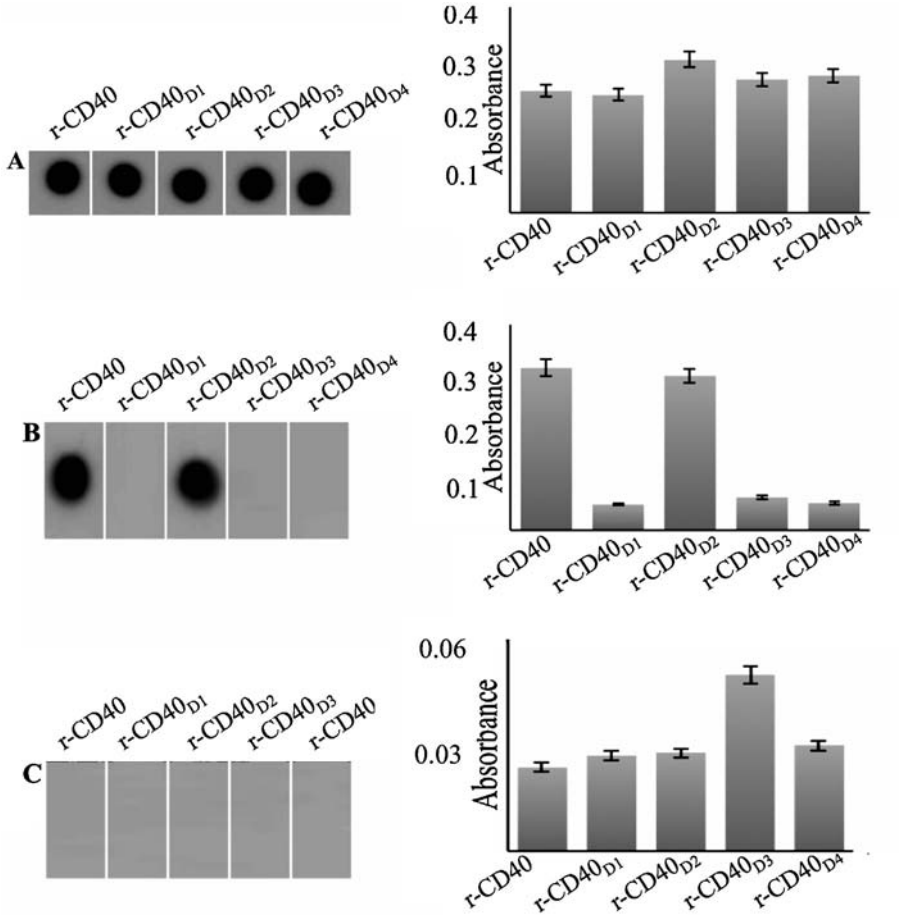

Fig. 7. Mapping of OspA binding site(s) on CD40. Input control (A) – presents results of western blotting and ELISA. CD40 fragments were immobilized on nitrocellulose membrane or in microtiter wells and identified with anti-Myc-HRP antibody. (B) Interaction between truncated CD40 proteins and $OspA_{18–249}$. CD40 fragments were fixed on nitrocellulose membrane or in microtiter wells and incubated with $OspA_{18–249}$. Interaction was detected by HisProbe-HRP. Negative control (C) – any non-specific binding of HisProbe-HRP conjugate to CD40 fragments was ruled out after incubation with HisProbe-HRP.

9), Val186Thr (mutation # 14) and Leu192Ser (mutation # 15) caused complete loss of affinity of OspA to CD40 (Fig. 6). Phe165, Ala172, Val186 and Leu192 are amino acids with hydrophobic side chain. It indicates the influence of hydrophobic interactions between the OspA and CD40. The overall results clearly show that these five amino acid residues are required for OspA–CD40 interaction.

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3.4. CRD2 of CD40 forms the binding site for OspA

CD40 is cell surface molecule belonging to the tumor necrosis factor receptor family. The extracellular region of CD40 contains four CRDs that participate in the binding of various ligands (Naismith and Sprang, 1998). To identify the CRDs that are involved in the binding with OspA, four truncated CD40 fragments (each encompassed a single CRD) were overexpressed in *L. tarentolae* expression system. CD40 fragments were incubated with $OspA_{18–249}$ of SKT-7.1 and the interaction was detected with HisProbe-HRP in both western blotting and ELISA. Among four CD40 fragments, only $CD40_{D2}$ (encompassed CRD2) showed binding ability to OspA in western blotting and ELISA (Fig. 7). Result of the western blotting suggests that CRD2 takes part in the formation of interface between OspA and CD40, besides identical binding affinities of CRD2 and r-CD40 observed in ELISA indicates that residues within CRD2 are sufficient to form entire interface between the OspA–CD40 complex.

4. Discussion

OspA is an important surface ligand employed in the adhesion process in vector as well as in the host. It helps *Borrelia* to attach to the tick gut endothelium (Pal et al., 2000), while in the host, it is important during the chronic stages of Lyme disease and development of neuroborreliosis (Rupprecht et al., 2008). OspA mediates adhesion of *Borrelia* to the neural cells (Rupprecht et al., 2006) and BMECs (Pulzova et al., 2011). Transient adhesion of *Borrelia* to endothelial cells evokes multiple signaling events in the endothelial cells, which may aid in the stationary adhesion of the spirochete and its extravasation. Interaction of OspA with CD40 receptor is required for transient tethering-type association of *Borrelia* on endothelial surface, a crucial step in the translocation of pathogen across the BBB (Pulzova et al., 2011).

OspA is abundantly expressed in tick midgut, which mediates attachment of *Borrelia* to endothelial cells *via* interaction between TGE binding domains and tick receptor for OspA (TROSPA) (Pal et al., 2004). OspA interacts with endothelial cells in ticks and mammalian hosts through different receptors, *i.e.* TROSPA and CD40, respectively. In this study we found that only HUVEC, but none of the TGE binding domains, possess binding ability to CD40. This finding suggests that OspA contains different domains for interaction with specific endothelial cell receptors. It will be interesting to find whether HUVEC binding domain interacts with TROSPA.

OspA contains 21 antiparallel β-strands and an α-helix at the C terminus (Li et al., 1997). Li et al. (1997) described that specific charged residues Arg139, Glu160 and Lys189 in the HUVEC

binding domain are crucial in the formation of potential binding pocket (hydrophobic cavity) for ligand with hydrophobic character. Based on this knowledge, truncated fragments of HUVEC binding domain were constructed and allowed to interact with CD40. In each fragment at least one of those three amino acids was absent. None of the truncated HUVEC fragments retained binding ability to CD40 (Fig. 5), which indicates that: (1) entire HUVEC binding domain is necessary to interact with CD40 and (2) all three amino acids, Arg139, Glu160 and Lys189, have to be present to form a potential CD40 binding pocket.

It is important to note that a trio of amino acids Arg139, Glu160 and Lys189 is conserved in the sequence of OspA among various *Borrelia* strains (Supplemental Fig. 3). However, it was shown that the binding of OspA to CD40 was distinct between neuroinvasive and non-neuroinvasive *Borrelia* strain (Pulzova et al., 2011). This suggests that formation of OspA–CD40 interface is not merely dependent on this trio. To identify the residues within the HUVEC binding domain accountable for CD40 binding, twenty amino acids were selected for site-directed mutagenesis based on the variations observed in the HUVEC binding domain of OspA of SKT-7.1 and SKT-2 (Table 1). Mutations at positions 149, 165, 172, 186 and 192 caused complete loss of binding ability to CD40 (Fig. 6). Moreover, in case of OspA from SKT-7.1, which shows strong binding affinity to CD40, hydrophobic residues are located at the positions 165 (Phe, hydropathy index 2.8), 172 (Ala, hydropathy index 1.8), 186 (Val, hydropathy index 4.2) and 192 (Leu, hydropathy index 3.8). Change in the hydrophobicity of these residues (165Tyr – hydropathy index −1.3, 172Thr – hydropathy index −0.7, 186Thr – hydropathy index −0.7 and 192Ser – hydropathy index −0.8) negatively influenced the binding ability of OspA to CD40, as observed in case of OspA of SKT-2 (Pulzova et al., 2011). These results confirm importance of hydrophobic cavity for the formation of OspA–CD40 interface.

Supplementary figure related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2014.09.003.

In the simultaneous experiments performed to explore OspA binding site(s) on CD40, we found that only $CD40_{D2}$ fragment (Glu55-Cys111, encompassing CRD2) is involved in the interaction with OspA (Fig. 7). CD40 is a transmembrane signaling protein expressed on the surface of B cells, monocytes, dendritic, epithelial and endothelial cells. CD40 is characterized by a repetitive amino acid sequence pattern of four CRDs, typically consisting of six cysteines forming three disulfide bonds. Previous study showed the involvement of CRD2 in the binding of CD40-ligand (CD40L) expressed on leukocytes (Bajorath et al., 1995). Ligation of CD40 with CD40L elevates production of adhesins (ICAM-1, VCAM-1) that increases cell binding (Hollenbaugh et al., 1995; Karmann et al., 1995), creates fenestrations (Tai et al., 2002) and alters MMPs expression (Mach et al., 1997; Schonbeck et al., 1997; Sukhova et al., 1999). Such orchestral cell signaling events take place during the translocation of leukocytes across endothelial barrier. Binding of OspA to CRD2 of CD40, suggests that *Borrelia* might mimic the interaction between CD40 and CD40L and might exploit cell-signaling events that take place during leukocyte extravasation.

To conclude, this work presents new insight into the molecular basis of ligand receptor interaction (OspA–CD40), which occurs during the transient adhesion of *Borrelia* to brain endothelial cell surface. Overall results indicate that amino acid variations in HUVEC binding domain affect interaction of OspA to CD40. That may explain the differential ability of *Borrelia* to adhere to the brain endothelial cell surface and transmigrate across the BBB. Exploitation of CRD2 of CD40 by *Borrelia* is also interesting finding that indicates possible mimicry of leukocyte translocation across endothelial barrier. Unfolding the ligand–receptor interface is essential in the development of effective vaccines, which may obliterate the first step in the translocation of pathogen, *i.e.* the transient adhesion.

5. Conclusion

Previously it was shown that OspA–CD40 complex mediates adhesion of *Borrelia* to the brain endothelial cells, which subsequently facilitates transendothelial migration of the spirochete and invasion of the brain tissue. Present work gives insight into the OspA–CD40 complex formation and identifies functional sites in both OspA and CD40. The work indicates the molecular basis of ligand–receptor interaction involved in the adhesion process, and may help in the development of novel therapeutics against neuroborreliosis.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

M.R.B., L.P. and P.M. conceived and designed the experiments. P.M., L.P., E.B., A.K., M.R.B., S.H. and M.A.D. performed experiments. P.M. and M.R.B. analyzed data. P.M. and M.R.B. wrote the paper and prepared the figures. All authors discussed the results and commented on the manuscript.

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