OspA-CD40 dyad: ligand-receptor interaction in the translocation of neuroinvasive *Borrelia* across the blood-brain barrier

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Lyme borreliosis is the most widespread vector-borne disease in temperate zones of Europe and North America. Although the infection is treatable, the symptoms are often overlooked resulting in infection of the neuronal system. In this work we uncover the underlying molecular mechanism of borrelial translocation across the blood-brain barrier (BBB). We demonstrate that neuroinvasive strain of *Borrelia* readily crosses monolayer of brain-microvascular endothelial cells (BMECs) *in vitro* and BBB *in vivo*. Using protein-protein interaction assays we found that CD40 of BMECs and OspA of *Borrelia* are the primary molecules in transient tethering of *Borrelia* to endothelium. OspA of neuroinvasive *Borrelia*, but not of non-neuroinvasive strain, binds CD40. Furthermore, only the neuroinvasive *Borrelia* and its recombinant OspA activated CD40-dependent pathway in BMECs and induced expression of integrins essential for stationary adhesion. Demonstration of the CD40-ligand interactions may provide a new possible perspective on molecular mechanisms of borrelial BBB translocation process.

Lyme borreliosis is the most commonly reported tick-borne infection in Europe and North America. If left untreated, *Borrelia* spreads systematically from the site of tick bite to various tissues, most probably skin, joints, heart and the central nervous system (CNS). Clinical symptoms of the neurological manifestation of acute Lyme neuroborreliosis include painful meningoradiculitis, lymphocytic meningitis, radicular pain (Bannwarth’s syndrome), and different forms of cranial or peripheral neuritis.

Invasion of CNS by *Borrelia* is a complex process, which requires successful crossing of the blood-brain barrier (BBB). The BBB is a regulatory interface between peripheral circulation and the CNS. It is composed of brain microvascular endothelial cells (BMECs), astrocytes, basement membrane, pericytes and neurons. The BMECs possess unique characteristics that distinguish them from peripheral endothelial cells (PECs). BMECs are connected via tight intercellular junctions that together with the lack of fenestration and reduced level of fluid-phase endocytosis limits free transport of solutes and protects the brain from the invasion of most of pathogens.

It’s still a matter of debate how the *Borrelia* crosses BBB. Some researchers favor a paracellular route (crossing of pathogen through intercellular space) of borrelial translocation, whereas others support a transcellular passage. Using state of the art real-time high-resolution 3D microscopy, Moriarty and co-workers have documented dissemination of *Borrelia* out of peripheral vasculature, suggesting a paracellular route of translocation. Borrelial dissemination in peripheral circulation is a multi-stage process that includes transient tethering-type associations, short-term dragging interactions, and a stationary adhesion. Stationary adhesion of *Borrelia* is commonly observed at endothelial junctions of PECs, and translational motility of spirochetes seems to play an integral role in trans-endothelial translocation. Spirochete interactions with endothelial cells, such as adhesion, crawling through intercellular space or exploitation of host-derived proteolytic enzymes (like plasminogen, matrix metalloproteinases etc.) to disrupt intercellular junctions are essential for crossing of the various barriers.

*Borrelia* is well equipped for the attachment to the host cells by expressing an array of adhesive molecules. Borrelial outer surface proteins (Osp) take part in adherence to endothelial cells like PECs and human umbilical vein endothelial cells (HUVECs). Other adhesive proteins like P66, ErpK, OspC and protein ligand for \(\beta_3\)-chain...
integrins also bind to the endothelial cells, whereas, Bgp, DbpA and BBK32 bind the glycosaminoglycans. In the CNS, BBA25 and BBA50 proteins of Borrelia mediate the adherence to glial cells. However, Borrelia regulates the expression of its surface proteins during various stages of dissemination in the host. Therefore the surface protein arsenal of Borrelia is different during the BBB translocation from that in the early stages of dissemination out of peripheral vasculature.

Several tight junction transmembrane proteins, including occludin, claudin-1, -3, -5 and -12, junctional adhesion molecules, zona occludens-1 etc., are expressed differently in BMEC and peripheral vascular endothelial cells (ECs). In addition, BMECs also express unique cell surface glycoproteins that are not found on other ECs, such as the cerebral cell adhesion molecule, BBB-specific anion transporter-1, CXC chemokines with Glu-Leu-Arg motifs etc. Thus the protein candidates involved in the transient tethering-type associations and a stationary adhesion of Borrelia with BMECs during BBB translocation might be different. So far there is no report available that lists adhesive molecules of Borrelia and receptors on BMECs responsible for such interactions.

Here, we explore the basic molecular mechanisms of translocation of Borrelia across BBB. Differential ability of neuroinvasive and non-neuroinvasive borreliae to cross the BBB and invade CNS was confirmed in vitro and in vivo. To understand the underlying molecular interactions in the transient attachment of Borrelia to BMECs, we used protein-protein interaction assays coupled with MALDI mass spectrometry. OspA protein of the pathogen and CD40 of BMECs were identified as potential interacting molecules. Together with experimental results derived from quantitative real time PCR assays performed to evaluate induction of CD40 mediated pathway in BMECs by neuroinvasive/non-neuroinvasive borreliae and their OspA proteins, we show that formation of OspA:CD40 dyad is an essential molecular step that further induces the expression of integrons (ICAM-1, PECAM and VCAM-1) and metalloproteinases (MMP-3 and MMP-9) necessary in the stationary adhesion of Borrelia and their translocation across BBB.

Results

Borrelial translocation across BMECs. Crossing of the BBB is a crucial step in the CNS invasion of Borrelia. To confirm translocation and neuro-invasiveness of Borrelia, an in vitro model of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of Borrelia was based on the amplification of fla gene (gene encoding Flagellin, also used as housekeeping gene), in the contents of luminal and abluminal chambers after incubation for 18 hours and 24 hours with two different strains – SKT-7.1 (neuroinvasive strain) and SKT-2 (non-neuroinvasive strain). Only for 18 hours and 24 hours with two different strains – SKT-7.1 in the contents of luminal and abluminal chambers after incubation with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae. Detection of BBB on Transwell cell culture inserts was prepared and infected with borreliae.

Interaction between CD40 and OspA was further confirmed by next round of co-immunoprecipitation in which recombinant CD40 protein tagged with Flag octapeptide (N-DYKDDDDK-CD40) was immobilized on anti-Flag agarose beads and hybridized with purified His-tagged OspA proteins of SKT-7.1 or SKT2. After stringent washings, proteins were eluted and separated by SDS-PAGE. Only r-OspA of SKT-7.1 was found in elute along with r-CD40 (Fig. 2d). The ~30 KDa protein observed in Co-IP was excised and subjected to MALDI-TOF based peptide mass fingerprinting. The Mascot search showed CD40 as the most probable protein candidate (6 peptides match, significance threshold <0.05, 74 score).

Expression of OspA in the brain and brain microvasculature. Unless the expression of OspA in the brain microvasculature is augmented, mere binding of OspA to CD40 is not sufficient to designate OspA as a major protein candidate in the transient adhesion of Borrelia to BMECs. Expression of outer surface proteins in Borrelia may vary during various phases of infection as well as in different host body compartments. Some authors have suggested downregulation of OspA in early phase of the infection, while others have reported expression of OspA in the unique environment of the brain and CSF, but not in the serum. Therefore, it was essential to determine whether OspA is expressed in borreliae that are present in the brain vasculature in vivo infected laboratory animals. PCR analysis of the brain and brain microvasculature of Wistar rats infected with SKT-7.1, revealed presence of SKT-7.1 and SKT-2 strains in both chambers after 24 hours of incubation, which confirms that Transwell membrane itself is not an obstacle for translocation of either of the Borrelia strains from luminal to abluminal chamber (Fig. 1; lanes 9 and 10, designated as positive controls).

Borrelial adhesion to BMECs: Candidate molecules. For successful BBB translocation the transient adhesion of Borrelia to BMECs surface is a crucial step. In order to identify the protein candidates engaged in this adhesion, electrophoretically separated membrane proteins of BMECs were allowed to hybridize with proteins of borreliae in a ligand capture assay (LCA). A ~32 kDa was the prominent protein found in the stripped protein fraction of LCA, in which proteins of SKT-7.1 were incubated with BMECs membrane proteins. No such protein candidate was found in LCA performed with whole cell lysate of SKT-2. To identify the ~32 kDa protein candidate, the protein band was excised, in-gel digested with trypsin and subjected for MALDI-TOF/MS analysis. A Mascot search of peptide mass fingerprint gave maximum identity with outer surface protein A (Ospa) of Borrelia.

To corroborate the binding affinity of OspA to the BMEC membrane receptors, recombinant His-tagged OspA proteins of both SKT-7.1 and SKT-2 were prepared and mixed with BMECs proteins in co-immunoprecipitation (Co-IP) as well as affinity ligand binding (ALBI) assays. In both assays, the binding ability of His-tagged OspA protein of SKT-7.1 to ~30 kDa protein of BMECs was confirmed. On the other hand, His-tagged OspA protein of SKT-2 did not interact with any of the BMEC proteins (Fig. 2b, c). The ~30 KDa protein observed in Co-IP was excised and subjected to MALDI-TOF based peptide mass fingerprinting. The Mascot search showed CD40 as the most probable protein candidate (6 peptides match, significance threshold <0.05, 74 score).
not only the presence but also the augmented expression of OspA (Fig. 3). This finding is crucial to support a role of OspA as an adhesive molecule in the transient tethering of Borrelia. No presence of non-neuroinvasive SKT-2 was found in the brain and brain microvasculature of infected rats (Fig. 3a). This strain was present in the ear punch, but the OspA protein was not expressed on its surface (Fig. 3).

**Induction of CD40 and its downstream pathway by Borrelia.** In endothelial cells, CD40 activation leads to triggering of the production of pro-inflammatory cytokines, matrix metalloproteinase, pro-coagulants, angiogenesis factors, and an abrupt increase in adhesion molecules like E-selectin, VCAM-1 and ICAM-1. In order to determine whether neuroinvasive and non-invasive strains invoke immune response differentially, BMECs were challenged with SKT-7.1 and SKT-2 strains *in vitro* and expression of CD40, CD80, integrins, matrix metalloproteinases and cytokines was analyzed. A remarkable difference was found between the inductions of CD40 in BMECs challenged with SKT-7.1 (ΔΔCT – 130.72) and SKT-2 (ΔΔCT – 1) (Fig. 4a). The difference in the upregulation of CD40 was reflected in the augmented expression of its downstream and associated molecules. Among major integrins, induction of VCAM was the highest (ΔΔCT – 155.1), followed by PECAM (ΔΔCT – 106.3) and ICAM-1 (ΔΔCT – 99.5) in BMECs infected with SKT-7.1. Expression of ELAM was unchanged in BMECs infected with SKT-7.1, however, surprisingly the ELAM was under expressed significantly in BMECs infected with SKT-2 (Fig. 4b).

To degrade extracellular matrix and intercellular junctions many pathogens exploit host matrix metalloproteinases. MMP-3 and MMP-9 metalloproteinases were found upregulated in BMECs challenged with SKT-7.1 (both ~ 160 ΔΔCT), but not in BMECs infected with SKT-2. Expression pattern of MMP-1 and MMP-2 remained unchanged in both non-infected and infected BMECs (Fig. 4c). Among interleukins we found 129 fold increase in the expression of pro-inflammatory IL-1 in SKT-7.1 infected BMECs. Similarly, 99.67 fold increase in the level of TNFα was observed in SKT-7.1 infected BMECs. As expected, no significant induction of the anti-inflammatory IL-6 was observed in both BMECs cultures challenged with SKT-7.1 and SKT-2 (Fig. 4d).

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**Figure 2 | Differential interaction of proteins of Borrelia garinii (SKT-7.1) and Borrelia burgdorferi sensu stricto (SKT-2) with BMECs.** Results of LCA are presented in panel a. BMEC proteins were fixed and separated on SDS-PAGE. Lane 1, albumine; lane 2, whole cell lysate of SKT-7.1; lane 3, whole cell lysate of SKT-2; lane 4, LCA negative control; lane 5, LCA with SKT-7.1; lane 6, LCA with SKT-2. Panel b and c depict interaction of r-OspA with BMEC surface protein. Panel b – Co-IP experiment presenting interaction between r-OspA and BMEC protein. r-OspAs of SKT-2 or SKT-7.1 were immobilized on Ni-NTA agarose and hybridized with BMEC proteins. Protein complex was eluted, fractionated on SDS-PAGE. Panel c – ALBI assay presents affinity between r-OspA of SKT-7.1 with ~ 30 kDa protein of BMEC. r-OspA proteins of SKT-7.1 and SKT-2 were hybridized with BMECs proteins immobilized on nitrocellulose membrane. Lanes SKT-7.1 (-) and SKT-2 (-), membranes were incubated only with TBSM (negative control). Panel d depicts Co-IP experiment with r-OspA and r-CD40. r-CD40 was immobilized on anti-Flag agarose beads and hybridized with r-OspAs of SKT-7.1 or SKT-2. Protein complex was eluted and fractionated on SDS-PAGE. Lane r-CD40, r-CD40 was bound on anti-Flag agarose and eluted (input control); lanes r-CD40 + r-OspA (SKT-2) and r-CD40 + r-OspA (SKT-7.1), Co-immunoprecipitation of r-CD40 either with r-OspA of SKT-2 or SKT-7.1.

**Figure 3 | Presence of Borrelia and expression of OspA by SKT-7.1 and SKT-2 strains in rat tissues assessed by PCR.** Panel a depicts detection based on PCR amplification of fla gene. Panel b depicts detection based on amplification of OspA gene. Detection of borreliae in the brain microvasculature of infected rats with SKT-7.1 (lane 1) or SKT-2 (lane 2); in the brain tissues (SKT-7.1 - lane 3; SKT-2 - lane 4); in the ear punch (SKT-7.1 - lane 5; SKT-2 - lane 6). Panel c - Expression of mRNA of OspA in borreliae was assessed by quantitative RT-PCR. fla served as housekeeping gene. + and ++ indicate expression levels of the gene, - depicts no expression.
Figure 4 | Induction of CD40 and its downstream pathway in BMEC infected with *Borrelia* strains. Expression of CD40 and CD80 (panel - a), integrins (panel - b), matrix metalloproteinases (panel - c), cytokines (panel - d), and VEGF and thrombomodulin (panel - e) in BMECs infected with non-neuroinvasive (SKT-2) or neuroinvasive (SKT-7.1) Borrelia strains. Error bars indicate the standard deviation of triplicate samples. *P* values were calculated by using a paired t test, comparing SKT-7.1 or SKT-2 infected BMECs with the non infected BMECs (*, *P* < 0.05).
We also assessed expression levels of vascular endothelial growth factor (VEGF) and thrombomodulin, which are crucial candidates respectively in angiogenesis and anticoagulant pathway, in the BMECs. No significant variations in the level of expression of these factors in non-infected and infected BMECs were observed (Fig. 4e).

**Induction of CD40 and its downstream pathway by r-OspA proteins.** To confirm the importance of CD40-OspA dyad in the induction of CD40 downstream cascade, BMECs were incubated with r-OspA proteins with or without pre-incubation with anti-CD40 antibody. As expected, only r-OspA from SKT-7.1 induced the expression of CD40 and CD80 (Fig. 5a), major integrins ICAM-1 and VCAM-1 (Fig. 5b), MMP-3 and MMP-9 (Fig. 5c), and pro-inflammatory cytokines IL-1 and TNFα (Fig. 5d). In the parallel experiment, the cells were incubated with anti-CD40 antibody to block the CD40 molecule and then challenged with *Borrelia* strains. This experiment was important to verify a crucial role of OspA mediated CD40 dependent activation of downstream and associated molecules required for successful BBB translocation of neuroinvasive *Borrelia*. We found that blocking of CD40 molecule on BMECs impeded upregulation of MMPs, integrins, IL-1 as well as TNFα (Fig. 5). This finding corroborates that CD40:OspA dyad is the unique structure capable to evoke cell-signaling events in BMECs, which permits neuroinvasive *Borrelia* to cross tight junctions.

**In-silico analysis of functional domains of OspA and amino-acid variations.** OspA is a multifunctional protein with lipid moiety. Central domain of OspA is polymorphic while the carboxy terminal region is relatively conserved. Amino acid variations in the central polymorphic region may alter the binding ability of OspA to various cell receptors. To map these functional sites in the OspA amino acid sequence, we retrieved data from protein databases (Uniprot, EMBL-EBI, Pfam etc.) and previous studies. Figure 6 depicts antibody binding sites, tick gut endothelium (TGE), and HUVEC binding domain, and hypervariable antigenically important (HAI) regions. The amino acid side chain around Trp-216 is predicted as an antigenically important site, and the amino-acid changes around this site contribute to the antigenic variation in OspA. Highly conserved tryptophan at residue 216 was also found in both SKT-2 and SKT-7.1, however, significant variation in its side chain (AANWSGT – SKT-2 and GKWDSKT – SKT-7.1) was noticed.

OspA possesses three conserved TGE binding regions. These sites were also conserved in OspA proteins of both strains, except one amino acid variation Ser91Ala with potential to change hydrophathy index (Fig. 6, Table 2). Four amino acid variations that cause change in hydrophathy indices were also found in the endothelial cell binding site (residues 143–183) of OspA of SKT-7.1 and SKT-2 (Fig. 6, Table 2). Thus, it is tempting to speculate that amino acid variations in the endothelial cell binding site might be the cause of the differential binding of OspA proteins to CD40.

**Discussion**

Current understanding of the pathogenesis of Lyme neuroborreliosis favors the theory of borrelial paracellular translocation through the tight junctions of BMECs. Translocation is evidently a multi-stage sequential process that begins with transient tethering-type associations, followed by induction of multiple signaling events and stationary adhesion on the endothelial cells, and ends with extravasation of *Borrelia*. Despite a plethora of reports and predictions hitherto, no experimental evidence is available that supports the theory that *Borrelia* exploits these translocation-dependent adhesion and endothelium penetration at sites of inflammation is a two-step process. Weak binding by oligosaccharides and members of the selectin family results in short-term interaction (rolling) of passing leukocytes. This is followed by firm adhesion and transmigration mediated by activated integrins and adhesion molecules, particularly VCAM-1 and ICAM-1. It can be postulated that *Borrelia* might mimic the events in leukocyte transmigration. Augmentation of the CD40 expression along with upregulation of pro-inflammatory cytokine IL-1, cell adhesive molecules (ICAM-1, PECAM and VCAM-1), and metalloproteinases (MMP-3 and MMP-9) observed only in BMECs infected with SKT-7.1, but not with SKT-2, strongly indicate the importance of initial transient tethering-type associations (OspA:CD40 dyad) for proper multiple signaling events in the endothelial cells required for borrelial crossing of the BBB.

Previous studies have also reported increased expression of VCAM-1 and ICAM-1 molecules in OspA treated vascular endothelium. Furthermore, mimicry of lymphocyte function-associated antigen 1 (LFA-1, a potent adherent partner of ICAM-1) by OspA ligand has been described elsewhere. Based on this we can predict the role of ICAM-1 and OspA in the firm (stationary) adhesion of *Borrelia* to BMECs.

OspA is undoubtedly a multifunctional protein that is absolutely necessary in the various stages of borrelial lifecycle and pathogenesis. OspA is abundantly expressed in tick gut as an important adhesive molecule. To avoid an inflammatory response, expression of OspA is downregulated in the early stages of Lyme disease. However, OspA expression in vivo can be significantly induced if the spirochetes are kept in an inflammatory environment. OspA plays an important role in binding to neuronal cells. These data indicate that OspA must be upregulated during the CNS invasion and acts as an important adherence factor, which is essential in the pathogenesis of Lyme neuroborreliosis. It is also well known that *Borrelia* can bind plasminogen via OspA on their surface. OspA also upregulates membrane urokinase-type plasminogen activator receptor (uPAR) leading to degradation of the extracellular matrix. The mammalian plasminogen-plasmin proteolytic system plays a crucial role in extracellular matrix degradation (intercellular junctions) and cell migration. Binding of host-derived proteinases (like plasminogen and MMPs) via OspA supports the theory that *Borrelia* exploits these proteinases to degrade the intercellular tight junctions. Owing to the hypervariability of OspA among several *Borrelia* strains, it is important to note that only expression of OspA is not sufficient, but its ability to interact with host’s receptors is crucial in the invasion processes.

Apart from OspA, other proteins like BBA25, BBA50 and DbpA adhere to cells in CNS. All these proteins are encoded by linear
Figure 5 | Induction of CD40 mediated downstream pathway after CD40:OspA dyad formation. Activation of CD40 mediated downstream pathway in BMECs is presented. BMECs were incubated with r-OspA of either SKT-7.1 or SKT-2 (bars in each graph - BMEC with rOspA SKT-2 and BMEC with rOspA SKT-7.1). Blockage of CD40 mediated pathway was achieved by incubation of BMECs with anti-CD40 antibodies prior to addition of r-OspAs ((bars in each graph - BMEC with rOspA SKT-2 + CD40Ab and BMEC with rOspA SKT-7.1 + CD40Ab). Not infected BMEC and BMECs incubated only with CD40 antibody served as controls. Expression of CD40 and CD80 (panel - a), integrins (panel - b), matrix metalloproteinases (panel - c), cytokines (panel - d), and VEGF and thrombomodulin (panel - e). Error bars indicate the standard deviation of triplicate samples. P values were calculated by using a paired t test, comparing gene expression in BMECs infected with r-OspA of SKT-7.1 and SKT-2. Comparison was also performed between gene expression in BMECs pre incubated with anti-CD40 antibodies and challenged with r-OspA of SKT-7.1 and SKT-2 (*, P < 0.05; **, P < 0.02).
**Table 1 | Primers and PCR conditions used in the study**

| Gene  | Primer 5'-3' | Tm (°C) | Product length (bp) |
|-------|--------------|---------|---------------------|
| OspA  | F- AAAATTTATGGAATAGGTCTA R- AAAGGCTTTTAAAGTCTACA | 50.1 | 811 |
| OspA  | F- TGAGGACTACTGCTGACAA R- TGAGGACTACTGCTGACAA | 53.0 | 260 |
| Fla   | F- ACGGCACTATTCAGATGCAGACAA R- TGAGGACTACTGCTGACAA | 55.0 | 173 |
| Fla   | F- CACATTTACAGACAGCAGACAG R- CCCGGACGCTGACAGCTTGGAG | 57.0 | 331 |
| β actin | F- TCTTCTCAGCCTCCTTCTCC R- TGGTCTTTACGGATGTCAACG | 65.4 | 100 |
| CD40  | F- CCAGGAGTCACTCAGGGAAAT | 58.2 | 178 |
| CD80  | F- ACCGACACTGCGAACTCAA R- GCAGGTCAGATTAGGGGCTGGATT | 48.6 | 222 |
| ELAM  | F- TCTCTTCCAGCCTTCCTTCCTCC | 46.9 | 238 |
| GADPH | F- GGAGCAGAAGCAAGTGGT R- CTGAAGGAACTCTAACTGCTGACA | 49.2 | 260 |
| ICAM-1 | F- GCCTTGAGGTTAGGGAGAAT R- GCAAGGACCTGCTGGTCAAG | 59.2 | 178 |
| IL-1  | F- ACCGACACTGCGAACTCAA R- GCAGGTCAGATTAGGGGCTGGATT | 48.6 | 222 |
| MMP 1 | F- CTGACAAAAGGGAAGAAATCTGGCTTCGT | 58.2 | 178 |
| MMP 2 | F- TGAGGACTACTGCTGACAA R- TGAGGACTACTGCTGACAA | 53.0 | 260 |
| MMP 3 | F- ACCGACACTGCGAACTCAA R- GCAGGTCAGATTAGGGGCTGGATT | 55.0 | 173 |
| MMP 9 | F- GCCTTGAGGTTAGGGAGAAT R- GCAAGGACCTGCTGGTCAAG | 59.2 | 222 |
| PECAM-1 | F- TCAGCAAGAGAGGCTAGTAAAAAAGGTGCACTGAGCGTCA | 55.2 | 212 |
| THBD  | F- CCAGGAGTCACTCAGGGAAAT R- GCAGGTCAGATTAGGGGCTGGATT | 48.6 | 222 |
| TNF-α | F- GCCTTGAGGTTAGGGAGAAT R- GCAAGGACCTGCTGGTCAAG | 59.2 | 248 |
| VCAM-1 | F- GCCTTGAGGTTAGGGAGAAT R- GCAAGGACCTGCTGGTCAAG | 59.2 | 212 |
| VEGF  | F- GCCTTGAGGTTAGGGAGAAT R- GCAAGGACCTGCTGGTCAAG | 59.2 | 212 |

**Table 2 | Changes in amino acid sequence of OspA between strain SKT-2 and SKT-1**

| Amino acid change | SKT-2 | SKT-1 |
|-------------------|-------|-------|
| I55 E             | 55 Le (4.5) | 55 Glu (−3.5) |
| V75 T             | 75 Val (4.2) | 75 Thr (−0.7) |
| V79 E             | 79 Val (4.2) | 79 Glu (−3.5) |
| A81 T             | 81 Ala (1.8) | 81 Thr (−0.7) |
| S91 A             | S91 Ser (−0.8) | 91 Ala (1.8) |
| S1116 L           | S1116 Ser (−0.8) | 116 Leu (3.8) |
| V132 T            | 132 Val (4.2) | 132 Thr (−0.7) |
| I136 T            | 136 Ile (4.5) | 136 Thr (−0.7) |
| F138 V            | F138 Thr (−0.7) | 138 Val (4.2) |
| Y165 F            | Y165 Tyr (−1.3) | 165 Phe (2.8) |
| V166 T            | V166 Val (4.2) | 166 Thr (−0.7) |
| T172 A            | T172 Thr (−0.7) | 172 Ala (1.8) |
| V179 K            | V179 Val (4.2) | V179 Lys (−3.9) |
| T186 V            | T186 Val (4.2) | 186 Val (4.2) |
| S192 L            | S192 Ser (−0.8) | 192 Leu (3.8) |
| E200 A            | E200 Glu (−3.5) | 200 Ala (1.8) |
| A208 Q            | A208 Glu (−3.5) | 208 Glu (−3.5) |
| A215 K            | A215 Ala (1.8) | 215 Lys (−3.9) |
| N251 A            | N251 Asn (−3.5) | 251 Ala (1.8) |

*Primers were designed based on GenBank sequences (gene ID: 171269, 25408, 25458, 25464, 24493, 25325, 24499, 80251, 20021, 30303, 81686, 171045, 81687, NM031591.1, 83580, 24835, 25361, 83785). Primers in bold letters were used for synthesis of recombinant OspA. Primers in italics for fla gene (331 bp product) were used to detect crossing of BMEC monolayer by Borrelia in invasion assay (Figure 1), other set of fla primers (173 bp product) was used in real-time assay.

In summary, this is the first report that presents differential adhesion of OspA ligand of neuroinvasive and non-neuroinvasive borreliae to the BMECs. The study unfolds the underlying protein:protein interaction in the transient tethering-type associations between *Borrelia* and BMECs via OspA-CD40 dyad. Results also show that OspA mediated CD40 dependent cell signaling events are necessary for borrelial translocation across BBB. Finally, owing to the augmented levels of integrins on the BMECs infected with neuroinvasive *Borrelia*, we postulate that ICAM-1 or VCAM may be the potential molecules involved in the stationary adhesion of *Borrelia* on BMECs.

**Methods**

Preparation of *in vitro* model of rat BBB. Primary cultures of rat BMECs were prepared from 2-week-old Wistar rats, as previously described. Briefly, forebrains were minced into pieces and digested with collagenase type II and DNase (Sigma, USA) for 1.5 h at 37°C. Microwell fragments were separated from myelin layer by gradient centrifugation in 25% bovine serum albumin-DMEM. Microwells were digested with collagenase-dispase (Roche, Switzerland) and DNase for 50 min at 37°C. Microwell endothelial cell clusters were separated on Percoll gradient and washed twice in DMEM-F12. Endothelial cell clusters were then plated on fibronectin (Sigma) and collagen type IV (Sigma) coated culture dishes or cell culture inserts.
Crossing of BMEC monolayer by *Borrelia* Invasion assay. 1 ml (~10^6) cells of the *Borrelia* cultures was centrifuged at 6500 × g for 5 min. Cell pellet was resuspended in supplemented DEMEM-F12 without gentamicin (DEMEM-F12-G). BMEC cells were washed twice with DEMEM-F12-G medium and 1 ml of *Borrelia* suspensions were added to luminal chamber of cell culture inserts containing confluent monolayer of BMEC. Borreliae were also added to luminal chambers of empty cell culture inserts to confirm the free passage of *Borrelia* across the membrane. Inserts were incubated at 37°C for 18 or 24 hrs. Contents of the luminal and abluminal chambers were centrifuged at 13000 × g for 15 min. Supernatant was discarded and DNA was isolated from the pellet with the help of DNAzol direct kit (Molecular research center, Cincinnati, Ohio). The presence of *Borrelia* was assessed by amplifying fla gene by PCR (Table 1). Invasion assay was performed in duplicate.

Preparation of Borella and BMEC cell lysates. *Borrelia* cultures were centrifuged at 13000 × g for 10 min. pellets were washed with PBS with 5 mM Na-azide (PH 7.4) and then resuspended in lysis buffer containing 1% of nuclease mix and 1% of protease inhibitor cocktail (GE Healthcare, USA). BMECs were scrubbed from culture dishes resuspended in ultra-pure water containing 1% of nuclease mix and protease inhibitor cocktail. *Borrelia* cell suspensions were sonicated on ice.

Membrane proteins of BMECs were enriched by using Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce, USA). Protein concentrations were measured by the Bradford method.

Detection of Borrelia-BMEC protein interactions: Ligand Capture Assay (LCA). BMEC cell lysate (300 μg of total protein) was fractionated by SDS-PAGE and proteins were electro-transferred on nitrocellulose membranes. Non specific binding sites were blocked with TBS buffer (10 mM Tris-HCl, pH 8.3 and 150 mM NaCl) containing 0.5% albumin fraction V (Sigma) for 1 hr at 37°C. Membrane bound BMEC proteins were then hybridized either with *Borrelia* lysates (900 μg of total protein) or TBS+BSA-V buffer (negative control) for 2 hrs at 37°C with agitation. Membranes were washed three times with TTBS (TBS with 0.05% Tween20) and incubated with protein capture buffer containing TBS containing 0.5% BSA and 0.05% Tween20 (patent pending-No.00127-2010, Slovak patent office) for 1 hr. Capture buffer containing proteins interacting with *BMEC* proteins was collected, desalted and concentrated with MWCO filters (PES-5000, Sartorius, Germany). Proteins were fractionated by SDS-PAGE and visualized by silver staining (Bio-Rad, USA). Protein occurred on the stained gel after LCAs was excised and processed for mass spectrometry. LCA was repeated at least four times.

MALDI mass spectrometry based detection of Borrelia and BMEC proteins. Excised Borrelia protein was digested as previously described21. An aliquot of digestion solution was mixed with an aliquot of t-cyano-4-hydroxycinamic acid (Bruker-Daltonics) in 33% acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto 600 μm AnchorChip (Bruker-Daltonics) and allowed to dry. MALDI-MS data were obtained in an automated analysis loop using an Ultraflex mass spectrometer (Bruker-Daltonics)34. Spectra were acquired in positive-ion mode at 50 Hz laser frequency, and 100 to 1000 individual spectra were averaged. Selected precursor ions were subject to fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode to obtain the corresponding MALDI-MS/MS spectra. Automated analysis of mass data was performed using the flexAnalysis (Bruker-Daltonics). MALDI-MS and MALDI-MS/MS data were combined through the BioTools (Bruker-Daltonics) to search a non-redundant protein database (NCBI) using Mascot software (Matrix Science, UK)35.

Preparation of recombinant OspA (r-OspA). Whole protein coding region of OspA of both neuro and non-neuroinvasive strains were amplified (Table 1). Amplicons were sequenced and then cloned into the pQE30-UA expression vector (His-tag at N-terminal) by using QIAexpress-UA cloning kit (Qiagen, USA). E. coli M15 host cells were transformed with recombinant pQE30-UA vectors and subsequent induction of r-OspA expression was carried out according to manufacturer’s instructions (Qiagen, USA). Recombinant His-tag OspA proteins were purified using Ni-NTA agarose (Qiagen, USA). Purified His-tag OspA proteins were immobilized on 100 μl of Ni-NTA agarose, subsequently agarose was washed twice with 1 ml of washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0). Whole cell lysate of BMEC (total protein load 1 mg) was added to agarose and incubated at 4°C for 10 hrs with constant shaking. After 4 washings proteins complex was eluted (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Eluted proteins were separated on SDS-PAGE and visualized with silver staining. BMEC protein interacting with His-tag OspA of SKT-7.1 was excised from the gel and subjected for MALDI-TOF based identification as described above.

Affinity ligand binding assay with r-OspA. Briefly, BMEC cell lysate (60 μg) was fractionated by SDS-PAGE and proteins were electro-transferred to nitrocellulose membrane. Non-specific binding sites were blocked with TBS containing 5% skim milk (TBSM) and membranes were incubated for 2 hrs either with r-OspA proteins (150 μg/ml in TBSM) or only TBS. r-OspA bound to BMEC proteins was detected using His-tag antibody conjugated with HRP (1:500 in TBSM, 2 hr incubation) and ECL western blustering substrate (Pierce, USA). Signals were captured on X-ray films.

Co-immunoprecipitation for confirmation of interaction between OspA of SKT-7.1 and CD40. Recombinant Flag-tagged CD40 of rat containing amino acid residues 9 to 282 (kind gift from Dr. Chakurkar, ICAR, India) was immobilized on anti-Flag agarose (Sigma, USA) as described above (protein load ~0.3 mg). As per manufacturer’s instructions, agarose was washed and purified r-OspAs of SKT-7.1 or SKT-2 were loaded on agarose (protein load ~0.3 mg). incubated overnight. Agarose beads were washed twice and protein complex was eluted (0.1 M glycine HCl, pH 3.5). Eluted proteins were separated on SDS-PAGE.

Presence of Borrelia and OspA expression in rat tissues. Six Wistar rats were infected with either SKT-2 or SKT-7.1 via intradermal route (approximately 1×10^6 spirochetes in mid-log phase). Rats were sacrificed by CO2 inhalation 60 days post infection. Brain and a piece of ear (ear punch) were dissected out, and the brain microvesSEL fraction was isolated on albumin gradient as described above. A part of tissue samples was subjected for total RNA isolation using Purezol-RNA kit (Bio-Rad, USA). RNA was treated with DNase I (Ambion, USA), reverse transcribed (scriptqDNA Synthesis Kit, Bio-Rad; 25°C 5 min; 42°C 30 min; 85°C 5 min; 4°C). An aliquot of the tissue sample was subjected for isolation of DNA using DNAzol reagent. Presence of Borrelia in tissues was confirmed by PCR targeting OspA and fla genes (Table 1).

Expression of OspA was assessed by quantitative real-time PCR (qQ5, Bio-Rad). The reaction mix contained 35 μM of target specific primers for OspA or fla genes (Table 1), iQSYBR Green Supermix (Bio-Rad), milliQ water and template cDNA. For negative control no cDNA template was added.

The experimental work on rats were done according to the guidelines and regulation led by ethical committee and commission for work with animals of University of Veterinary Medicine and Pharmacy, Kosice, Slovakia (number UVL Collo 4).

Activation of CD40-dependent downstream signaling in BMEC and its blockage. To assess whether *Borrelia* strains and their r-OspA proteins differentially activate the CD40 downstream signaling cascade, total RNA was isolated from BMECs infected with *Borrelia* (~10^6 cells) or incubated with r-OspA (0.25 mg/ml) for 24 hrs. In case of CD40 blocking, the BMEC proteins were pre-incubated with anti-CD40 antibody (Abcam) 30 min before addition of recombinant proteins. Non-infected BMECs and cells incubated only with anti-CD40 antibody served as controls. Total RNA was isolated and reverse transcribed. Quantitative measurement of mRNA expression for CD40, CD80, thomboModulin, TNF-α, and VEGF (Table 1) was done by real-time PCR. Expression was normalized (AQC) to the housekeeping genes GAPDH and β-actin with the help of iQ software (Biorad) by using following equation:

Nucleotide sequences of OspA of SKT-7.1 and SKT-2 were submitted to the GenBank (USA) under the accession numbers GU0598888 and GU320003. Nucleotide sequences were in-silico translated and amino-acid sequences were aligned (Geneious software, www.biomatters.com). Endothelium binding sites, antibody binding pockets and hypervariegated antigenically important regions in OspA were mapped by database search (Uniprot, SMART) and data mining12,26,27.

**Ospa Sequence alignment.** Nucleotide sequences of Ospa of SKT-7.1 and SKT-2 were submitted to the GenBank (USA) under the accession numbers GU0598888 and GU320003. Nucleotide sequences were in-silico translated and amino-acid sequences were aligned (Geneious software, www.biomatters.com). Endothelium binding sites, antibody binding pockets and hypervariegated antigenically important regions in OspA were mapped by database search (Uniprot, SMART) and data mining12,26,27.

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