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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)[1]. 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[2].

Invasion of CNS by *Borrelia* is a complex process, which requires successful crossing of the blood-brain barrier (BBB)[3,4]. The BBB is a regulatory interface between peripheral circulation and the CNS[3]. 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[5] 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[6,7], whereas others support a transcellular passage[8]. Using state of the art real-time high-resolution 3D microscopy, Moriarty and co-workers[9] 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[9]. 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[9]. 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[9–12].

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)[13]. Other adhesive proteins like P66, ErpK, OspC and protein ligand for β_3-chain

integrins also bind to the endothelial cells[14], whereas, Bgp, DbpA and BBK32 bind the glycosaminoglycans[15,16]. In the CNS, BBA25 and BBA50 proteins of *Borrelia* mediate the adherence to glial cells[17]. 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, zonula occludens-1 etc., are expressed differently in BMEC and peripheral vascular endothelial cells (ECs)[18]. 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.[19,20] 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 integrins (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 the SKT-7.1 strain was able to cross endothelial monolayers and was present in both luminal and abluminal chambers. SKT-2 strain remained in luminal chamber only (Fig. 1; lanes 3 and 4). This shows that only SKT-7.1 strain exhibits a potential for neuroinvasion. Neuro-invasiveness of SKT-7.1 was further confirmed *in vivo* in Wistar rats where both *fla* and *OspA* genes were detected in the brain and brain microvasculature (for details see below section: expression of OspA in the brain and brain microvasculature).

PCR performed on the contents of luminal and abluminal chambers of Transwells without endothelial monolayer showed the 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, electophoretically 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. (Fig. 2a; lanes 5 and 6). 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 BMEC 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).

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). This clearly indicates the differential affinity of OspA proteins to CD40 molecule.

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 BMEC. 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[21,22], while others have reported expression of OspA in the unique environment of the brain and CSF, but not in the serum[23,24]. Therefore, it was essential to determine whether OspA is expressed in borreliae that are present in the brain vasculature *in vivo* in infected laboratory animals. PCR analysis of the brain and brain microvasculature of Wistar rats infected with SKT-7.1, revealed

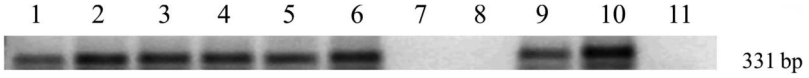


Figure 1 | Translocation of *Borrelia* across BMECs *in vitro*. Presence of Borrelia in luminal and abluminal chambers was detected by amplifying fla gene encoding flagellin. Lane 1 – SKT-7.1 luminal chamber after 18 hrs, lane 2- SKT-7.1 luminal chamber after 24 hrs, lane 3 – SKT-7.1 abluminal chamber after 18 hrs, lane 4- SKT-7.1 abluminal chambers after 24 hrs, lane 5 – SKT-2 luminal chamber after 18 hrs, lane 6 – SKT-2 luminal chamber after 24 hrs, lane 7– SKT-2 abluminal chamber after 18 hrs, lane 8 – SKT-2 abluminal chamber after 24 hrs, lanes 9 and 10 – positive controls SKT-7.1 and SKT-2 respectively (insert without BMEC monolayer) after 24 hrs, lane 11 – negative control.

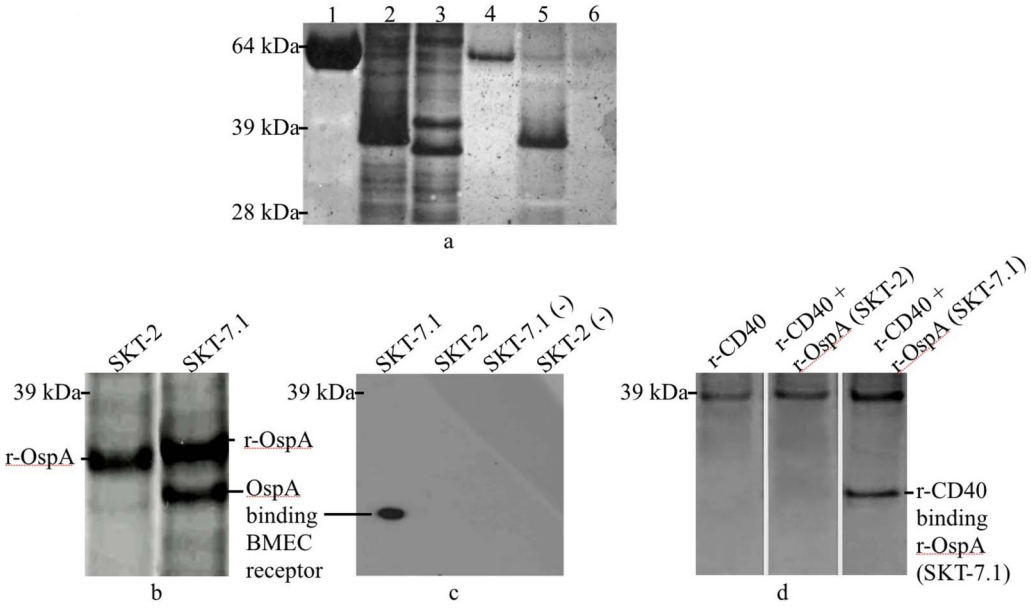
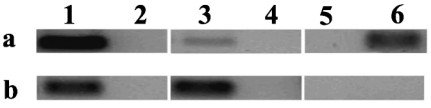


Figure 2 | Differential interaction of proteins of *Borrelia garinii* (SKT-7.1) and *Borrelia burgdorferi* sensu stricto (SKT-2) with BMECs. Results of LCA are presented in panel a. BMEC proteins were fixed on the nitrocellulose membrane and hybridized with Borrelia proteins. Interacting Borrelia proteins were stripped 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 BMEC 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.

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).



c

Borrelia strains	Brain microvasculature (*OspA/fla*)	Brain (*OspA/fla*)	Ear punch (*OspA/fla*)
SKT-7.1	+/+	++/+	-/-
SKT-2	-/-	-/-	-/+

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.

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[25–27]. 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-7.1infected 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).

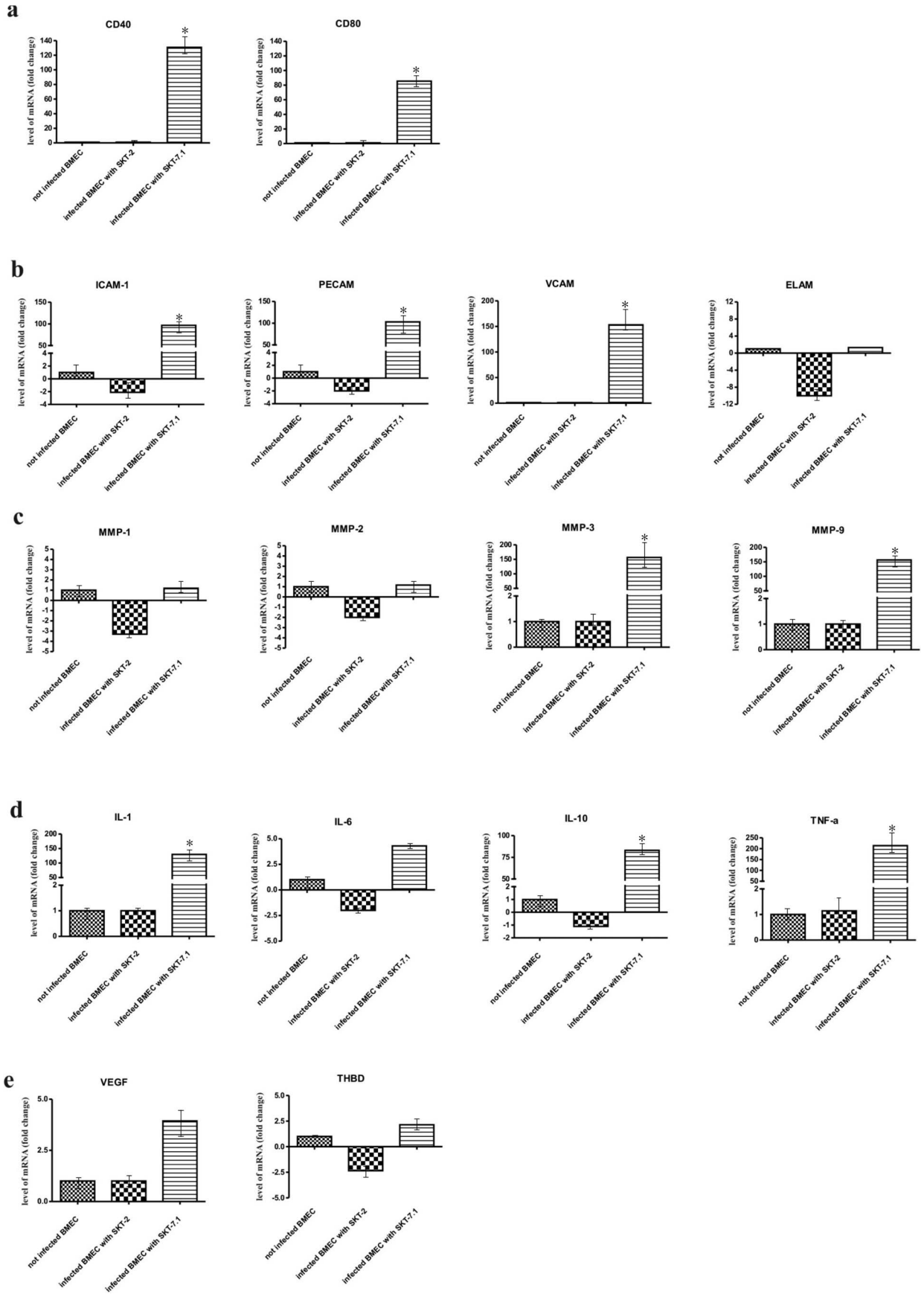


Figure 4 | Induction of CD40 and its downstream pathway in BMEC infected with *Borrelia strains.* Expression of CD40 and CD80 (panel - **a**), integrins (panel - **b**), matrix metalloproteinases (panel - **c**), cytokines (panel - **d**), and VEGF and thrombomodulin (panel - **e**) in BMECs infected 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[13,28–30]. 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[28]. Highly conserved tryptophan at residue 216 was also found in both SKT-2 and SKT-7.1, however, significant variation in its side chain (AA**W**NSGT – SKT-2 and GK**W**DSKT – 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 hydropathy index (Fig. 6, Table 2). Four amino acid variations that cause change in hydropathy 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 candidate molecules that take part in the tethering-type association were identified. Binding of protein ligand OspA of neuroinvasive strain SKT-7.1 to the BMEC surface protein CD40 might be essential for the transient tethering-type association.

Our results indicate that OspA is a major adherent molecule to the BMECs. OspA is also an important adherent molecule in the brain. *Borrelia* can adhere to murine neural and glial cell lines[31,32], primary neuronal cells from fetal mice[24], and primary rat brain cultures[31]. This adherence process appears to be mediated by borrelial OspA, proteoglycans or galactocerebrosides[24,33]. Previously it was shown that pre-treatment of borreliae with proteinase K (which cleaves OspA but does not affect borrelial viability and motility) decreases adherence by 70%. This finding suggests that OspA may contribute ~70% in the adhesion process[34] and there is requirement of additional borrelial ligands. It is noteworthy that not all OspA ligands can bind to BMEC surface proteins. In the present study OspA from SKT-7.1, but not from SKT-2 possessed CD40 binding ability.

Activation of CD40 in endothelial cells mediates downstream signaling that leads to production of pro-inflammatory cytokines[35] and enhanced expression of ICAM-1, E-selectin, VCAM-1 with consequent increase in cell binding[36,37], vascular endothelial growth factor (VEGF) and VPF, creates fenestrations[38], altered MMPs expression[39–41] and down-regulation of thrombomodulin (CD141)[42]. Such orchestered cell signaling events take place during the translocation of leukocytes across endothelial barrier. Leukocyte 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[43]. 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[44]. Furthermore, mimicry of lymphocyte function-associated antigen 1 (LFA-1, a potent adherent partner of ICAM-1) by OspA ligand has been described elsewhere[45]. 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[29]. 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[46]. 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 adhesion factor, which is essential in the pathogenesis of Lyme neuroborreliosis[23]. It is also well known that *Borrelia* can bind plasminogen via OspA on their surface[47]. OspA also upregulates membrane urokinase-type plasminogen activator receptor (uPAR)[48]. Plasminogen can be activated to plasmin[47,48] 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[49]. 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

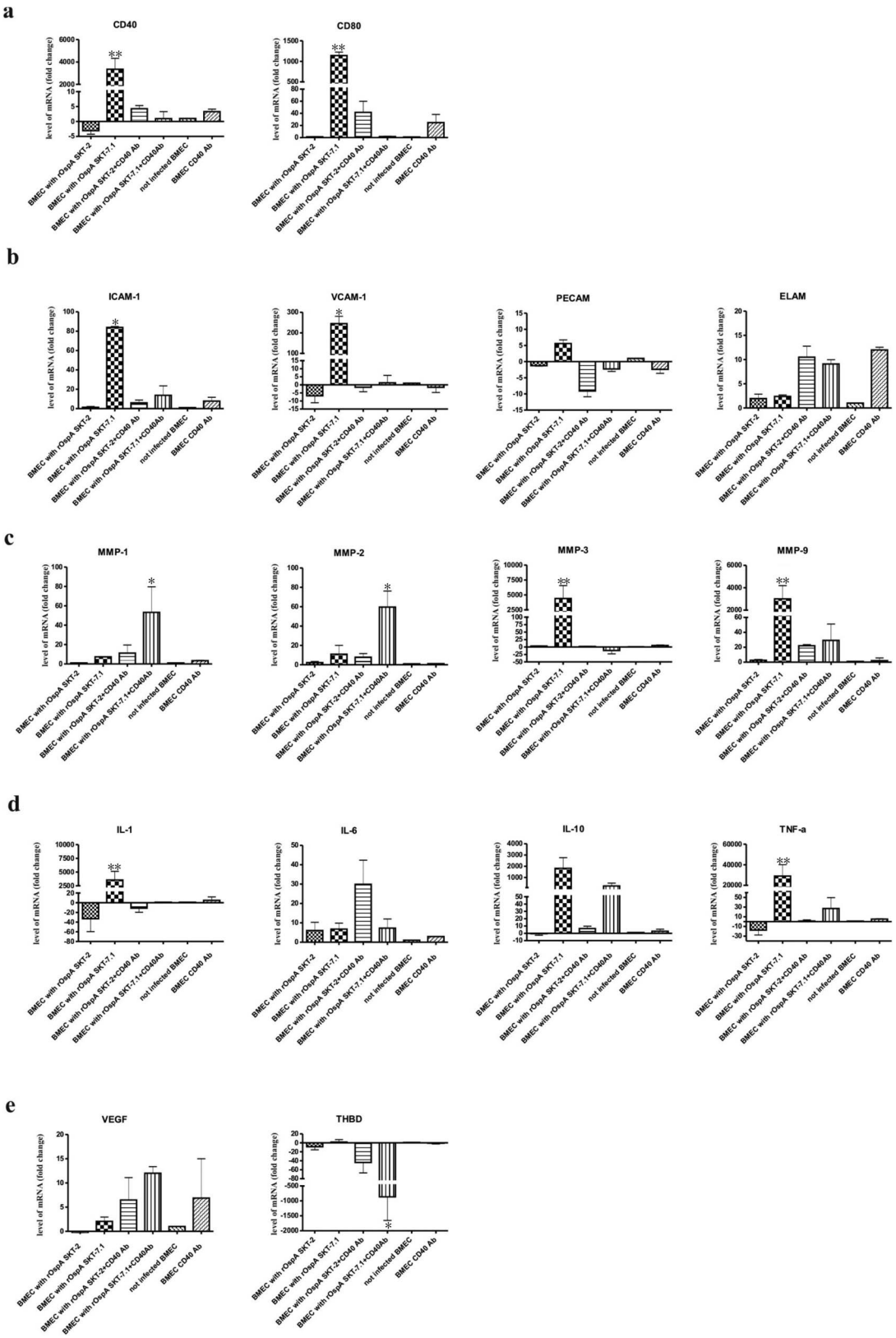


Figure 5 | Induction of CD40 mediated downstream pathway after CD40:OspA dyad formation. Activation of CD40 mediated downstream pathway in BMECs is presented. BMECs were incubated with r-OspA of either SKT-7.1 or SKT-2 (bars in each graph - BMEC with rOspA SKT-2 and BMEC with rOspA SKT-7.1). Blockage 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 (*, $P < 0.05$; **, $P < 0.02$).

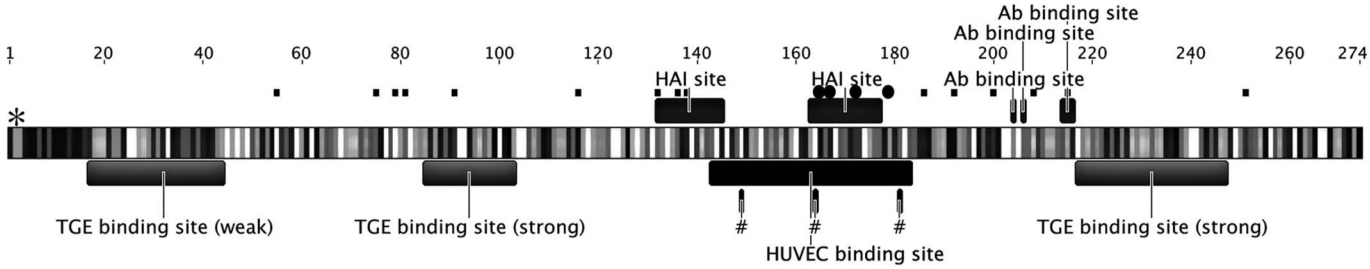


Figure 6 | Binding sites in OspA amino acid sequence. TGE – Tick gut endothelium, HUVEC – Human umbilical vein endothelial cells, Ab – antibody, HAI – hypervariable antigenically important site, * darker lines correspond to strong hydrophobic regions, while lighter (white) lines correspond to strong hydrophilic regions in OspA protein sequence. Dark squares indicate amino acid variations that lead to change in hydropathy indexin the OspA of SKT-7.1 and SKT-2. Dark circles depict amino acid variation in endothelial binding sites withchange in hydropathy index, while # indicates amino acid variation without change in hydropathy index.

Table 1 | Primers and PCR conditions used in the study

Gene	Primer 5′ - 3[a]	Tm (°C)	Product lenght (bp)
OspA	F- AAATATTTATTGGGAATAGGTCTA R- AAAGCGTTTTTAAGTTCATCA	**50.1**	**811**
OspA	F- TGAAGGAACTCTAACTGCTGACA R- TGGTGCCAKCTGAGTCGT	53.0	260
Fla	F- ACGGCACATATTCAGATGCAGACA R- TGCAGGCTGCATTCCAAGCT	55.0	173
Fla	*F- CACATATTCAGATGCAGACAGAGG* *R- CCGGTGCAGCCTGAGCAGTTTGAG*	*57.0*	331
β actin	F- TCTCTTCCAGCCTTCCTTCCT R- GAGGTCTTTACGGATGTCAACG	65.4	100
CD40	F- ACCGACACTGCGAACTCAA R- CATTGGAGAAGAATCCGACCG	53.8	222
CD80	F- GAAGACCCTCCTGATGAA R- CTTGTTTGTTTCTCTGCTTG	46.9	198
ELAM	F- GTGACAAAAAGAAACTGGCT R- CTGAAGGAGCAGGATGAAT	48.9	238
GADPH	F- GGAGCAGAAGCAAGTGGT R- CCCTCTCTTGATGTCTGC	59.2	178
ICAM-1	F- GCCTTGGAGGTGGATGGGAAGT R-GCAGGTCAGATTAGGGGCTGGATT	58.2	174
IL-1	F- GGACAGAATACATCTCAAAGC R- ATCTCCTTCAGCAACACAG	48.6	180
IL-6	F- CCAGAGTCATTCAGAGCAAT R- TGCCGAGTAGACCTCATAG	49.3	214
IL-10	F- ACTGGCTGGAGTGAAGACC R- TGGCAACCCAAGTAACCCTT	54.3	207
MMP 1	F- GCTCATACAGTTTCCCCGT R- TCATAGCACTCAGGGTTTCAG	51.5	213
MMP 2	F- TGACGGCTTCCTCTGGTG R- CATAATCCTCGGTGGTGCC	53.2	212
MMP 3	F- ACCCCACTCACATTCTCCA R- ACATCATCTGTCCATCGTTCA	52.6	188
MMP 9	F- TGAAAACCTCCAACCTCACGG R- GCCTTTAGTGTCTCGCTGTCC	54.6	178
PECAM-1	F- TCACAGACAAGCCCACCAGAGACA R- TCACAGAGCACCGAAGCACCATTT	59.5	187
THBD	F- GCTTGGGGTTTTCCTTCTGGGTGT R- GCCGTGAGTCCATACTACTGTCGC	59.2	248
TNF α	F- GCCTCAGCCTCTTCTCATTCCTGC R- CTCCTCCGCTTGGTGGTTTGCTAC	59.7	215
VCAM-1	F- GCCTCAGCCTCTTCTCATTCCTGC R- CTCCTCCGCTTGGTGGTTTGCTAC	52.1	195
VEGF	F- GCCTCAGCCTCTTCTCATTCCTGC R- CTCCTCCGCTTGGTGGTTTGCTAC	48.9	207

[a]Primers were designed based on GenBank sequences (gene ID - 171369, 25408, 25548, 25464, 24493, 25325, 24498, X02231, 300339, 81686, 171045, 81687, NM031591.1, 83580, 24835, 25361, 83785). Primers in bold letters were used for synthesis of recombinant OspA. Primers in italics letters 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.

plasmid 54 (*lp54*)[50]. *lp54* also encodes OspA therefore it can be predicted that this plasmid has immense importance in borrelial neuroinvasion.

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[51]. Briefly, forebrains were minced into pieces and digested with collagenase type II and DNase (Sigma, USA) for 1.5 h at 37°C. Microvessel fragments were separated from myelin layer by gradient centrifugation in 25% bovine serum albumin-DMEM. Microvessels were digested with collagenase-dispase (Roche, Switzerland) and DNase for 50 min at 37°C. Microvessel 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

Table 2 | Changes in amino acid sequence of OspA between strain SKT-2 and SKT-7.1

Change in the amino acid	SKT-2	SKT-7.1
I55 E	55 Ile (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)
A 81 T	81 Ala (1.8)	81 Thr (−0.7)
S 91 A	***91 Ser (−0.8)***	***91 Ala (1.8)***
S 116 L	116 Ser (−0.8)	116 Leu (3.8)
V 132 T	132 Val (4.2)	132 Thr (−0.7)
I 136 T	136 Ile (4.5)	136 Thr (−0.7)
T 138 V	138 Thr (−0.7)	138 Val (4.2)
Y 165 F	**165 Tyr (−1.3)**	**165 Phe (2.8)**
V 166 T	**166 Val (4.2)**	**166 Thr (−0.7)**
T 172 A	**172 Thr (−0.7)**	**172 Ala (1.8)**
V 179 K	**179 Val (4.2)**	**179 Lys (−3.9)**
T 186 V	186 Thr (−0.7)	186 Val (4.2)
S 192 L	192 Ser (−0.8)	192 Leu (3.8)
E 200 A	200 Glu (−3.5)	200 Ala (1.8)
A 208 Q	208 Ala (1.8)	208 Gln (−3.5)
A 215 K	215 Ala (1.8)	215 Lys (−3.9)
N 251 A	251 Asn (−3.5)	251 Ala (1.8)

Figures in the parenthesis indicate hydropathy index. Amino acid changes depicted in the bold letters are located in the endothelial cell binding domain. Amino acid change in the bold-italic letter is located in the TGE binding domain.

(Transwell, 1 cm^2; pore size 3 μm; Corning-Life Sciences, MA, USA) and maintained for the first 2 days in DMEM-F12 supplemented with 20% plasma, gentamicin, 2 mM L-glutamine, 100 μg/ml heparin (Sigma), 1 ng/ml basic fibroblast growth factor and 4 μg/ml puromycin. To fully induce BBB properties, BMEC were co-cultured with mixed glial cultures (3–4 days of co-cultivation). Integrity of monolayers was measured by transendothelial electrical resistance (ENDOHM-12 chamber, WPI Europe).

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 Cislo 44*).

Bacterial isolates. Two *Borrelia* strains SKT-7.1 (*B. garinii,* serotype 4, recently designated as *B. bavariensis* sp. nov.) and SKT-2 (*B. burgdorferi* s.s., serotype 1) were cultured in BSK-II medium with 6% rabbit serum at 33°C.

Crossing of BMEC monolayer by *Borrelia*: Invasion assay. 1 ml (~10^7 cells) of the *Borrelia* cultures was centrifuged at 6000 × g for 5 min. Cell pellet was resuspended in supplemented DMEM-F12 medium without gentamicin (DMEM-F12-*G*). BMEC cells were washed twice with DMEM-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 hr 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). The presence of *Borrelia* was assessed by amplifying *fla* gene by PCR (Table 1). Invasion assay was performed in duplicate.

Preparation of *Borrelia* 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 (patent pending–No.00127-2010, Slovak patent office) for 15 min. Capture buffer containing borrelial ligands 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 spectometry. LCA was repeated at least four times.

MALDI mass spectrometry based detection of *Borrelia* and BMEC proteins. Excised *Borrelia* protein was digested as previously described[52]. An aliquot of digestion solution was mixed with an aliquot of α-cyano-4-hydroxycinnamic 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)[53]. 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)[54].

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 proteins was carried out according to manufacturer's instructions (Qiagen, USA). Recombinant His-tag OspA proteins were purified using Ni-NTA agarose (Qiagen, USA). The presence of His-tag proteins was confirmed by SDS-PAGE and on western blot using anti-His antibody (Abcam, UK, 1:1500).

Co-immunoprecipitation assay with r-OspA. 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 NaH_2PO_4, 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 NaH_2PO_4, 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 biding 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 TBSM. r-OspA bound to BMEC proteins was detected with 6xHis tag antibody conjugated with HRPO (1:500 in TBSM, 2 hr incubation) and ECL western blotting 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 1×10^7 spirochetes in mid-log phase). Rats were sacrificed by CO_2 inhalation 60 days post inoculation. The 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 (iScriptcDNA Synthesis Kit, Bio-Rad; 25°C 5 min; 42°C 30 min; 85°C 5 min; 4°C).

Another part 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 (iQ5, Bio-Rad). The reaction mix contained 33 μM of target specific primers for *OspA* or *fla* genes (Table 1), iQ SYBR 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 Cislo 44*).

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* (~10^7 cells) or incubated with r-OspAs (0.25 mg/ml) for 24 hrs. In case of CD40 blocking, the BMECs 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, ELAM-1, VCAM-1, PECAM-1, ICAM-1, IL-1, IL-6, IL-10, MMP-1, MMP-2, MMP-3, MMP-9, thrombomodulin, TNFα and VEGF (Table 1) was done by real-time PCR. Expression was normalized (ΔΔCt) to the housekeeping genes GAPDH and β-actin with the help of IQ5 software (Biorad) by using following equation:

$$Normalized\ expression_{(gene\ x)} = \frac{Relative\ quantity_{(gene\ x)}}{Relative\ quantity_{(reference\ 1)}{}^{X} Relative\ quantity_{(reference\ 1)}{}^{X\ldots X} Relative\ Quantity_{(reference\ n)}{}^{1/n}}$$

$$Relative\ quantity_{(gene\ x)} = E_{gene\ x}{}^{(CT(control) - CT(sample))}$$

$$Where\ E = Efficiency\ of\ primer\ set (\%\ efficiency^{x}\ 0.01 + 1),\ where\ 100\% = 2$$

$$C_{T(control)} = Average\ C_T\ for\ control$$

$$C_{T(sample)} = Average\ C_T\ for\ the\ sample$$

OspA Sequence alignment. Nucleotide sequences of OspA of SKT-7.1 and SKT-2 were submitted to the GenBank (USA) under the accession numbers GU906888 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 hypervariable antigenically important regions in OspA were mapped by database search (Uniprot, SMART) and data mining[13,28–30].

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Author contributions

M.B. and A.K. planned the experiments. A.K. performed the *in vitro* cultivation of BBB model, *Borrelia* invasion assay and part of in-silico analysis of OspA protein. M.B. constructed plasmids for expression of r-proteins in Dr. Chakurkar's laboratory. R.M., P.M, E.B, L.P. and M.M. conducted real time experiments, LCA, Co-IP, ALBI assay, PCRs for detection of translocated *Borrelia* and part of in-silico analysis of OspA protein. L.P., A.K., M.N. and M.B. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

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