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A novel vascular targeting strategy for brain-derived endothelial cells using a TCR mimic antibody

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Abstract

Organ-specific vascular targeting, for example to the blood-brain barrier, requires the identification of unique molecular addresses on a subset of endothelial cells. The present study describes a crucial step towards tapping the exquisite specificity of the peptide/HLA class I system for this goal. We utilized a novel T-cell receptor (TCR) mimic antibody of high affinity and specificity, which is restricted by HLA-A2 and has been generated to recognize a peptide epitope derived from p68 RNA helicase (YLLPAIVHI). The parent protein is highly expressed by brain endothelial cells. Flow cytometry and confocal imaging showed that the antibody binds to HLA-A2 positive human brain derived endothelial cells, both immortalized hCMEC/D3 cells and primary cells. The TCR mimic antibody undergoes internalization into vesicles, where significant colocalization occurs with the early endosomal marker EEA-1, but barely with caveolin-1. To our knowledge internalization of neither MHC class I protein nor TCR mimics by brain endothelial cells has been previously observed. Knockdown of p68 protein expression by siRNA reduced the presentation of YLLPAIVHI-peptide/HLA-A2 complexes on the cell membrane by half as measured by flow cytometry 48h later. We also found that brain endothelial cells isolated from HLA-A2 transgenic mouse strains express the A2 transgene, and brain endothelial cells of one of these strains also present YLLPAIVHI-peptide/HLA-A2, making these mouse strains suitable models for studying TCR mimic antibodies in vivo. In conclusion, these data strongly support the notion that TCR mimic antibodies could be a new class of therapeutic targeting agents in a wide variety of diseases.

Keywords

major histocompatibility complex; blood-brain barrier; RNA helicase

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CONFLICT OF INTEREST STATEMENT

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INTRODUCTION

Monoclonal antibodies with specificity for peptide/MHC complexes copy the binding characteristics of T-cell receptors (TCR) and accordingly have been dubbed TCR mimics (TCRm) (Weidanz et al., 2006; Weidanz et al., 2007). The fact that TCRm have potentially much higher binding affinities than soluble TCR, generated interest in their use as diagnostic tools and therapeutic agents for viral infections and malignant tumors (Denkberg and Reiter, 2006; Reiter et al., 1997; Verma et al., 2010). Practical application of this approach was limited prior to recent progress in both identification of peptide epitopes for MHC molecules (Hawkins et al., 2008; Hickman et al., 2004) and in the efficient generation of TCRm to peptide/MHC complexes (Weidanz et al., 2007). We hypothesize that application of TCRm can be expanded to target differentiated, non-malignant cells, as the cellular proteome will be reflected in a distinct expression pattern of peptide/MHC complexes (Hickman et al., 2004). One important field that would benefit from the characterization of cell-type specific surface markers is vascular targeting, i.e. targeting of the vascular endothelial cells forming the interface between circulation and organ tissues. Here we focus on brain derived endothelial cells, which represent the morphological equivalent of the blood-brain barrier (BBB).

A strategy for targeting diagnostic agents or therapeutics to the BBB has been proposed, which is based on physiological transport mechanisms mediated by highly expressed endothelial receptor proteins. The potential of this approach is evident from numerous preclinical studies (for review see (Pardridge, 2007)), but unresolved challenges remain. For example, the receptors targeted to date, like the transferrin receptor (Bickel et al., 1993), insulin receptor (Pardridge et al., 1995), or LRP (Bertrand et al., 2009), are BBB enriched, but not brain specific. It is therefore desirable to identify unique “ZIP-codes” for the vascular bed of an organ, and to then generate highly specific targeting molecules. The combination of peptide/MHC targets and TCRm could provide that specificity. Before embarking on screening efforts with BBB endothelial cells, we wanted to use a well-characterized TCRm and obtain proof of principle that these cells interact with TCRm. For that purpose we selected the TCRm designated as RL6A (Verma et al., 2009) based on the following rationale: First, RL6A has been raised against a peptide/HLA-A2 complex, where the peptide epitope (YLLPAIVHI) had high likelihood of being expressed in brain endothelial cells. The peptide YLLPAIVHI was found in complex with the allele HLA-A2 (in the following abbreviated as YLL/A2). It is formed by proteasomal processing of the p68 RNA helicase protein, a multifunctional intracellular protein also known as DEAD box protein p68 (Bates et al., 2005; Iggo and Lane, 1989) and encoded by the DDX5 gene (Rossler et al., 2000). YLLPAIVHI has been independently isolated from breast tumor cells (Verma et al., 2009) and previously from transformed B-cells (Hunt et al., 1992). With respect to BBB endothelial cells, mRNA for p68 helicase has been identified among the most abundant transcripts in a genomic study of rat brain microvessels (Enerson and Drewes, 2006). Second, as documented by histological methods (Lassmann et al., 1991), genomic transcriptional analysis (Enerson and Drewes, 2006; Li et al., 2002; Male and Pryce, 1988), and proteomic techniques (Agarwal and Shusta, 2009) there is significant expression of the components of the MHC class I complex, heavy chain and beta microglobulin (light chain), by brain microvascular endothelial cells.

To date all TCRm including RL6A have been generated against human MHC complexes. Hence, a human endothelial cell line would facilitate testing of the concept outlined above by in vitro studies. Among the few human brain derived endothelial cell lines described to date, hCMEC/D3 cells (Weksler et al., 2005) provide a viable BBB model and appeared suitable for our studies, provided they express the HLA-A2 allele. HLA-A2 is the most abundant of the class I alleles with a frequency in the population of 40-50% (Middleton et

al., 2003). We further addressed the question, whether an animal model of BBB targeting by TCRm antibodies could be based on available HLA-A2 transgenic mouse strains.

MATERIALS AND METHODS

Endothelial cells

hCMEC/D3 cells were propagated in Microvascular Endothelial Cell Medium-2 (EGM-2 MV, obtained from Lonza, Allendale, NJ, USA) as described (Weksler et al., 2005). Primary human brain derived endothelial cells were obtained from ScienCell (San Diego, CA, USA) and grown for 1 passage in the endothelial cell medium supplied by ScienCell.

Animals and cell isolation from brain and spleen

All animal experiments were performed according to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center. Mice were kept under controlled temperature, light (12 h dark/ 12h light) and humidity and were fed standard rodent chow. Mice of the following HLA-A2 transgenic strains (with stock # in brackets) were obtained from Jackson Laboratories (Bar Harbor, ME, USA): C57BL/6-Tg(HLA-A2.1)1Enge/J [3475], B6.Cg-Tg(HLA-A/H2-D)2Enge/J [4191]; NOD/ShiLtDvs-Tg(HLA-A/H2-D/B2M)1Dvs/J [6604], NOD.Cg-Prkdcscid Emv30b Tg(HLA-A/H2-D/B2M)1Dvs/DvsJ [6605], NOD.129P2(B6)-B2mtm1Unc Tg(HLA-A/H2-D/B2M)1Dvs/DvsJ [6611]. The animals were euthanized under deep isoflurane anesthesia and brains and spleen were rapidly removed on ice for further processing. Brain-derived endothelial cells were isolated by MACS magnetic cell sorting (Miltenyi Biotec, Auburn, CA, USA) with positive selection by a PECAM-1 antibody, adapting a published protocol for rats (Demeule et al., 2001) to mouse brain tissue. Briefly, brain tissue was gently homogenized in a glass homogenizer, followed by density centrifugation in dextran solution and passage through 80µm Nylon mesh to obtain microvessels. These were digested by collagenase and DNase for 45min at 37°C, and passed through 30 µm Nylon mesh. The cell suspension was spun down and washed 3 times in PBS with 2mM EDTA and 0.5% BSA. Endothelial cells are obtained by magnetic cell sorting (MACS, Miltenyi Biotec, Auburn, CA, USA), using a biotinylated PECAM-1 antibody (MEC 13.3, BD-Pharmingen, San Diego, CA), streptavidin microbeads (Miltenyi Biotec) and positive selection on a MACS LS column, with a yield of $1-2 \times 10^5$ cells per brain and >95% purity. The spleen was treated similarly and dendritic cells were isolated by MACS separation with CD11c microbeads (Miltenyi Biotec).

Antibody sources

The TCRm RL6A was generated against YLLPAIVHI-peptide/HLA A2 complexes (YLL/A2) according to recently published methods (Verma et al., 2009; Weidanz et al., 2006; Weidanz et al., 2007). Briefly, female BALB/c mice were immunized with a solution containing 50 µg of purified YLL/A2 complex and Quil-A adjuvant (Sigma, St. Louis, MO) at 14-day intervals. Hybridoma supernatants were screened by ELISA followed by confirmation analysis using peptide-pulsed T2 cells. T2 cells and the BB7.2 hybridoma were obtained from ATCC (Manassas, VA, USA). Polyclonal goat antibody for EEA1, polyclonal rabbit antibody for caveolin-1, and Alexa Fluor-488 labeled chicken-anti goat or anti rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phycoerythrin (PE) labeled anti-mouse secondary antibodies for flow cytometry were purchased from Southern Biotech (Birmingham, Alabama, USA).

rt-PCR of p68 RNA helicase

Total RNA was isolated from hCMEC/D3 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers and probes for the completely spliced human DDX5 transcript were designed using Primer Express software (Applied Biosystems, Carlsbad, CA, USA) and the following combination was selected for custom synthesis (Eurofins MWG Operon, Huntsville, AL, USA): forward 5'-AAA CAT TGG TGC ACT TGA ACT GA-3', reverse 5'-TTT TTC ATC CTT TTC TAC GTC ATG AC-3', probe (FAM)-5'-TGC AAA CCA CAA CAT TCT TCA GAT TGT GG-3'- (TAMRA). PCR was run on a 7300 Real Time PCR system (Applied Biosystems).

Immunoblotting

25 µg of total protein lysate of siRNA treated hCMEC/D3 cells were run on 8% SDS-PAGE. Separated proteins were then transferred to a nitrocellulose membrane for detection of p68 RNA helicase protein by mouse monoclonal PAb204 anti-p68 antibody purchased from Upstate Millipore (Billerica, MA, USA). Detection of anti-p68 antibody was carried out using a goat-anti-mouse-HRP conjugate polyclonal antibody purchased from Southern Biotech. β-actin was detected using a rabbit polyclonal antibody obtained from Cell Signaling (Danvers, MA, USA).

Flow cytometry

hCMEC/D3 cells were grown to confluency in collagen coated dishes. Cells were detached with EDTA, centrifuged and resuspended in FACS buffer. Two-step staining at room temperature was performed on 2.5×10^5 cells in 200 µL with HLA-A2 antibody BB7.2 (500 ng/200 µL), RL6A (5 ng/200µL) or corresponding isotype controls (IgG2a or IgG2b). After 20 min the primary antibody was washed off and cells were incubated for 10 min with PE-labeled secondary antibody. For stimulation, IFNγ (200U/ml) was added for 24h before cells were detached with EDTA-buffer and stained. Flow cytometry analysis was performed on a FACScan or FACSAria (BD Biosciences, San Jose, CA).

siRNA knock down of p68

Human siGENOME siRNA for DDX5 and a control siRNA (non-targeting siRNA #2) were obtained from Dharmacon (Lafayette, Colorado, USA) and used to knock down the expression of p68 DEAD box protein. hCMEC/D3 cells were seeded at 2.5×10^4 cells/well in 6-plate wells and transfected at 50% confluency with 100 nM DDX5 siRNA or control siRNA using DHARMAFect (Dharmacon) according to the manufacturer's instructions. Transfected cells were incubated for 24h or 48h and then analyzed by flow cytometry with BB7.2 (500 ng/200 µL), RL6A (5 ng/200µL) or corresponding isotype control antibodies for expression of HLA-A2 or YLL/A2 complexes, respectively. Separate wells were processed for protein extraction and Western blotting of p68 protein.

Cellular uptake and internalization of antibodies

hCMEC/D3 cells were grown overnight on collagen coated coverslips to 50-80% confluency. RL-6A, BB7.2 and isotype controls were all labeled to similar specific fluorescence with Alexa Fluor-546 using a commercial kit according to manufacturer's protocol (Invitrogen, Carlsbad, CA) and incubated with 10µg/ml in complete media for different time periods at 37°C. After washing, the cells were fixed with 4% paraformaldehyde and the nuclear dye DRAQ5 was applied (Biostatus, Shephed, UK). EEA1 staining was performed with polyclonal goat IgG (0.4 µg/mL) and Alexa Fluor-488 labeled chicken-anti goat IgG. The anti caveolin-1 polyclonal rabbit IgG was used at 4 µg/mL and detected by Alexa Fluor 488 labeled chicken-anti rabbit IgG. Specificity of RL-6A was shown by pre-absorption of the Alexa Fluor-546 labeled antibody RL-6A with a 60-fold

molar excess of refolded, soluble peptide-HLA-A2 tetramers. For live imaging, cells were grown overnight on collagen-coated glass bottom wells and placed in a CO₂, temperature- and humidity-controlled incubator (PeCon, Erbach, Germany) mounted on the stage of the Leica IRE2 inverted microscope (Leica Microsystems, Heidelberg, Germany).

Confocal imaging was performed using a Leica plan-apochromat 63x lens (NA 1.4, oil) and the Leica TCS-SL spectral confocal scanner. Channel settings: Alexa 488 excitation 488 nm, emission bandwidth 500-535 nm; Alexa 546 excitation 546 nm, emission bandwidth 560-600; DRAQ5 excitation 633 nm, emission bandwidth 650-700 nm. Sequential scanning mode was chosen for fixed samples. The same settings for laser power and photomultiplier gains were applied for imaging samples stained with specific antibodies and isotype controls. Images were linearly adjusted for brightness and contrast using Leica Confocal Software offline, again using identical values for specific and control antibodies, then cropped and assembled in Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

For quantitative analysis of colocalization in subcellular compartments (e.g., endosomes and caveolae), Z-stacks of the corresponding channels were imported into ImageJ version 1.43u (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009). Global and objects-based analysis was performed using the Plug-in JACoP (Bolte and Cordelières, 2006). Prism 5c (GraphPad Software, La Jolla, CA, USA) was used for statistical comparison with Fisher's exact test.

RESULTS

hCMEC/D3 cells express HLA A2 and the YLL/A2 complex

We first tested the hCMEC/D3 cell line for expression of the MHC A*02 allele, as the first basic requirement of an in vitro model. Flow cytometry with the pan HLA-A2 antibody BB7.2 demonstrated that hCMEC/D3 is indeed positive for HLA-A2 (Fig. 1A). We next probed the expression of the endogenous source of the YLL peptide, namely p68 RNA helicase, as the second basic requirement. As shown in Fig. 1B, PCR with primers specific for human p68 resulted in a single amplicon of expected length (84 bp). These results allowed to then addressing/address? the question, whether the YLL peptide is presented on HLA-A2 molecules. Flow cytometric studies using the RL6A antibody are shown in Fig. 1C and indicate that untreated hCMEC/D3 cells in fact present YLL/A2 complex at a basal level, and that its expression is inducible by pretreatment of the cells with IFN γ . The affinity and specificity of RL6A for the YLL/A2 complex has previously been extensively characterized by Plasmon surface resonance, in ELISA assays with plated peptide/HLA-A2 tetramers, and by loading varying amounts of either specific YLL peptide or unrelated peptides onto T2 cells (Verma et al., 2009). Here we chose another control experiment to obtain additional proof of specificity by selectively knocking down the endogenous p68 protein, thereby (partially) depleting the supply of YLL-peptide available to load the HLA-A2 protein. The immunoblots in Fig. 2 illustrate that treatment with specific siRNA decreased the cellular p68 protein levels at 24h and 48h. At the 24h time point flow cytometry of the cells did not yet reveal a difference in the expression of the YLL/A2 complexes compared to control (cells treated with non-targeting siRNA), nor was there a change in total HLA-A2 expression (fluorescence intensity with BB7.2). By 48h of treatment the median fluorescence intensity of hCMEC/D3 cells treated with p68 specific siRNA decreased by half compared to cells treated with control siRNA, while the BB7.2 signals remained unchanged. These data are compatible with reduced cellular p68 protein levels resulting in diminished proteasomal YLL-peptide generation and MHC loading. A lag time between reduction in the p68 protein itself and decreased cell surface expression of YLL/A2 complex was expected due to the half-life of peptide/MHC complexes on the cell surface and intermediate steps of peptide processing, transport by ER-associated TAP

transporters, MHC loading and trafficking of peptide/HLA to the cell surface. Steady levels of total HLA-A2 displayed on the cell membrane, as indicated by the unaltered signal with BB7.2, were also expected, because YLL/A2 complexes constitute only a small percentage of all HLA-A2 complexes.

hCMEC/D3 and primary brain derived endothelial cells internalize peptide-HLA complexes

We next used confocal microscopy to visualize the interaction of the RL6A TCRm and control antibodies with endothelial cells. As shown in Fig. 3A, by 15 min of incubation RL6A decorated the plasma membrane and was internalized into vesicles. These intracellular vesicles were positive for the early endosomal marker EEA1 (Fig. 3B). The pan A2 antibody BB7.2 was also bound and internalized, while hCMEC/D3 cells neither bound nor internalized corresponding isotype control antibodies applied under identical conditions as RL6A and BB7.2. Further, when an antibody to caveolin-1 was used instead of EEA1, there was virtually no colocalization with RL6A or BB7.2 detectable. This indicates that caveolae appear not to be involved in the internalization process. We substantiated the visual colocalization of the TCRm RL-6A with subcellular compartments as shown in Figure 3B by quantitative image analysis with ImageJ on the entire Z-stacks from which the optical sections were taken. Pearson's correlation coefficient for RL-6A and EEA1 was $r = 0.66$, compatible with partial colocalization (Bolte and Cordelières, 2006). Furthermore, objects-based analysis with plug-in JACoP (Bolte and Cordelières, 2006) resulted in particle coincidence of 23 out of 50 RL-6A positive particles with EEA1 positive structures (equal to 46% colocalization). Only marginal quantitative colocalization was found for RL-6A and caveolin-1, with 5 out of 60 RL-6A positive objects (8.3%) coinciding with caveolin-1 positive structures ($p < 0.0001$ vs. RL-6A/EEA1, Fisher's exact test).

To further corroborate the findings of binding and internalization, which were obtained with a protocol involving fixation of the cells after incubation with the labeled TCRm or control antibodies, we repeated the experiments under live imaging conditions. As shown in Fig. 3C, equivalent results were obtained.

Visual evidence for the specificity of binding/internalization of the RL6A TCRm was achieved by competition experiments with soluble YLL/A2 complexes. Preincubation of the antibody with a 60-fold molar excess of soluble YLL/A2 tetramer almost completely abolished binding/internalization of RL6A (Fig. 3D). An unrelated peptide/A2 tetramer (KLM) had no effect on binding and internalization of the TCRm (Fig. 3D).

The isotype antibody controls used in both the flow cytometry studies and in the confocal experiments, as well as the blocking experiments with peptide/A2 tetramers confirmed that the binding interaction of anti-HLA antibodies was antigen specific and not due to nonspecific adsorption or binding to other receptors with IgG-binding properties. To address potential differences in expression and/or processing of p68 helicase and derived MHC class I complexes between primary cells and immortalized cell lines like hCMEC/D3, we sought to confirm the key findings in primary brain derived endothelial cells. Therefore, flow cytometry and confocal microscopy were applied to a sample of primary human cells. As shown in Fig. 4A, for the batch of primary brain ECs used in our experiments, flow cytometry revealed HLA-A2 expression and staining with RL6A. In accordance with the data obtained using hCMEC/D3, confocal microscopy showed internalization of the RL6A antibody by these primary ECs (Fig. 4B). Hence the primary cells and the cell line have equivalent characteristics with respect to YLL/A2 expression and function.

HLA-A2 transgene expression on brain endothelial cells

Before TCRms can be explored as diagnostic tools and/or targeting vectors to endothelial cells of the vascular system in vivo, a suitable animal model is required. A number of transgenic mouse strains have been described carrying the human HLA A2.1 allele on different genetic background. All gene constructs are under the control of the human HLA-A2.1 promoter and are expected to be broadly expressed. However, to our knowledge, actual transgene expression in endothelial cells has not been studied or reported. We screened 5 of those strains (Newberg et al., 1996; Pascolo et al., 1997; Serreze et al., 2007; Takaki et al., 2006) for the level of transgene expression using flow cytometry on isolated brain endothelial cells. Fig 5 A depicts that in naïve mice, endothelial cells from 3 strains showed a weakly positive signal for BB7.2 (stock 3475, 4191, 6605). After stimulation with IFN γ for 2 days, all strains expressed the transgene as indicated by a 2-3-fold shift in median fluorescence intensity compared to the isotype controls. One of the strains (4191) also expressed detectable levels of the YLL/A2 complex after in vivo stimulation of MHC by IFN γ (Fig. 5B). Dendritic cells isolated from spleen served as a positive control in these FACS studies. As expected, dendritic cells from all strains showed robust signals with the BB7.2 antibody, which could be stimulated by IFN γ pretreatment. An example (stock 3475) is shown in Fig. 5C. Interestingly, dendritic cells also stained with RL6A for YLL/A2 in all strains except stock 6605. Taken together these results unequivocally establish that the YLL/A2 complex, derived from p68 RNA helicase, is formed in the murine system.

DISCUSSION

The results of the present study support with the following conclusions: First, p68 helicase protein expression by brain derived endothelial cells results in presentation of the same YLL/A2 complex that has been recently detected in various breast cancer cell lines (Verma et al., 2009). Second, binding and internalization of high affinity TCRm like RL6A via peptide/MHC complexes on vascular endothelial cells highlights potential diagnostic or therapeutic applications of these antibodies for cell types other than epithelial tumors or viral infected cells. Third, HLA-A2 transgenic mice express the transgene on brain microvascular endothelial cells and hence offer an animal model for evaluation of brain targeting approaches.

Using TCRm RL6A, expression of the cognate YLL/A2 complex has been validated both on a cell line (hCMEC/D3) and on primary cells. The presentation of YLL/A2 by human brain endothelial cells could not have been predicted with certainty, as the expression of a parent protein is a necessary, but not a sufficient condition for expression of peptide/MHC complexes derived from that protein. Further, as described for a different TCRm and its cognate peptide/HLA-A2 complex, no quantitative correlation could be established between the levels of protein expression and cell surface expression of the peptide/MHC complex (Weidanz et al., 2006).

Considering the binding of IgG to endothelial cells, it is noteworthy in this context that endothelial cells in peripheral organs and in brain express the neonatal Fc receptor (FcRn) (Borvak et al., 1998; Deane et al., 2005; Schlachetzki et al., 2002). FcRn have been implied in the transport of IgG at the BBB (Zhang and Pardridge, 2001). However, Zhou et al. (Zhou et al., 2005) reported that IgG binding to the human FcRn is highly species specific and that human FcRn recognizes murine IgG poorly (IgG2b) or not at all (IgG1, IgG2a). BB7.2 belongs to the mouse IgG2b subclass, RL6A is a mouse IgG2a antibody. Therefore, no binding by FcRn on hCMEC/D3 would be expected, which is consistent with the present data for IgG2a and IgG2b isotype controls.

To our knowledge the confocal imaging data in the present work represent the first evidence of MHC class I internalization by brain-derived endothelial cells and the first study to show TCRm internalization by non-malignant cells. Internalization of MHC class I complexes has previously been reported for a number of other cell types, including dendritic cells (Lizee et al., 2005), lymphoid cells (Machy et al., 1987; Tse and Pernis, 1984), fibroblasts (Huet et al., 1980), and sinusoidal endothelial cells of the liver (Limmer et al., 2000). We have recently found endocytosis of the RL6A TCRm by the breast carcinoma cell line MDA-MB-231 (unpublished). With respect to the endocytic mechanisms involved in uptake of TCRm, our results are in good agreement with current perceptions of the cellular trafficking of MHC class I molecules (Donaldson and Williams, 2009). Naslavsky et al. demonstrated that the small GTPase arf6-dependent pathway, which involves neither clathrin and dynamin nor caveolin-1, internalizes certain membrane proteins including MHC class I (Naslavsky et al., 2003; Naslavsky et al., 2004). This pathway partially overlaps downstream with early endosomes as indicated by EEA1 staining (Naslavsky et al., 2003), consistent with the present colocalization of 46% of RL-6A positive endosomes with EEA1 positive vesicles. While caveolar and endosomal pathways have been found to intersect (Pelkmans et al., 2004), this appears to be largely irrelevant in the current case, due to insignificant colocalization (8%) of RL-6A with caveolin-1-positive structures. Future studies are required to address the fate of TCRm after entering the early endosomal compartment.

Screening available HLA-A2 transgenic strains revealed that these mice could be viable animal models for brain targeting with A2-restricted antibodies, in particular with TCR mimics. Based on the flow cytometric analysis of transgenic mice, we expect strain B6.Cg-Tg(HLA-A/H2-D)2Enge/J (stock 4191) to be a promising model for in vivo experiments with the RL6A TCRm. The generation of YLL/A2 complexes in the murine system is made possible by the fact that human and murine p68 protein are 98% identical at the amino acid level, and most importantly, the YLLPAIVHI sequence is 100% conserved between the species. While we could not detect YLL/A2 on brain endothelium of the other transgenic strains studied here, these could still be valuable models when screening for the expression of new peptide/HLA-A2 complexes.

Proteomic approaches including peptide phage display (Arap et al., 2002), combinatorial antibody libraries (Jones and Shusta, 2007), or expression cloning efforts (Agarwal and Shusta, 2009) are underway with the goal to characterize specific membrane proteins as targets on endothelial cells. The present results introduce an exciting novel strategy to identify unique vascular targets. We hypothesize that peptide/HLA complexes can be isolated, which are highly specific for a given type of vascular endothelial cell. The subsequent generation of TCRm specific for such peptide/HLA combinations will allow targeting to select organ vascular beds for diagnostic or therapeutic purposes. Moreover, the fact that peptide/HLA expression patterns change with alterations of cellular state (e.g., viral infection, inflammatory response, cell proliferation) invokes the possibility of targeting modified endothelial cells vs. normal, resting endothelium. This option is highlighted in the present study by the shifts of fluorescence intensity observed for the model TCRm, RL-6A, both in vitro (Fig. 1) and in vivo (Fig. 5) after stimulation with the pro-inflammatory cytokine IFN γ . Applications in brain may include targeting the vasculature affected by neuroinflammatory disease (e.g., multiple sclerosis, ischemia/reperfusion injury) or tumor neovasculature.

In conclusion the presented data show for the first time that a TCRm specifically binds peptide/MHC complexes on brain-derived endothelial cells and that these cells internalize the antibody in endosomal vesicles. The concept of TCRm as targeting vectors can be explored in transgenic mice expressing human HLA in brain endothelium. These findings

stimulate the development of a strategy to identify novel and highly specific targets at the BBB and in other organ vascular beds.

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ABBREVIATIONS LIST

BBB	blood-brain barrier
TCRm	T cell receptor mimic

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Figure 1.

Panel A shows the result of flow cytometry of hCMEC/D3 cells stained with the antibody BB7.2. Detection was performed using a 2-step technique with unlabeled primary antibody and PE-labeled secondary antibody.

Panel B depicts agarose gel electrophoresis after PCR of total RNA isolated from hCMEC/D3 cells. The obtained amplicon runs as a single band of predicted length (84 bp) compared to a standard ladder.

Panel C shows the flow cytometer data after staining of hCMEC/D3 with RL6A. Median channel fluorescence shifts to the right after stimulation of hCMEC/D3 cells with IFN γ overnight.

Figure 2.

Flow cytometry and Western blot of hCMEC/D3 cells after treatment with siRNA specific for p68 RNA helicase (abbreviated p68) or non-targeting control siRNA (abbreviated ctrl). There is a significant reduction of the p68 protein in whole cell extract compared to the reference housekeeping protein, β -actin, after 24 h or 48h of transfection with siRNA (100 nM). Treatment with specific or control siRNA does not cause a difference in FACS staining for BB7.2 at both time points. While staining with RL6A at 24h also shows no difference, at 48h the signal is significantly decreased. MFI = median fluorescence intensity.

Figure 3.

Panel A shows confocal images demonstrating membrane binding (arrows) and internalization (arrow heads) of Alexa Fluor 546 (A546) labeled TCRm RL6A by hCMEC/D3 cells (red fluorescence signal). Cells were paraformaldehyde-fixed after 15 min incubation with the antibodies at 37°C. No staining was detectable with an A546 labeled isotype control antibody. In this panel and all other panels nuclei were stained with the far-red DNA dye DRAQ5 and pseudo-colored in blue. DIC = differential interference contrast. Panel B indicates that the TCRm RL6A and the pan HLA-A2 antibody BB7.2 (both A546 labeled, red fluorescence signal) were internalized into endosomal compartments: the arrows and arrowheads in the top group of confocal images highlight colocalization of RL6A or BB7.2, respectively, with the early endosomal marker EEA1 labeled by Alexa Fluor 488 (A488, green fluorescence signal), as evident from the merged images (yellow color). In contrast, the bottom group of images shows lack of colocalization with caveolin 1 (CAV1) by labeling of distinct structures, which do not overlay in the merged images. The hCMEC/D3 cells were incubated with A546 labeled antibodies RL6A or BB7.2 for 30 min at 37°C, followed by fixation and staining for EEA1 or CAV1 (both detected by A488 labeled secondary antibodies).

Panel C depicts images of live hCMEC/D3 cells after 30 min of incubation with A546-labeled RL6A, BB7.2 or isotype control antibody. Both RL6A and BB7.2 bind to the cell membrane (arrows) and undergo internalization (arrowheads), while the isotype control showed no interaction with the cells.

Panel D compares cellular uptake of RL6A after pre-incubation of the antibody with either an irrelevant peptide/A2 complex in solution (denoted KLM tetramer) or with the cognate YLL/A2 complex (YLL tetramer). Uptake is virtually abolished by YLL tetramer, but unaffected by the irrelevant epitope. The Z-stack provides further evidence for the internalization process of the labeled TCRm (here after 30 min of incubation with the cells). The arrowhead highlights one of the vesicles with clear intracellular localization. Scale bars in all panels = 5 μ m.

Figure 4.

Studies with primary human brain-derived endothelial cells. The cells were positive for HLA-A2 and for expression of YLL/A2 as shown by flow cytometry (panel A). Panel B illustrates binding and internalization of RL6A, which was labeled by AlexaFluor 546 (A546), as highlighted by arrowhead and arrows in the single optical section and the Z-stack, respectively. In contrast, A546 labeled isotype control antibody showed no interaction with the endothelial cells. DIC, differential interference contrast. Scale bars = 5 μ m.

Figure 5.

Flow cytometry of brain derived endothelial cells (panels A and B) and dendritic cells from the spleen (panel C) isolated from different mouse strains carrying transgene constructs of human HLA-A2. Cells were isolated by magnetic cell sorting either from naïve mice or after systemic treatment with IFN γ for 48h. The brain EC of 3 strains (3475, 4191, 6605) showed weak spontaneous signals with BB7.2. After IFN γ all strains were positive (A). In strain 4191, after IFN γ treatment a subpopulation of EC (gate in lower right corner, dot blot) expressed not only HLA-A2, but also the specific YLL/A2 complex (B). Panel C shows the strong positive staining for BB7.2 and RL6A on dendritic cells in strain 3475. Similar results were seen with dendritic cells from 3 other strains.