Chapter 4

4 Modulation of PP2A and VE-cadherin in Human Microvascular Endothelial Cells in a Macrophage Co Model

4.1 Introduction

Neurotropic and non-neurotropic infections such as pneumonia [661], multiple sclerosis, Alzheimer's disease and stroke [662-664] are associated with disruption of the blood brain barrier (BBB). A key component of this being mediated by proinflammatory M1 macrophages, which are the major source of the proinflammatory cytokines released during an inflammatory attack. In particular, within the brain, M1 macrophages inhibit brain repair [665, 666] and disrupt integrity of the blood brain barrier [667].

Integrity of the blood brain barrier in maintained through cell-cell contact involving tight junctions and adherens junctions [43, 54, 668, 669]. Interestingly, pro-inflammatory cytokines decrease VE-cadherin abundance in endothelial cells [670], and can alter its internalisation or association with its adaptor proteins, P120 and β-catenin through phosphorylation [260]. Regarding VE-cadherin, phosphorylation is critical in regulating its function [671] and contains multiple phosphorylation sites; most of which have been discovered from high throughput mass spectrometry [672-676]. The more frequently investigated are the Tyr^{645}, Tyr^{658}, Tyr^{685}, Tyr^{731}, Tyr^{733} and more recently Ser^{665} [256, 325, 327, 677-679]. The pro-inflammatory chemokine CCL2, causes Src-dependent Tyr phosphorylation of VE-cadherin and induces dissociation from the accessory protein β-catenin independently of altering paracellular permeability [291, 680]. Despite this, the involvement of phosphatases, and in particular PP2A in inflammation-mediated regulation of VE-cadherin is largely unknown. Although, a micro-array study by Lopez-Ramirez *et al.* characterising cytokine induced gene expression in hCMEC/D3s, found PP2Ac and VE-cadherin to be down-regulated [253].

In study 1, I established that inhibition of PP2A caused a loss of VE-cadherin in brain microvascular endothelial cells and was associated with an increase in paracellular permeability. The present chapter builds upon this to investigate if macrophages alter VE-cadherin through PP2A in a co-culture model of BBB inflammation. The aims of the present chapter were to: 1) established a proinflammatory *in vitro* hBMEC and

macrophage co-culture model, 2) investigate the effect of Mθ and M1 macrophages on PP2A and VE cadherin abundance in hBMECs and 3) determine if overexpression of PP2A can alter the effect of Mθ and M1 on VE-cadherin and paracellular permeability in brain endothelial cells.

4.2 Materials and Methods

4.2.1 Cell culture

Human brain microvascular endothelial cells (hBMECs; cell systems, WA, USA) and HCMEC/D3 (gifted from Dr. M. Campbell, Trinity College Dublin) were cultured in EndoGRO-MV culture medium containing 5 % foetal bovine serum, supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. hBMECs medium also contained ciprofloxacin (10 μg/mL). Cells were maintained at 37 ^{o}C in a humified atmosphere containing 5 % CO_2. hBMECs were used until passage 10.

Peripheral Blood Mononuclear cells (PBMCs) were isolated from human blood packs (gifted from the Irish Blood Transfusion Service, National Blood Centre, Dublin) by density gradient centrifugation. Blood packs were diluted 50:50 with PBS and carefully layered onto histopaque 1077 (Sigma Aldrich, Dublin) followed by centrifugation at 1650 rpm for 25 min and allowed to decelerate with the brakes switched off. The buffy layer was removed, diluted in PBS and centrifuged at 2000 rpm for a further 8 min. PBMCs were seeded in a 12 well plate (at a density of 2 x 10^7 cells/well) and cultured in RPMI 1640, 10% human serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. The PBMCs were left for 6 days, after which the attached cells were washed with PBS and replaced with serum free media for 24 h maintaining the unpolarised Mθ phenotype. The PBMCs were differentiated into M1 macrophages following exposure to LPS (100 ng/mL) and IFN-γ (20 ng/mL) for 24 h prior to the co-culture experiment.

All experiments were performed under serum free conditions.

4.2.2 Co-culture model

For the co-culture experiments, the inserts of a 12 well Millicell hanging plate (0.4 μM pore size) were coated with matrigel and dried at 37 ^{o}C for 30 mins. hBMECs and

hCMEC/D3s were seeded (2 x 10^5 cells/well) onto the matrigel, cultured for 24 h and a second layer was seeded over the initial layer. The endothelial cells and either Mθ or M1 macrophages were combined as a co-culture after 24 h in the EndoGRO supplemented media, minus serum for 24 h.

4.2.3 Bradford Protein Assay

The protein concentration in cell lysate was determined using a Bradford assay. Bovine serum albumin standards were prepared by serial dilution ranging in concentration from 0 – 1500 μg/mL. 5 μL of standards, a blank control and protein samples were added to a 96 well plate before combining with 250 μL of Bradford reagent (Sigma, Dublin). Colour was allowed to develop for 15 min at room temperature, before measuring absorbance at 595 nm using a spectrophotometer (BIO-TEK EL808). All samples were measured in duplicate and protein concentration was determined by interpolation of the mean absorbance against concentration of the standard curve.

4.2.4 Western blotting

Cells were lysed in a modified RIPA buffer (Tris-base 50 mM, NaCl 150 mM, EDTA 2mM and NP-40 0.5 % v/v), supplemented with the protease inhibitor cocktail SIGMAFAST™, sodium orthovanadate (2 mM) and sodium fluoride (5mM). Samples (20 μg protein) were boiled for 1 min in LDS sample buffer (LDS 5%, Tris HCl 1M, glycerol 50%, bromophenol blue 2.5 mg, phenol red 2.5 mg, ficoll 400 5% and β-mercaptoethanol 10 %). Samples were kept on ice for 3 min prior to loading on an 8% polyacrylamide gel. Samples were separated by electrophoresis using a Tris/glycine running buffer (Tris-base 25 mM, glycine 192 mM and SDS 0.1%). Following separation, the protein was transferred to a PVDF membrane using a semi-dry transfer system and transfer buffer (Tris-base 50 mM, glycine 40 mM, methanol 20% v/v, SDS 0.037% w/v, dH_2O). Membranes were blocked in TBS-T (Tris-base 10 mM, NaCl 100 mM and HCl 1 M and 0.1% Tween-20) containing 5% marvel for 1 h, prior to probing overnight at 4 ^oC with 1^o antibodies against VE-cadherin, PP2Ac, pPP2Ac, demethyl-PP2Ac, PME-1 or LCMT-1 (Santa Cruz). Membranes were extensively washed in TBS-T and probed for 1 h with a polyclonal goat anti-mouse HRP conjugated 2^o Ab (Dako). Following washing, bands were visualised using chemiluminescence detection (3.2 μL of 30% hydrogen peroxide/ 6 mL of 250 mM

Luminol, 90 mM 4-iodophenylboronic acid and 100 mM Tris-HCl)[681] and captured on a Fusion FX imaging system (Vilber Lourmat). Membranes were stripped using (62.5 mM Tris- HCl, 2% SDS and < 0.02% β-mercaptoethanol) and re-probed with an anti-β-actin HRP conjugated antibody. All antibodies were diluted (1:1000, excluding anti-β-actin (1:3000)) in blocking buffer. Densitometry was analysed using Bio1D software and samples normalised to β-actin. Protein molecular weight was determined using the EZ-RUN™ molecular weight ladder (Fischer Scientific, Dublin, Ireland).

4.2.5 Cell transfection and Overexpression

hBMEC were seeded in 6-well plates (2 x 10^5 cells per well) 24 h prior to transfection. In brief, following formation of the transfection complex (30 min at room temperature), cells were transfected using TransIT-X2 (10 μL; Mirus) and 1μg of pCMV6 PP2Ac and pCMV6 (empty vector) in serum free opti-mem (250 μL; Sigma) for 72 h. A mock transfection (transfection reagent only) was included as a control. The transfection protocol was optimised using a CY3 transfection control to obtain an efficiency of ~80%. PP2Ac over-expression confirmed by Western blot.

4.2.6 Semi-Quantitative Real-Time PCR

Total RNA was isolated from endothelial cells using guanidinium thiocyanate-phenol-chloroform extraction (TriReagent, Sigma-Aldrich, Dublin). RNA was treated with DNase I (Sigma-Aldrich, Dublin) and reverse transcribed using random hexamers and MMLV reverse transcriptase (RevertAid, ThermoFisher, Dublin). mRNA expression was determined using target specific primers Table 4.1 designed from the transcript sequence using the primer quest tool on the IDT website (https://eu.idtdna.com/Primerquest/Home/Index) and specificity confirmed by nucleotide search (Primer-BLAST; NCBI). cDNA was amplified using a real time thermocycler (Stratagene Mx3000P, Agilent Technologies) and GoTaq DNA polymerase (Promega). The comparative Ct method [$2^{-\Delta\Delta ct}$] was used to quantify gene expression. A no template control and no reverse transcriptase control were included for each primer set. Amplicons were interrogated using a dissociation curve and size confirmed following agarose gel electrophoresis under UV transillumination