Endothelial cell activation by interleukin-1 and extracellular matrix laminin-10 occurs via the YAP signalling pathway

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Abstract

Laminin-10 (LM-10) is a key regulator of blood-brain barrier (BBB) repair after hypoxia and inflammation. Here we investigated the signalling mechanisms regulated by LM-10 in human brain endothelial cell line hCMEC/D3 in response to interleukin(IL)-1beta(β) in vitro. LM-10 promoted endothelial proliferation and repair of an endothelial monolayer after scratch injury, and upregulated IL-1β-induced ICAM-1 and VCAM-1 expression. IL-1β and LM-10 regulated YAP signalling pathway in endothelial cells leading to differential expression of YAP target genes, ctgf and serpine-1, providing evidence that the YAP signalling pathway could be a new therapeutic target for the treatment of BBB dysfunction in CNS diseases.

1. Introduction

In physiological conditions, the extracellular matrix (ECM) of the central nervous system (CNS) provides a structural and functional environment for the cells of the neurovascular unit that is essential for maintenance of blood-brain barrier (BBB) integrity and brain homeostasis. However, after cerebral ischaemia, the BBB undergoes profound changes associated with the breakdown of tight junctions, remodelling of the ECM and enzymatic degradation of ECM proteins (Lu et al., 2011; Wang et al., 2007). The early events of BBB breakdown are also associated with leukocyte infiltration, mediated by cell adhesion molecules and chemokines (Thornton et al., 2010). The cytokine interleukin(IL)-1 (that includes the agonists IL-1α and IL-1β) is an established mediator of the pro-inflammatory response associated with BBB dysfunction and subsequent tissue damage (Denes et al., 2011). Although IL-1 is known to exert detrimental actions during the acute phase of stroke, increasing evidence suggests a biphasic action of IL-1 that exhibits neuroprotective properties during the sub-acute phase (Rodriguez-Grande et al., 2015). Furthermore, increasing evidence suggests that the ECM plays a dynamic role during the detrimental phase of stroke, whilst promoting repair during the later stages post-stroke. Interestingly, a novel function of the ECM as a regulator of IL-1β-induced signalling in astrocytes and cerebral endothelial activation has been demonstrated in vitro (Summers et al., 2013; Summers et al., 2010). ECM remodelling after CNS injury is associated with BBB repair, and IL-1β has been shown to mediate repair mechanisms, leading to the hypothesis that BBB repair driven by IL-1β could be regulated by ECM remodelling. Indeed, we have recently demonstrated LM-10 as a key ECM molecule involved in BBB repair after hypoxic injury and IL-1β-induced inflammation in vitro (Kangwantas et al., 2016). However, the role of LM-10 as a regulator of inflammation and angiogenesis had not yet been determined.

The mechanisms underlying the dynamic crosstalk between the ECM and inflammation are poorly understood. Although it has been previously demonstrated that different components of the ECM alter IL-1β signalling pathways in astrocytes and endothelial cells in vitro (Summers et al., 2013; Summers et al., 2010), the crosstalk between signalling pathways downstream of the IL-1 receptor and other key signalling pathways has not been fully characterised. The YAP/Hippo pathway has recently gained significant interest as an extremely dynamic pathway implicated in ECM remodelling, as seen in cancer and inflammatory diseases (Wang et al., 2020; Warren et al., 2018). It is well established that ECM-integrin signalling is a key step in the initiation of the YAP

Abbreviations: BBB, blood-brain-barrier; CNS, central nervous system; ECM, extracellular matrix; ELISA, Enzyme-linked immunosorbent assay; ERK1/2, extracellular-regulated kinase 1/2; ICAM-1, intercellular cell adhesion molecule-1; IL, interleukin; LM-10, laminin-10; NF-κB, nuclear factor kappa B; VCAM-1, vascular cell adhesion molecule-1.

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pathway. Critically, recent evidence has demonstrated a key role of YAP in tumour necrosis factor-a-induced endothelial activation (Choi et al., 2018). However, the role of YAP in endothelial cells after IL-1β treatment had not yet been determined, and whether LM-10 could modulate this response is currently unknown. Using in vitro approaches, we demonstrate here that LM-10 modulates key in vitro hallmarks of angiogenesis and modulates endothelial cell activation after IL-1β treatment through the upregulation of key adhesion molecules, and demonstrate a novel mechanism whereby YAP signalling pathway is involved in endothelial activation by IL-1β, providing new signalling mechanisms of cerebrovascular activation regulated by IL-1 and the ECM.

2. Materials and methods

2.1. Tissue culture plate coating

Tissue culture plates (Corning, UK) were coated at 4 °C overnight with human (recombinant) LM-10 (Biologam, Sweden) diluted in phosphate-buffered saline (PBS) with calcium and magnesium (PBS-+) at concentrations of 0.1, 1, 2.5, 5, 10 μg/ml. Control wells were incubated with filter sterilised 0.22 μm pore size filter (Starlab, UK) 0.1% (w/v) low endotoxin bovine serum albumin (BSA) in PBS+. After overnight incubation, LM-10 solutions were discarded. PBS+ was added to control and LM-10 coated wells to prevent drying out. Tissue culture plates were stored at 4 °C. For experiments using Matrigel, neat Matrigel (9.16 mg/ml) or LM-10 (10 μg/ml) was added to Dulbeccos Modified Eagles Medium (DMEM) (Invitrogen, UK) to a concentration of 20 μg/ml, and tissue culture plates were pre-coated at 4 °C overnight. Plates were washed in PBS+ before cell seeding.

2.2. hCMEC endothelial cell line culture

The immortalised human cerebral microvascular endothelial cell line, hCMEC/D3, was purchased from Merck (UK). Cells were cultured in 75 cm² tissue culture flasks pre-coated with rat tail collagen type I (Merck, UK), (1:100 in PBS) at 37 °C for 1 h, and maintained in EndoGro-MV complete culture media (5% fetal bovine serum, 1-glutamine (10 mM), EndoGro-LS supplement (0.2%), heparin sulphate (0.75 U/ml), ascorbic acid (50 μg/ml), hydrocortisone hemisuccinate (1 μg/ml), recombinant human epidermal growth factor (5 ng/ml), recombinant human basic fibroblast growth factor (1 ng/ml; Merck) and 1% penicillin-streptomycin) at 37 °C in a humidified atmosphere containing 5% CO2. Cells were passaged at 80-90% confluency using 0.25% trypsin/ 1 mM EDTA solution, and were used in experiments until passage 10.

2.3. Cell culture treatments

hCMEC/D3 cells were seeded at a density of 200,000 cells/well in 24-well plates pre-coated with Matrigel (20 μg/ml) or LM-10 (10 μg/ml) in EndoGro-MV complete culture medium for 4 h. For inflammatory mediator release experiments, cultures were treated with recombinant human IL-1β (R&D Systems, UK) at 0.1, 0.3, 1, 3, 10 or 100 ng/ml diluted in EndoGro-MV complete culture medium, for 24 h. Control cultures were treated with vehicle (EndoGro-MV complete culture medium alone) for 24 h. For signalling pathway and qPCR experiments, cultures were treated with IL-1β (10 ng/ml) for 5, 15, 30, 60,120 or 240 min.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ICAM-1, VCAM-1, phospho-p38a and phospho-ERK1/2 levels in cell lysates, and IL-8 levels in supernatants, were quantified by human ELISA (R&D Systems, UK) according to the manufacturer’s instructions. Absorbance was measured at 450 nm and corrected at 570 nm using a plate reader (Synergy HT, BioTek, UK). The standard curve was fitted by a sigmoidal 4PL equation in GraphPad Prism. Concentrations in the samples were calculated by interpolating the values from the standard curve. Levels of all cytokines were corrected for cell number and expressed as pg/ml per μg/ml of total protein (pg/μg of protein), using total protein determined by the BCA assay.

2.5. Western blot analyses

hCMEC/D3 cells were lysed in RIPa buffer (50 mM Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) supplemented with 1% protease inhibitor cocktail and phosphatase inhibitors. Lysates were denaturated in Laemmli buffer (2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol in 60 mM Tris-HCl, pH 6.8) at 95 °C for 5 min, and equal amount of protein were resolved on 10% SDS polyacrylamide gel, then transferred onto polyvinylidene difluoride (Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad). Non-specific binding sites were blocked with 5% (w/v) BSA in PBS 0.1% (v/v) Tween 20 (PBST) for 1 h at room temperature (RT). Membranes were then incubated (4 °C overnight in primary antibody in PBST 5% BSA, as follows; anti β-Actin (Cell Signalling UK, 1/1000); anti phospho-p65 (Ser536) (Cell Signalling UK, 1/1000); anti p-YAP127 (Cell Signalling UK, 1/1000); anti p-YAP397 (Cell Signalling UK, 1/1000); anti YAP (Cell Signalling UK, 1/1000); anti β-actin (Abcam UK, 1/1000). Membranes were washed in PBST and incubated with secondary anti rabbit anti-IgG (Agilent UK, 1/1000) antibody in PBST 1% BSA for 1 h at RT. Membranes were washed and incubated in ECL. Prime Western Blotting Detection Reagent (GE Life Sciences, UK) before exposure with G:BOX (Syngene) and Genesys software. Densitometry was determined using ImageJ software, and detected intensities were normalised against β-actin. Finally, ratios of pYAP/YAP were calculated. Full Western blot images are provided in Supplementary figures.

2.6. Quantitative polymerase chain reaction (qPCR)

RNAs were extracted using Purelink RNA minikit (Thermo Fisher Scientific, UK) according to the manufacturer’s instructions, with the use of PureLink™ DNase Set (Thermo Fisher Scientific). Extracted RNAs were assessed for yield and purity using Nanodrop 1000. Messenger RNA (1 μg) was converted to cDNA using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific), according to the manufacturer’s instructions. Quantitative polymerase chain reaction (qPCR) was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) in 384-well format using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Three microliters of 1:20 diluted cDNA was loaded with 200 mmol/L of primers in triplicate. Primers used were as follows; Ctgf FWD: CAGCATGGACGTTCGTCTG, REV: AACCAAGTGGTTGTTGCTTTGG; Serpine 1 FWD: ACCGCAACGTGGTTGTTTCTCA, REV: TTGAAATCCATAGCTGCTTGAAAT; GAPDH FWD: GCACAGGTTGAACG; REV: AGGGATTGCGTCCTGAG. Data were normalised to the expression of the housekeeping gene GAPDH. Expression levels of genes of interest were calculated as follows: relative mRNA expression = E–(Ct of gene of interest)/ E–(Ct of housekeeping gene), where Ct is the threshold cycle value and E is efficiency.

2.7. Scratch wound assay

hCMEC/D3 cells were seeded at 40,000 cells/well in 96-well ImageLock plate (Essen BioScience) and left to adhere for 4 h. Scratch wound injury was carried out using a 96-pin IncuCyte WoundMaker Tool (Essen BioScience). Cells were then washed twice with PBS and replaced with fresh media. Phase contrast images were acquired at 2 h intervals for a period of 24 h on an Incucyte Zoom Live Cell Analysis system using a 4×/3.05 Plan Apo OFN25 objective. The 96-well Cell Migration Software Application Module (Essen BioScience) was used to quantifiy relative wound density. Relative wound density is a measure
2.8. Tube formation assay

Fifty µl of undiluted Matrigel (9.16 mg/ml) was added to individual wells of an ice-cold 96-well plates, and the plate incubated at 37 °C for 45 min to allow gelation. Seventy µl of either LM-10 (10 µg/ml) or PBS (control) was added to the Matrigel pre-coated wells, and plates were incubated for a further 2 h at 37 °C. The PBS and LM-10 aqueous layers on top of the Matrigel were aspirated. hCMEC/D3 cells were resuspended in media supplemented with 3 µg/ml bFGF and were seeded at a density of 10,000 cells/well. Phase contrast images were acquired at 0 and 6.5 h on an Incucyte Zoom Live Cell Analysis system using 4x/0.1 Plan Apo OFN25 objective. The angiogenesis analyser macro in ImageJ was used to quantify total number of branches and total branching length.

2.9. Statistical analyses

All data were analysed with GraphPad Prism 8.1.2 (GraphPad Software Inc) using the statistical tests stated in the figure legends. Homoscedasticity of the standard deviations were evaluated with a Brown-Forsythe and Bartlett’s test, alongside the use of homoscedasticity plots (predicted vs residual) and QQ plots to assess equal variance and normality. Appropriate transformations were applied where necessary. Data are presented as mean ± standard error of mean (SEM). Details of replicates are indicated in the figure legend. Statistic significance was accepted at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

3. Results

3.1. LM-10 regulates angiogenic phenotype and IL-1β-induced activation of hCMEC/D3 cells

We previously reported that LM-10 plays a key role in the maintenance of BBB integrity, and reverses most of the key hallmarks of BBB dysfunction induced by acute hypoxia and IL-1β (Kangwantas et al., 2016). In the current study we have used hCMEC/D3 cells, a cell line that has been extensively characterised as a valid brain endothelial phenotype and used as a model of human BBB function (Wekslar et al., 2013). To confirm the cellular effects of LM-10 on hCMEC/D3 cells, we investigated the effect of LM-10 on angiogenic responses using a scratch assay (Fig. 1A) and tube formation assay (Fig. 1B). LM-10 reduced width of scratch in a time-dependent manner compared to Matrigel (Fig. 1Ai). The tube formation assay, LM-10 increased the number of branches (1.35-fold increase, p = 0.0263) and total branching length (1.57-fold increase, p = 0.0144, Fig. 1 Bi and ii) compared to Matrigel, showing that LM-10 increased tube-like structures and their length. These data suggest that LM-10 aids endothelial cell migration, an initial step in the processes of angiogenesis and BBB repair.

We next aimed to examine if LM-10 acts as a regulator of IL-1β-induced endothelial cell activation and IL-1β-induced signalling pathways in hCMEC/D3 cells. IL-1β treatment had a significant effect on ICAM-1 (p < 0.0001, Fig. 2 Ai), VCAM-1 (p < 0.0001, Fig. 2 Aii) and IL-8 (p < 0.0001, Fig. 2 Aii) expression in a concentration-dependent manner. The ECM had a significant effect on expression of VCAM-1 (p = 0.0245, Fig. 2 Aii) demonstrating an elevated expression of VCAM-1 in hCMEC/D3 cells when grown on LM-10 compared to Matrigel, whilst a trend towards a statistically different effect of LM-10 on ICAM-1 expression was detected (p = 0.0576, Fig. 2 Ai). Specifically, LM-10 induced a higher IL-1β-induced expression of ICAM-1 in hCMEC/D3 cells treated with IL-1β at 100 ng/ml (1.16 fold, p = 0.0089) and VCAM-1 at 10 ng/ml (1.17 fold, p = 0.0058), 30 ng/ml (1.16 fold, p = 0.0013) and 100 ng/ml (1.22 fold, p < 0.0001) compared to corresponding IL-1β concentrations on Matrigel. No significant effect of ECM coating on IL-8 expression was observed (p = 0.6916, Fig. 2 Aii), suggesting that LM-10 does not influence IL-8 expression in hCMEC/D3.

We next investigated whether LM-10 alters IL-1β signalling pathways, namely ERK1/2, p38 and NF-κB, as these are key signalling elements downstream of IL-1 receptor activation in endothelial cells (Summers et al., 2013; Thornton et al., 2016). IL-1β had no significant effect on phospho-ERK1/2 levels in hCMEC/D3 cells on Matrigel and LM-10 after 5 and 15 min of IL-1β treatment, whereas a significant decrease (p = 0.0012) in phospho-ERK1/2 levels was detected after 30, 60 and 120 min of IL-1β treatment on both matrices (Fig. 2 Bi). Importantly no significant effect of LM-10 on IL-1β-induced phosphorylated ERK1/2 levels was detected. Furthermore, IL-1β treatment significantly increased (p = 0.0002) phospho-p38 levels in a time-dependent manner both on Matrigel and LM-10, with maximum activation detected 30 min after IL-1β treatment followed by a decrease in phospho-p38 levels at 60 and 120 min of IL-1β treatment (Fig. 2 Bii). No effect of LM-10 on IL-1β-induced p38 activation was detected. Finally, IL-1β treatment significantly increased (p = 0.0002) the levels of phospho-NF-κB p65 in a time-dependent manner in cells on Matrigel and LM-10 (Fig. 3A). In hCMEC/D3 cells seeded on Matrigel, a significant increase in phospho-NF-κB p65 levels was detected 5 min after IL-1β treatment compared to baseline (15.5-fold increase, p = 0.0002), which dropped slightly but remained significantly elevated at 15 min (11-fold increase, p = 0.0081), 30 min (13-fold increase, p = 0.0011) and 60 min (11-fold increase, p = 0.0063), before markedly decreasing at 120 min and 240 min after IL-1β treatment. In hCMEC/D3 cells seeded on LM-10, a significant increase in phospho-NF-κB p65 levels was detected 5 min after IL-1β treatment compared to baseline (12.8-fold increase, p < 0.0001), which slightly dropped but remained significantly elevated at 15 min (8.9-fold increase, p = 0.0002) and 30 min (7-fold increase, p = 0.0046), before markedly decreasing at 60 min after IL-1β treatment. Interestingly the ECM had a significant effect on phospho-NF-κB p65 levels, with significantly higher levels of phospho-NF-κB p65 observed at 5 min after IL-1β treatment in cells seeded on LM-10 compared to Matrigel (1.5-fold increase, p = 0.0425).

We next assessed IκBα expression after IL-1β treatment in hCMEC/D3 cells (Fig. 3B). There was a marked, significant reduction in IκBα levels in hCMEC/D3 cells seeded on Matrigel after 5 min (84%, p = 0.0021), 30 min (95%, p = 0.0001) and 60 min (87%, p = 0.0016) after IL-1β treatment. A marked and significant reduction in IκBα levels at 5 min (82%, p = 0.0013), 30 min (92%, p = 0.0001) and 60 min (88%, p = 0.0005) after IL-1β treatment was also observed in hCMEC/D3 cells seeded on LM-10. Although IL-1β treatment (p < 0.0001) affected IκBα in a time-dependent manner both on Matrigel and LM-10, no effect of ECM was determined and statistical comparison between corresponding time-points showed no significant differences in IκBα levels were detected in cells on Matrigel and LM-10.

3.2. LM-10 regulates YAP signalling after IL-1β-induced activation in hCMEC/D3 cells

Having demonstrated that not all classical IL-1β downstream signalling pathways were modulated by LM-10, we investigated alternative signalling pathways that may be modulated by LM-10 in IL-1β-induced
Fig. 1. Effect of LM-10 on hCMEC/D3 cell migration and cell tube formation.

(Ai) Representative images and (Aii) quantification of the scratch assay. 96-well Essen ImageLock plates were pre-coated with Matrigel (20 μg/ml) or LM-10 (10 μg/ml). 40,000 hCMEC/D3 cells were seeded for 4 h and the 96-pin IncuCyte WoundMaker Tool was used to create uniform cell-free zones. Phase contrast images were acquired every 4 h on an Incucyte Zoom Live Cell Analysis system using a 123 ×/3.05 Plan Apo OFN25 objective. Arrows indicate boundaries of the scratched regions. Scale bar 400 μm. The Incucyte scratch wound assay analysis software was used to calculate relative wound confluence. (Bii) Representative images and quantification (Bii-iii) of the tube formation assay. 30,000 hCMEC/D3 cells were seeded on top of the Matrigel layer. Phase contrast images were acquired at 0 and 6.5 h on an Incucyte Zoom Live Cell Analysis system using a 123 ×/3.05 Plan Apo OFN25 objective. Scale bar 800 μm. The angiogenesis analyser macro in ImageJ was used to quantify number of branches (Bii) and total branching length (Biii).

Data are represented as mean ± SEM of 3 technical replicates and 3 biological replicates (n = 3, A) and of 2 technical replicates and 4 biological replicates (n = 4, B). Data were assessed by a two-way RM ANOVA followed by Sidak’s post-hoc analysis comparing the means of wound confluence at corresponding time points on Matrigel vs LM-10 (A). Significant main effects of time and the ECM coating are indicated on the top left of the graphs (A). Data were assessed by paired t-test to compare coatings (B). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
activated endothelial cells. The ECM has been recently implicated as a regulator of the YAP/Hippo pathway (Warren et al., 2018), and evidence suggests that inflammation can modulate this signalling mechanism (Choi et al., 2018). Therefore, we hypothesised that IL-1β and/or LM-10 alters YAP signalling in endothelial cells. To address this hypothesis, we initially measured the phosphorylation of YAP at S127 (Fig. 4A) and S397 (Fig. 4B) after IL-1β treatment. These phosphorylation sites play differential roles in YAP signalling whereby p-YAP(S127) induces cytoplasmic retention of YAP (Zhao et al., 2008; Zhao et al., 2007), and p-YAP(S397) creates a phospho-degron motif for proteasomal degradation (Zhao et al., 2010). The p-YAP(S127)/YAP and p-YAP(S397)/YAP ratios are an indicator of levels of dephosphorylated YAP in the cell that is able to translocate into the nucleus and activate associated genes.

When analysing the p-YAP(S127)/YAP ratio after IL-1β treatment in hCMEC/D3 cells, we found that IL-1β treatment significantly (p < 0.0001) affected p-YAP(S127) levels in a time-dependent manner in cells on either Matrigel or LM-10 (Fig. 4A); In hCMEC/D3 cells seeded on...
Matrigel, phosphorylation of YAP(S127) significantly decreased at 5 min (54%, \( p = 0.0164 \)) and 15 min (54%, \( p = 0.0053 \)) compared to baseline levels, and then slowly return to baseline levels at 120 min and 240 min. In cells seeded on LM-10, a significant decrease in p-YAP(S127) was observed at 15 min (46%, \( p = 0.0038 \)), which then returned to baseline levels at 120 min and 240 min. No significant effect of ECM on p-YAP(S127) levels was determined, and no significant differences between Matrigel and LM-10 were observed at each time point after IL-1\( \beta \) treatment. When hCMEC/D3 cells were seeded on Matrigel, there was a small, no significant, initial reduction in p-YAP(S397) levels at 5 min (35% \( p = 0.7288 \)) and 30 min (15%, \( p = 0.9978 \)) after IL-1\( \beta \) treatment compared to baseline. This was followed by a marked increase in p-YAP(S397) levels between 30 min and 240 min after IL-1\( \beta \) treatment, and significant increases were observed at 120 min (2.7-fold increase, \( p = 0.0063 \)) and 240 min (4.1-fold increase, \( p < 0.0001 \)) compared to baseline. In contrast, the signalling pattern of p-YAP(397) expression in hCMEC/D3 seeded on LM-10 was markedly different to cells on Matrigel, in that, a consistent, significant reduction in p-YAP(397) levels in hCMEC/D3 cells on LM-10 was observed between 15 min and 120 min after IL-1\( \beta \) treatment compared to basal levels, with a significant reduction at 15 min (55%, \( p = 0.0349 \)), 30 min (72%, \( p = 0.0089 \)) and 60 min (69%, \( p = 0.0073 \)), followed a small increase in levels observed at 240 min (36%, \( p = 0.4310 \)). Interestingly, the ECM had a significant (\( p = 0.0112 \)) effect on the levels of p-YAP(S397), demonstrating elevated levels of p-YAP(S397) on Matrigel compared to LM-10.

To investigate the nuclear activity of YAP as a transcriptional co-activator, we analysed the mRNA levels of its target genes Ctgf (Fig. 5A) and Serpine 1 (Fig. 5B). There was a significant marked reduction (97%, \( p = 0.0197 \)) of Ctgf mRNA levels in hCMEC/D3 cells on
Matrigel after IL-1β treatment, whilst reduction of Ctgf mRNA levels in cells on LM-10 was marginal and not significant (22%, $p = 0.6031$). On direct comparison of baseline Ctgf mRNA levels between cells on Matrigel and LM-10, there was notably elevated levels on LM-10 (1.6-fold increase, $p = 0.2515$). After IL-1β treatment, there was a significant difference in levels of Ctgf mRNA levels between hCMEC/D3 cells grown on Matrigel and LM-10 (42-fold increase, $p = 0.0127$). In contrast, we observed a different IL-1β effect on Serpine 1 mRNA expression levels whereby no overall effect of IL-1β was observed, whilst there was a trend towards a significant effect of ECM ($p = 0.0667$), suggesting potentially elevated levels of Serpine 1 mRNA in cells on LM-10.

Fig. 4. Effect of LM-10 on YAP signalling after IL-1β-induced activation in hCMEC/D3 cells. hCMEC/D3 cells were seeded at a density of 200,000 cells/well in a 24-well plate for 4 h. Cells were treated with IL-1β (10 ng/ml) for various length of time (from 5 min to 240 min). Cell lysates were then collected and assayed for p-YAP(S127) (A), p-YAP(S397) (B) and total YAP by Western blot. p-YAP(S127), p-YAP(S397), YAP and β-actin were quantified using densitometry on ImageJ. p-YAP(S127), p-YAP(S397) and YAP were normalised to β-actin, and the ratio of normalised p-YAP (S127)/total YAP and p-YAP (S397)/total YAP was calculated. The blots are representative images. Data are represented as mean ± SEM of 4 biological replicates ($n = 4$). Data were assessed by a two-way RM ANOVA followed by Sidak’s post-hoc analysis. *represents significance comparing the mean ratio of p-YAP(S127)/total YAP at corresponding time points on Matrigel vs LM-10 and # represents significance within ECM to baseline. Significant main effects of IL-1β treatment time and the ECM coating are indicated on the top left of the graphs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Full images of Western blot are provided in supplementary figures.
Relative expression of IL-1β in hCMEC/D3 cells

ECM p<0.05

# ML M - 1 0
0.0
0.5
1.0
1.5
2.0
2.5
Serpine1
Relative expression
IL-1/g69 ++
A) B)
/g13

Fig. 5. Effect of LM-10 genes expression downstream of YAP after IL-1β-induced activation in hCMEC/D3 cells.

Recent research has demonstrated a novel role of the ECM as a regulator of IL-1β-induced endothelial inflammation in vitro (Summers et al., 2013) and LM-10 has been identified as a potential mediator of BBB repair in vitro (Kangwanats et al., 2016). However, the specific role of LM-10 as a regulator of angiogenesis and IL-1β-driven endothelial inflammation in vitro remained unknown. Furthermore, the signalling crosstalk between IL-1β and YAP/hippoc signalling in endothelial cells has not been investigated. Here we present novel evidence of a dynamic cross-talk between IL-1β and YAP signalling in endothelial cells regulated by LM-10, providing a potential mechanism underlying cerebrovascular inflammatory responses regulated by ECM remodelling in CNS diseases.

We next sought to determine the signalling mechanisms by which LM-10 may modulate the inflammatory activation of endothelial cells. Previous studies have shown a novel regulatory mechanism by which the ECM modulates ERK1/2 signalling after IL-1β treatment in rat astrocytes and rat brain endothelial cells in vitro (Summers et al., 2013; Summers et al., 2010). However, IL-1β treatment failure to induce ERK1/2 and p38 MAPK pathways in hCMEC/D3 cells, and therefore no effect of LM-10 was observed. Lack of effect of IL-1β treatment on ERK1/2 in endothelial has already been shown previously (Thornton et al., 2010), however our results are contradictory to those reported by Ni and colleagues (Ni et al., 2017) who detected an increase in phospho-p38α in response to IL-1β treatment. In contrast, IL-1β treatment transiently activated NF-kB, a response previously observed by us in rat endothelial cell culture (Summers et al., 2013), and IL-1β-induced NF-kB activation was further potentiated by LM-10, suggesting that NF-kB activation may regulate IL-1β-induced adhesion molecule expression in hCMEC/D3 cells. In contrast our study demonstrates for the first time that regulation of IL-1β-induced endothelial cell activation by LM-10 may be mediated by the YAP pathway, a recently described new signalling pathway shown to have critical roles in vascular systems, contributing to vessel homeostasis, vascular development, angiogenesis (Park and Kwon, 2018).

Specifically, we demonstrate here a novel temporal pattern of YAP phosphorylation at S127 and S397 regulated by IL-1β and LM-10. The subcellular distribution of YAP is controlled by the reversible phosphorylation of S127, resulting in binding and cytoplasmic retention, hence the inability to bind to TEADs (Zhao et al., 2008; Zhao et al., 2007), whilst the phosphorylation of S397 creates a phospho-degron motif for β-TrCP binding resulting in proteosomal degradation, providing an irreversible longer-term mechanism of YAP inhibition (Zhao et al., 2010). After IL-1β treatment in hCMEC/D3 cells, a similar decrease in pYAP(S127) followed by an increase back to respective basal levels was observed in cells on Matrigel and LM-10, whereas the temporal pattern of pYAP(S397) phosphorylation after IL-1β treatment was significantly impaired in cells on Matrigel compared to LM-10. These findings suggest that IL-1β-induced phosphorylation of YAP at S127 and S397 is coupled when cells are seeded on Matrigel, whilst the phosphorylation of S127 and S397 is uncoupled in cells on LM-10, and that transient reductions in pYAP(127) and pYAP(397) on LM-10 are indicative of longer dephosphorylation of YAP, and hence translation into...
the nucleus. In support of this, we show heavily decreased levels of Ctgf mRNA in hCMEC/D3 cells on matrigel 2 h after IL-1β treatment, reflective of decreased activation of genes downstream of YAP. In comparison, no significant change in Ctgf mRNA was observed 2 h after IL-1β treatment in hCMEC/D3 cells on LM-10, supporting our previous findings that YAP was not sequestered and degraded to the same degree in cells on Matrigel. However, we did not detect any significant change in Serpine1 mRNA levels. Since Serpine1 is typically associated with mechano-signalling (Liu et al., 2015), it is likely that this gene is not regulated via YAP in hCMEC/D3 cells after IL-1β treatment.

5. Limitations and translation of findings

Our study identifies for the first that LM-10 is critical regulator of angiogenic responses and IL-1β / YAP signalling cross-talks in endothelial cells in vitro. Whilst those findings suggest that LM-10 could be a regulator of cerebrovascular remodelling and functional recovery after central inflammatory conditions, the translatability of findings to clinical settings needs to be explored further and significant limitations exist. The use of a single cell type culture system, although informative to understand the endothelial-specific response to ECM / inflammatory stimuli in our study, may not directly translate to in vivo response since IL-1β /LM-10 may also induce responses of other cells of the neurovascular unit, i.e. astrocytes and pericytes. Most importantly, a fine tuning of LM-10/IL-1β-induced YAP signalling and angiogenic response in cells of the neurovascular unit might be critical to promote beneficial angiogenesis as opposed to aberrant pathogenic angiogenic responses that could impair functional recovery. Finally, the spatio-temporal expression and actions of IL-1β/LM-10 needs to be investigated, as this will be critical to promote beneficial angiogenic responses for optimum tissue repair and functional recovery.

6. Conclusions

In conclusion, we demonstrate here for the first time a novel signalling mechanism whereby YAP signalling pathway is critically regulated by IL-1β and LM-10 in endothelial cells, providing new signalling mechanisms of cerebrovascular activation regulated by IL-1 and the ECM that could be targeted for the treatment of inflammatory CNS disease.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2022.577993.

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