TNF α and MMP1 in brain microvascular endothelial cells regulate blood-brain barrier dysfunction in psychotic disorders

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Abstract

In schizophrenia and bipolar disorder, tight junction deficits in brain microvascular endothelial cells (BMECs) lead to blood brain barrier (BBB) dysfunction. Using an *in vitro* model of human BBB function, we show that BMECs derived from human induced pluripotent stem cells of schizophrenia patients show reduced BBB integrity and increased small molecule permeability when compared to healthy control BMECs. Stratification based on BBB function in schizophrenia and bipolar disorder patients identified a BBB-deficit subtype with reduced barrier function, increased permeability to larger molecules, and decreased claudin-5 (CLDN5) levels. BMECs from the BBB-deficit group show increased MMP1 activity, which correlated with reduced CLDN5 levels and worse BBB function, which were rescued by tumor necrosis factor α (TNF α) and MMP1 inhibition. These results show intrinsic deficits in BMECs in psychotic disorders that result in BBB disruption and further identify TNF α and MMP1 as potential targets for ameliorating BBB deficits.

Introduction

Schizophrenia (SZ) and bipolar disorder (BD) account for a large proportion of morbidity, mortality, and global burden associated with severe mental disorders¹. Despite a significant genetic overlap between SZ and BD², little clarity exists regarding their pathogenesis. Although there is convergent evidence for blood brain barrier (BBB) dysfunction and peripheral inflammation in SZ and BD, it remains to be determined whether BBB deficits are 1) intrinsic to brain microvascular endothelial cells (BMECs) which results in increased peripheral inflammation or 2) whether peripheral inflammation from other sources impair BBB function³. The BBB protects the brain by regulating molecular and cellular trafficking between the bloodstream and brain parenchyma through physical and enzymatic transport, as well as by regulating immunological processes that are vital for brain homeostasis and response to injury³. Notably, the BBB is composed of a monolayer of BMECs that line blood vessels and provide strict regulation of paracellular permeability through protein junctional complexes between BMECs that limit passive diffusion and paracellular transport⁴.

Findings from peripheral inflammatory cytokine, transcriptomic, genetic, and neuroimaging studies point to an important role for BMECs in the pathogenesis of SZ and BD³. For example, circulating peripheral inflammatory cytokines have been shown to negatively impact the BBB⁵. Conversely, transcriptomic and genetic studies indicate the presence of disruptions in paracellular⁶ and transcellular^{7–13} mechanisms related to BMEC function. There is also increasing evidence for increased BBB permeability in subgroups of patients with SZ^{7,14,15} and BD^{16,17}. These findings suggest BMEC involvement in the pathogenesis of SZ and BD in specific subgroups of patients, but there is no study to date that has directly investigated the role of BMECs in SZ and BD. While, there is no consensus on which models are best for interrogating BBB function, stem cell-based BBB models are promising avenues since they are able to recapitulate the intrinsic contribution of disease- and patient-specific genetic backgrounds^{3,18}. We recently adapted and validated a method to model BBB function *in vitro* using human induced pluripotent stem cell (iPSC)-derived BMECs¹⁹, in order to gain new insights into the biology underlying SZ and BD.

In this study, we employ human iPSC-derived BMECs to investigate BBB function in SZ and psychotic BD (collectively referred to as psychotic disorders). We report here the presence of increased BBB permeability in BMECs from SZ patients compared to healthy control BMECs and show that stratification based on BBB function can be used to identify a “BBB-deficit” subtype of psychosis patients that have BMEC biology that is distinctly different from BBB-nondeficit patients or healthy individuals. This BBB-

deficit group demonstrated increased MMP1 levels and activity, which was associated with impaired BBB function. Pharmacological inhibition of MMP1 with piroxicam or biological inhibition of TNF α with infliximab, an upstream regulator of MMP1, were able to restore BBB function in BBB-deficit patients.

Results

Morphological and functional properties in an *in vitro* human BBB model. The impervious nature of the human BBB is attributed primarily to the structure and function of BMECs, comprising physical, enzymatic, transport, and immunological processes that are vital for proper BBB functioning³. We had previously adapted and optimized a protocol for differentiating human iPSCs to generate BMEC-like cells with morphological and molecular marker expression (TJP1, OCLN, CLDN5, CD31 and SLC2A1), physical barrier properties with high transendothelial electrical resistance (TEER) $\geq 2000 \Omega \times \text{cm}^2$, enzymatic transport activity for ABCB1 and ABCC1, and angiogenic potential in the sprouting assay that are characteristic features of BMECs^{19–21}. Human iPSCs were grown in Essential 6 (E6) medium for four days to prime the cells for differentiation and then switched to human endothelial serum-free culture medium (hESFM) supplemented with 20ng/mL basic fibroblast growth factor (bFGF), 10 μM retinoic acid (RA), and B27 supplement to induce BMEC differentiation¹⁹. On the sixth day, cells were sub-cultured onto a collagen IV and fibronectin 1 matrix hESFM plus B27, bFGF, and RA resulting in a purified BMEC population by day 8²¹ (Fig. 1a).

We utilized a transwell system to examine the physiological properties of the BBB and quantify TEER, a standard measure of barrier properties (Fig. 1b). BMECs were differentiated from human iPSCs of four healthy control (HC), four psychotic bipolar I disorder (BD), and four schizophrenia (SZ) patients that were matched for age and sex (Supplementary Table 1). In order to establish the reliability of TEER measurements both within and across different cell lines, TEER experiments were conducted with technical as well as biological replicates over the course of six days. TEER was peaked on day 2 (TEER $\geq 2,000 \Omega \times \text{cm}^2$) and plateaued on day 3 (TEER $\sim 1,000 \Omega \times \text{cm}^2$), as had been described previously for iPSC-derived BMECs^{22,23}. Additionally, low standard errors ($10.4\text{--}339 \Omega \times \text{cm}^2$) were observed across technical and biological replicates, demonstrating consistent TEER function across HC, BD or SZ cell lines (Supplementary Fig. 1a). TEER experiments were performed for each of the 12 cell lines, with an average of three biological replicates and across six days. The results demonstrate similar individual variation in TEER function across all patients in the study with average standard errors of $45.8 \Omega \times \text{cm}^2$, $75.6 \Omega \times \text{cm}^2$, and $77.9 \Omega \times \text{cm}^2$ for HC, BD, and SZ, respectively (Supplementary Fig. 1b). This demonstrates that BMECs across all groups have a maximal peak TEER on day 2, followed by sustained TEER elevations over 6 days, as well as minimal variation within and between experimental conditions, supporting the use of TEER as a primary functional outcome for measuring BBB function.

BBB barrier dysfunction in SZ. We sought to determine whether BMECs could recapitulate BBB disruption described in human postmortem and *in vivo* studies^{7,16,24}. We found robust evidence of BBB disruption in SZ and BD BMECs, with significant reduction in peak TEER values primarily when compared to HC BMECs (Fig. 1c). Studies examining BBB function in similar iPSC derived BMEC usually collect TEER values 2-3 days after subculturing^{20,22,23}. Thus, we compared TEER values on day 2 and found a significant reduction in the SZ BMECs compared to the HC BMECs. There was no difference identified between the BD group and SZ or HC groups (Fig. 1d). Since the BBB is also characterized by impermeability^{20,21}, we performed a paracellular permeability assay to determine BBB leakiness using fluorescently labeled molecules ranging from 0.1kDa to 80kDa (Fig. 1e)²⁵. SZ BMECs showed greater permeability to 0.1kDa and 10kDa molecules when compared to HC or BD BMECs (Fig. 1f). Interestingly, SZ BMECs showed higher permeability to 70kDa and 80kDa molecules compared to BD but not to HC

BMECs (Fig. 1f). These results demonstrate that BMECs from SZ patients result in poorer BBB integrity as evidenced by lower TEER and higher permeability compared to HC or BD BMECs.

Paracellular transport is controlled by a complex arrangement of tight junction proteins in BMECs including CLDN5, OCLN, and TJP1 that are vital for maintaining BBB integrity³. Several studies have identified paracellular deficits in SZ, particularly as it relates to CLDN5³. CLDN5 is deleted in 22q11 deletion syndrome (22qDS) which carries a 30% risk for developing SZ²⁶ and 60% risk for developing BD²⁷. Also, BMECs from patients with 22qDS + SZ showed impaired BBB integrity²³. In animal models CLDN5 knockdown results in increased BBB leakage^{6,26}, and BBB leakage has also been reported in human postmortem samples of individuals with SZ⁸ or 22qDS + SZ²³. We performed RT-qPCR in BMECs and found that CLDN5 mRNA levels were significantly lower in SZ and BD BMECs compared to HC BMECs, but there was no difference in mRNA levels of OCLN or TJP1 (Fig. 1g). Protein levels of CLDN5 and OCLN, but not TJP1, were reduced in SZ BMECs when compared to HC and BD BMECs (Fig. 1h). To determine the cellular attributes that accompany the decreased levels of these tight junction proteins in SZ, we examined morphological features relevant to BBB integrity by quantifying the length (contiguous junctional area), width (health of junctional area), and total intensity of OCLN and CLDN5 in the BMECs²⁰. Both the intensity and width of CLDN5 were significantly reduced in SZ compared to HC (Fig. 1i and 1j), showing a correlation between reduced CLDN5 expression and aberrant BBB-related morphological attributes in SZ. Additionally, OCLN width and length were reduced in SZ and BD BMECs compared to HC BMECs (Fig. 1i and 1j). These results show that reduced CLDN5 results in morphologies consistent with impaired tight junctions in the SZ BMECs.

BBB deficit subtypes in psychosis. Subtypes of psychosis defined by biosignatures have been pivotal in the pathophysiological understanding of a disorder that is solely defined by clinical syndromes^{28,29}. We had previously identified a high inflammatory subtype of psychosis in chronic and first episode psychosis patients using peripheral markers of inflammation and vascular disruption. Studies of postmortem tissue^{7,30}, cerebral spinal fluid³¹, peripheral markers³², and in vivo brain imaging^{16,33} suggest that BBB disruption exists in SZ and/or BD and that it can be identified in a subgroup of individuals with psychosis. In order to identify patient subgroups with significantly higher levels of BBB (peak TEER) dysfunction, an outlier analysis was performed (based on the mean minus two standard deviations) in HC individuals and used to stratify the SZ and BD groups. Using this method, three of the eight psychosis patients were identified as outliers (peak TEER < 1000 $\Omega \times \text{cm}^2$). The outlier group consisted of 2 SZ and 1 BD patients making up 37.5% of the sample and were labelled the “BBB-deficit” group. The psychosis group with better BBB function included 2 SZ and 3 BD patients making up 62.5% of the sample and were labelled “BBB-nondeficit” group. The BBB-deficit group exhibited more extensive BBB disruption as indicated by extremely low TEER values across 6 days of culture (Supplementary Fig. 1b; Fig. 2a) and an absence of day 2 TEER peak (Fig. 2b) compared to HC and BBB-nondeficit groups. The BBB-deficit group also had greater permeability to 0.1kDa, 4kDa, and 10kDa compared to HC individuals and BBB-nondeficit patients (Fig. 2c). Furthermore, analysis of tight junction proteins revealed a reduction in CLDN5 mRNA expression in both the BBB-deficit and the BBB-nondeficit groups (Fig. 2d and e), but there was no difference at the mRNA level for OCLN or TJP1 and no notable change in tight junction protein levels across the groups. However, using immunostaining techniques, we found that CLDN5 and OCLN intensity, width, and length were significantly reduced in BBB-deficit BMECs compared to HC BMECs (Fig. 2f and g). Compared to the BBB-nondeficit group, BBB-deficit BMECs had lower CLDN5 intensity and width, as well as lower OCLN intensity (Fig. 2f and g). Lastly, the BBB-nondeficit BMECs showed lower CLDN5 and OCLN width when compared to HC BMECs (Fig. 2f and g). Similar to existing clinical studies^{7,15–17,30,31}, our results demonstrate there is a BBB-deficit subgroup in

psychotic disorders characterized by more extensive BBB disruption as evidenced by decreased TEER, increased permeability, lower CLDN5 intensity, and aberrant tight junctional morphology.

MMP1 impairs BBB function. Studies suggest that peripheral inflammation may be causally related to psychosis³⁵, but the source of inflammation is currently unknown. BMECs are a potential source of inflammation in psychotic disorders given their role in immune and inflammatory pathways^{3,32,36,37}. In order to test the hypothesis that BMECs secrete factors that can alter BBB function, we performed cross-culture experiments to determine: 1) whether secreted factors from HC BMECs can improve BBB function (i.e., improve TEER) in BBB-deficit BMECs and 2) whether secreted factors from BBB-deficit BMECs can impair BBB function in HC BMECs. For the first scenario, HC BMECs were seeded in the bottom chamber of a transwell plate and BBB-deficit or -nondeficit BMECs were seeded in the upper chamber (Fig. 3a). Using this experimental design, we observed that exposure to media from HC BMECs improved peak TEER values in 2 out of the 3 BBB-deficit lines (Fig. 3b) and this effect was sustained over 6 days (Supplementary Fig. 2a). In the second scenario (HC BMECs seeded on top and BBB-deficit or non-deficit BMECs seeded on bottom), we observed a decrease in TEER values for the HC BMECs on day 2 (Fig. 3a) and a delayed peak on day 3 with a decreasing trend over time (Supplementary Fig. 2a). These data provide evidence that BMECs secrete factors that impact BBB function as evidenced by media from HC BMECs rescuing BBB function in BBB-deficit BMECs while media from BBB-deficit BMECs impaired BBB function in HC BMECs.

BMECs are known to release several cytokines, chemokines, and growth factors in response to a variety of environmental stimuli, and as part of BBB homeostasis³⁸⁻⁴¹. Prior studies of peripheral inflammation in psychosis have linked inflammation with the development of psychosis³⁵. We performed a targeted proteomic screen of 48 markers related to inflammation^{39,41} in BMEC-conditioned media. Of 48 markers, only 27 proteins had sufficient data for analysis that passed quality control metrics (Supplementary Table 2). CXCL12 levels were increased in conditioned media from SZ BMECs when compared to HC and BD individuals (Supplementary Fig. 2b and c). Conditioned media from BBB-deficit BMECs showed increased CXCL8 and CCL2 levels when compared to conditioned media from HC and BBB-nondeficit BMECs, while CCL19 and MMP1 levels were higher in the BBB-deficit group compared to the BBB-nondeficit group (Fig. 3c and d). Inflammatory marker topology showed overall greater inflammatory/chemokine activation in BMECs in SZ and BD when compared to HC (Supplementary Fig. 2d). To determine whether CCL19, CXCL8, CCL2 or MMP1 were related to BBB phenotypes (TEER, cadaverine, dextran, transferrin, CLDN5 expression, CLDN5 protein level), we performed a correlational analysis which demonstrated that higher levels of MMP1 were associated with reduced TEER ($r=-0.79$, $p \leq 0.01$) and CLDN5 protein levels ($r=-0.61$, $p \leq 0.05$), as well as increased permeability to cadaverine 0.1 kDa ($r=0.64$, $p \leq 0.05$) across the whole study sample (Fig. 3e). In addition, higher levels of CXCL8 and CCL2 were associated with reduced TEER and higher CCL19 level was associated with increased permeability to dextran 10 kDa (Fig. 3e). These data suggest that MMP1 may be responsible for the BBB deficits observed in a subgroup of patients with SZ or BD.

In BMECs, matrix metalloproteinase (MMP) proteins regulate vascular remodeling through degradation of the extracellular matrix³⁸. MMP1 cleaves extracellular components such as collagen, gelatin, and laminin⁴². At the same time, MMP1 can also activate other MMPs such as MMP2 and MMP9, which in concert can reduce expression levels of tight junction proteins such as TJP1, OCLN, or CLDN5⁴³. Moreover, TNF α and interleukin 6 (IL-6) are pro-inflammatory cytokines that have been directly linked to increased MMP1 levels and can synergistically modulate MMP1 expression⁴⁴. To determine whether MMP1 was differentially expressed in BMECs between groups, we quantified mRNA levels of MMP1 and found no changes in MMP1 expression between diagnostic (Supplementary Fig. 2e) or BBB-deficit

categories (Fig. 3f). MMP1 protein levels were increased in the SZ (Supplementary Fig. 2e) and BBB-deficit group (Fig. 3g) compared to HC or BD groups, but this difference was not significant. Since MMP1 is initially released from the cytosol in an inactive form and is subsequently activated by proteolytic cleavage⁴⁵, we quantified MMP1 activity in the BMEC culture media. MMP1 activity was significantly higher in the BBB-deficit group compared to HC (Fig. 3h), but there was no significant difference between the diagnostic categories (Supplementary Fig. 2f). Furthermore, while increased MMP1 activity was associated with reduced TEER ($r=-0.477$), CLDN5 expression ($r=-0.349$) and CLDN5 protein levels ($r=-0.359$), the effect was not significant (data not shown). These data suggest that factors affecting MMP1 release and activation in the BBB-deficit group may be responsible for the aberrant BBB function.

Inhibition of MMP1 restores BBB function. Since increased MMP1 activity was observed in the BBB-deficit groups, we sought to test whether inhibition of MMP1 can restore BBB function in the BBB-deficit group. Piroxicam is a non-steroidal anti-inflammatory drug that broadly inhibits several MMPs but is highly selective for MMP1 compared to other MMPs⁴⁶. Infliximab is an immunosuppressive drug that specifically targets TNF α ⁴⁷ and has been shown to decrease MMP1 levels, endothelial cell necroptosis, and to prevent BBB leakage⁴⁸. BMECs from BBB-deficit and HC lines treated with infliximab or piroxicam demonstrated a persistent improvement in TEER values over 6 days, but no such effect was seen in BMECs from the BBB-nondeficit groups (Fig. 4a). BMECs from the BBB-deficit group demonstrated an improvement in peak TEER when treated with infliximab (170 %) or piroxicam (166 %) when compared to untreated conditions (Fig. 4b). HC BMECs also demonstrated an increase in peak TEER with piroxicam (145%), but not with infliximab (Fig. 4b). In order to confirm that these drugs are engaging the target in BBB-deficit BMECs, we measured MMP1 activity in the presence or absence of infliximab or piroxicam. We found a trend towards reduction in MMP1 activity when BBB-deficit BMEC were treated with piroxicam (78%) as opposed to infliximab (55%) though the difference was not statistically significant (Fig. 4c). Additionally, both drugs increased CLDN5 gene expression, but had no effect on CLDN5 intensity in BBB-deficit BMECs (Fig. 4d and Supplementary Fig. 3). Taken together, these data suggest that inhibition of TNF α or MMP1 leads to specific effects and modulates BBB-related features in BBB-deficit lines, but not in BBB-nondeficit lines.

Discussion

In this study, we utilized *in vitro* models of BBB function using iPSC-derived BMECs from individuals with SZ, BD and HC to demonstrate that BBB properties (integrity and permeability) and molecular markers (CLDN5 and OCLN) are most impaired in BMECs from SZ patients compared to BMECs from HC individuals, with BMECs from BD patients showing an intermediate phenotype. Notably, we identified a subgroup of patients with SZ or BD that demonstrated substantial impairments in BBB function. BMECs from this BBB-deficit group showed reduced barrier integrity and increased permeability as well as decreased CLDN5 and OCLN levels compared to BMECs from HC and BBB-nondeficit individuals. Cross-culture experiments suggested that factors released by BBB-deficit BMECs can impair BBB function in HC BMECs, and that the secretome from HC BMECs can restore BBB function in BBB-deficit BMECs. Proteomic screening of BMEC-conditioned medium showed greater levels of active MMP1 in BBB-deficit lines. Higher level of MMP1 activity was subsequently found to be associated with reduced barrier integrity, increased permeability, and decreased CLDN5 protein levels. Pharmacologic inhibition of MMP1 with piroxicam and inhibition of TNF α , an upstream regulator of MMP1, with infliximab, restored BBB function in BBB-deficit BMECs.

Our experimental data using human iPSC-derived BMECs offer direct evidence for intrinsic deficits in BMECs in SZ that potentially explain neuroanatomical changes described in psychotic disorders. Ultrastructural changes in brain endothelial cells have been described in aborted embryos from mothers

with SZ⁴⁹, as well as in postmortem tissue from SZ patients⁵⁰, including increased endothelial surface area, cytoplasmic vacuolization, granulation, thickening of the basal lamina, and plasma membrane disruption. Additionally, transcriptional profiling of brain endothelial cells in SZ patients have identified differences in expression of genes related to immune, inflammation, angiogenesis, and cell adhesion^{8,51}. While these studies are of major importance and had implicated BMEC dysfunction in SZ, they did not delineate the specific effects of brain endothelial cells and instead examined the brain as a whole. Some of these studies have also described changes related to astrocyte, microglia, oligodendrocyte, or neurons, making it difficult to interpret the cellular source of BBB dysfunction in SZ pathogenesis^{50,52,53}. In an effort to elucidate this complex interplay between the different cell types, a study using human iPSC-derived neural stem cells demonstrated that the secretion of several angiogenic factors from SZ patients reduced endothelial cell migration and angiogenesis, suggesting a dysregulated cross-talk between neural stem cells and endothelial cells during neurovascular development in SZ⁵⁴. Conversely, two studies examining human iPSC-derived BMECs in patients with 22qDS and SZ demonstrated showed the presence of impaired barrier integrity^{23,55}, a phenotype that had been demonstrated in animal models of 22qDS²⁶, suggesting a primary role of BMECs in BBB dysfunction in SZ pathogenesis.

Our CLDN5 results are consistent recapitulate findings of decreased CLDN5 levels in postmortem studies of patient with SZ⁸. Higher levels of IgA against CLDN5 have been described in the blood of patients with SZ, suggesting the presence of paracellular and BMEC disruption⁵⁶. Furthermore, 22qDS syndrome, which includes haploinsufficiency for CLDN5, is a well-established risk factor for the development of SZ and BD²⁶. A genetic variant of CLDN5 (rs10314) has been associated with a 50% reduction in BMEC-specific CLDN5 expression and is weakly associated with SZ or BD risk^{26,57-62}. Mouse models of CLDN5 knockdown show the presence of learning and memory impairments, sensorimotor gating deficits, and *in vivo* BBB leakage, further supporting the notion that CLDN5 plays a role in BBB function and SZ phenotypes²⁶. Rodent studies also show a dose-dependent increase in CLDN5 levels with exposure to lithium or antipsychotics, suggesting that the therapeutic effects of these drugs may involve their effects on the neurovasculature²⁶.

The identification of a BBB subtype (37.5%) in this study is consistent with prior evidence in human studies that had identified subgroups of patients with elevated peripheral inflammation and/or BBB disruption. There are several studies, including our own, which show the presence of peripheral inflammation in 30-50% of patients with chronic psychosis^{17,63} and in first-episode schizophrenia¹⁵ which was associated with *in vivo* evidence of BBB disruption^{15,17}. Additionally, studies show blood-cerebrospinal fluid barrier (BCSFB) disruption in 22-41% of patients with SZ and/or BD disorder^{31,64}. This work was further expanded by our group and others demonstrating enlarged choroid plexus volume, a key BCSFB structure, in chronic or first-episode psychosis patients, and that greater choroid plexus volume was associated with greater IL-6 levels⁶⁵ or allostatic load⁶⁶, suggesting a complex interplay between systemic inflammation and BBB breakdown. BBB subtypes have also been described using lymphocyte (41%)⁶⁷ and perivascular macrophage (40%)⁷ density in patients with SZ, the latter of which was found in a subgroup of patients of high cortical inflammation. While these studies have expanded our understanding of the role of inflammatory-immune-BBB nexus in SZ and BD, they do not point to specific cellular mechanisms priming this response.

In addition to demonstrating that intrinsic properties BMECs may be the source of BBB deficits in a subgroup of patients with SZ or BD, we also show evidence that MMP1 is involved in mediating BBB disruption in these lines. MMP1 has not been previously studied or implicated in SZ or BD. However, MMP1 has been linked to MMP9 activation and MMP1 can be regulated by TNF α , both of which have been shown to play a role in SZ and/or BD pathogenesis⁶⁸⁻⁷⁰. While it is well established that MMP1 can

reduce expression of CLDN5⁴³, which could provide a mechanistic link for understanding BBB in BD and SZ, this effect was not observed in our study. With this mechanism in mind, we examined the effects of infliximab (TNF α biologic) and piroxicam (MMP1 inhibitor) which were found to partially rescue BBB function in BBB-deficit BMECs. While infliximab has not been used for the adjunctive treatment of SZ or BD, a recent review examining the association between antibody immunotherapy for immune/inflammatory disorders and psychosis, found that 4 out of 8 antibody therapies suppressing TNF α (including infliximab) correlated with a decreased risk of psychosis⁷¹. However, cases describing acute psychosis after the administration of TNF α antibody therapy have also been reported^{72,73}. Piroxicam has not been used as an adjunctive treatment for SZ or BD, but studies of non-selective COX inhibitor (aspirin) and selective COX-2 inhibitor (celecoxib) in SZ^{74,75} have reported symptomatic improvement in SZ patients, with the greatest benefit during a first episode of psychosis⁷⁶. These studies demonstrate the potential clinical importance of using drugs that target TNF α /MMP1 for the treatment of patients with SZ or BD. Our study provides a scientific basis for the hypothesis that patients in the BBB-deficit subgroup may be more likely to respond to therapies targeted at TNF α or MMP1.

Taken together, our work shows the existence of BBB-deficit subgroup in SZ and BD and offers a mechanistic link between inherent BMEC dysfunction and peripheral inflammatory activation. Our findings provide a strong rationale using specific pharmacological or biological interventions targeting TNF α or MMP1 mediated BBB disruption as potential therapeutic approaches.

Data availability

Additional data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Code availability

Any code used to support the findings of this study is available from the corresponding authors upon reasonable request.

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Author contributions

P.L. conceived the idea. R.K. assisted with study design. P.L. developed/adapted the BMEC protocol and collected pilot data for the study. S.P. performed and analyzed TEER experiment, permeability, RT-qPCR, immunocytochemistry, co-culturing, and MMP1 inhibition assays. P.L. and S.P. analyzed Olink proteomic assays. S.P. and S.S. performed and analyzed western blots. D.B. contributed Matlab code and processed ICC images for tight junction proteins quantification. P.L. and S.P. wrote the first draft of this manuscript. P.L., S.P., S.S., D.B., and R.K. edited and revised the manuscript.

Competing interests

None of the other authors declare any competing interests.

Methods

Cell lines. Human induced pluripotent stem cells (iPSC) were generated from fibroblasts using mRNA-based genetic reprogramming (Cellular Reprogramming Inc.) from age- and sex-matched chronic schizophrenia (n=4), psychotic bipolar I disorder (n=4), and healthy control (n=4) patients, as approved by the Institutional Review Boards (IRB)⁷⁷⁻⁷⁹. Two male and two female patient lines were included for each group. All human iPSCs were maintained in xeno-free conditions in NutriStem medium (ReproCell, 01-0005), expressed appropriate pluripotency characteristics, and were found to be karyotypically normal using the KaryoStat Assay⁷⁷⁻⁷⁹. Information on these cell lines is provided in Supplementary Table 1.

Differentiation of iPSCs to BMECs. iPSCs were differentiated into BMEC-like cells following a protocol we have described earlier^{19,20}. In brief, iPSCs were washed with DPBS and dissociated using Accutase for 5 min at 37°C. The cells were collected via centrifugation and seeded onto Geltrex-coated (ThermoFisher Scientific, A1413202) 6-well plates (Corning, 353046) at a density of 146,000/cm² in NutriStem medium supplemented with 10 μM rho-associated kinase (ROCK) inhibitor Y-27632. After 24 hours (day 0), NutriStem medium was changed to E6 medium, which was changed every day for 4 days. On day 4, E6 medium was replaced with human endothelial serum-free medium (hESFMS) (ThermoFisher Scientific, 11111044) supplemented with B27 supplement (1:200 dilution), 20 ng/mL basic fibroblast growth factor (bFGF), and 10 μM retinoic acid (RA). On day 6, cells were washed with DPBS and treated with Accutase for 30-45 min at 37°C. These cells were collected by centrifugation and sub-cultured onto plates coated with a mixture of 400 μg/mL collagen IV (COL4, Sigma, C5533-5MG) and 100 μg/mL fibronectin I (FN1, ThermoFisher Scientific, 33016015). Cells were plated using a ratio of 1 well of a 6-well plate to 3 wells of a 12-well filtered transwell plate (0.4 μm pores, Fisher Scientific, 07-200-161) and 6 wells of a 24-wells plastic-bottom plate (Corning, 353047). On day 7, medium was changed to hESFM with B27 supplement without bFGF and RA.

TEER measurement and analysis. On day 7, TEER was measured using STX2 chopstick electrodes and an EVOM2 voltohmmeter (World Precision Instruments) every day for six days. Raw values were corrected for the resistance of an empty transwell filter plate. TEER measurements were performed in triplicate for each well and for each condition. The average value from the blank wells (i.e. empty wells without any cells) were subtracted from the raw values for each condition. The subtracted values (in Ω) were multiplied by 1.12 cm², the surface area of the transwell inserts in the 12-transwell plate and the transformed values were used for the analyses. To identify cell lines with higher level of BBB disruption (BBB-deficit group), we first calculated the mean (3037 Ω x cm²) and the standard deviation (1051 Ω x cm²) of peak TEER values in the HC group (n=4). Any peak TEER value that was more than two standard deviations below the mean (i.e. any value <1000 Ω x cm²) was considered as exhibiting higher level of BBB disruption and was categorized into the BBB-deficit group.

Permeability studies. Cells were plated using a ratio of 1 well of a 6-well plate to 3 wells of a 12-well filtered transwell plate (0.4 μm pores, Fisher Scientific, 07-200-161). After 48-72 hours of subculturing (day 8-9), permeability assays were conducted using 4 kDa, 10 kDa and 70 kDa dextrans labeled with fluorescein isothiocyanate (Sigma, 46944, FD10S, 46945), transferrin (Thermo Fisher Scientific, T-13342), or Alexa Fluor 555 cadaverine (Thermo Fisher Scientific, a30677) in hESFM supplemented with B27 supplement (1:200 dilution). 1.5 mL of fresh medium was added to the bottom of the transwell and 0.5

mL of dye and medium were added to the top. The wells were then incubated at 37 °C for 1 hour. Medium from the bottom section of the transwell chamber was collected and analyzed using a 2103 EnVision Multilabel Reader (Perkin Elmer). Averaged values from a blank well, which did not contain any cells, were subtracted from the raw values of the triplicate wells for each condition. The subtracted values (in relative fluorescent unit or RFU) were used for the analyses.

mRNA and protein analysis. RNA was extracted from BMECs that had been subcultured for 48-72 hours (day 8-9) using trizol to lyse the cells and the RNeasy Mini Kit (Qiagen, 74104) for RNA purification. For RT-PCR, 500ng of RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814). SYBR green (Mangobio, 08-25-00020) assay was run on Roche light cycler 480II. In 364-well plates, each well contained 2 μ L of Evagreen qPCR Mix, 1 μ L forward/reverse primers mix, 5 μ L RNase free water, and 2 μ L cDNA. For each gene of interest, two technical replicates and three independent biological replicates per cell line were tested. Each gene of interest was normalized to a reference gene, GAPDH. Analysis was performed on Excel spreadsheet and graphs prepared with GraphPad version 8.0. Primers were obtained from KiCqStart™ Primers (Millipore Sigma) as listed in Supplementary Table 3. For protein extraction, cells were washed with DBPS and lysed with RIPA buffer. Protein concentration was measured with a BCA assay. 10 μ g of protein of each sample was run on a Criterion TGX Precast gel 4–20% (BIO-RAD, 5671094) at 120 V for 1.5 hours. Gels were transferred to a Immobilon-P Transfer Membrane (Millipore IPVH00010, pore size: 0.45 μ m, PVDF) and blocked in Odyssey Blocking Buffer (Li-Cor, 927-40,000) for 1 hour at room temperature. Membrane was probed overnight with primary antibodies at 4 °C (Supplementary Table 4), followed by washes with 1x TBST, then incubated with secondary antibody using donkey anti-rabbit (Li-Cor 1:10,000, IRDye 680, 925-68073, lot #C70601-01) or anti-mouse (Li-Cor 1:10,000, IRDye 800, 925-32212, lot #C70502-03) for 1 hour at room temperature. Images were analyzed using an Odyssey Clx Imaging System (Li-Cor Biosciences) and quantified using Image J.

Immunocytochemistry (ICC). After 48 -72 hours of sub-culturing (day 8-9) on 24-wells plastic-bottom plates (Corning, 353047), cells were washed twice with DPBS. Cells were then fixed with 4% paraformaldehyde (PFA) and pre-blocked in DPBS with 5% donkey serum and 0.2% Triton X-100 for 1 hour at room temperature. Fixed cells were incubated with primary antibodies: rabbit anti-human-TJP1 (1:200), mouse anti-human-CLDN5 (1:200), rabbit anti-human-OCLN (1:200) in DPBS containing 5% donkey serum overnight at 4°C. Cells were washed five times with a minimum of 5 minutes per wash using DPBS. Then, they were incubated in secondary antibodies for 1 hour at room temperature. Primary and secondary antibodies are listed in Supplementary Table 4. Cells were then incubated in Hoechst 33342 solution (1:1000), for approximately 1 min before visualizing on IN Cell Analyzer 6000 (GE Healthcare Life Sciences) using fluorescence microscopy settings.

Tight junction protein expression quantification. Raw ICC images were exported in 16-bit TIF format and analyzed using a custom MATLAB script adapted from Neal et al. 2019²⁰. Six different fields of view were selected for each cell line per condition. Fields of view were selected based on the following criteria: field of few had cells covering the entire field of view, low background noise, and images that were clear and not blurry. In the junction (dsRed and FITC) images, individual pixels were compared to the average pixel intensity within a 10-pixel square radius, resulting in binarized junction images. The binarized images were used to extract tight junctional length, width, and total intensity measures for each tight junction protein (CLDN5 and OCLN) in all cell lines and each field of view. The total number of junction-positive pixels was divided by the number of Hoechst-positive pixels in each field of view as a confluency-normalized metric of junctional density. Individual nuclei were detected using a custom FIJI script that identified DAPI-positive pixels within each image. For this process, raw images were exported

in a 16-bit TIF format and first converted to 8-bit images. A Kuwahara filter was used with a sampling window width of 11 pixels for smoothing and noise reduction. Images were auto-thresholded using Li's method^{80,81} and converted to a binary mask. A watershed algorithm was used to segment out nuclei, and the number of nuclei were then extracted. Normalized values from each field were averaged and appropriate statistical tests performed to determine differences between each diagnostic group.

Cross-culture assay. Cells were plated using a ratio of 1 well of a 6-well plate to 3 wells of a 12-wells filtered transwell plate (0.4 μ m pores, Fisher Scientific, 07-200-161). For the first assay, a HC cell line was seeded on the top chamber of the transwell while a BBB-deficit or BBB-nondeficit cell line was seeded on the bottom chamber of the transwell (Figure 3a). In a complementary assay, these conditions were reversed. TEER measurements were collected in triplicate for each well and for each condition and analysis of the TEER measurements were performed as described above.

Olink analysis of BMEC conditioned medium. Conditioned medium was collected from each cell line after 48-72 hours of subculturing and analyzed by Olink Proteomics, Inc. (Watertown, MA) using the Target 48 cytokine platform. Samples were assigned a randomized 4-digit ID number and the assays were run by technicians without any knowledge of the clinical status of the samples. Processing and data quality control were performed by Olink Proteomics. All data were delivered as excel spreadsheet along with the certificate of analysis. Further information on how Olink determines limits of detection (LOD) and sample normalizations can be found online (<https://www.olink.com/>). Markers with more than 25% of quantity not sufficient data values were excluded from the analysis. Biomarkers with <25% of quantity not sufficient data were imputed to the mean of the samples (n=12) for each respective marker. Among the 48 markers, 27 were detected and selected for further analysis. The full list of biomarkers can be found in Supplementary Table 2. Raw concentration values (pg/mL) were normalized to the average concentration in the HC group. Statistical tests were performed as described in the figure legends.

Active MMP1 Fluorokine E assay. Conditioned medium collected from each cell line were examined for levels of active MMP1 protein using standard protocols for the Human Active MMP1 Fluorokine E Kit (R&D Systems, F1M00). Assays was done in technical duplicates and a standard curve was generated. Concentration of active MMP1 protein in the conditioned medium was calculated based on the standard curve. Any value with a negative concentration was imputed to zero ng/ μ L (n=7).

MMP1 inhibition assay. Infliximab (Sigma Aldrich, CAS#170277-31-3) and piroxicam (Sigma Aldrich, CAS# 36322-90-4) were used as TNF α and MMP1 inhibitors, respectively. Stock solution of infliximab was diluted with hESFM to 300ng/mL working concentration and piroxicam was diluted to 10 μ M working concentration. Technical triplicates of the MMP1 inhibition assay were conducted for each condition in three independent biological replicates. Cells were treated with each inhibitor for 24 hours during subculturing before being removed and replenished with fresh medium. TEER measurements, immunocytochemistry, mRNA and protein analysis were conducted as described above.

Statistical Analysis.

All representative graphs were analyzed and created in R studio and GraphPad Prism 8. Any statistical analysis such as One-Way or Two-Way ANOVA and post-hoc Tukey test are reported in the figure legends. Correction for multiple comparisons were included when needed. All reported p-values are two-sided. Outlier removal was applied when needed using the ROUT method in which Q was set to 0.5% (i.e. at least 99.5% are actual outliers). Univariate analysis of variance via Kruskal-Wallis rank sum test was used to identify any moderating effects of sex, iPSCs passage numbers, and cell viabilities (%)

on peak TEER measurements across all three biological replicates. Reported chi-squares and p-values from this analysis can be found in the Supplementary Table 5. Bivariate correlations were performed between peripheral markers and BBB phenotypes using Spearman correlations.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Figures

Figure 1

Human iPSC-derived BMECs from SZ patients demonstrate aberrant barrier function in a BBB model. a) Schematic for differentiating human iPSCs into BMECs over an 8-day period. **b)** Experimental set-up for TEER measurements. **c)** Average TEER values collected over 6 days for healthy control (HC, n=4), bipolar

disorder (BD, n=4) and schizophrenia (SZ, n=4) cell lines. Significance was determined by Two-Way ANOVA and post-hoc Tukey multiple comparison test (*p=0.011 comparing SZ to HC on day 2, *p=0.0405 comparing SZ to HC on day 3). **d)** Average peak (day 2) TEER values for HC, BD, and SZ BMECs. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test (**p=0.0094, comparing SZ to HC). TEER values were collected for three biological replicates each and shown as mean $\pm$ s.e.m. **e)** Schematic of permeability assay using fluorescently labeled substrates of varying molecular weight. **f)** Permeability of fluorescently labeled substrates across a confluent monolayer of BMECs from HC (n=4), BD (n=4), and SZ (n=4) patients. Permeability assays were conducted at the time of peak TEER values (day 2, Fig.1c). Permeability readings were performed in triplicate using three biological replicates. Permeability values (RFU) were reported using three biological replicates with mean $\pm$ s.e.m. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison tests. Cadaverine 0.1kDa **p=0.0022 comparing SZ to HC and **p=0.0029 comparing SZ to BD; dextran 4kDa *p=0.0268 comparing SZ to BD; dextran 10kDa *p=0.0358 comparing SZ to HC and *p=0.0338 comparing SZ to BD; dextran 70kDa *p=0.0308 comparing SZ to BD; transferrin 80kDa **p=0.0087 comparing BD to HC and *p=0.0448 comparing SZ to BD. 2 outliers were removed using the ROUT method (q= 0.5). **g)** Normalized mRNA levels in BMECs for TJP1, OCLN, and CLDN5 for HC, BD, and SZ BMECs. mRNA expression for each gene was normalized to the housekeeping gene GAPDH. Each normalized value was then calibrated to the average mRNA expression from the HC group (n=4) using the $\Delta\Delta C_t$ method; RT-qPCR was performed in technical duplicates; each dot represents a biological replicate. Mean $\pm$ s.e.m is shown for each group. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test (***p=0.0008 comparing CLDN5 mRNA expression in BD to HC, ***p=0.0001 comparing CLDN5 mRNA expression in SZ to HC). 7 outliers were removed. **h)** Normalized protein levels from HC, BD, and SZ groups; each dot represents a biological replicate. Means $\pm$ s.e.m is indicated for each group. Band intensities from western blots were quantified using Fiji by Image J and normalized to a reference protein (GAPDH). Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test (*p= 0.0405 comparing CLDN5 protein level in SZ to HC, **p= 0.007 comparing OLCN protein level in SZ to HC and ****p<0.0001 comparing OCLN protein level in SZ to BD). 4 outliers were removed. **i)** Representative images are shown of immunostaining for CLDN5 (green), OCLN (red), and nuclei (blue) in HC, BD, and SZ patients. Images were processed using Matlab, assessing for fluorescent intensity; scale bar is 50 μ m. **j)** Normalized total fluorescent intensity, width intensity, and length intensity for OCLN and CLDN5 in BMECs from HC, BD, and SZ lines. Six fields of view for each subject were used for quantification. Raw intensity values were normalized to the total number of nuclei in each respective field of view. Each dot represents one field of view. Mean $\pm$ s.e.m is shown for each group. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test. ****p< 0.0001 comparing CLDN5 width in SZ to HC, **p = 0.0027 comparing CLDN5 width in BD to HC, **p= 0.0012 comparing CLDN5 total intensity in SZ to HC, *p= 0.0337 comparing OCLN width in BD to HC, **p=0.001 comparing OCLN width in SZ to HC, **p=0.0082 comparing OCLN length in BD to HC, **p=0.0071 comparing OCLN length in SZ to HC. All reported p-values are two-sided.

Figure 2

A psychosis subgroup shows extensive BBB leakage. a) Average TEER across 6 days with significant differences observed between HC (n=4), BBB-nondeficit (n=5) and BBB-deficit group (n=3). BBB-nondeficit group consists of BD (n=3) or SZ (n=2) patients with TEER values greater than $1000 \Omega \times \text{cm}^2$; BBB-deficit group consists of BD (n=1) or SZ (n=2) patients with TEER of less than $1000 \Omega \times \text{cm}^2$. Significance was determined by Two-Way ANOVA and post-hoc Tukey multiple comparison test (**p=0.0097 (day 1),

**** $p < 0.0001$ (day 2), *** $p = 0.0009$ (day 3), ** $p = 0.0053$ (day 4), ** $p = 0.0068$ (day 5), * $p = 0.0116$ (day 6) comparing BBB-deficit group to HC; and ## $p = 0.0012$ (day 1), #### $p < 0.0001$ (day 2), #### $p < 0.0001$ (day 3), ## $p = 0.0020$ (day 4), ### $p = 0.0009$ (day 5), ### $p = 0.0008$ (day 6) comparing BBB-deficit group to BBB-nondeficit group). **b)** Average peak (day 2) TEER values for HC, BBB-nondeficit, and BBB-deficit groups. Significance was determined by One-Way and post-hoc Tukey multiple comparison test (**** $p < 0.0001$ comparing BBB-deficit to HC or BBB-nondeficit groups). TEER values were collected using three biological replicates and shown as mean $\pm$ s.e.m. **c)** Permeability of fluorescently labeled substrates for HC, BBB-nondeficit, and BBB-deficit groups. Permeability values (RFU) were collected using three biological replicates and shown as mean $\pm$ s.e.m. Significance was determined by One-Way and post-hoc Tukey multiple comparison test. Cadaverine 0.1kDa * $p = 0.0107$ comparing BBB-deficit to HC and * $p = 0.0249$ comparing BBB-deficit to BBB-nondeficit; dextran 4kDa ** $p = 0.0022$ comparing BBB-deficit to HC and * $p = 0.0124$ comparing BBB-deficit to BBB-nondeficit; dextran 10kDa **** $p < 0.0001$ comparing BBB-deficit to HC and *** $p = 0.0001$ comparing BBB-deficit to BBB-nondeficit; transferrin 80kDa * $p = 0.0418$ comparing BBB-nondeficit to HC). **d)** Normalized mRNA levels for CLDN5, OCLN and TJP1 from HC, BBB-nondeficit, and BBB-deficit BMECs; mRNA expression for each gene was normalized to the housekeeping gene GAPDH. Each normalized value was then calibrated to the average mRNA expression from the HC group ($n = 4$) using the $\Delta\Delta C_t$ method; RT-qPCR was performed in technical duplicates and each dot represents a biological replicate. Mean $\pm$ s.e.m is shown for each group. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test (*** $p = 0.0007$ comparing CLDN5 mRNA expression in BBB-deficit to HC, *** $p = 0.0003$ comparing CLDN5 mRNA expression in BBB-nondeficit to HC). 5 outliers were removed using the ROUT method ($q = 0.5$). **e)** Normalized levels of CLDN5, OCLN and TJP1 protein in HC, BBB-nondeficit, and BBB-deficit BMECs; each dot represents a biological replicate. Mean $\pm$ s.e.m is shown for each group. Band intensities were quantified using Fiji by Image J and were normalized to GAPDH. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test and no significant differences were observed between any of these groups. 3 outliers were removed. **f)** Representative images of immunostaining for CLDN5 (green), OCLN (red), and nuclei (blue) in BMECs from HC, BBB-nondeficit, and BBB-deficit groups; images were processed using Matlab, assessing for membrane and cytosolic fluorescence intensity; scale bar is 50 μm . **g)** Quantification of normalized total fluorescence intensity for width and length for CLDN5 and OCLN in HC, BBB-nondeficit, and BBB-deficit BMECs; 6 fields of view for each cell line were used for quantification. Raw intensity values were normalized to the total number of nuclei in each respective field of view. Each dot represents a field of view. Mean $\pm$ s.e.m are shown for each group. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test. **** $p < 0.0001$ comparing CLDN5 width in BBB-deficit to HC, ** $p = 0.0046$ comparing CLDN5 width in BBB-nondeficit to HC, * $p = 0.0251$ comparing BBB-deficit to BBB-nondeficit; * $p = 0.0404$ comparing CLDN5 length intensity in BBB-deficit to HC; **** $p < 0.0001$ comparing CLDN5 total intensity in BBB-deficit to HC, *** $p = 0.0004$ comparing CLDN5 total intensity in BBB-deficit to BBB-nondeficit; **** $p < 0.0001$ comparing OCLN width in BBB-deficit to HC, * $p = 0.042$ comparing OCLN width in BBB-nondeficit to HC, ** $p = 0.0040$ comparing OCLN length in BBB-deficit to HC, ** $p = 0.0012$ comparing OCLN total intensity in BBB-deficit to HC, *** $p = 0.0003$ comparing OCLN total intensity in BBB-deficit group to BBB-nondeficit. All reported p -values are two-sided.

3a)

3b)

3c)

3d)

3e)

3f)

3g)

3h)

Figure 3

MMP1 secreted by SZ BMECs impairs BBB function. **a)** Schematic of cross-culture assays. In condition 1, BMECs from a HC individual was placed in the bottom chamber while BMECs from either a BBB-deficit or -nondeficit group was placed in the upper chamber. In condition 2, BMECs from a HC individual was placed in the top chamber while BMECs from either BBB-deficit or -nondeficit groups was placed in the bottom chamber. **b)** Peak (day 2) TEER values comparing HC subjects cultured alone to HC subjects co-

cultured with either BBB-nondeficit or BBB-deficit subjects and comparing BBB-deficit subjects cultured alone to BBB-deficit subjects co-cultured with HC subjects. TEER values were collected using three biological replicates and shown as mean $\pm$ s.e.m. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test (* p = 0.0466 comparing HC Top/BBB-nondeficit Bottom to HC cultured alone, ** p =0.0019 comparing HC Top/BBB-deficit Bottom to HC cultured alone, **** p < 0.0001 comparing BBB-deficit Top/ HC Bottom to BBB-deficit cultured alone. **c)** Heatmap depicting normalized proteomic marker levels detected in conditioned media collected from HC (n =4), BBB-nondeficit (n =5), and BBB-deficit (n =3) groups during peak TEER (day 2) values. **d)** Normalized levels of CCL19, CXCL8, CCL2, and MMP1 in HC, BBB-nondeficit, and BBB-deficit BMECs. Raw concentration values were normalized to the average concentration of individuals in the HC group. Each dot represents the normalized cytokine level for each subject. Mean $\pm$ s.e.m is shown for each group. Significance was determined using a general linear hypothesis test (* p = 0.0143 comparing CCL19 in BBB-deficit to BBB-nondeficit, * p =0.0453 comparing CXCL8 in BBB-deficit to HC, [#] p = 0.0289 comparing CXCL8 in BBB-deficit to BBB-nondeficit, * p =0.0280 comparing CCL2 in BBB-nondeficit to HC, ^{##} p =0.00369 comparing CCL2 in BBB-deficit to BBB-nondeficit, [#] p =0.0345 comparing MMP1 in BBB-deficit to BBB-nondeficit, and p =0.0516 comparing MMP1 in BBB-deficit to HC. **e)** Correlation matrix between peak TEER values, CLDN5 mRNA and protein levels, fluorescein accumulations from permeability assay, and the concentrations of markers from proteomic analysis. Correlations were performed using Spearman correlations (CCL19-Dextran 10kDa r =0.678, * p =0.0118; CXCL8-TEER r =-0.734, ** p =0.0091; CCL2-TEER r =-0.69, * p =0.0324, MMP1-TEER r =-0.790, ** p =0.0036; MMP1-CLDN5 protein level r =-0.608, * p =0.040; MMP1-Cadaverine 0.1kDa r =0.643, * p =0.0280). **(f-h)** Quantification of MMP1 mRNA, protein, and proteolytic activity in HC, BBB-nondeficit, and BBB-deficit groups. Mean $\pm$ s.e.m is shown for each group. Significance was determined by One-Way ANOVA and post-hoc Tukey multiple comparison test (* p = 0.419). RT-qPCR was conducted in three technical and biological replicates for each subject. MMP1 mRNA expression was normalized to the housekeeping gene GAPDH. Each normalized value was then calibrated to the average MMP1 mRNA expression from the HC group (n =4) using the $\Delta\Delta C_t$ method. Western blots were performed in two biological replicates for each subject and band intensities were quantified using Fiji by Image J and band intensities were normalized to GAPDH. MMP1 protein activity assay (human Active MMP1 Flurokine E kit) was performed in technical triplicates using conditioned medium from HC, BBB-nondeficit, and BBB-deficit BMECs. All reported p -values are two-sided.

Figure 4

Inhibition of MMP1 and TNF α restores BBB function. **a)** Percent change in TEER across six days in HC (n=1), BBB-nondeficit (n=1), and BBB-deficit (n=3) groups treated with pharmacologic MMP1 inhibitor (Piroxicam) or biologic TNF α inhibitor (Infliximab). BMECs were exposed to Infliximab (300ng/mL) or Piroxicam(10uM) for 24 hours during subculturing (day 6). Percent change was calculated based on the average TEER value in infliximab or piroxicam condition from each day and then normalized to the

average TEER value in the control condition. Significance determined by Two-Way ANOVA and Uncorrected Fisher's LSD multiple comparison test (HC group: day 3 $^{\#}p=0.0437$ comparing piroxicam to control, day 4 $^{\#}p=0.0112$ comparing piroxicam to control, day 5 $^{\#}p=0.0395$ comparing piroxicam to control; BBB-nondeficit group: day 1 $^{\#}p=0.0489$ comparing piroxicam to control; BBB-deficit group: day 2 $^{\ast}p=0.0371$ comparing infliximab to control and $^{\#}p=0.0413$ comparing piroxicam to control, day 3 $^{\ast}p=0.0494$ comparing infliximab to control, day 5 $^{\#}p=0.0441$ comparing piroxicam to control, day 6 $^{\ast}p=0.0153$ comparing infliximab to control and $^{\#}p=0.0309$ comparing piroxicam to control). **b)** Peak (day 2) TEER values of BMECs from HC, BBB-nondeficit, and BBB-deficit groups with infliximab or piroxicam treatment. Significance was determined by One-Way ANOVA and Uncorrected Fisher's LSD multiple comparison test (HC group: $^{\ast}p=0.0161$ comparing piroxicam to control; BBB-deficit group: $^{\ast}p=0.0385$ comparing infliximab to control and $^{\ast}p=0.0487$ comparing piroxicam to control). TEER values were collected using three biological replicates and shown as mean $\pm$ s.e.m. **c)** Quantification of MMP1 protein activity in conditioned medium from BMECs of BBB-deficit subjects with infliximab or piroxicam treatment. MMP1 protein activity assay was performed in technical duplicates. **d)** Quantification of CLDN5 mRNA expression in BMECs from BBB-deficit subjects treated with infliximab or piroxicam; RT-qPCR was conducted in three technical and biological replicates for each line. Mean $\pm$ s.e.m is shown for each group. By using One-Way ANOVA and post-hoc Tukey multiple comparison test, no significances were observed in MMP1 activity or CLDN5 mRNA levels. All reported p-values are two-sided.

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