Brain microvascular endothelial cells exhibit lower activation of the alternative complement pathway than glomerular microvascular endothelial cells

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Sarah E. Sartain1, Nancy A. Turner2, and Joel L. Moake3

From the 1Department of Pediatrics, Section of Hematology-Oncology, Baylor College of Medicine, Texas Children’s Hospital, Houston, Texas 77030 and the 2Department of Bioengineering, Rice University, Houston, Texas 77005

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Atypical hemolytic uremic syndrome (aHUS) and bone marrow transplantation-associated thrombotic microangiopathy (TA-TMA) are associated with excessive activation of the alternative complement pathway (AP) and with severe renal, but rarely cerebral, microvascular damage. Here, we compared AP activation and regulation in human glomerular and brain microvascular endothelial cells (GMVECs and BMVECs, respectively) unstimulated or stimulated by the proinflammatory cytokine, tumor necrosis factor (TNF). Compared with GMVECs and under both experimental conditions, BMVECs had increased gene expression of the AP-related genes C3, CFB, and C5 and decreased expression of CFD. This was associated with increased expression in BMVECs (relative to GMVECs) of the genes for surface and soluble regulatory molecules (CD46, THBD, CD55, CFH, and CFI) suppressing formation of the AP C3 and C5 convertases. Of note, unlike GMVECs, BMVECs generated extremely low levels of C3a and C5a and displayed decreased activation of the AP (as measured by a lower percentage of Ba generation than GMVECs). Moreover, BMVECs exhibited increased function of CD141, mediating activation of the natural anticoagulant protein C, compared with GMVECs. We also found that the C3a receptor (C3aR) is present on both cell types and that TNF greatly increases C3AR1 expression in GMVECs, but only slightly in BMVECs. Higher AP activation and C3a generation in GMVECs than in BMVECs, coupled with an increase in C3aR production in TNF-stimulated GMVECs, provides a possible explanation for the predominance of renal damage, and the absence of cerebral injury, in individuals with episodes of aHUS and TA-TMA.

Thrombotic microangiopathies (TMAs)2 are disorders characterized by microvascular thrombosis, microangiopathic hemolytic anemia, thrombocytopenia, and microvascular endothelial injury (1–3). Atypical hemolytic uremic syndrome (aHUS) and bone marrow transplantation-associated TMA (TA-TMA) are TMAs associated with abnormalities in the alternative complement pathway (AP) as well as severe renal damage (3–6).

The AP is one of three pathways of the innate immune complement system responsible for protecting the host from unwanted pathogens or foreign invaders. It is initiated when C3b is cleaved from complement component C3 and attaches to an activating surface, releasing a soluble C3a fragment (7, 8). Factor B (FB) then binds to C3b (9, 10), and factor D (FD) cleaves FB in this complex to form C3bb (the active C3 convertase of the AP) (11), releasing the activation product Ba. The C3 convertase is stabilized by factor P (properdin) (12–14). The C3bBb cleaves C3 to generate additional C3b and C3a in an activation amplification loop (15, 16); as the ratio of C3b to Bb increases, C3bbBbC3bb (the C5 convertase of the AP) forms and cleaves C5 to C5b, releasing the soluble C5a activation fragment (11, 17). AP activation is likely to occur on ultra-large von Willebrand factor (ULVWF) multimeric strings secreted by, and attached to, human endothelial cells. The attachment of AP components to the ULVWF multimeric strings and AP activation has been previously demonstrated on human umbilical vein endothelial cells (HUVECs) (18–20). The classical complement pathway (CP) component, C4, does not attach to the strings; therefore, this pathway is not activated by ULVWF (18). The potent inflammatory cytokine, tumor necrosis factor (TNF), likely promotes AP activation by inducing ULVWF secretion (21).

C3a and C5a function as pro-inflammatory polypeptides (anaphylatoxins) that cause neutrophil chemotaxis, histamine release from basophils and mast cells, and increased vascular permeability (22). C3a and C5a are generated by activation in each complement pathway: the AP, the lectin pathway, or the CP. The activation product, Ba, is unique to the AP and is not generated with activation of the lectin pathway or CP. No receptor for the Ba protein has been identified. Receptors for vascular endothelial cell; TNF, tumor necrosis factor; C3aR, complement C3a receptor; FB, factor B; FD, factor D; ULVWF, ultra large von Willebrand factor; HUVEC, human umbilical vein endothelial cell; CP, classical complement pathway; C5aR, complement C5a receptor; FH, factor H; FI, factor I; THBD, thrombomodulin; PC, protein C; BSA, bovine serum albumin; VWF, von Willebrand factor; Ab, antibody; qPCR, quantitative PCR; PE, phycoerythrin.
The vulnerability of the kidney to AP-mediated injury in aHUS and TA-TMA led us to hypothesize that there is a difference in AP activation and regulation in GMVECs (the cell type that is predominantly involved in these two types of TMA) compared with brain microvascular endothelial cells (BMVECs), a microvascular endothelial cell type that is not affected. To achieve our objectives, we compared AP activation and regulation in GMVECs and in BMVECs that were either unstimulated or, as a model for inflammation/infection, stimulated by TNF. These MVECs serve as ideal models for our studies as they produce and secrete all AP components and regulators, as well as VWF (18, 55).

### Results

#### Gene expression of AP components in unstimulated and TNF-stimulated BMVECs relative to GMVECs

We compared in BMVECs and GMVECs the expression of genes that encode essential proteins in the activation of the AP: C3, CFB, C5, CFP, and CFD. Although the classical pathway is not activated on ULVWF strings, we included the gene expression of C4 as a control. Unstimulated BMVECs had 14-fold higher mRNA levels for C3, ~3-fold higher levels for both CFB and C5, and ~5-fold higher mRNA levels for C4 compared with levels in unstimulated GMVECs. Unstimulated BMVECs had ~4-fold lower mRNA levels for both CFP and CFD compared with unstimulated GMVECs (Fig. 1A). Expression levels of C3, CFB, C5, and C4 were ~7-, ~4-, ~5-, and ~8-fold lower, respectively, and CFD expression levels were 3-fold lower in TNF-stimulated BMVECs compared with TNF-stimulated GMVECs (Fig. 1B).

#### Quantitative gene expression of AP components by TNF-stimulated GMVECs and BMVECs

We additionally quantified changes in gene expression of each AP component in BMVECs and GMVECs after exposure to TNF from unstimulated gene levels in each MVEC type (Fig. 2). AP component expression of both cell types changed with TNF stimulation in a similar pattern (but not in magnitude). Both GMVECs and BMVECs had increased gene expression of C3 (150- and 50-fold, respectively) and of CFB (60- and 80-fold, respectively) and reduced expression of CFP (10- and 2-fold respectively). CFD expression changed minimally in GMVECs and BMVECs with TNF stimulation (<2-fold increases), and C5 and C4 mRNA levels did not change substantially in either cell type with TNF.

#### Gene expression of AP surface and soluble regulatory protein genes in unstimulated and TNF-stimulated BMVECs relative to GMVECs

Gene expression levels of surface (CD46, THBD, and CD55) and soluble (CFI and CFH) AP regulatory protein genes in BMVECs were calculated relative to levels in GMVECs. BMVECs had significantly higher levels of mRNA encoding each of these five AP regulatory proteins studied, compared with GMVECs. The largest relative differences were in CD46, CD55, and CFI, where expression levels were 6-, 14-, and 18-fold higher, respectively (Fig. 3A and Table 2). TNF-stimu-
lated BMVECs also had higher expression levels of all five AP regulatory protein genes studied relative to TNF-stimulated GMVECs, and TNF magnified the relative differences for THBD, CD55, CFI, and CFH, which were 10-, 18-, 23-, and 7-fold higher, respectively (Fig. 3B and Table 2).

Surface AP regulatory proteins in unstimulated and TNF-stimulated BMVECs and GMVECs

Protein levels of the surface AP regulators, CD46 and CD55, on unstimulated and TNF-stimulated BMVECs were determined by flow cytometry and compared with GMVECs (55). Under both conditions CD55 receptors were ~2-fold higher on BMVECs compared with GMVECs (Fig. 4A). CD46 protein presence on BMVEC and GMVEC surfaces was similar (Fig. 4A), even though CD46 gene expression was higher in BMVECs compared with GMVECs (Fig. 3). A possible explanation for this enigma is that the mAb used to detect surface CD46 does not bind and detect the complete array of BMVEC CD46 isoforms. CD46 has multiple isoforms as a result of alternate gene splicing, and identical CD46 isoforms are not present in cerebral and renal tissue (56). TNF resulted in statistically significant increases of detectable CD46 on the surface of both cell types (1.6-fold on GMVECs and 2.1-fold on BMVECs) but did not result in significant changes in CD55 on either cell type (Fig. 4A).

Our previous study demonstrating a 6.7-fold down-regulation of GMVEC THBD expression by TNF (55) led us to compare CD141 receptor function for thrombin binding and protein C activation on both cell types under nonstimulating and TNF-stimulating conditions. TNF stimulation resulted in decreased activation of PC in both GMVECs and BMVECs. Without TNF stimulation, BMVECs generated 3.3-fold more activated PC than unstimulated GMVECs, and with TNF stimulation, BMVECs generated 10-fold more activated PC than TNF-stimulated GMVECs (Fig. 4B).

Soluble AP regulatory protein, FH, in BMVEC and GMVEC supernatant with and without TNF stimulation

FH levels measured from the supernatant of both unstimulated and TNF-stimulated BMVECs were ~1.5-fold higher than levels measured from the supernatant of unstimulated and TNF-stimulated GMVECs, respectively (Fig. 5).

Levels of complement proteins and activation products in unstimulated and TNF-stimulated BMVEC and GMVEC supernatants

C3 protein released from unstimulated and TNF-stimulated BMVECs were ~5- and ~4-fold higher, respectively, than C3 released from unstimulated and TNF-stimulated GMVECs (Fig. 6A). FB released from unstimulated BMVECs and GMVECs was undetectable, and with TNF stimulation, BMVECs released sub-
We studied differences in AP activation between GMVECs and BMVECs with and without TNF stimulation by measuring complement activation products, C3a, C5a, and Bb, in the cell supernatant. Without or with TNF stimulation, GMVECs produced much higher concentrations of C3a and C5a than did BMVECs. Unstimulated and TNF-stimulated BMVECs did not generate detectable C3a and produced only minute amounts of C5a, demonstrating that GMVECs have increased complement activation compared with BMVECs under both experimental conditions (Fig. 6B). In contrast to C3a and C5a, Bb was generated by both cell types under both experimental conditions and increased significantly in the supernatant of both cell types with TNF stimulation (consistent with TNF-mediated AP activation as demonstrated previously (55)). Bb is an AP-specific activation product that is released from C3b-bound FB after cleavage by FD to form C3bBb (11). The Bb in this convertase can be displaced by FH or CD46 (without changing released Bb levels), followed by inactivation by co-factor FI to iC3b (57).

As shown in Fig. 6A, TNF-stimulated BMVECs released about 4-fold more FB than TNF-stimulated GMVECs. Thus, under conditions of uncontrolled TNF-mediated AP activation and 100% conversion, BMVECs would be capable of generating 4-fold more Ba than GMVECs. However, we found that only ~2% of the FB produced by TNF-stimulated BMVECs was cleaved/activated to Ba. In contrast, in TNF-stimulated GMVECs, ~4% of the FB produced was cleaved/activated to Ba (Fig. 6C). Therefore, with TNF stimulation, BMVECs converted half as much Ba from FB than GMVECs. This indicates substantially more FB (~4-fold) relative to TNF-stimulated GMVECs (Fig. 6A).

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that although TNF-stimulated BMVECs released higher concentrations of both C3 and FB than TNF-stimulated GMVECs, a smaller percentage of possible C3 convertases was formed in BMVECs. The undetectable levels of C3a in BMVECs (Fig. 6B) also may indicate that the C3 convertases that did form were subsequently inactivated (by FH and CD46). Consequently, further AP activation (C5 convertase formation) was suppressed.

**Gene expression of C3a and C5a receptors, C3AR1 and C5AR1, in unstimulated and TNF-stimulated BMVECs and GMVECs**

BMVEC mRNA levels of C3AR1 and C5AR1, the genes encoding C3ar and C5ar, were calculated relative to levels in GMVECs. Under both unstimulated and TNF-stimulated conditions, GMVECs expressed considerably higher mRNA levels of C3AR1 than BMVECs. Unstimulated BMVECs had 4.8-fold fewer C3AR1 mRNA copies relative to unstimulated GMVECs (Fig. 7A). TNF-stimulated BMVECs had C3AR1 mRNA levels that were 23-fold lower relative to levels in TNF-stimulated GMVECs (Fig. 7B). TNF stimulation induced a 5.8-fold increase in C3AR1 expression in GMVECs and only a 1.4-fold increase in BMVECs (Fig. 8), accounting for the 23-fold relative difference in the TNF-stimulated cell types. Expression levels for C5AR1 were undetectable in both BMVECs and GMVECs without and with TNF stimulation (Table S1).

**C3aR and C5aR proteins on the surface of unstimulated and TNF-stimulated BMVECs and GMVECs**

C3aR was ~2-fold higher on BMVECs under both unstimulated and TNF-stimulated conditions. TNF stimulation increased C3aR detection by ~1.5-fold on the surface of both cell types, although this was only statistically significant in GMVECs (Fig. 9A). This detection of more C3aR on BMVEC compared with GMVEC surfaces, along with the finding that the receptor only increased by 1.5-fold on TNF-stimulated GMVEC surfaces, was unexpectedly different from the gene expression results (Figs. 7 and 8). Analysis of the single-gated cell populations by flow cytometry showed that a considerable percentage of both BMVECs (~73%) and GMVECs (~85%) were negative for surface C3aR, although saturating concentrations of the detection antibody were used (Fig. 9B). Similar results showing a reduction in C3a receptors have been previously reported by others to be caused by receptor complex (C3a-bound C3ar) internalization (58, 59).

C5ar was not detected by flow cytometry on the surfaces of unstimulated or TNF-stimulated BMVECs or GMVECs (Fig. S1).

**Discussion**

We compared AP activation and regulation in BMVECs to GMVECs under nonstimulating and TNF-stimulating conditions in an effort to elucidate relative cerebral and renal injury in aHUS and TA-TMA. Our data indicate that BMVECs are more effective in resisting TNF-mediated AP activation than GMVECs, possibly contributing to the explanation for the absence of cerebral microvascular injury and the prominence of renal damage in patients with inflammation/infection-induced episodes of aHUS and TA-TMA.

Under both experimental conditions, the AP component gene expression levels of C3, CDF, and C5 in BMVECs were much higher than the expression levels in GMVECs. Conversely, gene expression levels of CDF were ~3-fold lower in unstimulated and TNF-stimulated BMVECs compared with GMVECs under the same conditions (Fig. 1). The relatively reduced quantity of FD produced in BMVECs may contribute to the diminished cleavage of FB into active Bb (restricting C3 convertase formation and AP activation) in BMVECs relative to GMVECs.

Compared with GMVECs, BMVECs demonstrated increased gene expression for the negative AP regulatory protein genes, CD46, THBD, CD55, CFI, and CFH (Table 2 and Fig. 3) under both unstimulated and TNF-stimulated conditions. TNF did not affect levels of CD55 protein in either cell type, but it did result in statistically significant increases of detectable CD46 receptors on both cell types (with a greater increase in BMVECs, Fig. 4A). Because BMVECs had higher levels of gene expression for all five AP regulatory proteins than GMVECs under basal conditions, control of AP activation would be predicted to be more effective in BMVECs than in GMVECs with an AP-activating agent, such as TNF. To assess this functionally, we measured AP activation products C3a, C5a, and Ba in the supernatant of unstimulated and TNF-stimulated BMVECs and GMVECs and found that control of AP activation was, in fact, more effective in BMVECs. With provocation of AP activation by TNF, BMVECs displayed significantly decreased AP activation by converting half as much Ba from FB than GMVECs under the same conditions. TNF-stimulated BMVECs had C3aR mRNA copies relative to unstimulated GMVECs (~73%) and GMVECs (~85%) were negative for surface C3aR, although saturating concentrations of the detection antibody were used (Fig. 9B). Similar results showing a reduction in C3a receptors have been previously reported by others to be caused by receptor complex (C3a-bound C3ar) internalization (58, 59).

C5ar was not detected by flow cytometry on the surfaces of unstimulated or TNF-stimulated BMVECs or GMVECs (Fig. S1).

**AP regulation and activation in BMVECs and GMVECs**

![Figure 5](link) Levels of the soluble AP regulatory protein, FH, in the supernatant of unstimulated and TNF-stimulated GMVECs and BMVECs. Super-natant from unstimulated GMVECs (n = 4) and BMVECs (n = 4) in T-75 flasks was collected after 24 h in serum-free media and assayed for FH by ELISA. The same flasks were then stimulated for 48 h with 10 ng/ml TNF (24 h in complete media and 24 h in serum-free media), and the 24-h supernatant was again collected and assayed for FH. *, p < 0.05.
Supernatant from unstimulated BMVECs and GMVECs in T-75 flasks was collected and assayed by ELISA for complement components C3 and FB (A) and AP activation products C3a, C5a, and Ba (B and C). The same flasks were then stimulated for 48 h with 10 ng/ml TNF (24 h in complete media and 24 h in serum-free media), and the 24-h supernatant was again collected and assayed for the same proteins. A, n = 4 for BMVECs and n = 6 for GMVECs for C3, and n = 6 for both GMVECs and BMVECs for FB; FB levels in unstimulated BMVECs and GMVECs were below detection limits (bdl) of the assay. B, n = 6 for GMVECs and n = 4 for both BMVECs and BMVECs for C3a experiments, n = 6 for both BMVECs and GMVECs for C5a experiments, and n = 4 for both BMVECs and GMVECs for Ba experiments; C3a levels in unstimulated and TNF-stimulated BMVECs were below detection limits of the assay. C, percentage of Ba generated from FB under TNF-stimulating conditions was calculated from Ba levels in B and FB levels in A; n = 4 for both BMVECs and GMVECs. *, p < 0.05; **, p < 0.001.
condition. This is likely because of the following two reasons: 1) this study did not generate detectable levels of C3a under either simulation (55). In contrast to GMVECs and HUVECs, BMVECs in this study did not generate detectable levels of C3a under either condition. This is likely because of the following two reasons: 1) the ample quantities of soluble and surface BMVEC-negative regulatory molecules available to suppress AP initiation and active C3 convertase formation (Figs. 3–5 and Table 2); and 2) binding of C3a to C3aR, preventing detection in the cell supernatant (which likely also occurs in GMVECs). Finally, BMVECs generated barely detectable levels of C5a under (~0.1 pg/ml) and with (~2 pg/ml) TNF stimulation. In contrast, C5a levels generated by GMVECs were about 50 pg/ml under both experimental conditions (Fig. 6B). The low level of C5a generated by BMVECs is compatible with minimal formation of C3 and C5 convertases by this cell type because of ample AP regulatory molecules to suppress AP activation. Conversely, the vigorous generation of C3a and C5a by GMVECs is likely the result of abundant formation of these convertases due to insufficient amounts of AP regulatory molecules (compared with BMVECs).

Because of the higher production of FH and CD46, and the nearly negligible production of C5a by TNF-stimulated BMVECs, the elevated Ba generated by BMVECs during TNF stimulation (Fig. 6B) likely does not indicate increased AP activation compared with GMVECs. FB that is bound to C3b can be cleaved/activated by FD to produce Bb and release Ba during the formation of the AP C3 convertase (C3bBb). The cleaved Bb can be displaced by FH (57) or CD46 (60), which function as co-factors of FI to generate iC3b, to reduce further amplification of C3b. Without enough active C3 convertase formation to increase the affinity of C5 binding, the C5 convertase will not be generated (61). Therefore, C5 convertase generation in BMVECs is suppressed without affecting the quantity of Ba released. Additionally, the higher amounts of Ba in TNF-stimulated BMVEC supernatant are unlikely to have much effect on BMVEC membranes without a known receptor or function for Ba.

Functionally, BMVECs, compared with GMVECs, generated ~3- and 10-fold higher levels of activated PC under unstimulated and TNF-stimulated conditions, respectively, indicating a higher amount of CD141 on BMVEC surfaces under both conditions (Fig. 4B). CD141, as both a complement regulator and a natural anticoagulant, has the potential to play a role in host cell protection from AP-mediated injury (by acting as a co-factor for FI-mediated proteolysis of C3b (30, 31)) and from thrombotic injury (by increasing activation of PC (33)). Because of the strong relationship between low activated PC levels and thrombosis, GMVECs are likely more prone to thrombus generation, which in vivo could lead to occlusion of the microvessels and renal dysfunction.

Gene expression levels of C3AR1 were many-fold lower in both unstimulated and TNF-stimulated BMVECs than in GMVECs (Fig. 7), and were increased to a greater extent by TNF stimulation in GMVECs (Fig. 8). In contrast, BMVECs had more C3aR surface protein under both experimental conditions compared with GMVECs (Fig. 9), and TNF stimulation only resulted in a 1.5-fold increase of the receptor on GMVEC surfaces (versus 5.8-fold gene level increase). The higher amounts of C3a generated by GMVECs (compared with BMVECs) likely leads to increased C3a–C3aR binding, followed by functional internalization of the C3aR–C3a complex and therefore lower detection of C3aR on GMVECs by flow cytometry under both unstimulated and TNF-stimulated conditions. This interpreta-

![Figure 7. Gene expression levels of C3AR1 in unstimulated and TNF-stimulated BMVECs and GMVECs.](image)

![Figure 8. Quantitative gene expression of C3AR1 in TNF-stimulated BMVECs and GMVECs.](image)
Figure 9. C3aR protein on surfaces of unstimulated and TNF-stimulated BMVECs and GMVECs. BMVECs and GMVECs were incubated with or without TNF (10 ng/ml)-supplemented media for 48 h. Samples of $2 \times 10^5$ BMVECs ($n = 5$) or GMVECs ($n = 5$) were labeled with saturating amounts of PE-conjugated mAbs to C3aR or with PE-conjugated isotype control antibodies alone (to measure background fluorescence), fixed, and analyzed by flow cytometry. A, mean fluorescence intensities (plus S.D.) of C3aR on BMVEC and GMVEC surfaces without and with TNF. B, single gated population of GMVECs (upper panel) or BMVECs (lower panel) detected by forward and side scatter was analyzed in quadrant plots of PE fluorescence (y axis) and forward scatter (FSC, x axis). Fluorescent detection of C3aR within this single gate showed two distinct cell populations, positive or negative for C3aR, in each cell type and under both experimental conditions (unstimulated and TNF-stimulated). The same quantity of isotype antibody and monoclonal mouse antibody to C3aR was used in both cell types. Quadrant plots are representative of at least five experiments on each cell type, with and without TNF stimulation. *, $p < 0.05$; **, $p < 0.001$. 

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Expression of the C5aR gene, C5AR1, as well as presence of C5aR, was below the levels of detection for BMVECs and GMVECs under unstimulated and TNF-stimulated conditions (Table S1 and Fig. S1). The absence of C5aR confirmed that generated C5a is not partially receptor-bound, the measured C5a levels are accurate, and that the extremely low C5a levels measured in BMVECs are the result of limited AP activation.

We have shown that TNF activates the AP in GMVECs and BMVECs, evidenced by the increases in Bα protein with TNF stimulation. However, our data do not show parallel increases in C3α and C5α (Fig. 6B). C3α likely does increase with TNF stimulation, but the corresponding rise of C3αR prevents detection of the increase because of C3α binding and C3α–C3αR complex internalization. TNF stimulated GMVECs still generated enough additional C3α to exceed the saturation of their increased numbers of C3αR but not enough to detect a significant rise in C3α in their supernatant. The TNF-stimulated BMVECs, even with 20-fold fewer C3αR and 7-fold more C3 synthesis (based on gene expression data), did not generate even enough C3α to exceed C3αR saturation, and therefore, C3α was not detected in TNF-stimulated BMVEC supernatant. Because neither the gene expression nor the C5αR protein was detected in either BMVECs or GMVECs, the detected levels of generated C5α were more accurate. C5α most likely did not increase in TNF-stimulated BMVECs because the increased amounts of FH and CD46 prevented sufficient C3b amplification to form C5 convertases. Alternatively, the production of C5α in GMVECs with TNF stimulation may have been limited by the synthesis rate of C5α; as shown in Fig. 2, TNF stimulation resulted in extensive increases in C3 and CFB gene expression levels, without changes in C5 gene expression.

C3α and C5α are potent anaphylatoxins implicated in many disease states, including sepsis, asthma and allergy, cancer, neurodegenerative diseases, and autoimmune diseases, among others (22, 62–73). Some studies have reported that these proteins may contribute to renal damage (74–77). These data, along with the data reported here on GMVEC generation of C3α and increased production of C3αR, suggest that C3α may be involved in the pathogenesis of renal injury in aHUS and TA-TMA. Our in vitro findings of increased alternative complement pathway activation along with vigorous C3α generation in GMVECs, compared with BMVECs, correspond strongly to the in vivo clinical observation that injury to the kidneys is much more frequently observed than injury to the brain in patients with aHUS and TA-TMA.

Plasma AP protein concentrations are much higher than the levels generated by the MVECs in this study. In vivo, circulating plasma complement proteins may promote supplementary assembly/activation of AP components on these two types of MVECs. However, these circulating plasma proteins are unlikely to alter the number or function of membrane-bound regulatory receptors (CD141, CD55, and CD46), which provide the most effective host cell defense. Additionally, in our experiments, BMVECs and GMVECs express and produce their own AP components and AP regulatory proteins. Because there was no external addition of complement proteins, our models provide an ideal system for the elucidation of basic molecular mechanisms of AP activation and regulation.

In summary, we compared AP activation and regulation in BMVECs and GMVECs and demonstrated that GMVECs produce decreased amounts of the AP regulatory proteins, activate the AP to a greater extent, and generate more C3α and C5α than BMVECs under both nonstimulating and TNF-stimulating conditions. The data may contribute to the susceptibility of the kidneys and relative resistance of the brain to AP-mediated injury in aHUS and TA-TMA.

Experimental procedures

Cells

BMVECs and GMVECs—Pooled primary human BMVECs (ACBRI-376 V) and GMVECs (ACBRI-128 V) were purchased from Cell Systems. BMVECs and GMVECs were grown in complete media (CM131, MCD-131 medium (Sigma), supplemented with penicillin/streptomycin/L-glutamine (Life Technologies, Inc.), plus microvascular growth supplement (Life Technologies, Inc.)). These MVECs serve as an ideal model for our studies as they produce and secrete all AP components and regulators, as well as VWF (18, 55). Additionally, GMVECs are the cells that are primarily affected in TMA, whereas BMVECs serve as an optimal control, because the brain is an organ that has no pathologic association with aHUS and TA-TMA.

Seeding—GMVECs and BMVECs were seeded in T-25 flasks for flow cytometry experiments and in T-75 flasks for gene expression experiments and AP component/activated PC measurements.

VWF detection in Weibel-Palade bodies (WPBs)—Characterization of the cells as endothelial was confirmed by immunofluorescence that showed 95% of the GMVECs or BMVECs positive for VWF in Weibel-Palade bodies (Fig. S2). BMVECs and GMVECs were grown to confluence (8–12 days) on coverslips. For internal detection of VWF in Weibel-Palade bodies, the ECs were first washed with PBS, fixed for 10 min with 2% p-formaldehyde in PBS, and made permeable with 0.2% Triton X-100 for 5 min. After further PBS washes, the ECs were stained with polyclonal rabbit anti-human VWF (Ramco Laboratories) plus secondary Alexa Fluor 488 (green) chicken anti-rabbit IgG (Invitrogen catalog no. A21441) for 15 min. Cell nuclei (blue) were detected with 1 nm 4,6 diamidino-2-phenylindole (DAPI) in the mounting solution (Fluoro-gel II). Fluorescent images were acquired using IP Lab software version 3.9.4r4 (Scanalytics, Inc., Fairfax, VA) on a Nikon Diaphot TE300 microscope equipped with a CFI Plan Apo Lambda ×100 oil N.A. 1.45 objective plus ×10 projection lens (Nikon, Garden City, NY), SensiCamQE CCD camera (Cooke Corp., Romulus, MI), motorized stage and dual filter wheels (Prior) with single band excitation, and emission filters for FITC/TRITC/CY5/DAPI (Chroma, Rockingham, VT).
Reverse transcription real-time quantitative PCR (RT-qPCR)

RNA was extracted from unstimulated and TNF-stimulated BMVECs and GMVECs using TRIzol and isolated using chloroform extraction and isopropyl alcohol precipitation. RNA integrity was verified by 260:280 optical ratios and 1% agarose/formaldehyde electrophoresis. RNA samples were reverse-transcribed using SuperScript VILO MasterMix (Invitrogen), and the resulting cDNA samples (100 ng) were amplified in triplicate by real-time qPCR under the following conditions: 95 °C for 3 min, 40 cycles of 10 s at 95 °C, 10 s at 55 °C, and 30 s at 72 °C, and 95° for 10 s (CFX96, Bio-Rad). Amplified products were detected using TaqMan Gene Expression Assays (with probes that span target exon junctions) and Perfect FastMix II (Quanta).

Quantitative changes in gene expression of BMVECs and GMVECs in the presence of TNF were calculated using the method developed by Pfaffl et al. (78), which uses specific primer efficiencies to evaluate the amount of cDNA amplification per each PCR cycle. Gene efficiencies were determined by amplification of 100 to 0.01 ng of cDNA and calculating the slope of the line after plotting the quantification cycle (Cq) versus nanograms of cDNA. The relative quantification of gene expression with/without TNF stimulation in BMVECs and GMVECs was calculated as described by Livak and Schmittgen (79). The standard deviation in gene expression assays (s) was determined by Equation 1,

\[ S = \sqrt{S_1^2 + S_2^2} \]

(Eq. 1)

where \( s_1 \) and \( s_2 \) are the standard deviations of triplicate Cq measurements for the reference and target genes.

Genes studied include the complement surface regulatory proteins CD46, CD55, and THBD (gene for CD141); soluble regulatory proteins CFH and CFI; AP components C3, C5, CFB, CFD, and CFP; the classical component C4; anaphylatoxin receptors C3AR1 and C5AR1; and the reference gene GAPDH. Assay probe ID numbers and gene efficiencies are listed in Table S2. The Minimum Information for publication of Quantitative real-time PCR Experiment (MIQE) guidelines state that using a single reference gene is permissible if there is consistent quantification in the quantification cycle of the reference gene under untreated and treated experimental conditions (80). Data in Table S3 show the consistent quantification cycle of GAPDH in untreated and TNF-treated BMVECs and GMVECs, thus satisfying these criteria.

Flow cytometry

TNF stimulation of GMVECs and BMVECs—Once confluent in T-25 flasks, control cells were incubated for 24 h in serum-free media (MCDB-131 plus insulin–transferrin–selenium, Life Technologies, Inc.), and experimental cells were incubated for 24 h with CM131 plus TNF (10 ng/ml, Life Technologies, Inc.) and then incubated for an additional 24 h in serum-free media plus 10 ng/ml TNF (total TNF exposure 48 h). Both control and experimental flasks were incubated in serum-free media for a total of 24 h to eliminate any proteins derived from serum that might affect surface receptor detection. The specific concentration and duration of exposure of TNF were chosen based on our previous experiments (55).

Cell-surface labeling of CD46, CD141, CD55, C3aR, and C5aR—Control and experimental cells were detached by a 10-min incubation with 5 mM EDTA in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS (to retain surface proteins) and centrifuged (10 min at 400 × g). Cells were counted using the TC10 automated cell counter (Bio-Rad) and resuspended in 1% BSA/Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS at 10\(^6\) cells/ml. Samples of 2 × 10\(^4\) GMVECs or BMVECs (20 μl) were labeled individually with saturating amounts of each fluorescently conjugated mAb (see below) or labeled with saturating amounts of FITC-conjugated (CD46 and CD55) or PE-conjugated (CD141, C3aR, and C5aR) isotype controls (see below) to measure background fluorescence. Saturating amounts of antibody were as follows: 1 μl of CD46, CD141, CD55, or C5aR antibody + 1 μl of corresponding isotype control per 20-μl cell suspension, and 4 μl of C3aR antibody + 4 μl of corresponding isotype control per 20-μl cell suspension. Samples were incubated for 20 min in the dark. Cells were then fixed with 0.5 ml of 1% formaldehyde/Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS. Cells expressing higher fluorescence than background (isotype control alone) were considered positive, and background fluorescence was subtracted from positive fluorescence. Experiments were repeated with increasing passage number (passages 5–7 for GMVECs and passages 4–6 for BMVECs) to exclude the possibility that any differences appreciated were related to passage differences.

Abs to human AP regulatory proteins (flow cytometry)—Abs include the following: CD46, clone E4.3, mouse monoclonal IgG2a,κ conjugated to FITC (BD Biosciences, 555949); CD141, clone IA4, mouse monoclonal IgG1,κ conjugated to PE (BD Biosciences, 559781); and CD55, clone IA10, mouse monoclonal IgG2a,κ conjugated to FITC (BD Biosciences, 556963). Isotype controls included PE mouse, clone IgG1 (BD Biosciences, 349043), and FITC mouse, clone IgG2a,κ (BD Biosciences, 55573).

Abs to human C3aR and C5aR (flow cytometry)—Abs include the following: C3aR, clone hC3aR, mouse monoclonal IgG2b,κ conjugated to PE (BD Biosciences, 551178), and C5aR, clone D53-1473, mouse monoclonal IgG1,κ conjugated to PE (BD Biosciences, 550494). Isotype controls included PE mouse, clone IgG1 (BD Biosciences, 349043), and FITC mouse, clone IgG2a,κ (BD Biosciences, 55573).

Acquisition and analysis

Samples were acquired using FACScan (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences) or FlowJo Collector’s Edition software (Cytec). The instrument settings of the forward scatter and side scatter profiles were log mode. MVEC samples appeared as single populations and were gated based on their forward and side-scatter profiles. Five thousand gated events were analyzed for each sample.

Measurement of AP components, regulators, and activation products from BMVEC and GMVEC supernatant

In these experiments, AP components C3 and FB, AP regulator FH, and AP activation products, C3a, C5a, and B, were
assayed by ELISA from the supernatant of unstimulated or TNF-stimulated BMVECs. These experiments assessed nondirectional release of AP components and regulators from the surface of the two types of MVECs.

**Supernatant collection and TNF stimulation**—Once confluent in T-75 flasks, GMVECs and BMVECs were incubated for 24 h in 1 ml of serum-free media per T-75 flask to concentrate the components in the cell supernatant. The supernatant was collected after 24 h and immediately frozen in liquid nitrogen for 20 s (to prevent further activation of the AP) prior to storing at −80 °C until assayed. These samples were designated the unstimulated controls. After at least 24–48 h of recovery in CM131, the same flasks were then incubated for 24 h in CM131 plus 10 ng/ml TNF and incubated an additional 24 h in 1 ml per T-75 flask of serum-free media plus 10 ng/ml TNF (total TNF exposure of 48 h). The concentrated supernatant was again collected, flash-frozen, and stored at −80 °C until assayed. These samples were designated as the TNF-stimulated samples. GMVECs in these experiments were studied as passages 3 and 4 and BMVECs were used at passages 3–5.

**Sample preparation**—Samples were rapidly thawed and kept on ice prior to use to prevent spontaneous complement activation, and then analyzed for the various complement pathway components, regulators, or activation products.

**C3 fluorescence immunoassay**—Black 96-well plates were coated with 50 ng/ml polyclonal rabbit anti-human C3a (detects human C3a and C3, Complement Technologies, catalog no. A218) in 100 nM bicarbonate buffer (pH 9.6) overnight at 4 °C. TBST-washed wells were blocked overnight with 1% Ig-free BSA in PBS (BSA/PBS), followed by a 50-min incubation with 100 μl/well test samples (MVEC supernatant from unstimulated or TNF-stimulated cells, no dilution) or purified C3 protein (Complement Technologies, catalog no. A113) for the standard curve (with a range of 9.4–600 ng/ml). TBST-washed wells were next incubated with 31 ng/ml goat polyclonal Ab to human C3 (Complement Technologies, catalog no. A213) for 25 min, followed by incubation with 100 ng/ml secondary donkey anti-goat IgG-HRP (Pierce, catalog no. PA 1-28664). Fluorescence was measured in a Tecan Infinite M200 Pro plate reader 25 min after the addition of HRP substrate 10-acetyl-3,7-dihydroxyphenoxazine (AnaSpec, Fremont, CA) with excitation of 530 nm and emission of 590 nm.

The high range of sensitivity of the C3 immunoassay (as well as the others detailed below) is based on the 10-acetyl-3,7-dihydroxyphenoxazine, a substrate for HRP that reacts with hydrogen peroxide to produce a highly fluorescent product. The raw fluorescent readings for the standards range from 1000 to 45,000. Reciprocal plots of standard dilutions (1/concentration) versus fluorescence intensity at 590 nm (1/590 intensity) produce linear equations that allow the interpolation of complement component concentrations from 0 to the lower limit of the standard curve, in addition to values between the lower limit and higher limit of the standard curve (55).

**FB, C3a, and C5a fluorescence immunoassays**—FB, C3a, and C5a levels were measured in unstimulated and TNF-stimulated GMVEC and BMVEC supernatant samples with FB, C3a, and C5a ELISAs using the same protocol as the C3 immunoassay. The Abs and standard curves used were as follows: 1) FB: capture, 100 ng/ml polyclonal goat anti-human FB (Complement Technologies, catalog no. A235); standard, FB protein (Complement Technologies, catalog no. A135) with a range of 12.5–800 ng/ml; detection Abs, 0.1 μg/ml monoclonal mouse antibody to human FB (Quidel Corp., catalog no. A225) that was generated using purified human FB as the Ag and is reactive with both FB and the Ba fragment, and 0.1 μg/ml secondary donkey anti-mouse IgG-HRP (Pierce, catalog no. PA1-28748). 2) C3a: capture, 50 ng/ml polyclonal rabbit anti-human C3a (Complement Technologies, catalog no. A218); standard, purified C3a des-Arg protein (Complement Technologies, catalog no. A119) with a range of 61 pg/ml to 39 ng/ml; detection Abs, 100 ng/ml mouse mAb to human C3a (Pierce, Thermo Fisher Scientific, catalog no. GAU 013-16-02) and 0.25 μg/ml secondary goat anti-mouse IgG-HRP (Rockland Immunodiagnostic Chemicals, Limerick, PA). 3) C5a: capture, 100 ng/ml polyclonal rabbit anti-human C5a (Complement Technologies, catalog no. A221); standard, C5a des-Arg protein (Complement Technologies, catalog no. A145) with a range of 0.156–10 ng/ml; detection Abs, 0.1 μg/ml mouse monoclonal anti-human C5a (Pierce, Thermo Fisher Scientific, catalog no. MA 1-40162) and 0.25 μg/ml secondary goat anti-mouse IgG-HRP.

**Ba and C5a ELISA kits**—Ba levels were measured in unstimulated and TNF-stimulated GMVEC and BMVEC supernatant samples using an ELISA kit (Quidel, San Diego, catalog no. A033). C5a levels were measured in unstimulated and TNF-stimulated BMVEC (but not GMVEC) supernatant samples by an ELISA kit (Quidel, catalog no. A021) as a means to confirm the results from the C5a fluorescence immunoassay.

**Measurement of activated PC from BMVEC and GMVEC supernatant**

In these experiments, activated PC was assayed by ELISA from the supernatant of unstimulated or TNF-stimulated GMVECs and BMVECs after the addition of PC and thrombin.

**PC/thrombin supplementation, supernatant collection, and TNF stimulation**—GMVECs and BMVECs confluent in T-75 flasks were incubated for 24 h in serum-free media. The cells were then washed with PBS and supplemented with 0.2 μM human PC (Hematologic Technologies, catalog no. HCP-0070) and 10 nM human α-thrombin (Hematologic Technologies, catalog no. HCT-0020) in 1 ml of activated PC buffer (0.1% BSA, 3 mM CaCl₂, 0.6 mM MgCl₂ in Ca²⁺/Mg²⁺-free PBS) (33) and incubated at 37 °C for 60 min (33, 81). Further activation of PC to activated PC was inhibited with the addition of 10 nM hirudin (1.5 units/ml, Sigma, catalog no. H7016) (33). Supernatants were collected and frozen in liquid nitrogen for 20 s prior to storage at −80 °C until analysis. These samples were designated as the unstimulated controls. After allowing recovery for at least 24–48 h in complete media (CM131), the same flasks were incubated for 24 h in CM131 plus 10 ng/ml TNF and then for an additional 24 h in serum-free media plus 10 ng/ml TNF. The flasks were again washed with PBS and supplemented with 0.2 μM human PC and 10 nM human α-thrombin in 1 ml of activated PC buffer and incubated at 37 °C for 60 min. Supernatant was collected, flash-frozen, and stored at −80 °C. These samples were designated as the TNF-stimulated samples. The 24–48 h recovery time in CM131 after collection of control
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