

C3a Mediates Endothelial Barrier Disruption in Brain-Derived, but Not Retinal, Human Endothelial Cells

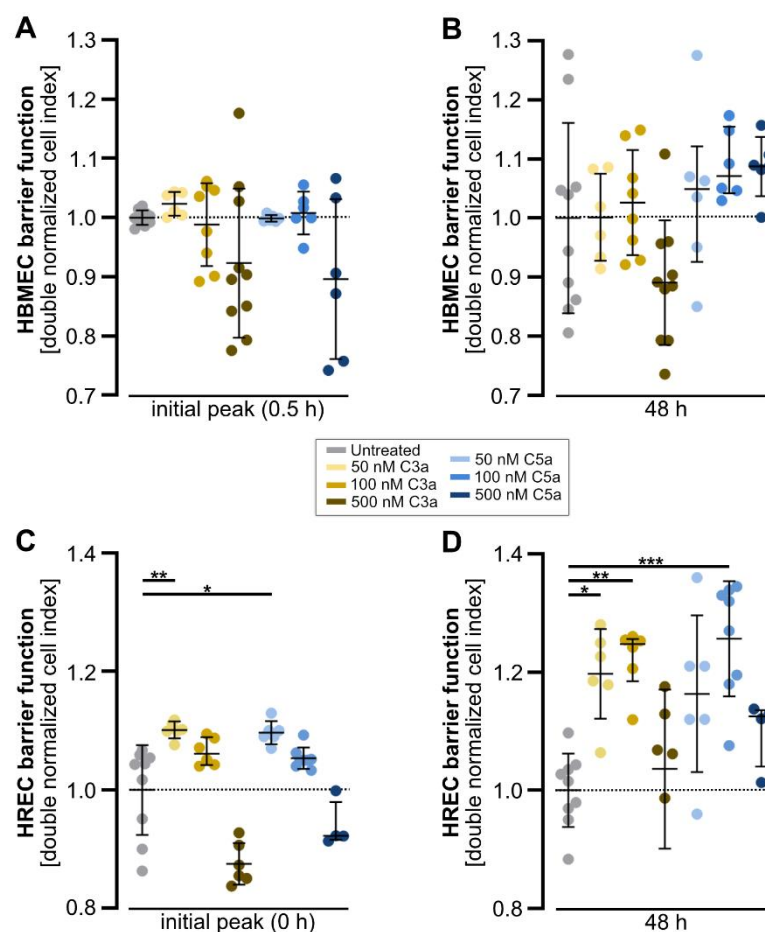
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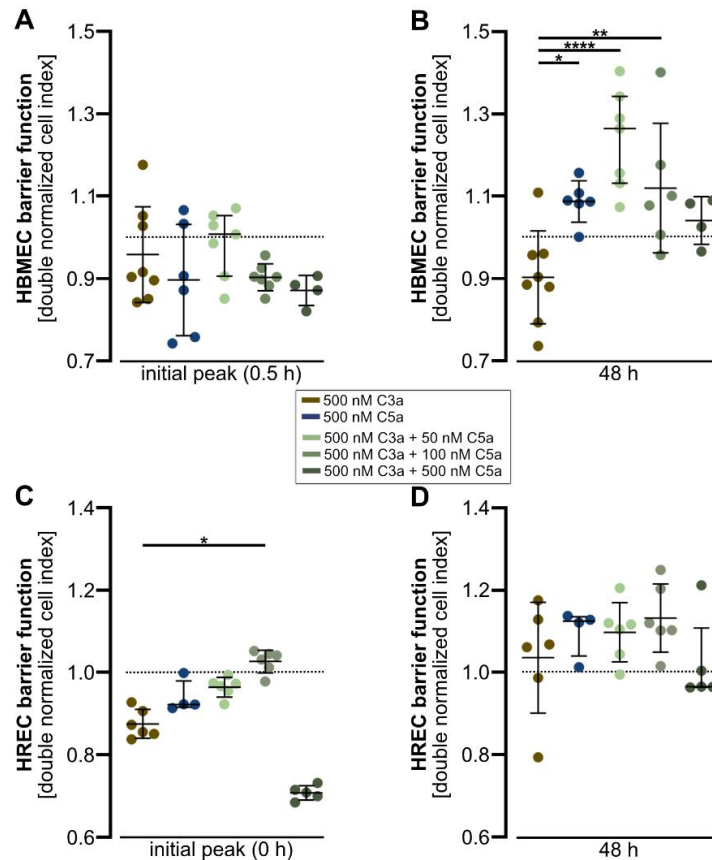
† These authors contributed equally to this work.

SUPPLEMENTARY MATERIAL

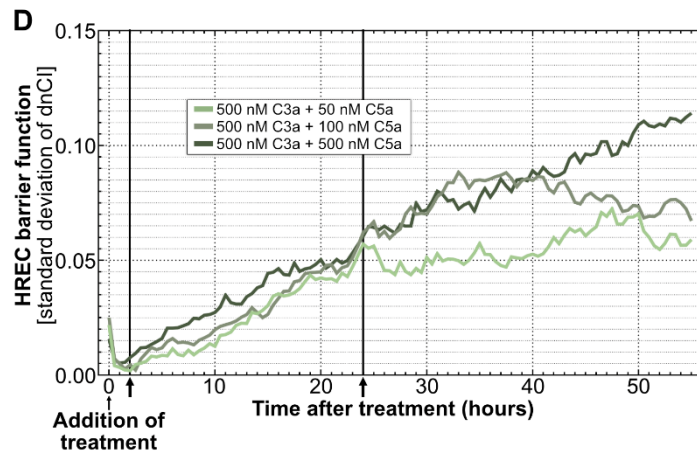
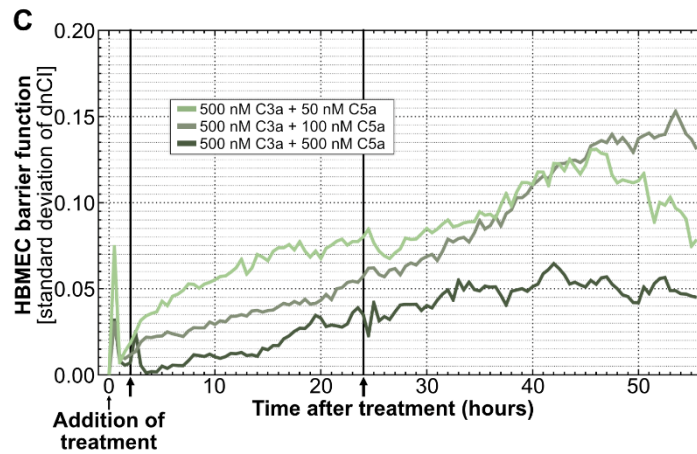
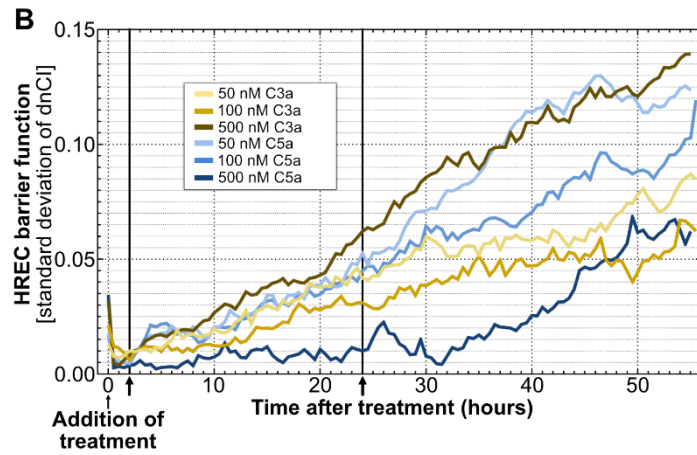
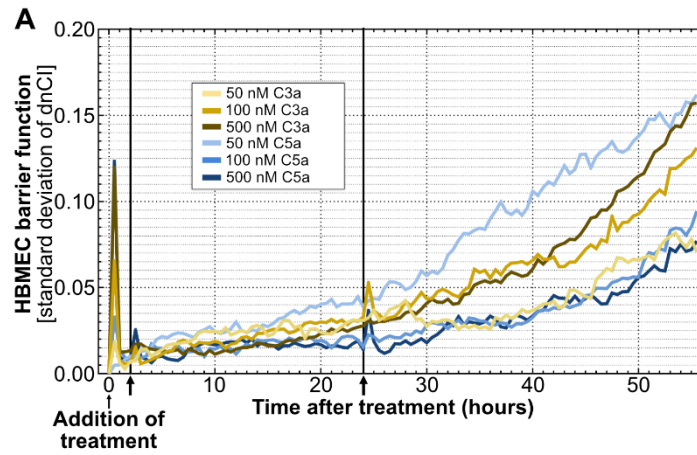


Supplement Figure S1 **Low-dose stimulation of HREC with anaphylatoxins C3a and C5a increase the dnCI.** (A) DnCI in HBMEC remained unaltered after exposure to 50 nM, 100 nM and 500 nM C3a and C5a for 0.5 hours. (B) After 48 hours of exposure to anaphylatoxins, there was no change in dnCI between treatment groups. (C) Immediately after treatment initiation, 50 nM C3a and 50 nM C5a significantly increased the dnCI in HREC when compared with the untreated control. (D) 48 hours after exposure to anaphylatoxins, the dnCI was significantly increased in 50 nM C3a, 100 nM C3a and 100 nM C5a treated HREC when compared with the untreated control. (A, B) RTCA graph represents data from $n = 10$ for untreated and 500 nM C3a, $n = 6$ for 50 nM C3a and 50, 100 and 500 nM C5a and $n = 8$ for 100 nM C3a treated cells, respectively. (C, D) RTCA graph represent data from $n = 9$ for

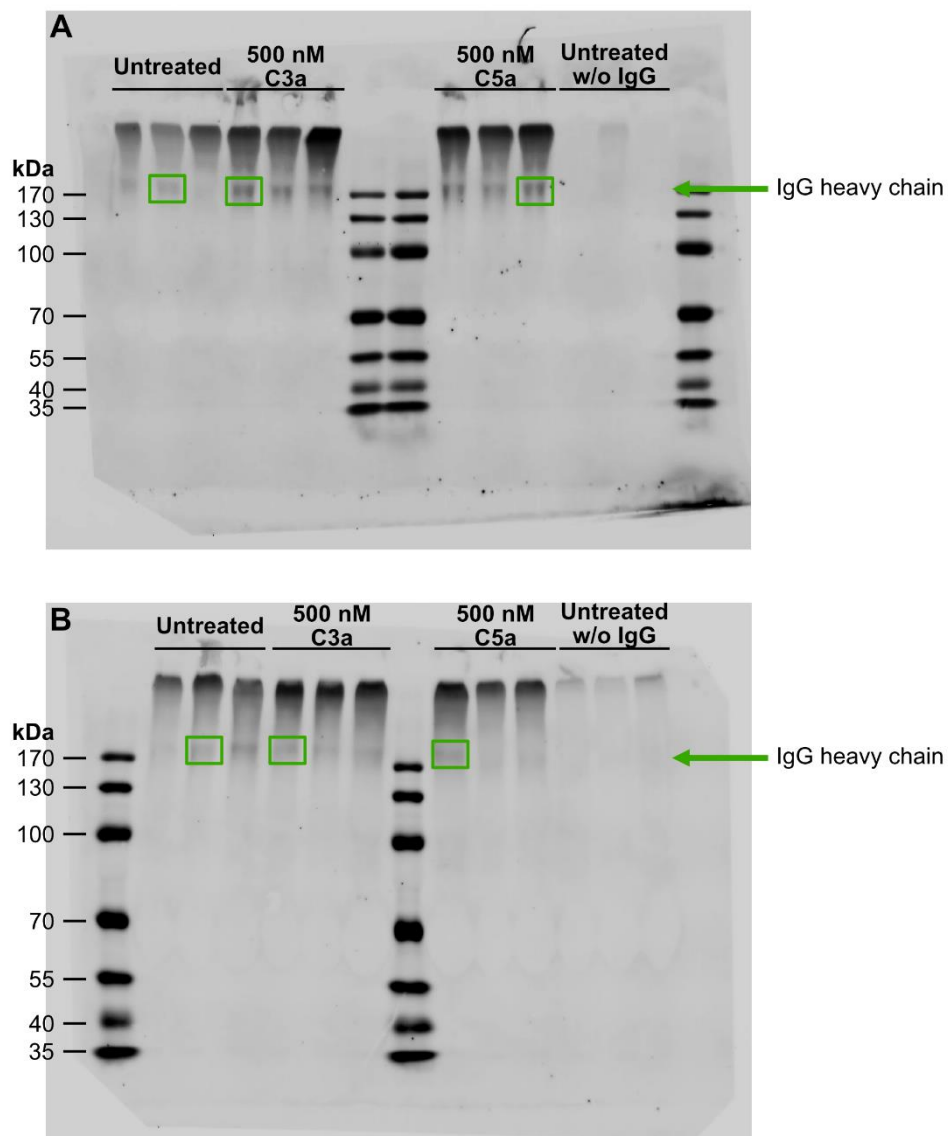
untreated, $n = 6$ for 50 nM C5a and 50, 100 and 500 nM C3a, $n = 8$ for 100 nM C5a and $n = 4$ for 500 nM C5a, respectively. **(A, B, C, D)** Two and 24 hour time points were analysed as separate datasets. Kruskal-Wallis Test with Dunn's multiple comparisons test was used for datasets including non-parametric data. One-way ANOVA with Dunnett's T3 post hoc test for parametric data sets. * $p < .05$, ** $p < .01$, *** $p < .001$.



Supplement Figure S2 **C5a attenuates the C3a-mediated disruptive effect on the endothelial monolayer after 48 hours of exposure in HBMEC.** **(A)** 500 nM C3a + 50 nM C5a, 500 nM C3a + 100 nM C5a and 500 nM C3a + 500 nM C5a altered the dnCI in HBMEC 0.5 hours after treatment initiation when compared to 500 nM C3a treated HBMEC. **(B)** After 48 hours exposure to 500 nM C3a + 50 nM C5a and 500 nM C3a + 100 nM C5a significantly attenuated of the dnCI compared to 500 nM C3a treatment in HBMEC. **(C)** Immediately after treatment initiation, 500 nM C3a + 100 nM C5a increased the dnCI in HREC compared to 500 nM C3a treated cells. **(D)** The dnCI remained unaltered in all treatment groups after 48 hours of anaphylatoxin treatment in HREC when compared with 500 nM C3a treated cells. **(A, B)** RTCA graph represents the mean of data from $n = 9$ for 500 nM C3a, $n = 6$ for 500 nM C5a and $n = 7$ for 500 nM C3a + 50 nM C5a and 500 nM C3a + 100 nM C5a and $n = 4$ for 500 nM C3a + 500 nM C5a treated cells, respectively. **(C, D)** RTCA graph represents the mean of data from $n = 6$ for 500 nM C3a, 500 nM C3a + 50 nM C5a and 500 nM C3a + 100 nM C5a and $n = 4$ for 500 nM C5a and $n = 5$ for 500 nM C3a + 500 nM C5a. **(A, B, C, D)** Two and 24 hour time points were analysed as separate datasets. Kruskal-Wallis Test with Dunn's multiple comparisons test was used for datasets including non-parametric data. One-way ANOVA with Dunnett's T3 post hoc test was used for parametric data sets. * $p < .05$, ** $p < .01$, *** $p < .0001$.



Supplement Figure S3 **The standard deviation of dnCI positively correlates with cultivation time across all treatment groups in the RTCA.** (A) The dnCI standard deviation peaked during the initial search in dnCI and increased over time in all HBMEC treatment groups, particularly after the 24 hour analysis time point. (B) DnCI increased in all HREC treatment groups, with 500 nM C5a maintaining a very low standard deviation until 40 hours after treatment. (C) The combination treatment of C3a and C5a showed an increasing standard deviation in dnCI over the time of HBMEC cultivation. (D) In HREC, the standard deviation among all combination treatments was similar until the 24 hours analysis timepoint, after which it remained stable in 500 nM C3a + 50 nM C5a treated cells. (A) RTCA graph represents data from $n = 10$ for untreated and 500 nM C3a, $n = 6$ for 50 nM C3a and 50, 100 and 500 nM C5a and $n = 8$ for 100 nM C3a treated cells, respectively. (B) The RTCA graph represent data from $n = 9$ for untreated, $n = 6$ for 50 nM C5a and 50, 100 and 500 nM C3a, $n = 8$ for 100 nM C5a and $n = 4$ for 500 nM C5a, respectively. (C) The RTCA graph represents the mean of data from $n = 9$ for 500 nM C3a, $n = 6$ for 50 nM C5a and $n = 7$ for 500 nM C3a + 50 nM C5a and 500 nM C3a + 100 nM C5a and $n = 4$ for 500 nM C3a + 500 nM C5a treated cells, respectively. (D) The RTCA graph represents the mean of data from $n = 6$ for 500 nM C3a, 500 nM C3a + 50 nM C5a and 500 nM C3a + 100 nM C5a and $n = 4$ for 500 nM C5a and $n = 5$ for 500 nM C3a + 500 nM C5a.



Supplement Figure S4 **Uncropped Western blots for (A) HBMEC and (B) HREC in Figure 8B.**

Supplement Table S1 In-silico validation of in-house designed primers.

General information		in silico primer validation								
Transcript	Primer sequence	UCSC in silico PCR					NCBI Primer blast			Primer Design
		Product(s)	Ensembl ID	no. of amplicons	amplicon size	Target sequence (Top Hit)	NCBI blast (Top Hit)	amplicon size	mismatch	Category
CDH5	GGACCGAGAGAAGCAGGCCA	CDH5	ENST00000341529.8	3	148	GGA CCGAGAGAAGCA GCCCAggt at gagat cgt ggt ggaagcgcg agat gccaggccctccggg gggactcgggcacgccaccg tgctggtcactctgcaagacat caatgacaacttccctTCTT CACCCAGACCAAGTAC ACA	NM_001795.5 Homo sapiens cadherin 5 (CDH5)	148	--	Ex5 Ex6 spanning
	TGTGTACTTGGTCTGGGTGAAGA									
C3	AAGAACCGCTGGGAGGACCC	C3	ENST00000695652.1	8	123	AAGAACCGCTGGGAG GACCCtggttaagcagctcta caacgtggaggccacatccta tgccctcttggccctactgcagc taaaagactttgactttgtgcc tcCCGTCTGTGCGTTGG CTCAAT	C3	123	--	Within exon 29
	ATTGAGCCAACGCACGACGG									
C3AR1	TTCCGAATGCAAAGGGGCCG	C3AR1	ENST00000307637.5	1	62	TTCCGAATGCAAAGG GGCCGcttgccaagtctca ggcaaaACCTTTCGAGT GGCCGTGGT	NM_001326477.2 Complement C3a receptor 1 (C3AR1), transcript variant 3 -1	62	--	Within exon 2
	ACCACGGCCACTCGAAAGGT									
GAPDH	CCCCACCACACTGAATCTCC	GAPDH	ENST00000229239.10	4	105	CCCCACCACACTGAAT CTCCctctctcacagttgcc tgtagacccttgaaagggg aggggcctagggagccgcacC TTGTCATGTACCATCA ATAAAGTACC	NM_001357943.2 GAPDH, transcript variant 7	105	--	Ex1 Ex3 spanning Ex2 Ex4 spanning
	GGTACTTTATTGATGGTACATGACAAG									

Supplement Table S2 Wet lab validation of in-house designed primers.

Primer set	PCR product size	Forward Primer	Sequence (bold nucleotides align with NCBI Top Hit)	Reverse Primer	NCBI Blast (Top Hit)	Sequence ID
CDH5	161	GGACCGAGAGAAGCAG GCCA	GGATTTGTAGTAGCGCGAG ATGCCCAGGGCCTCCGGGG GGACTCGGGCACGGCCACC GTGCTGGTCACTCTGCAAG ACATCAATGACAACTTCCCC TTCTTCACCCAGACCAAGTA CACAAACTC		cadherin 5 (CDH5)	NM_001795.5
			GGATTGTATTGATGTCTTGC AGAGTGACCAGCACGGTGG CCGTGCCCCGAGTCCCCCG GAGGCCCTGGGCATCTCGC GCTTCCACCACGATCTCAT ACCTGGCCTGCTTCTCTCG GTCCAAGCGTACCCCT	TGTGTACTTGGTCTGGGTGA AGA	cadherin 5 (CDH5)	NM_001795.5
GAPDH	105	CCCCACCACACTGAAT CTCC	AGGGAAGGGGGCCTAGGG AGCCGCACCTTGTCTATGTA CCATCAATAAAGTACCAATT CG		glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001289745.3
			CTCGTCTCAAGGGGGTCTA CATGGCAACTGTGAGGAGG GGAGATTCAGTGTGGTGGG GAAT	GGTACTTTATTGATGGTA CATGACAAG	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001289745.3