Calcineurin inhibitor-induced endothelial cell injury – A role for complement

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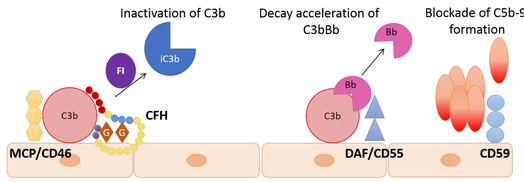
INTRODUCTION

- Calcineurin inhibitors (CNI) are widely used immunosuppressive agents
- CNI use is associated with development of thrombotic microangiopahy (TMA)
  - Incidence 3.5 - 14% (*Zarifian et.al. Kidney Int. 1999; Bren et.al. Transplant Proc 2002; Al-Nouri et.al. Blood 2015)*
  - Can lead to organ failure, graft loss and death but can also remain subclinical
- Evolving *in-vivo* and *in-vitro* evidence suggest a role for complement dysregulation in the pathogenesis of CNI-induced TMA via induction of endothelial cell (EC) release of complement-activating microparticles leading to bystander EC injury *(Renner et.al. JASN 2013)*
- Mechanism of CNI-induced complement-mediated EC injury remains poorly understood

OBJECTIVES

- To determine whether cyclosporine (CsA) induces complement activation on EC
- To unravel the mechanism of CsA-induced complement activation on EC



Complement regulation on EC surfaces is provided by complement factor H (CFH) and membrane-bound regulators MCP/CD46, DAF/CD55 and CD59.

We hypothesize that CsA impairs complement regulation on EC surfaces by impairing the function of CFH and downregulation of MCP/CD46, DAF/CD55 and CD59.

METHODS

- Blood outgrowth endothelial cells (BOEC) derived from peripheral blood of healthy donors and an aHUS patient (MCP/CD46 frameshift variant c.747dupA (p.Ala250Serfs*6) were cultivated to confluence in EBM-2 media (passages 3-10)
- Experimental conditions:
  - Various concentrations/duration of CsA
  - 50% normal human serum (NHS; complement active) served as complement source
  - A previously established method of complement fixation on EC using anti-CD59 (blocking) antibody (*Noone et.al. Kidney Int 2016, Riedl et.al. Kidney Int Rep 2016)*

RESULTS

Cyclosporine leads to dose & time dependent endothelial cell cytotoxicity (data not shown).

Cyclosporine leads to dose-dependent EC complement deposition

The sequence of CsA incubation for 24 h followed by 50% NHS for 30 min resulted in a dose-dependent C3c deposition on EC surface. This effect was exacerbated by serum starvation and by additional sensitization with anti-CD59 antibody.

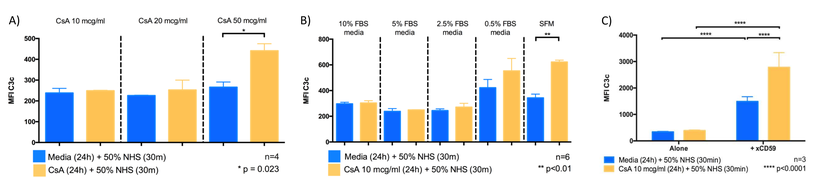


Figure 1: C3c deposition on EC surface by flow cytometry. Non-viable cells excluded from analysis with Fixable Viability Dye eFluor® 780. **A)** Incubating EC with increasing doses of CsA resulted in significantly higher C3c deposition. **B)** Incubating EC with CsA reconstituted in media supplemented with decreasing amounts of fetal bovine serum (FBS) resulted in significantly higher C3c deposition. **C)** Addition of anti-CD59 enhanced CsA-induced C3c deposition. This previously established strategy combines the effect of complement induction via sensitization (classical pathway) and amplification (alternative pathway).

Finding 1:
Cyclosporine-induced complement C3c deposition on EC is dose-dependent - can be enhanced by serum starvation and by anti-CD59 sensitization

RESULTS

Cyclosporine leads to enhanced expression of surface complement regulators

To protect against complement activation, ECs express surface complement regulators membrane cofactor protein (MCP/CD46), decay accelerating factor (DAF/CD55) and protectin (CD59). The sequence of CsA incubation for 24h resulted in increased expression of MCP/CD46, DAF/CD55 and CD59 on EC surface.

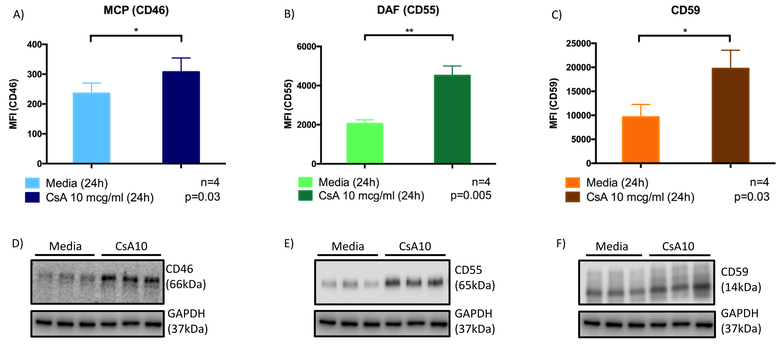


Figure 2: (A, B, C) EC surface complement regulators by flow cytometry. Non-viable cells excluded from analysis with Fixable Viability Dye eFluor® 780. **(D, E, F) Complement regulators in cell lysate by western blot.** Compared to media, incubating ECs in CsA 10 µg/ml for 24 h resulted in enhanced expression of complement surface regulators: **A & D)** MCP/CD46, **B & E)** DAF/CD55, and **C & F)** CD59.

Cyclosporine leads to reduced complement factor H surface binding and cofactor activity

Complement factor H (CFH) is a cofactor to complement factor I (CFI) and MCP in inactivating surface-bound C3b. This surface regulatory effect of CFH requires its binding to EC surfaces. CsA incubation for 24 h led to reduced CFH surface binding (Fig. 3F) and regulation of C3b as evidenced by the late appearance of the α'2 (46 kDa) and α'2 (43 kDa) fragment (Fig. 3E).

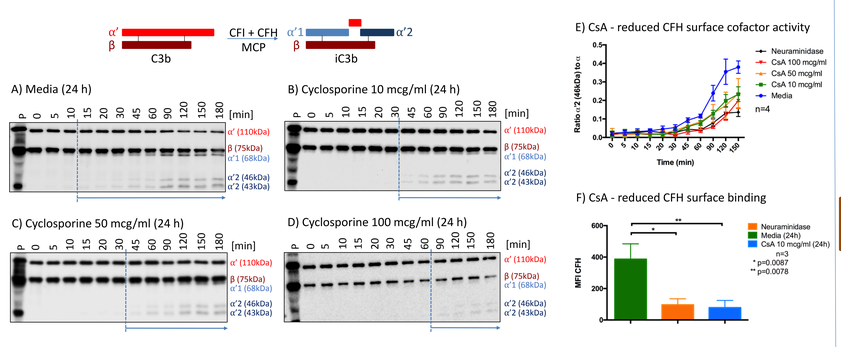


Figure 3: CFH surface cofactor activity. ECs were treated with **A)** media, **B)** CsA 10 mcg/ml, **C)** CsA 50 mcg/ml or **D)** CsA 100 mcg/ml for 24h. They were then incubated with CFH 10 mcg/ml (1h), washed with phosphate-buffered saline (PBS) and incubated with CFI 9mcg/ml and C3b 3.3mcg/ml. Supernatant was transferred into sample buffer at various time-points, separated by SDS-PAGE and C3b degradation fragments were detected by Western blotting. **E)** CsA led to reduced CFH surface cofactor activity. **F)** CsA led to reduced surface-bound CFH-Alexa Fluor® 488 by flow cytometry (n=3, *p=0.0087, **p=0.0078). Neuraminidase cleaves sialic acid groups from EC surfaces. EC were pre-incubate with Neuraminidase 500 mU/ml for 1h.

Finding 2:
Cyclosporine leads to increased expression of surface complement regulators and reduced CFH surface binding and cofactor activity

RESULTS

Cyclosporine leads to diminished endothelial cell glycocalyx

CFH surface regulation requires its binding to glycosaminoglycans and sialic acid residues on EC glycocalyx. Because we found that CsA impaired CFH surface binding and regulation of C3b, we assessed the effect of CsA on EC glycocalyx. CsA incubation for 24 h led to diminished EC glycocalyx (Figure 4).

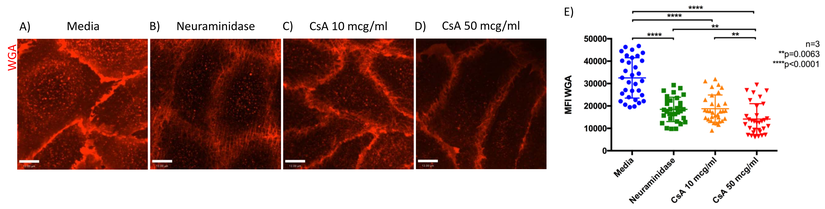


Figure 4: Live cell imaging of EC glycocalyx with Alexa Fluor® 594-conjugated wheat germ agglutinin (WGA). ECs were treated with **A)** media, **C)** CsA 10 mcg/ml, or **D)** CsA 50 mcg/ml for 24h. **B)** EC were pre-incubated with neuraminidase 500 mU/ml for 1h. Neuraminidase cleaves sialic acid groups from EC glycocalyx. WGA binds to sialic acid and N-acetylglucosaminyl residues on EC glycocalyx. **E)** CsA led to less intense staining with Alexa Fluor® 594-conjugated WGA (n=3, **p=0.0063, ****p<0.0001). Mean fluorescence intensity was measured with ImageJ. Total of 3 sets of experiments with 10 representative images taken per condition (each dot represents 1 image).

Finding 3: Cyclosporine leads to diminished endothelial cell glycocalyx

MCP BOEC - deficient in MCP/CD46, and are more susceptible to CsA-induced complement deposition

BOEC isolated from a patient with aHUS secondary to a frameshift variant in the MCP/CD46 gene were characterized and their EC phenotype confirmed by immunofluorescence (vWF, p-selectin) and flow cytometry (VE-cadherin/CD144, PECAM-1/CD31) (data not shown). MCP BOECs were deficient in membrane-bound MCP/CD46 (Figure 5A-C) and were more susceptible to CsA-induced complement C3 deposition (Figure 5D).

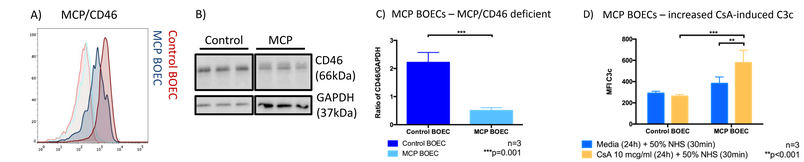


Figure 5: MCP BOEC. A, B, C) MCP BOECs had reduced expression of MCP/CD46 by **A)** flow cytometry and **B, C)** Western blot (n=3, ***p=0.001) when compared to control BOECs. **D)** MCP BOECs demonstrated higher CsA-induced C3c deposition on EC surface by flow cytometry when compared to control BOECs (n=3, **p<0.001). Non-viable cells were excluded with Fixable Viability Dye eFluor® 780.

Finding 4: MCP BOECs demonstrate endothelial phenotype, have reduced expression of MCP/CD46, and are more susceptible to cyclosporine-induced complement deposition

CONCLUSION

- Our findings suggest a role for cyclosporine-induced, complement-mediated EC injury
- Key role for CFH surface dysregulation in CsA-induced complement activation on EC surfaces
- CsA-induced injury to EC glycocalyx may be the key mechanism leading to alternative pathway dysregulation – warrants further studies
- BOECs from aHUS patient with pathogenic MCP/CD46 variant are deficient in membrane-bound MCP/CD46 and have genetically predisposed susceptibility to CsA-induced complement deposition