

Title: Protein Kinase C- θ interacts with mTORC2 and vimentin to limit Regulatory T-cell function.

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Abstract: max 3,800 characters (spaces not included)

Regulatory T-cells (Tregs) play a critical role in preventing autoimmune and alloimmune reactions, including graft-versus-host disease (GVHD). Two recent clinical trials demonstrated that in patients undergoing hematopoietic stem cell transplantation, adoptive transfer of Tregs significantly reduced the incidence of grades II-IV GVHD. While Tregs significantly reduced GVHD severity, they did not eliminate GVHD. One potential way to augment Treg-mediated inhibition of GVHD is to increase Treg suppressive potency. We showed previously that Treg-specific inhibition of protein kinase C- θ (PKC- θ) enhances Treg function (*Science* 328:372, 2010). However, it is unclear whether PKC- θ inhibition can boost Treg function in a systemic inflammatory condition like GVHD. Furthermore, the mechanism by which PKC- θ inhibition augments Treg function is unknown. In this study, we address these unanswered questions.

Using a mouse MHC class I/II disparate acute GVHD model, we found that freshly isolated Tregs treated for 30 minutes with 10uM of the clinically available PKC- θ inhibitor AEB071 suppressed GVHD mortality (**Fig 1A**) and severity significantly better than DMSO treated Tregs. As Tregs exert much of their protective effect against GVHD early in the course of the disease, we analyzed proliferation of GVHD-causing conventional T-cells (Tcon) on D4 after transplant. We observed a significant reduction in Tcon proliferation in mice given AEB071 treated Tregs compared to DMSO treated Tregs. We then performed multi-photon microscopy on D4 after transplant using TE α -GFP Tcon, CD11c-eYFP antigen presenting cells (APCs) and wild-type Tregs. Compared to DMSO, AEB071 treated Tregs significantly increased Tcon velocity and displacement from APCs. Increased velocity and displacement are indicative of decreased Tcon-APC interactions, suggesting reduced priming when AEB071 Tregs are present.

Mechanistically, AEB071 vs DMSO treatment of Tregs resulted in augmented expression of the suppressive molecules Neuropilin-1 (Nrp1) and Lymphocyte activation gene 3 (Lag3) after *in vitro* activation (**Fig 1B, C**) and in Tregs isolated from acute GVHD mice. Antibody blockade of Nrp1 and Lag3 in *in vitro* transwell suppression assays reduced the effect of AEB071 treatment, suggesting that these molecules may play a role in enhancing Treg function after PKC- θ inhibition. Flow cytometry analysis of phosphorylated proteins in activated Tregs revealed that PKC- θ inhibition resulted in reduced phosphorylation of the mTORC2 target FoxO3a, but not mTORC1 targets S6 and 4E-BP1. In addition, the mTORC2-specific phosphorylation site on Akt, serine 473, was reduced, whereas the mTORC1 specific site, threonine 308, was unaltered. Together, these data suggest reduced mTORC2 activity. Reduced phosphorylation increases Foxo3a nuclear translocation, which may result in increased Nrp1 and Lag3 expression, since Foxo3a has binding sites in both gene promoters. As both mTORC1 and 2 are involved in T-cell metabolism, we investigated the effect of AEB071 treatment on Treg oxygen consumption rate (OCR). Compared to DMSO, AEB071 treatment significantly increased Treg baseline and maximal OCRs after activation (**Fig 1D**). Increased OCR has been associated with increased Treg function.

To identify additional alterations in phosphorylated proteins after PKC- θ inhibition, we performed a phosphoproteomic screen using *in vitro* expanded human Tregs treated with AEB701 or DMSO. We identified significant alterations in phosphorylation sites on 72 proteins, including reduced phosphorylation of an adaptor molecule that links PKC- θ to the intermediate filament vimentin. We found that vimentin is highly upregulated in Tregs compared to Tcon and that in Tregs, vimentin interacts with PKC- θ after activation. AEB071 treatment reduced the interaction between vimentin and PKC- θ . As with AEB071 treatment, Vimentin siRNA significantly increased Treg suppression *in vitro* compared to control transfected Tregs (**Fig 1E**), and augmented expression of Nrp1 and Lag3. AEB071 treatment of vimentin siRNA transfected Tregs did not further augment Treg function, suggesting an overlapping mechanism.

In summary, our data demonstrate that PKC- θ interacts with mTORC2 and vimentin to modulate multiple aspects of Treg function, and that a brief incubation of Tregs with a PKC- θ inhibitor may be a viable method to enhance the efficacy of Treg therapeutics.

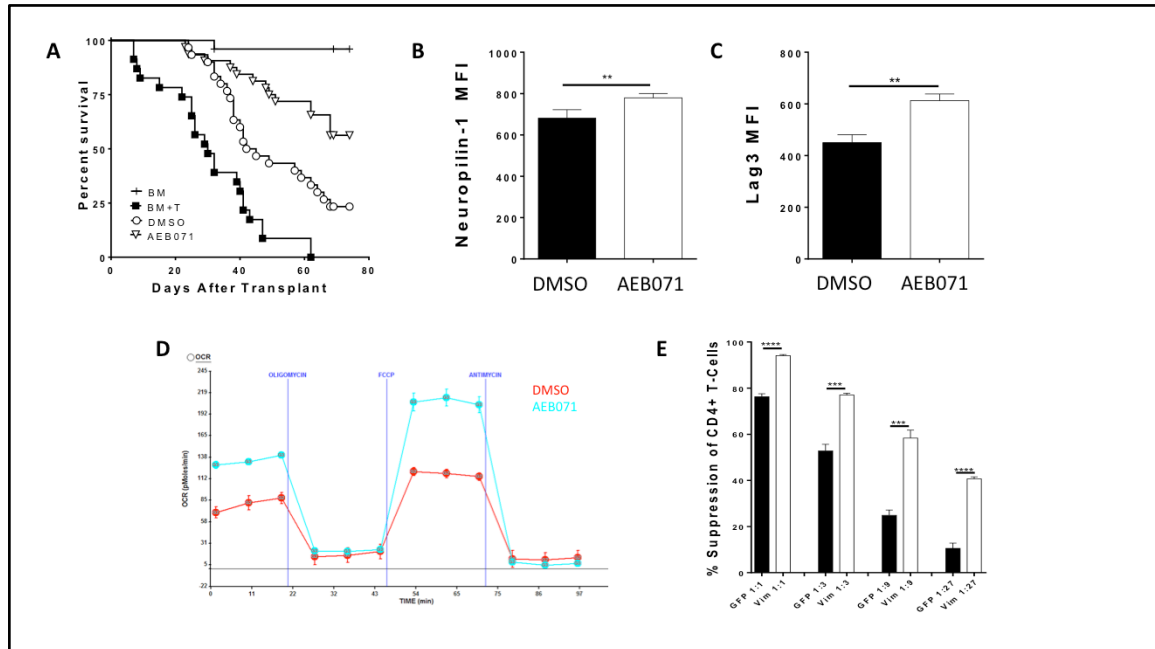


Figure 1. PKC- θ inhibition and vimentin siRNA treatment increase Treg function. **A) Acute GVHD:** Lethally irradiated BALB/c mice were given 10e6 C57BL/6 bone marrow only (BM) only, or BM with 2e6 CD4+25-/CD8+25- Tcon cells without (BM+T) or with 1e6 Tregs treated with DMSO (DMSO) or 10uM AEB071 (AEB071) for 30 minutes. Compared to DMSO, Tregs treated with AEB071 significantly increased **(A)** recipient survival ($p = 0.0036$). **B and C) Treg activation:** Tregs were treated with DMSO or AEB071 as above, and activated overnight with plate bound anti-CD3/28 and IL-2. AEB071 treatment significantly increased **(B)** Neuropilin-1 and **(C)** Lymphocyte activation gene 3 (Lag3) surface expression. **D) Treg metabolic activity:** Tregs were treated with DMSO or AEB071 and activated as above. Oxygen consumption rate (OCR) analysis revealed that AEB071 treatment significantly increased baseline Treg OCR (time 0-21 min) and maximal OCR (time = 44 - 68 min). **E) Treg suppression:** Tregs were transfected with vimentin siRNA (vim) or control (GFP), and activated for 36 hours. CFSE labeled Tcon were mixed with T-cell depleted splenocytes and soluble anti-CD3 mAb. Treg:Tcon ratios of 1:1 – 1:27 were plated and CFSE dilution assessed after 3 days.