# Inhibition of Integrin αvβ8 enhances immune checkpoint induced anti-tumor immunity by acting across immunologic synapse in syngeneic models of breast cancer

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## INTRODUCTION

We explored whether integrin αvβ8 inhibition potentiates immune checkpoint blockade (ICB) in syngeneic orthotopic models of breast cancer. Integrin αvβ8 mediates cell type specific and tissue localized activation of TGFβ1/3 to regulate the immune system. For example, αvβ8 expressed on dendritic cells (DC) in the intestine has been shown to be a key mediator of tolerance, maintaining gut immunologic homeostasis. αvβ8 on regulatory T cells (Tregs), macrophages, cancer cells, and fibroblasts sustains an immunosuppressive tumor micro-environment. Herein we propose that inhibition of αvβ8 can reverse anti-tumor tolerance and sensitize tumors to ICB-therapy.

## AIMS and METHODS

**Study Aims**

- Evaluate efficacy of αvβ8 inhibitor in combination with ICB in checkpoint -resistant syngeneic models of breast cancer
- Understand mechanism of action of αvβ8 antagonism

**Methods**

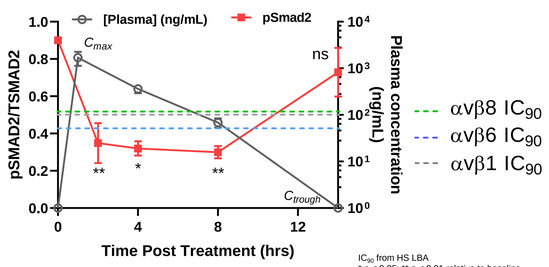
Efficacy was evaluated in combination with anti-PD-1 in EMT6 and PyMT breast cancer syngeneic mouse models. A potent integrin small molecule inhibitor (integrin SMi) was orally administered at 60 mg/kg BID for 21 days. Anti-αvβ8 or non-isoform specific anti-TGFβ mAbs were dosed TIWx3 at 7 and 10 mg/kg, respectively. Tumor volumes are presented as mean±SEM. CD8 depletion studies were performed with αCD8 clone 2.43 dosed at day 5, 8, 13, 20 as described in Mariathasan, et al.; 2018. Mice were euthanized at tumor volume of 2000 $mm^3$, or combined volume of 2000 $mm^3$ if two tumor cell lines were implanted. Statistics were performed by t-test, one-way ANOVA, or log-rank test. Flow cytometry and transcriptome analysis on bulk and single-cell levels were used to assess the mechanism of action in EMT6.

## INTEGRIN SMALL MOLECULE INHIBITOR

| Cyclobutylaminoacid |
|---|
| αvβ8 $IC_{50}$ HS LBA: 30 nM |
| αvβ6 $IC_{50}$ HS LBA: 10 nM |
| αvβ1 $IC_{50}$ HS LBA: 30 nM |

HS LBA -serum shifted ligand binding assay

### Integrin SMi inhibits TGF-β canonical pathway

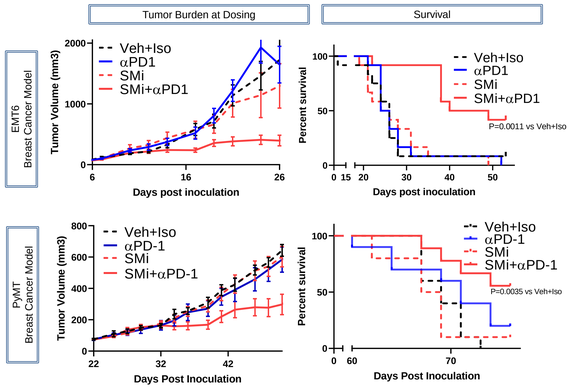



$IC_{90}$ from HS LBA
* p < 0.05; ** p < 0.01 relative to baseline

The pharmacodynamic (PD) effect on pSmad2 correlated with systemic integrin SMi pharmacokinetics (PK). Oral integrin SMi evaluated in EMT6 model showed a significant reduction in pSmad2 between 2h and 8h after single-dose administration. The inhibition of pSmad2 correlated with full target coverage for 8h (based on HS LBA) and returned to the level observed in untreated tumors by 14h when the drug plasma level was below the detection limit. Similar levels of inhibition were seen with a pan-αv small molecule inhibitor and anti-TGFβ mAb (data not shown).

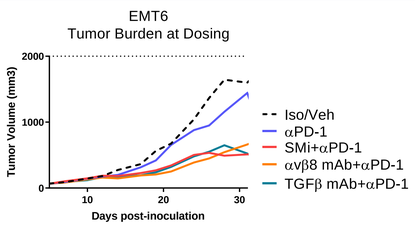
## RESULTS

### Integrin SMi enhances the effect of ICB therapy in EMT6 and PyMT models



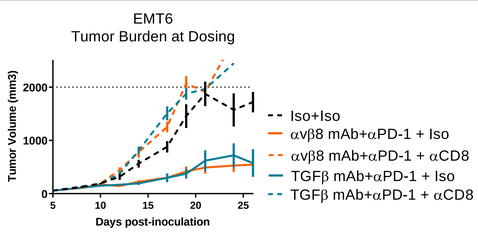

Efficacy of integrin SMi as monotherapy or combined with αPD1 against syngeneic models of breast cancer. Combination of oral integrin SMi with αPD-1 was efficacious in the primary ICB resistant EMT6 model and resulted in superior tumor regression during treatment (p=0.0011) and improved survival with 5/12 complete responders relative to 0/12 in αPD-1 alone (upper panel). Efficacy was confirmed in the transplanted PyMT breast cancer model (lower panel).

### Integrin SMi phenocopied anti-tumor activity of αvβ8 and TGFβ mAbs




A side-by-side comparison of integrin SMi, αvβ8 mAb and TGFβ mAb in combination with αPD-1 in EMT6 syngeneic model of breast cancer was conducted. All treatments resulted in comparable responses, as measured by tumor burden during the dosing window, confirming that anti-tumor activity is driven by αvβ8 inhibition (n = 3 independent studies).

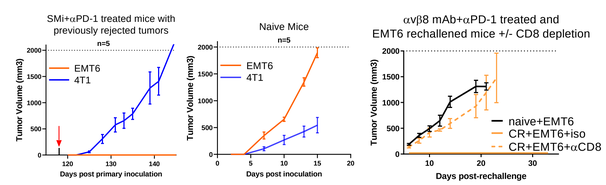
### CD8 T cells mediate the anti-tumor activity of αvβ8 antagonism




A CD8 depletion study was performed to gain insight into the mechanisms of αvβ8 inhibition in anti-tumor activity. CD8 depletion resulted in enhanced tumor outgrowth suggesting that the anti-tumor effect was driven by adaptive immunity rather than by direct anti-proliferative effect on tumor cells. These findings suggest a CD8+ T-cell responses is crucial to tumor eradication. A similar effect was observed for TGFβ mAb + αPD-1, as published (Mariathasan, et al.; 2018).

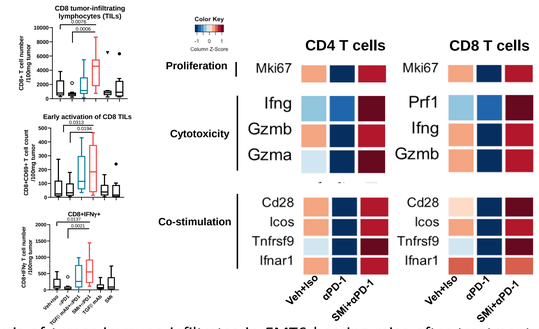
## RESULTS

### Long term immunity is established after αvβ8 antagonism



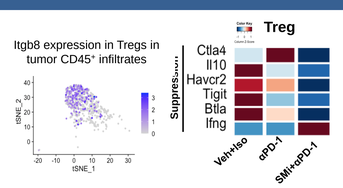

Long-term tumor immunity was established following integrin SMi treatment in EMT6 model. Complete responders (CRs) from EMT6 efficacy study re-challenged (↓) 89 days after treatment with the same tumor showed no outgrowth. In contrast, another tumor type (4T1) was established the same as in naive mice. In an analogous study, EMT6 CRs from αvβ8 mAb+αPD-1 treated mice were re-challenged >50 days after treatment. In this study CD8 T cell depletion led to tumor re-growth further establishing the mechanism of long term tumor immunity.

### T-cells drive anti-tumor immunity during αvβ8 antagonism




Analysis of tumor immune infiltrates in EMT6 bearing mice after treatment with integrin SMi and TGFβ mAb alone and in combination with αPD-1 was performed at 5 days of treatment. Integrin SMi+αPD-1 increased the density of CD8 T cell tumor-infiltrating lymphocytes (TILs) (p=0.0006), early activated CD8 T cells (CD8+CD69+, p=0.0194) and CD8 T cells expressing IFNγ (CD8+IFN-γ+, p=0.0021) relative to αPD-1 alone. The effect was comparable to the treatment with TGFβ mAb+aPD-1 (left panel). Single-cell RNA sequencing was used to further analyze the immune cell composition of tumor infiltrates. Integrin SMi+αPD-1 increased T cell proliferation (Ki67) and expression of effector cytokines including IFNγ, granzymes, and perforins. Moreover, combination treatment increased activation marker signatures like Cd28, Tnfrsf9 (CD137), and Icos further confirming enhanced T cell co-stimulation and activation (right panel).

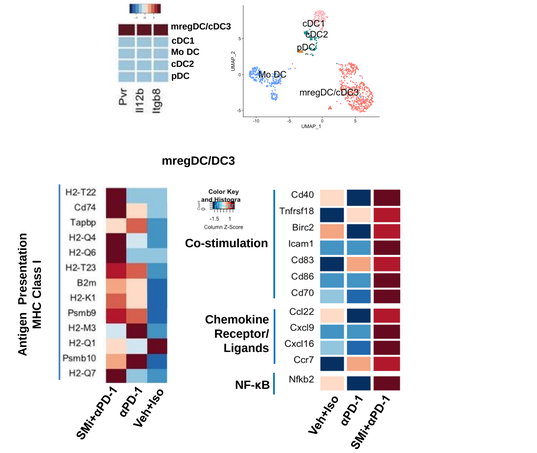
### αvβ8 antagonism attenuates Tregs




Single-cell transcriptomic analysis of tumor CD45+ populations identified that the majority of infiltrating Tregs expressed Itgb8 (left panel). Integrin SMi+αPD-1 treatment attenuated Treg function, demonstrated by downregulation of suppressive cytokines and checkpoint receptors, including Ctla4, Havcr2 (TIM-3) Tigit, Il10, and Btla. Interestingly αvβ8 inhibition increased IFNγ signature in Tregs, further confirming their dysfunction (right panel).

## RESULTS

### αvβ8 regulates tumor tolerance




Single-cell transcriptomic analysis of lymph nodes identified that Itgb8 was specifically expressed on subsets of conventional DCs, co-expressing Ccl22 and Fscn1. αvβ8 was detected in mesenteric lymph nodes consistent with findings showing the crucial role of αvβ8 in tolerance (Paidassi et al., 2011; Esterházy et al., 2016). Based on transcriptional characteristics, and comparison to DCs found in human tumors, αvβ8-positive DCs have had the features of recently described mregDCs/DC3 (Maier, et.al., 2020; Zilionis, et. al, 2019, Garris et.al, 2018) (left panel). Integrin SMi+αPD-1 reversed the tolerogenic phenotype of these cells that had an improvements in MHC class I antigen presentation, and signatures for migration (Ccr7, Cxcl16, Ccl22) and co-stimulation (Cd40, Cd83/6; middle and right panels).

## CONCLUSION

- Morphic integrin SMi in combination with checkpoint inhibitor showed efficacy in ICB resistant models of breast cancer (EMT6 and PyMT)
- Anti-tumor activity was mediated through adaptive immunity, and was dependent on CD8 T cells
- αvβ8 inhibition resulted in increased T cell infiltrates within tumor stroma
- Examination of immune cell gene signatures on immune cell populations within tumor and lymphoid organs provided evidences for reduced tolerance, demonstrated by attenuation of Treg functions and increased activation of DC
- These results show that an orally administered αvβ8 targeted inhibitor is a potent modulator of anti-tumor immune response acting across the immunologic synapse and is a promising therapeutic approach for ICB refractory tumors