Pharmacodynamic Biomarkers in Syngeneic Tumor Models Treated by a GITR Agonist (GITRL-Fc) and Prevalence of GITR Expression

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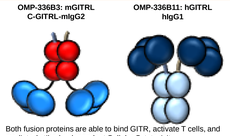
INTRODUCTION

GITRL (Glucocorticoid-Induced Tumor Necrosis Factor Receptor Ligand, TNFSF18) is a member of the tumor necrosis factor (TNF) ligand superfamily. GITRL binds and activates the co-stimulatory surface receptor GITR, which promotes proliferation and activation of effector T cells (T eff) and inhibits suppressive activity of regulatory T cells (T reg), both important to promote anti-tumor immunity.

We generated a novel single-gene GITRL trimer fused to an immunoglobulin Fc domain (GITRL-Fc) that shows robust single agent antitumor efficacy and immune effects in multiple syngeneic mouse models, suggesting its potential benefit in cancer immunotherapy.



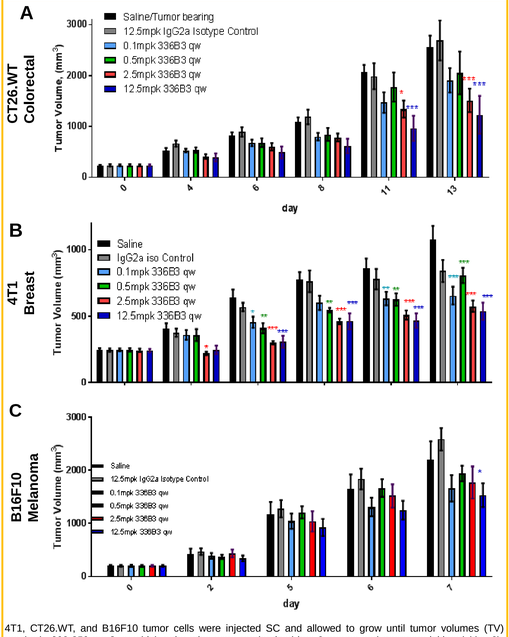
Both fusion proteins are able to bind GITR, activate T cells, and mediate Antibody- dependent Cellular Cytotoxicity.

MATERIALS AND METHODS

In Vivo Studies: 6-8 weeks old female Balb/C and Foxp3.GFP (for 4T1 and CT26.WT) or C57BL/6J (for B16F10) were purchased from Envigo, Charles River Laboratories, or Jackson Laboratories. When tumors were established, mice were randomized into treatment groups and dosed weekly (IgG2a, saline, and 336B3 at 0.1, 0.5, 2.5 or 12.5 mg/kg (mpk)). At the designated time points, tumors, blood, and spleens were harvested and processed for flow cytometry, IHC, and gene expression analysis.
Flow Cytometric Analysis: Single cell suspensions of splenocytes, dissociated tumors, or PBMC were used for staining with fluorochrome labelled antibodies and their isotype controls for membrane markers or intracellular cytokines. Prior to staining for the detection of intra-cellular cytokines, cells were stimulated for 4 hours at 37°C in a CO_2 incubator with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO), 1 mg/ml ionomycin (Sigma, St. Louis, MO) in the presence of Monensin and Brefeldin A (eBioScience, San Diego, CA). The data were acquired and analyzed on a Fortessa X-20 flow cytometer using the FACS DIVA software (BD Biosciences, San Jose, CA).
Immunohistochemistry (IHC): GITR staining in human tumor tissues was performed at QualTek Laboratories (Newtown, PA) using an anti-human GITR antibody generated at OncoMed Pharmaceuticals. Quantification of GITR staining and immune cell populations was done using Definiens Tissue Studio on slide images scanned by Aperio. IHC to quantify Cd4 and Cd8 cells on murine tumor tissues was performed at OncoMed.
Gene Expression Analysis: Global gene expression in tumor and in whole blood samples were performed on Affymetrix Mouse Genome 430 2.0 microarrays (Affymetrix, Santa Clara, CA) at Almac Diagnostics (Craigavon, UK). Genes differentially expressed between GITRL-Fc (336B3) and the saline groups were identified with Bayesian t-test (Cyber-T). qPCR analysis was performed on the QuantStudio 7 Flex (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA).
RNASeq data of GITR Expression: The normalized counts in the TCGA files (rnaseqv2_rsem.genes.normalized_results) were used in the prevalence and correlation analysis of GITR expression.

RESULTS

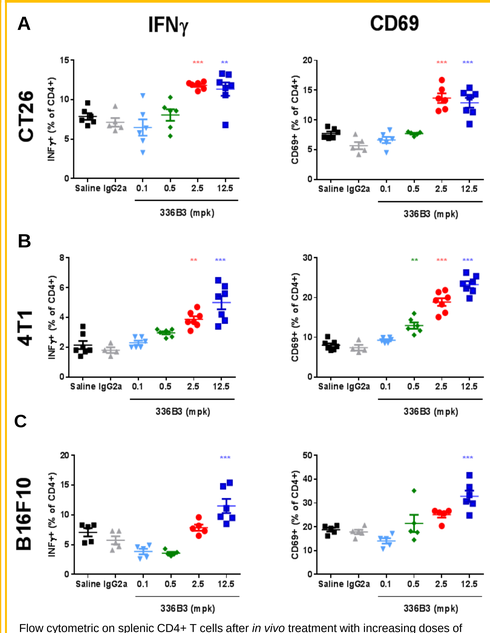
GITRL-Fc treatment shows single agent antitumor efficacy in multiple syngeneic models



4T1, CT26.WT, and B16F10 tumor cells were injected SC and allowed to grow until tumor volumes (TV) reached ~200-250 mm3, at which point mice were randomized into 6 groups and treatment initiated (day 0). Mice received either IP saline solution (black), IgG2a Isotype control (gray), or 336B3 (12.5mpk, blue; 2.5 mpk red; 0.5 mpk green and 0.1 mpk light blue). *,**,*** p<0.05, p<0.01, p<0.001 2-way ANOVA versus saline followed by Bonferroni post-test.

Panels A, B, and C show efficacy graphs of *in vivo* dose-response studies, demonstrating GITRL-Fc robust single agent antitumor activity on large established tumors from different syngeneic models.

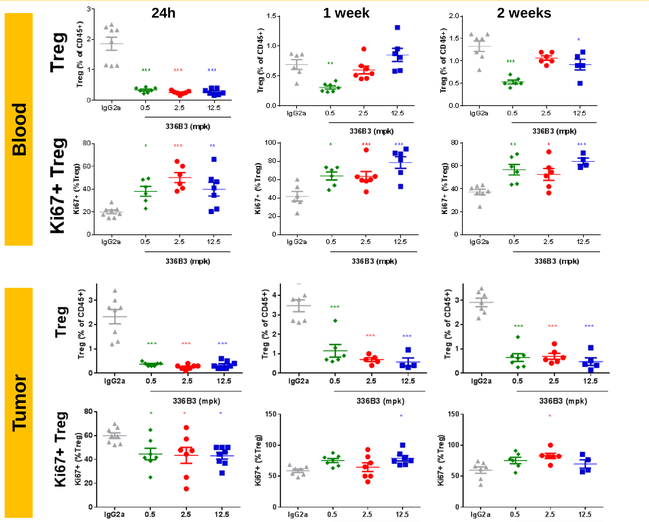
GITRL-Fc promotes dose dependent increase of IFNγ and T cell activation markers in splenocytes



Flow cytometric on splenic CD4+ T cells after *in vivo* treatment with increasing doses of 336B3 in mice bearing 4T1 (panel **A**) CT26, WT (panel **B**) or B16F10 (panel **C**) syngeneic models . After ~9-2 weeks, tumors were collected and processed into single cell suspension for staining of immune cells and of intracellular cytokines. Mean±SEM; n=5-6 animals per group. *, **, *** indicate P <0.05, 0.1, 0.001 vs saline by 1 way ANOVA and Bonferroni post-test.

Panels A, B, and C show flow cytometric results confirming that 336B3 promotes dose-dependent activation of CD4+ T cell as demonstrated by the increases in intracellular release of IFNγ and in expression of the T cell activation marker (Cd69).

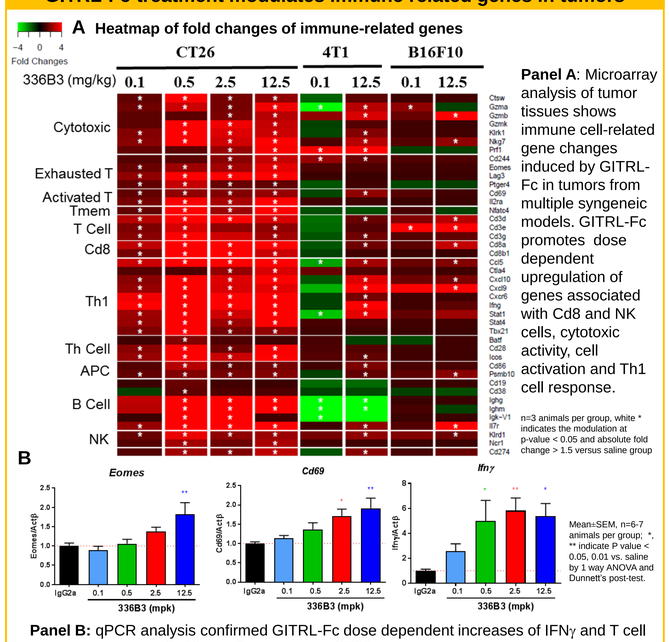
GITRL-Fc treatment promotes proliferation and depletion of Treg cells in tumor and blood



Foxp3GFP mice bearing CT26.WT tumors were randomized when tumors were established.
Mean±SEM; n=6-8 animals per group. *, **, *** indicate P <0.05, 0.1, 0.001 vs saline by 1 way ANOVA and Bonferroni post-test.

Flow cytometry analysis shows that 336B3 induces early and sustained depletion of Treg cells and promotes their proliferation in both tumor and in peripheral blood.

GITRL-Fc treatment modulates immune related genes in tumors



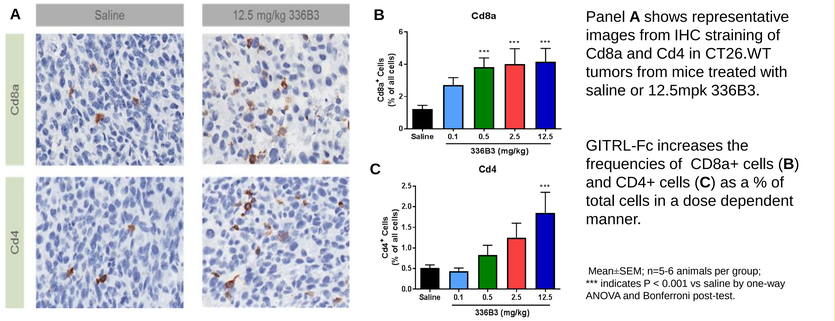
Panel A: Microarray analysis of tumor tissues shows immune cell-related gene changes induced by GITRL-Fc in tumors from multiple syngeneic models. GITRL-Fc promotes dose dependent upregulation of genes associated with Cd8 and NK cells, cytotoxic activity, cell activation and Th1 cell response.

n=3 animals per group, white * indicates the modulation at p-value < 0.05 and absolute fold change > 1.5 versus saline group

Panel B: qPCR analysis confirmed GITRL-Fc dose dependent increases of IFNγ and T cell activation markers (Cd69 and Eomes) in the CT26 tumors.

Mean±SEM, n=6-7 animals per group; *, ** indicate P value < 0.05, 0.01 vs. saline by 1 way ANOVA and Dunnett's post-test.

GITRL-Fc promotes infiltration of T cells in tumor

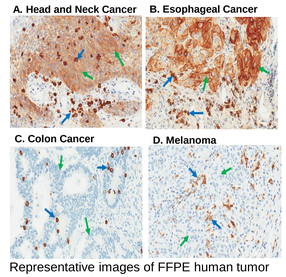


Panel **A** shows representative images from IHC straining of Cd8a and Cd4 in CT26.WT tumors from mice treated with saline or 12.5mpk 336B3.

GITRL-Fc increases the frequencies of CD8a+ cells (**B**) and CD4+ cells (**C**) as a % of total cells in a dose dependent manner.

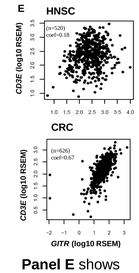
Mean±SEM; n=5-6 animals per group; *** indicates P < 0.001 vs saline by one-way ANOVA and Bonferroni post-test.

Prevalence of GITR expression in human cancer tissues



Representative images of FFPE human tumor tissues were stained for GITR immunoreactivity by IHC and evaluated for localization of GITR+ cells (20X). **Green** arrows are tumor cells while **blue** arrows are immune cells. Note that in some tumors GITR is expressed in both immune and tumor cells (**A, B**) while in other tumor types GITR only expressed in immune cells (**C, D**).

We generated a human anti-GITR antibody and profiled 17 cancer indications to evaluate patterns of GITR expression in different cell types across tumors. Sections were scored for GITR staining in: 1) immune cells closely associated with tumor tissue 2) immune cells in the stroma; 3) tumor cells with expression on the plasma membrane.



Panel E shows that in some human tumors (from TCGA), *GITR* expression is highly correlated with immune cells markers (e.g. *CD3e*) while in other tumors the correlation is very low, suggesting that *GITR* is expressed on non-immune cells.

F GITR REACTIVITY IN TUMOR-ASSOCIATED IMMUNE CELLS — Percentage of cases for each indication with GITR-Positive Immune Cell Abundance ≥2 in Tumor

Group	Tumor Indication	% of Cases with Abundance ≥2
High when range = 44-100% of cases ≥2	H&N	70%
	Gastric CA	55%
	Melanoma	55%
	TNBC	48%
	RCC	44%
Moderate when range = 30-43% of cases ≥2	Cervical CA	40%
	NSCLC Adeno	40%
	NSCLC Squam	40%
	T-cell Lymphoma	39%
	Colon CA	38%
	Ovarian CA	38%
	Esophageal CA	33%
	Endometrial CA	31%
Low when range = 0-29-% of cases ≥2	Bladder CA	22%
	Leukemia	15%
	Pancreatic CA	12%
	Prostate CA	9%

5 indications (H&N, Gastric, Melanoma, TNBC, and RCC) show high prevalence of GITR expression in tumor-associated immune cells

G GITR REACTIVITY IN TUMOR EPITHELIUM — Percentage of cases for each Indication with ≥10% of tumor cells with GITR staining at ≥2+:

Group	Tumor Indication	% of Cases with Staining ≥10% at ≥2+
High when range = 40-100% of cases with ≥10% at ≥2+	T-cell Lymphoma	61%
	Esophageal CA	60%
	H&N CA	50%
Moderate when range = 10-39% of cases with ≥10% at ≥2+	Ovarian CA	21%
	Cervical CA	20%
	NSCLC Adeno	20%
	NSCLC Squam	15%
Low when range = 1-9% of cases with ≥10% at ≥2+	TNBC	10%
	Gastric CA	5%
	Pancreatic CA	4%
Negative when range = 0% of cases with ≥10% at ≥2+	Bladder CA	0%
	Colon CA	0%
	Endometrial CA	0%
	Leukemia	0%
	Melanoma	0%
	Prostate CA	0%
	RCC	0%

Table G shows 3 tumor indications (T cell lymphoma, esophageal, and H&N) with high prevalence of GITR expression on tumor cells.

CONCLUSIONS

- Using a surrogate GITRL-Fc molecule, we show potent single agent dose-dependent anti-tumor efficacy on large established tumors in multiple murine syngeneic models.
- GITRL-Fc treatment increased IFNγ release in tumors, peripheral blood, and splenocytes in syngeneic mouse models while increasing T cell activation markers.
- GITRL-Fc promoted proliferation and depletion of Treg cells in both tumors and peripheral blood.
- Consistent with its mechanism of action, GITRL-Fc treatment upregulated immune genes associated with activation of T and NK cells and Th1 response in tumors.
- GITRL-Fc promoted CD4 and CD8 T cell infiltration, while decreasing Treg cell frequencies and increasing CD8/Treg ratios in tumor tissues.
- IHC analysis showed that GITR is not only expressed in immune cells, but also in tumor cells of some tumor types including HNSC, lung, esophageal, cervical, uterine, breast, and bladder tumors.
- PD biomarkers were identified in tumor tissues and peripheral blood samples of multiple *in vivo* syngeneic models. The PD biomarkers are consistent with GITRL-Fc mechanism of action and can be used in the clinic to demonstrate activity of our GITRL-Fc clinical candidate, OMP-336B11.