#5621 Prevalence of GITR Expression and Pharmacodynamic (PD) Biomarkers in Syngeneic Tumor Models Treated by a GITR Agonist (GITRL-Fc)

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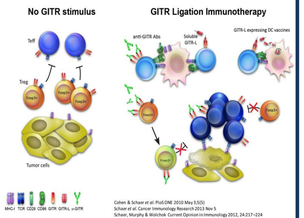
Abstract

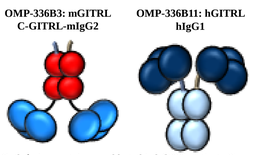
GITRL (Glucocorticoid-Induced Tumor Necrosis Factor Receptor Ligand, TNFSF18) is a member of the tumor necrosis factor (TNF) ligand superfamily. GITRL binds and activates the co-stimulatory surface receptor GITR, which promotes proliferation and activation of effector T cells (Teff) and inhibits suppressive activity of regulatory T cells (Treg). It is thus hypothesized that co-stimulation of GITR by agonist agents will 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.

To investigate the prevalence of GITR expression in human tumors, RNA-Seq data analyses of 33 tumor types in TCGA showed GITR is highly expressed in a subset of solid tumors, including head & neck, lung, breast, esophageal, and bladder cancers. In most solid tumors, GITR expression correlated poorly with T cell markers, implying that GITR may not be exclusive to immune cells and may be expressed in tumor cells as well. Similar findings emerged from RNA-Seq data analysis of patient-derived xenograft (PDX) samples from 24 tumor types. The gene expression data was corroborated by immunohistochemistry (IHC) analysis of GITR expression in 17 tumor types which showed that in addition to immune cells, GITR was expressed on tumor cell membranes.

A multi-platform approach was taken to investigate GITRL-Fc pharmacodynamic (PD) biomarkers in tumors and in matched whole blood samples from mice bearing CT26 colon, 4T1 breast, and B16F10 melanoma carcinoma tumors. Global gene expression levels were profiled by microarray on treated and control tissues. We also monitored the changes of immune cell populations and cytokine secretions by flow cytometry, Luminex and IHC. Immune gene changes were more robust in tumors than in blood samples. In tumor samples, GITRL-Fc increased gene expression associated with T cells and their activation, Th1 response, cytotoxicity, natural killer cells, and interferon gamma (Ifnγ). These gene changes were validated by quantitative real-time PCR. Similarly, flow cytometry analysis showed that GITRL-Fc promoted activation of CD4+ effector cells, decreased Treg frequency, and increased the ratio of CD8+ T cell/Treg in tumors. GITRL-Fc also modulated secretion of cytokines in splenocytes, including an increase in IFNγ.

Taken together, the PD biomarker changes in immune-related gene expression, immune cell populations, and cytokine secretions observed in these preclinical tumor models are consistent with GITRL-Fc mechanism of action and demonstrated target engagement of GITRL-Fc.





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

Materials and Methods

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. The FPKM values of human GITR expression in PDX models were obtained from the CrownBio HuBase RNASeq database (http://hubase.crownbio.com, San Diego, CA).

In Vivo Studies: 6-8 weeks old female Balb/C (for 4T1 and CT26.WT) or C57Bl/6J (for B16F10) were purchased from Envigo or Charles River 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). At the designated time points, tumors, blood, and spleens were harvested and processed for flow cytometry, cytokine secretion assays, IHC, and gene expression analysis.

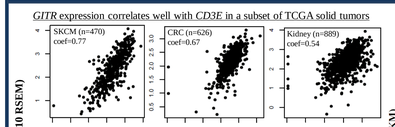
Flow Cytometric Analysis: Single cell suspensions of splenocytes, tumors or PBMCs were used for staining with fluorochrome labelled antibodies for membrane markers or intracellular cytokines. Prior to staining for the detection of intra-cellular cytokines, cells were stimulated for 4 hours at 37°C in a CO_2 incubator with 50ng/ml phorbol 12-myristate 13-acetate, 1μg/ml ionomycin in the presence of Monensin and Brefeldin A. The data were acquired and analyzed on a Fortessa X20 flow cytometer using the FACS DIVA software (BD Biosciences).

Immunohistochemistry (IHC): GITR staining in human tumor tissues was performed at QualTek Laboratories using an anti-human GITR antibody generated at OncoMed Pharmaceuticals. Scoring of GITR staining on immune cell populations in the tumor and in the stroma was performed by two pathologists. IHC to quantify CD4+ and CD8+ cells on murine tumor tissues was performed at OncoMed, and quantification was done using Definiens Tissue Studio on slide images scanned by Aperio software.

Gene Expression Analysis: Global gene expression in tumor and whole blood samples was performed on Affymetrix Mouse Genome 430 2.0 microarrays (Affymetrix) at Almac Diagnostics. Genes differentially expressed between GITRL-Fc (336B3) and saline groups were identified with Bayesian t-test. qPCR analysis was performed on the QuantStudio 7 Flex (Applied Biosystems/Thermo Fisher Scientific).

Correlation of GITR with T Cell Markers and Its Expression in Tumor Cells

GITR expression correlates well with *CD3E* in a subset of TCGA solid tumors



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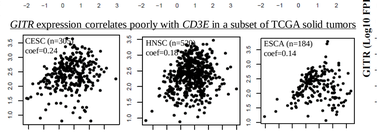
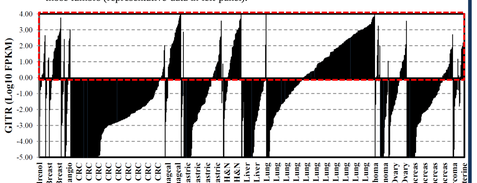
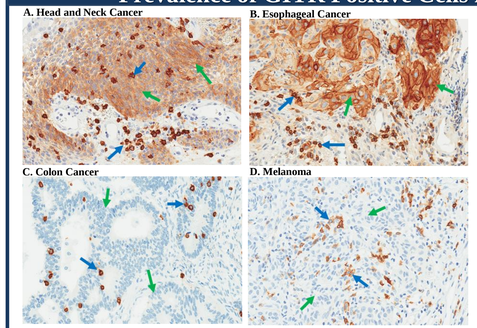


GITR was expressed in certain tumor types (from TCGA) and a subset of PDX tumor models (log10 FPKM >0, e.g., breast, lung, esophageal, head & neck, CrownBio). In some human tumors, there is a high correlation of *GITR* with immune cells (e.g. *CD3*) while other tumors show low correlation with immune cells suggesting *GITR* is expressed on non-immune cells in those tumors (representative data in left panel).



Prevalence of GITR Positive Cells in Human Cancer Tissues



FFPE human tumor tissues were stained for GITR immunoreactivity by IHC and evaluated for localization of GITR+ cells (20X). **Green** arrows are tumor cells and **blue** arrows are immune cells. GITR was expressed in both immune and tumor cells in some tumor types, e.g., H&N (A), Esophageal (B) tumors, while GITR was only expressed in immune cells in other tumor types, e.g., colon (C) and melanoma (D) tumors.
Tumors were scored for GITR staining in the immune cells in tumors and in tumor cells with expression on the plasma membrane. The score used an abundance scale of 0-3, where 0=no GITR staining, 1=few GITR+ cell staining, 2=moderate amount of GITR+ cell staining, and 3=high amount of GITR+ cell staining.

GITR expression on immune cells in tumors

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	0%

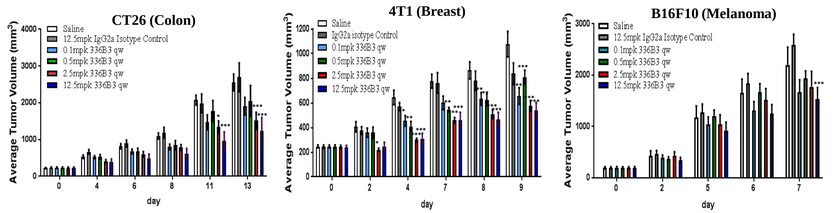
Head and neck cancer, gastric cancer, melanoma, TNBC, and RCC show high prevalence of GITR expressions in tumor-associated immune cells.

GITR expression on tumor cell plasma membrane

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%
	TNBC	10%
Low when range = 1-9% of cases with ≥10% at ≥2+	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%

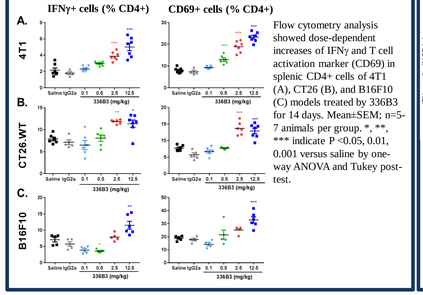
T cell lymphoma, esophageal cancer, and head and neck cancer show high prevalence of GITR expressions in tumor cells.

Anti-Tumor Efficacy of GITRL-Fc (336B3) in Multiple Syngeneic Mouse Models



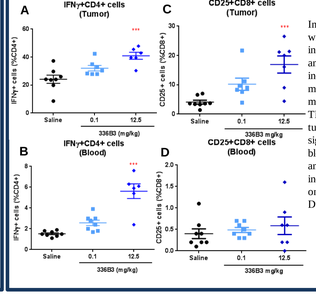
CT26.WT, 4T1, and B16F10 tumor cells were injected subcutaneously and allowed to grow until tumor volumes reached ~200-250 mm³, at which point mice were randomized into 6 groups and treatment initiated (day 0). Mice received either IP saline solution (white), IgG2a Isotype control (gray), or 336B3 (12.5mpk,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

Dose-dependent Increases of IFNγ and T Cell Activation Marker in Splenocytes



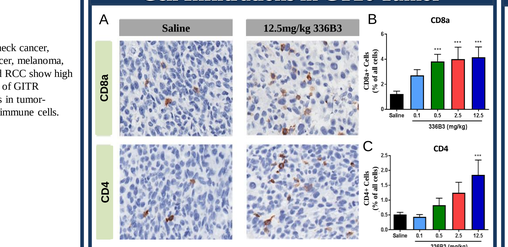
Flow cytometry analysis showed dose-dependent increases of IFNγ and T cell activation marker (CD69) in splenic CD4+ cells of 4T1 (A), CT26 (B), and B16F10 (C) models treated by 336B3 for 14 days. Mean±SEM; n=5-7 animals per group. *, **, *** indicate P <0.05, 0.01, 0.001 versus saline by one-way ANOVA and Tukey post-test.

GITRL-Fc Increased IFNγ and T Cell Activation Marker in CT26 Tumor and Blood



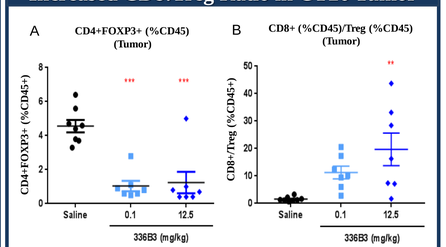
In the CT26 model treated with 336B3 for 7 days, IFNγ in CD4+ cells in tumor (A) and peripheral blood (B) was increased in a dose dependent manner. T cell activated marker (CD25) in CD8a+ TILs was upregulated in tumor (C) but not significantly in peripheral blood (D). Mean±SEM, n=6 animals per group. *** indicates P<0.001 vs saline by one-way ANOVA and Dunnett's post-test.

GITRL-Fc Increased CD4+ and CD8+ Cell Infiltrations in CT26 Tumor



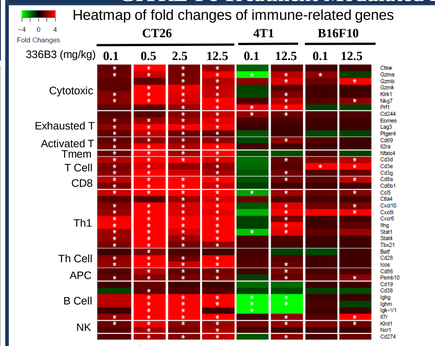
IHC staining of CD8a and CD4 (A) in the tumor tissues of saline or 12.5mg/kg 336B3 treated animals from the CT26.WT model. CD8a+ cell (B) and CD4+ cell (C) frequencies (% of total cells) showed increases in a dose dependent manner. Mean±SEM; n=5-6 animals per group; *** indicates P < 0.001 versus saline by one-way ANOVA and Bonferroni post-test.

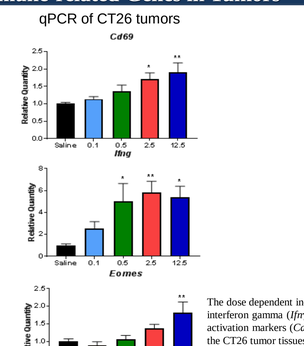
GITRL-Fc Decreased Treg Frequencies and Increased CD8:Treg Ratio in CT26 Tumor



The decreased frequency of Tregs (% of CD45+ cells) (A) and the increased CD8/Treg ratio (B) in the CT26.WT tumor model treated by 336B3 for 7 days implies an increase in the cytotoxic environment of the tumor in response to 336B3 treatment. Mean±SEM; n=7-8 animals per group; **, *** indicate P< 0.01, 0.001 versus saline by one-way ANOVA and Dunnett's post-test.

GITRL-Fc Treatment Modulated Immune-related Genes in Tumors





Microarray analysis identified immune cell-related genes modulated by GITRL-Fc (336B3) in tumors from multiple syngeneic models. Genes associated with T-cells, cytotoxic cell activity, NK cells, and Th1 response were upregulated by GITRL-Fc in a dose-dependent manner. n=3 animals per group, white* indicates modulation at P<0.05 and absolute fold change > 1.5 versus the saline group.

The dose dependent increases of interferon gamma (*Ifnγ*) and T cell activation markers (*Cd69* and *Eomes*) in the CT26 tumor tissues by GITRL-Fc were confirmed by qPCR. Mean±SEM, n=6-7 animals per group; *, ** indicate P< 0.05, 0.01 versus saline by one-way ANOVA and Dunnett's post-test.

Summary

- Gene expression and IHC analysis showed that GITR is expressed in multiple solid tumors including head and neck squamous cancer (HNSC), lung, esophageal, cervical, uterine, breast, and bladder tumors.
- GITR is not only expressed in immune cells, but also in tumor cells in certain tumor types.
- GITRL-Fc fusion protein showed potent single agent dose-dependent antitumor efficacy on large established tumors in multiple murine syngeneic mouse models.
- GITRL-Fc treatment increased IFNγ release and T cell activation markers in tumors, in peripheral blood, and in splenocytes in syngeneic mouse models.
- GITRL-Fc increased CD4 and CD8 T cells infiltration while decreasing Treg frequencies and increasing CD8/Treg ratios in tumor tissues.
- PD biomarkers were identified in tumor tissues and in peripheral blood from multiple *in vivo* syngeneic models. These biomarkers are consistent with GITRL-Fc mechanism of action and can be used in the clinic to demonstrate activity of GITRL-Fc.