FG-3165 is a Novel Galectin-9 Neutralizing Antibody that Inhibits Galectin-9-Mediated Dimerization of TIM-3 and Galectin-9-Induced Apoptosis of CD4+ and CD8+ T Cells

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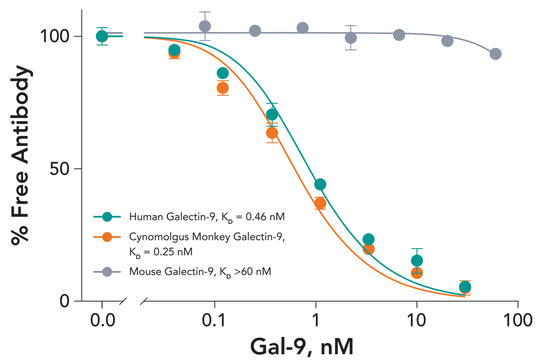
Background

- Galectin-9 (Gal-9) is a β-galactoside binding lectin that contains two conserved carbohydrate-recognition domains (CRD).
- Gal-9 is produced by a variety of cell types and binds to cell-surface receptors on immune cells to promote an immunosuppressive phenotype within the tumor microenvironment.[1] One of the ways by which Gal-9 is reported to promote immunosuppression is inducing T cell apoptosis.[2]
- Gal-9 binding to T cell receptors such as T cell immunoglobulin mucin domain-containing protein-3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA) can cause receptor dimerization.[3] This dimerization triggers a signaling cascade leading to T cell exhaustion and apoptosis.[4-6]
- Here, we describe the *in vitro* characterization of FG-3165, a humanized monoclonal anti-Gal-9 antibody, that is being developed for the treatment of solid tumors.

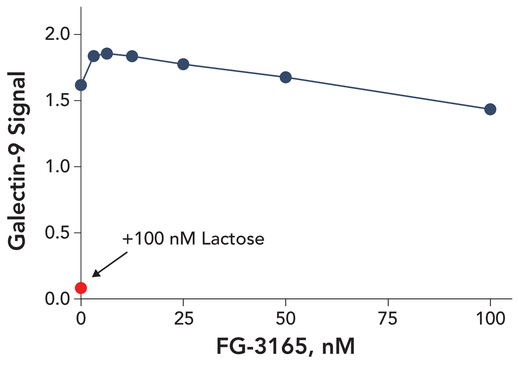
Results

Figure 1. FG-3165 Binds to Human Gal-9 with Sub-nanomolar Affinity



- FG-3165 binding to Gal-9 was assessed in a solution equilibrium assay.
- FG-3165 binds to human Gal-9 with a K_D of 0.46 nM.
- FG-3165 binds human and monkey Gal-9 with similar affinity but shows weak binding to mouse Gal-9.

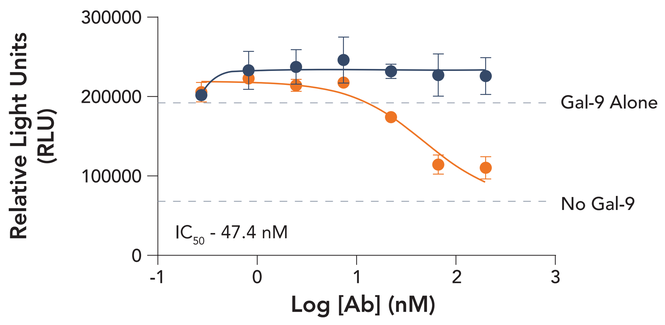
Figure 2. FG-3165 Does Not Block Gal-9 Binding to TIM-3 in an ELISA Format



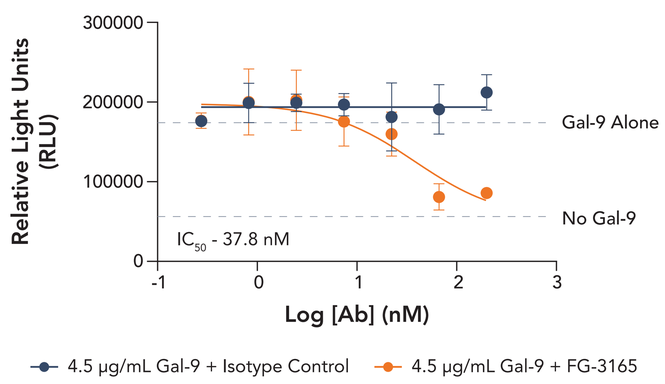
- The effect of FG-3165 on the binding of Gal-9 to TIM-3 was tested by ELISA by mixing Gal-9 with FG-3165 and adding to TIM-3 coated plates.
- Gal-9 binds TIM-3 despite the presence of excess FG-3165.

Figure 3. FG-3165 Prevents Gal-9-Induced Signaling by Inhibiting TIM-3 Dimerization and VISTA Dimerization

A. TIM-3 Dimerization Assay



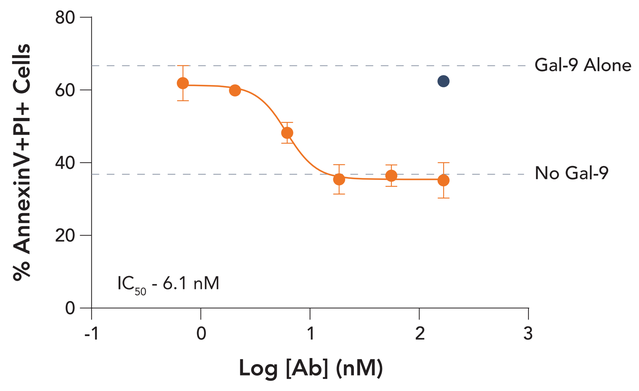
B. VISTA Dimerization Assay



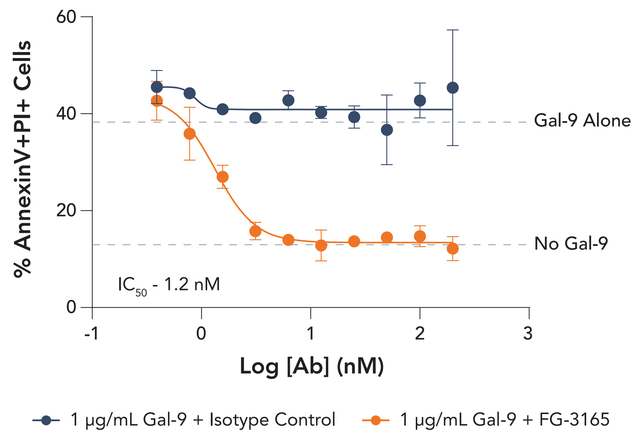
- The effects of FG-3165 on Gal-9-induced TIM-3 (**A**) or VISTA dimerization (**B**) were assessed using Eurofins DiscoverX PathHunter® eXpress Dimerization kits.

Figure 4. FG-3165 Inhibits Gal-9-Induced Apoptosis of CD4+ and CD8+ T Cells

A. CD4+ T Cell Apoptosis

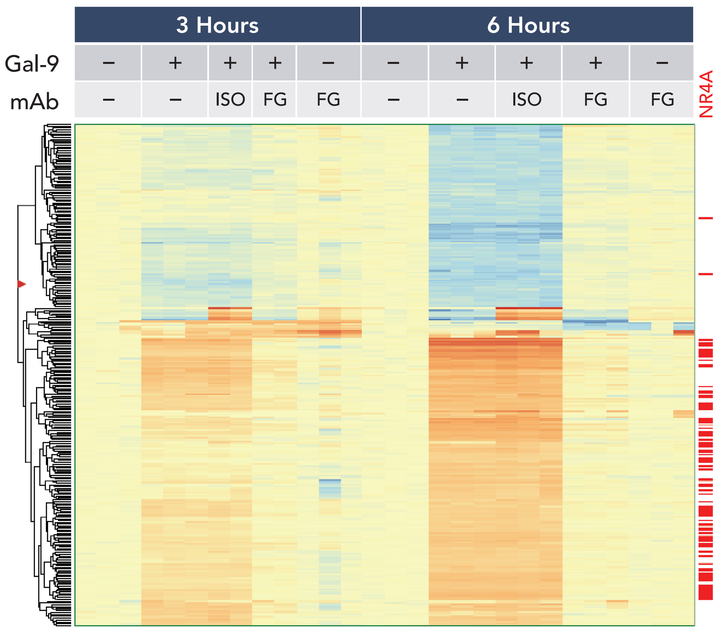


B. CD8+ T Cell Apoptosis



- Human CD4+ (**A**) or CD8+ (**B**) T cells were treated with Gal-9 +/- antibody for 48 hours and apoptosis was measured by Annexin V and Propidium Iodide (PI) staining.

Figure 5. FG-3165 Blocks the Early Transcriptional Response to Gal-9 in CD8+ T Cells



- Human CD8+ T cells were treated with Gal-9 +/- FG-3165 (or isotype control) for three or six hours and evaluated by RNA sequencing.
- The early transcriptional response to Gal-9 was almost entirely normalized by FG-3165, including resolution of inflammation, proliferation, and apoptosis signaling genes.

Table 1. Transcriptional Effects of Gal-9 ± FG-3165

	3 Hours			6 Hours		
Gene	Gal-9 vs no Tx, Fc	Gal-9 + FG-3165 vs no Tx, Fc	Gal-9 + FG-3165 vs Gal9, %Nm	Gal-9 vs no Tx, Fc	Gal-9 + FG-3165 vs no Tx, Fc	Gal-9 + FG-3165 vs Gal9, %Nm
IL11	2.8*	1.4	79.3*	9.7*	1.3	97*
IFITM5	2.1*	1.1	94.8*	8.7*	1.9*	88.5*
EGR1	2.7*	-1.1	103.5*	8.2*	-1.1	101.6*
EGR4	2.7*	-1.1	103.6	7.3*	1	101.5*
FOS	2*	1.1	94.5*	6.3*	1.1	98.4*
CYR61	3.5*	1.4*	84.6*	6.1*	1	97.5*
PTGS2	2.1*	1.1*	87.3*	5.6*	1.2*	95.2*
DUSP8	2.5*	1.4*	74.9*	5.5*	1.3*	92.4*
JUN	2.3*	1.1	94.8*	5.5*	1.2	96.1*
FOSB	1.9*	1.2*	80.6*	5*	1	98.9*
GEM	1.8*	1.1*	83*	4.7*	1.2*	94.9*
TMEM88	2.9*	1.2	91.1*	4.6*	1.3	91.1*
NR4A1	2.1*	1.1	90.7*	4.6*	1.1	97.1*
EGR3	1.5*	-1.3*	149.5*	4.5*	-1.4*	108.5*
ARC	1.6*	1	96*	4.3*	1.1	90.9*
CCL3L3	2.4*	1.6*	53.4*	4.3*	1.3	92.1*
ZSCAN12P1	2.5*	1.2	86*	4.2*	1.3*	90.6*
TNF	2.5*	1.4*	73.2*	4*	1.4*	86.2*
OVGP1	2.5*	1.6*	62.7*	3.9*	1.5*	84.4*
TNFRSF12A	1.8*	1.3*	63.3*	3.8*	1.5*	82.8*

*p<0.05

Figure 6. FG-3165 Reverses Gal-9-Mediated Induction of NR4A Nuclear Receptors and PD-1 in CD8+ T Cells

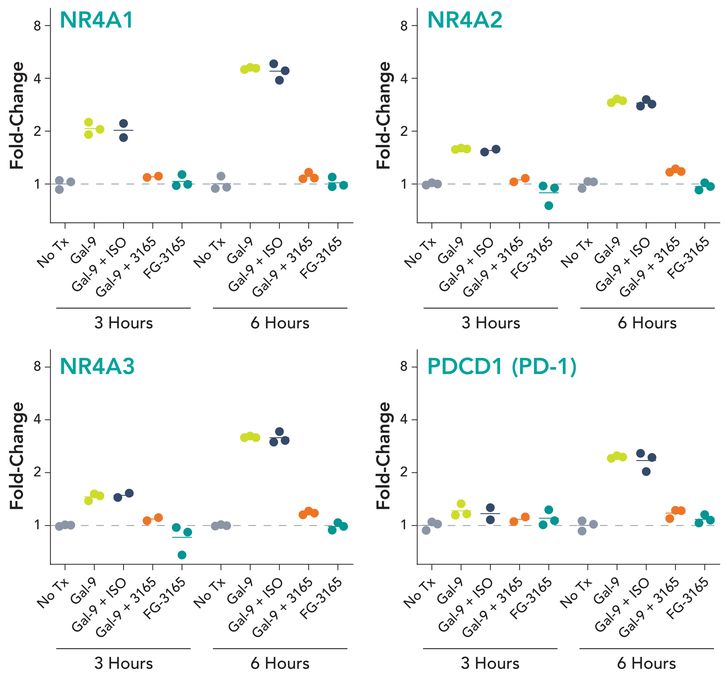
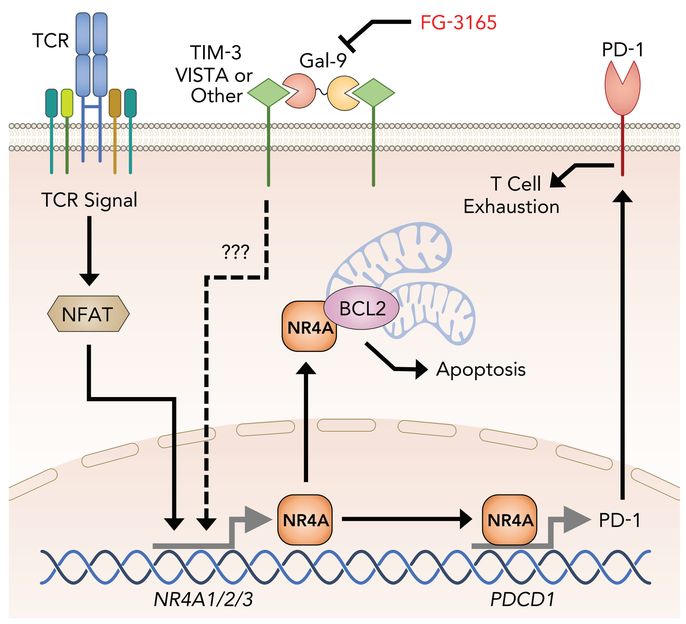


Figure 7. Model: FG-3165 Inhibits Dimerization of Cell Surface Receptors and Blocks Gal-9 Signaling, including Gal-9-mediated Upregulation of the Pro-Apoptotic NR4A Nuclear Receptor Family



Summary

- FG-3165 binds with sub-nanomolar affinity to human Gal-9 but does not block binding of Gal-9 to TIM-3.
- However, FG-3165 disrupts Gal-9-induced dimerization of TIM-3 and VISTA.
- FG-3165 inhibits Gal-9-induced apoptosis of both cytotoxic T cells and T helper cells.
- FG-3165 blocks the early transcriptional response to Gal-9.
- These data indicate that neutralization of Gal-9 by FG-3165 may overcome an important immunosuppressive mechanism in the tumor microenvironment, positioning FG-3165 as a promising candidate for the treatment of solid tumors.

Methods

- **Solution Equilibrium Assay:** FG-3165 (10 pM) was equilibrated for 22 hours at room temperature with multiple concentrations of Gal-9 in PBS/10% BSA. Free FG-3165 was captured for 30 minutes on Gal-9-coated MSD plates and detected by binding SulfoTAG-labeled goat anti-human IgG followed by electrochemiluminescence generation. KD values were obtained by fitting to the equation defined by Ducata, D., et al.[7]
- **TIM-3/Gal-9 Binding Assay:** Gal-9 (3.5 nM) was incubated with various concentrations of FG-3165 and the mixtures were applied to a microtiter plate coated with 1 µg/mL Fc(Tim-3)$_2$. Plate-bound Gal-9 from FG-3165/Gal-9 mixtures was detected using a biotinylated anti-Gal-9 antibody (R&D Systems, BAF2045) with streptavidin-HRP detection.
- **TIM-3 and VISTA Dimerization Assays:** Dimerization assays were performed per manufacturer instructions with 4.5 µg/mL Gal-9 +/- FG-3165 or an isotype control antibody for 16 hours.
- **T Cell Apoptosis:** Human CD4+ T cells were activated with human CD3/CD28 activator beads and simultaneously treated with 1 µg/mL Gal-9 +/- FG-3165 or an isotype control antibody for 48 hours. Human CD8+ T cells were activated with CD3/CD28 activator beads for three days before treatment with 1 µg/mL Gal-9 +/- FG-3165 or an isotype control antibody in the presence of CD3/CD28 activator beads for 48 hours. CD4+ and CD8+ T cell apoptosis was measured by Annexin V and Propidium Iodide (PI) staining.
- **RNA Sequencing:** Human CD8+ T cells were activated with human CD3/CD28 activator beads for three days before treatment with 1 µg/mL Gal-9 +/- FG-3165 or an isotype control antibody in the presence of human CD3/CD28 activator beads for three or six hours. RNAseq libraries were prepared from isolated RNA using an Illumina TruSeq Stranded mRNA Library Prep Kit and analyzed on High Output Flow Cells on an Illumina NextSeq 500 Sequencer. Downstream analyses were performed using Strand NGS software (Strand Life Sciences). Enrichment analysis was performed using Enrichr (https://maayanlab.cloud/Enrichr/).

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