Although the interaction between GITR (Glucocorticoid-Induced Tumor Necrosis Factor Receptor) and GITR (its ligand) is important for the development of immune responses, the cellular mechanisms underlying anti-tumor immunity including both innate and adaptive immunity is not fully understood. We generated a novel single-gene linkerless GITR-trimer fused to an immunoglobulin Fc domain (GITR-Fc), and investigated its effect on controlling tumor growth and immune responses in preclinical tumor models. Treatment with GITR-Fc significantly reduced tumor growth in several preclinical tumor models by inducing Th1 biased anti-tumor immunity and reducing Treg-mediated immune suppression. Immune cell depletion studies showed that anti-tumor immunity induced by GITR-Fc depended on CD8\(^+\) T cells as well as NK cells. Furthermore, the combination of GITR-Fc with PD-1 blockade significantly reduced the tumor growth in the Ria6 murine kidney adenocarcinoma tumor model. Taken together, these results suggest that GITR-Fc can improve cancer treatment as a single agent or in combination therapy by enhancing innate and adaptive cellular immunity. 

**Materials and Methods**

**GITR-Fc protein.** DNA constructs were generated that encoded murine GITR, extracellular domain concatenated to form a single-gene trimer and fused to the N- or C-terminus of a murine IgG Fc domain as indicated in the diagram.

**Luciferase Reporter assay.** The ability of GITR activation of NRFluc signal was determined by in vivo luciferase reporter assays. HEK-293 cells were stably transduced with an expression vector encoding a full-length human or mouse GITR as well as plasmids encoding a NRFluc-dependent luciferase reporter construct. GITR and NRFluc luciferase reporter expressing HEK-293 cells were plated to 96 well plates and incubated overnight. Serially diluted recombinant fusion proteins or antibodies were added to the appropriate wells and incubated overnight. Luciferase levels were measured 18 hours later using a Steady Glow luciferase assay kit (Promega).

**In Vivo Studies.** The murine colon carcinoma (CT26.WT, ATCC CRL-2638), the murine renal cortical adenocarcinoma (Renca, ATCC CRL.22610), and the murine skin melanoma (B16.F10, ATCC CRL.6447) were obtained from American Type Culture Collection. Single cell suspensions of CT26 or Renca tumor cells were injected subcutaneously into the flanks of 7-8 week-old Balb/c or C57BL/6 mice. One or two weeks following tumor inoculation, mice with palpable tumors were injected i.p. individually with GITR-Fc, anti-GITR (DTA-1), anti-PD-1, or combination according to the schedule described in the results and conclusions. Tumor volumes were measured by measuring two bisecting diameters of each tumor with electronic calipers. Tumor volumes were calculated using the formula: V=π/6 x (a x b\(^2\)) with a as the larger diameter and b as the smaller diameter.

**ELISPOT.** Splenocytes were cultured in the presence and absence of tumor specific CD8 T-cell peptide (1μg/ml final concentration of AH1-1 peptide for CT26 model) in T cell for 48 hrs followed by the ELISPOT assay as described in manufacture’s instructions.

**T cell and NK cell Cytotoxicity assay:** Ten million splenocytes were cultured with tumorspecific CD8 T cell peptide AH1 for 7 days, washed and counted. These effector T cells were co-cultured with euthanized CT26 tumor cells labeled with CFSE (Celltrace CFSE Kit, ATCC) and the expressed T cells were collected and measured for the release of calcein from the tumor cells. For NK cell cytotoxicity assays, E:T ratio of labeled Calcein AM labeled as NK cell target (E:T of 25:1). Specific lysis is calculated using the formula: Specific lysis (%) = (Experimental release - spontaneous release) / (maximum release - spontaneous release) x 100.

**Treg Suppression Assay** CD4+CD25+ regulatory T cells (from each treatment group) were isolated in 2 steps using a Treg Isolation Kit (Miltenyi). T cells were isolated from spleens of naive mice by negative selection and labeled with VIT dye. T cells were then stimulated with anti-CD3 and anti-CD28 antibodies and incubated with Treg for 3 days. Cells were then stained with anti-CD4 or anti-CD8 and the dilution of VIT was used to calculate T-cell proliferation.

**Results and Conclusions**

**Structure and statistical function of GITR-Fc engineered using a novel single-gene linkerless GITR trimer**

**GITR-Fc mediates robust anti-tumor activity through T and NK cells**

**GITR-Fc and PD-1 blockade significantly reduces tumor growth**

**Summary**

- GITR-Fc is active and more potent than agonist GITR mAb.
- GITR-Fc induces a potent anti-tumor immunity by promoting Th1 type immune response and inhibiting Treg suppressive function.
- GITR-Fc significantly reduces tumor growth in a T cell- or NK cell-dependent manner.
- GITR-Fc enables complete eradication of some tumors as a single agent.
- Combination of GITR-Fc with anti-PD1 blockade significantly reduces tumor growth.