Optimal target saturation of ligand-blocking anti-GITR antibody IBI37G5 dictates FcγR-independent GITR agonism and antitumor activity

Graphical abstract

Highlights
- Activated/exhausted tumor-infiltrating CD8+ T cells co-express PD1 and GITR
- Anti-GITR antibody IBI37G5 shows FcγRs-independent agonism and antitumor effect
- IBI37G5 synergizes with anti-PD1 antibody to control established murine tumors
- IBI37G5 exhibits best antitumor efficacy at doses near-saturating target receptors

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In brief
Determining the optimal in vivo efficacious doses is essential for the success of anti-GITR antibody development. Liu et al. propose a dose-finding strategy by monitoring the target saturation levels of GITR receptors to predict antitumor efficacy. This finding also has implications for therapeutic antibodies targeting other co-stimulatory receptors.
Optimal target saturation of ligand-blocking anti-GITR antibody IBI37G5 dictates FcγR-independent GITR agonism and antitumor activity

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SUMMARY

Glucocorticoid-induced tumor necrosis factor receptor (GITR) is a co-stimulatory receptor and an important target for cancer immunotherapy. We herein present a potent FcγR-independent GITR agonist IBI37G5 that can effectively activate effector T cells and synergize with anti-programmed death 1 (PD1) antibody to eradicate established tumors. IBI37G5 depends on both antibody bivalency and GITR homo-dimerization for efficient receptor cross-linking. Functional analyses reveal bell-shaped dose responses due to the unique 2:2 antibody-receptor stoichiometry required for GITR activation. Antibody self-competition is observed after concentration exceeded that of 100% receptor occupancy (RO), which leads to antibody monovalent binding and loss of activity. Retrospective pharmacokinetics/pharmacodynamics analysis demonstrates that the maximal efficacy is achieved at medium doses with drug exposure near saturating GITR occupancy during the dosing cycle. Finally, we propose an alternative dose-finding strategy that does not rely on the traditional maximal tolerated dose (MTD)-based paradigm but instead on utilizing the RO-function relations as biomarker to guide the clinical translation of GITR and similar co-stimulatory agonists.

INTRODUCTION

Although substantial clinical success has been achieved by cancer immunotherapy in recent years, the relatively low overall response rate and inevitable drug resistance in most treated patients are still major challenges in this field.1 Amplifying the T cell receptor (TCR) downstream signal through activating the co-stimulatory receptors holds great promise to further enhance the efficacy of immunotherapy.2 Glucocorticoid-induced tumor necrosis factor receptor (GITR) is an important immune co-stimulatory receptor belonging to the tumor necrosis factor receptor superfamily (TNFRSF). It is constitutively expressed on regulatory T (Treg) cells and has low expression on naive T cells or T memory cells. However, when T cells are activated, GITR expression will be significantly upregulated.3 Through binding to GITR ligand (GITRL) expressed on the surface of activated antigen-presenting cells (APCs), GITR can activate downstream nuclear factor κB (NF-κB) signals via tumor necrosis factor (TNF) receptor-associated factors (TRAFs), release pro-inflammatory cytokines, and promote T cell proliferations.4

In the tumor microenvironment (TME), after tumor specific T cells recognize their cognate tumor antigens through TCR, GITRL functions as a co-stimulatory factor to enhance the effector function and clonal expansion of GITR+ T cells.5 Exogenous provision of GITRL or GITR agonist antibodies can mimic the role of GITRL in the TME and cross-link GITR receptors to increase the function of CD8+ T cells, thereby enhancing anti-tumor immunity.6–8 In addition, GITR signals stimulated by GITR agonists can also alleviate the immunosuppressive function of Treg cells by either down-regulating Foxp3 expression or de-differentiation of Treg cells into CD4 effector T cells.9–11 By Fc-mediated antibody-depedent cellular cytotoxicity (ADCC) functions, GITR antibodies can also selectively deplete GITR high-expressing Treg cells in the TME, thereby skewing the balance in favor of tumor-killing T cells.12 A number of pre-clinical studies have also proved that GITR agonists combined with other immunotherapies can rescue dysfunctional CD8+ T to promote rapid tumor killing and stimulate the proliferation of precursor effector memory T cells for long-lasting responses in mice.6,13,14

Based on these studies, many companies have developed GITR agonists with different mechanisms of action (MOAs),
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(legend on next page)
and some of them have entered clinical trials. Despite great anti-tumor potential and excellent safety profiles shown in preclinical studies, the clinical efficacy of GITR agonists is usually quite limited in cancer patients. Some pharmacodynamic responses have been observed in early-phase clinical studies; however, objective tumor responses are rarely seen in patients treated with monotherapy. Other clinical studies combining GITR agonist and anti-programmed death 1 (PD1) antibodies failed to show synergistic activity, except for one small cohort of immune checkpoint inhibitor (ICI)-naïve (but none in ICI-pre-treated) melanoma patients. These clinical disappointments have unveiled significant knowledge gaps when extrapolating data from animal models to human patients. Conceivably, the successful clinical translation of GITR agonists may require better understandings of GITR biology in the context of human tumors and demand in-depth mechanistic studies to uncover the key determinants for optimal GITR activation in vivo using human-relevant models.

To study these issues, we have characterized an anti-GITR agonist antibody IBI37G5 and investigated its immune-stimulation mechanisms and the pharmacokinetics (PK)/pharmacodynamics (PD) relationships in mouse and monkey models. We have also determined the proper antibody-receptor stoichiometry required for IBI37G5-induced GITR activation and defined the therapeutic window for optimal anti-tumor efficacy based on simulated receptor occupancy-function relations in vivo. These findings provide insights into the paradigm of using receptor occupancy (RO)-activity relation for dose selection and facilitate the translation of therapeutics targeting GITR and other co-stimulatory receptors.

RESULTS

Dynamic regulations of GITR and PD1 expression on tumor-infiltrating lymphocytes (TILs) from human CRC

Previous studies have shown that GITR expression is low on human naïve and memory T cells but can be significantly upregulated upon TCR activation, a phenomenon analogous to PD1 expression on T cells. This leads us to speculate that GITR and PD1 may share a similar regulation pattern following T cell activation. Indeed, we observed that activation of human peripheral blood mononuclear cells (PBMCs) in vitro induced upregulation of both markers (Figures S1A and S1B). To examine the intricate regulation of GITR and PD1 expressions in the TME, we analyzed the publicly available single-cell RNA sequencing (scRNA-seq) data from 14 tumor samples collected from 7 treatment-naïve, stage I to IV colorectal cancer (CRC) patients (Figure S1C) and clustered T cells into 8 distinctive subtypes (Figures 1A–1C). CD8+ T cell clusters show a clear T cell activation trajectory (naïve → pre-effector → activated → exhausted) (Figure 1D). As expected, naïve markers (CCR7, IL7R, and LEFT) decreased along the cell order, while exhaustion markers (HAVCR2, LAG3, and TIGIT) showed the opposite trend (Figure 1E). Effector markers (GZMB, IFNG, and PRF1) increased in parallel with exhaustion markers and reached their peaks near the end of the trajectory, consistent with previous reports that terminally exhausted cells are the primarily cytotoxic CD8+ T cells in the TME. In addition, both GITR (TNFRSF18) and PD1 expression increased along the CD8+ T cell activation trajectory, consistent with our in vitro finding (Figure 1E). Parallel upregulation of GITR and PD1 was also observed in CD4+Foxp3+ T cells (Figure S1E). By contrast, GITR and TIGIT expressions in Treg cells continued to rise from already high basal levels while other exhaustion and effector markers remained mostly unchanged (Figure S1E), consistent with the previously reported roles of GITR and TIGIT in Treg cells.

To evaluate the co-expression patterns of GITR and PD1 on TILs in situ, we performed multiplex fluorescence staining of 75 CRC samples on the tumor microarray chip. CD8+ T cells and CD4+ Foxp3+ T cells account for an average of 53.6% and 42.4% of all T cells, respectively. With an average of only 4%, Treg numbers were inversely correlated with the rate of CD8+ T cell infiltrations (Figure S1F). These results are in line with our scRNA-seq analysis and the previously reported tumor immunohistochemistry data (Figure 1B). GITR+ or PD1+ CD8+ T cells were distributed both in the tumor parenchyma and tumor-associated stroma and were correlated with each other on their respective infiltration levels. Moreover, a substantial proportion of these infiltrated lymphocytes showed PD1+GITR+ double positivity (Figures 1F and 1G). Similar trends were also observed in CD4+Foxp3+ T and Treg cells in these tumor samples (Figures S1G–S1I).

Collectively, we found that the regulation of GITR and PD1 expression was highly orchestrated during the activation/exhaustion processes of T cells in CRC. We also noted that...
GITR may complement PD1 in identifying tumor-specific CD8+ T cells in both mouse and human tumors (Figure S2). These findings underscore the critical function of the co-stimulatory GITR signal, in concert with the inhibitory signals of immune checkpoints, to modulate TCR activation and cytotoxic activities of T cells. Indeed, TCgaarda data analysis predicts better prognosis in cancer patients with high TNFSF18 (GITRL) expression in the TME (Figure S1J), further supporting the notion that activating the GITR-GITRL axis is beneficial for anti-tumor immunity.13,32

**Characterization of IBI37G5, a ligand-mimetic anti-GITR agonist antibody**

We previously disclosed an anti-GITR agonist antibody IBI37G5 derived from mouse hybridoma technology.34 IBI37G5 is a humanized immunoglobulin G1 (IgG1) with high affinity to human GITR (hGITR) (Kd = 2.5 nM) (Figures 2A and 2B) and GITRls from non-human primates (NHPs) (Figures S3A–S3C), but it does not cross-react with species like canines and rodents (Figure S3D). To understand the mechanisms of antibody-mediated GITR agonism, we compared the full-length hlgG1 with the Fc-silent (hlgG1-PGLALA) or -absent (F(ab')2) versions of IBI37G5 in an hGITR-Jurkat reporter assay and found that all of them showed robust agonistic activities (Figure 2C), indicating an Fc-independent function in vitro. In the ligand competing experiment, we showed that IBI37G5 (regardless of hlgG1 or F(ab')2) abrogated GITRL binding to hGITR-expression cells at half-maximal effective concentration [EC50] < 1 nM (Figure 2D) due to its much higher affinity than GITRL (Kd > 5 μM as reported).34 Moreover, the epitope-binning assay confirmed competitive binding of IBI37G5 with GITRL (Figure 2E). To determine the epitope of IBI37G5, we performed an alanine-scanning experiment by introducing a mutation to each residue on all possible surface regions of GITR’s extracellular domain (ECD) and evaluated how they affect the bindings to antibody. Mutations on nineteen residues lead to reduced binding to IBI37G5 by 50% or more, and all the residues, except R90 and S107, are also required for GITRL binding (Figures 2F and S3E).

To investigate how subtle differences in epitope between IBI37G5 and GITRL influence the binding affinity and agonistic activity, we computationally modeled the complex structure of variable fragments (Fv) of IBI37G5 and GITR’s ECD based on the recently solved GITR crystal structures33 and our alanine-scanning result (Figures 2G, 2H, and S3F). Our model of the Fv-GITR binding interface highlighted four key amino acids (R90, K105, F106, S107) on GITR that form extensive interactions with residues in CDR3s of both variable heavy (VH) and variable light (VL) regions (Figure 2G). Superimposition of IBI37G5-Fv on the structure of GITRL-GITR complex (PDB: 7KHD) reveals a large overlapping binding area within the conformational epitopes located on the surfaces of CRD2 domain (Figure 2H). The binding interface with total SASA of ~1,274 Å² comprises an extensive network of hydrophobic interactions, van der Waals (VDW) interactions, and hydrogen bonds that may contribute to the marked difference (~1,000-fold) in affinities, despite a substantially shared binding epitope, between antibody and GITRL (Figure 2I). By computer modeling, we predicted that the bivalent IBI37G5 was able to engage two GITR receptors simultaneously (one GITR by each Fab arm) on the cell membrane based on the statistical analyses of IgG1 antibody conformations.35 The most probable distance between the C termini of IBI37G5-bound GITR is about 120 Å (range from 90 to 200 Å) (Figure 2J), which is close to the distance of 108 Å observed in the trimeric GITR-GITRL structure, to allow sufficient spacing of GITR intracellular domains for TRAF trimer engagement.

Collectively, these data show that IBI37G5 is a high-affinity ligand-blocking GITR agonist antibody that mimics the mode of action of GITRL to facilitate efficient GITR activation independent of FcγR.

**IBI37G5 combined with PD1 blockade synergistically activates T cells in vitro and in vivo**

To investigate the function of IBI37G5 on T cell activation, we first conducted an in vitro human T cell co-stimulation assay. In 2/4 healthy donors, single-agent IBI37G5 induced modest but significant (p < 0.05) activation of T cells as measured by interferon (IFN-γ) release (Figure 3A). When combined with anti-PD1 antibody sintilimab (Tyvvt),36 IBI37G5 significantly enhanced the activity of sintilimab in 3/4 donors. To examine the immune-modulating activities of human-specific antibodies in vivo, we used the immunocompetent hPD1/hGIT double knockin mice to test whether IBI37G5 alone or in combinations could promote anti-tumor immunity in syngeneic tumor models. We found that

**Figure 2. Characterization of IBI37G5, a ligand-mimetic anti-GITR agonist antibody**

(A) Binding of IBI37G5 to CHOS-hGITR cells using fluorescence-activated cell sorting (FACS) analysis.
(B) Kinetic analysis of IBI37G5 binding to hGITR using surface plasmon resonance.
(C) Agonistic activities of different IBI37G5 formats in Jurkat-hGITR NF-κB reporter assay. Graph shows representative results of at least 3 replicate experiments.
(D and E) IBI37G5 competes GITRL binding to GITR in FACS and bio-layer interferometry (BLI) analysis.
(D) Competitive binding of IBI37G5 with hGITRL-mFc on Jurkat-hGITR cells. Mean ± SD is presented.
(E) Sandwich ligand-blocking assay showing hGITR/hGITRL interaction blocked by IBI37G5. Representative sensorgrams from duplicate measurements are shown.
(F) Alkaline scanning on GITR shows residues required for IBI37G5 or GITRL binding (red) and residues only required for IBI37G5 binding (purple).
(G) Modeled structure of hGITR and Fv (IBI37G5) complex shown in cartoon. hGITR, VH, and VL are colored in yellow, marine, and blue, respectively. Interface residues included in the epitope and CDR3 regions are shown as sticks.
(H) hGITRL, Fv, and overlapped binding regions on hGITR.
(I) Superimposed structures of hGITR/Fv (IBI37G5) and hGITR/hGITRL complexes (left). Schematic diagram elucidated the significant interactions between hGITR (gray) and IBI37G5-VL (magenta) and IBI37G5-VH (yellow) and hGITRL (cyan). Hydrogen bonds, salt bridges, and van der Waals interactions are indicated in orange dashed lines, purple lines, and green lines, respectively (right). The table listed the information of interactions (bottom).
(J) Comparison of modeled receptor-antibody (left) and receptor-ligand (right) complexes shown in surface representation. hGITR/IBI37G5 was modeled based on the most probable conformation of hlgG1. One GITR receptor dimer was masked from hGITR/hGITRL complex to show receptor-ligand interaction. The distance was measured between the C termini of modeled hGITR.
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in both MC38 and CT26 tumors, the efficacy of either antibody alone was relatively weak, showing only modest tumor-growth delay. However, in the combination-treatment group, we observed significant tumor regressions, suggesting a synergistic effect (Figures 3B and 3C). To better understand the underlying mechanism, we analyzed the phenotypes and functionalities of TILs in MC38 tumors 1 week post drug treatment. The tumor weight changes were consistent with the above efficacy studies during the short treatment cycle, showing best activities in the combination group (Figure 3D). In addition, the proportions of total tumor-infiltrating CD8+ T cells and cytotoxic GzmB+CD8+ T cells after combination therapy were both highest among all treatment groups (Figure 3E). However, no such changes were observed in the tumor-infiltrated CD4+ T cells (Figure 3F). To examine how different therapy affects the polyfunctionalities of tumor-specific T cells, we isolated the TILs from MC38 tumors and analyzed their intracellular production of effector molecules after stimulation with the autologous tumor cells ex vivo. Increased productions of IFN-γ and TNF-α by CD8+ and CD4+ TILs were detected, to various extents, in all treatment groups compared with IgG control, with TILs after combination therapy showing highest induction levels (Figures 3G and 3H). Despite the comparable expressions at baseline, both the proportions and absolute numbers of CD8+ TILs positive for single (GzmB) or double effector parameters (IFN-γ and TNF-α) by CD8+ and CD4+ TILs were detected, to various extents, in all treatment groups compared with IgG control, with TILs after combination therapy showing highest induction levels (Figures 3E–3H and S4). Together, these data suggest that the tumor-specific CD8+ TILs, as well as a smaller CD4+ subset, exhibited the potential for activation, infiltration, and cytotoxicity after combination therapy of anti-GITR and anti-PD-1 antibodies, which led to the in vivo anti-tumor efficacy. Previous studies have reported that GITR activation could down-regulate Foxp3 expression and inhibit the immunosuppressive activity of Treg cells and that GITR antibodies could also deplete GTRhigh Treg cells in the TME through Fc-mediated ADCC functions. As IBI37G5 has a strong GITR activation effect and adopts an ADCC–competent hIgG1–Fc, we speculate that IBI37G5 may exert anti-tumor efficacy (at least partially) by modulating Treg functions. But, unexpectedly, IBI37G5 treatment did not affect either Foxp3 levels or Treg percentages in CD4+ T cells from mouse tumors or spleens (Figure 3I). A small, but significant, decrease of Treg percentage was observed only in the combination group, possibly due to some indirect effects that negatively affected the Treg availability in the pro-inflammatory TME after immune stimulation. Collectively, we show that IBI37G5 could work in conjunction with PD1 antibody to directly activate effector T cells, especially cytotoxic CD8+ T cells, but less likely function through depleting Treg cells, to exert anti-tumor effects.

**GITR homodimerization and antibody bivalency are both required for the agonistic activity of IBI37G5.**

Recently, Wang et al. reported that both mouse- and hGITR have pre-ligand assembly domain (PLAD)-like sequences at the membrane-proximal CRD3 domain to enable the formation of...
homodimer structures through non-covalent interactions, which resembles the N-terminal PLAD-mediated receptor clustering in other TNFR family members. Homodimer structure is necessary for ligand-dependent formation of high-order GITR clustering and downstream signal amplification. Given the mechanistic similarities between IBI37G5 and GITRL, we speculate that IBI37G5 also requires GITR-receptor dimerization to exert its agonistic activity. To test this hypothesis, we introduced amino-acid mutations on two critical phenylalanine residues (F137 and F139) simultaneously into alanines (AA), arginines (RR), or aspartic acids (DD) to reduce or abolish the receptor-receptor hydrophobic interface and examined how these changes might affect the activity of GITR agonists. In silico, we modeled three GITR mutants and analyzed their interface binding energy. These GITR dimer mutants had higher overall energy and dG separated/dSASA (the binding energy per unit interface surface area) and were more difficult to form stable homodimers than wild-type GITR (Figure 4A). In Jurkat NF-κB reporter cells expressing GITR receptors (wild type [WT] or mutants) (Figure S6A), the capability of IBI37G5 to activate downstream signaling was positively correlated with the feasibility of GITR variants to form homodimers: GITR(WT)>GITR(AA)>GITR(RR)~GITR(DD) (Figure 4B, top). A similar trend was also observed with GITRL trimer and high-valency GITR agonist MED1873 (Figure 4C). Although FcγR-mediated cross-linking was not required for function, the agonistic activity of IBI37G5 in reporter cells expressing all GITR variants was dramatically amplified by FcγR-expressing Raji cells (Figure 4B, bottom). Notably, signal transductions of GITR interface mutants were largely rescued to the activity levels approaching WT-GITR by Raji co-cultures, suggesting that FcγR-mediated secondary cross-linking can overcome the defects of mutant GITRs in insufficient oligomerization after IBI37G5 stimulation. In comparison, the structure of the membrane-proximal domain of another TNFRSF member 4-1BB is highly homologous to GITR but lacks the hydrophobic interface needed for receptor homodimerization (Figure S6C). After replacing this hydrophobic region on CRD3 of GITR with a corresponding 4-1BB fragment, we found a near-complete loss of IBI37G5 activity, which was rescuable by Raji cross-linking (Figures S6D and S6E), reinforcing the notion that, in contrast to 4-1BB, the ability of receptor homodimer formation was indispensable for GITR activation.

Previous studies have shown that, unlike antagonist antibodies, TNFRSF agonist antibodies usually require bivalency or even higher valency to exert activity. Considering the similarities shared by TNFRSF members, we postulate that IBI37G5 also requires antibody bivalency for activity. To this end, we designed a monovalent form of IBI37G5 (mvIBI37G5) and tested its activity on different GITR variants. As expected, the GITR agonistic activity was almost completely abolished in the mvIBI37G5 form. Even with Raji co-culturing, the activity of mvIBI37G5 could only be slightly restored in WT-GITR-expressing reporter cells but not in other homodimer-interface mutants (Figure 4D). Moreover, we used confocal microscopy to directly visualize and quantify the capability of IBI37G5 to induce receptor oligomerization. Jurkat cells expressing WT-GITR-GFP fusion proteins showed strong induction of receptor clustering as measured by increased GFP foci numbers and fluorescence intensities after IBI37G5 treatment (Figure 4E). In contrast, neither GITR interface mutant-expressing nor mvIBI37G5-treated cells exhibited measurable foci formation, except scattered foci of smaller sizes and weaker intensities were detected in IBI37G5-treated GITR(-AA)-Jurkat cells, in line with its weak residual activity in the reporter assays.

Collectively, we show that IBI37G5 promotes GITR oligomerization and activation in a way that highly resembles GITRL, which is dependent on both receptor homodimerization and antibody bivalency for high-order receptor clustering and signal amplifications in T cells.

**IBI37G5 induces a bell-shaped dose response in vitro**

In the Jurkat reporter assay, we observed that when the concentration of IBI37G5 (or GITRL trimer) exceeded that of peak activity, the NF-κB signal began to decrease rather than reaching a plateau, presenting a bell-shaped response that is reminiscent of some previously reported agonist antibodies. We speculated that this phenomenon was due to the intrinsic properties of bivalent IBI37G5 rather than being Fc related (Figure S7A). As the NF-κB reporter system is an artificial system that cannot truly reflect the immediate changes of GITR downstream signal in human T cells, we isolated CD4^+ T cells from PBMCs and then co-stimulated these *Staphylococcus enterotoxin B* (SEB)-primed T cells with IBI37G5 before detection of NF-κB p65 phosphorylation. With the rise of IBI37G5 concentrations, the phosphorylation levels of NF-κB in GITR^+ CD4^+ T cells gradually elevated and later peaked at 37 nM. But after the peak, a continued trend of decreasing activities was observed despite the increase of drug concentrations up to 1,000 nM. In contrast, the phosphorylation of NF-κB in GITR^+ CD4^+ T cells, as an internal control, remained unchanged during the treatment course, suggesting a target-specific effect of IBI37G5-induced bell-shaped GITR activation in vitro (Figure 5A). Similar results of phosphorylation on two different sites (SS36 and SS29) of NF-κB p65 in Jurkat-GITR cells were also observed (Figure S7B).

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Figure 4. GITR homodimerization and antibody bivalency are both required for the agonistic activity of IBI37G5

(A) The cartoon models of WT dimeric hGITR and mutants when mutating two critical interface residues F137 and F139 into alanines (AA), arginines (RR), or aspartic acids (DD). The mutated residues are shown as sticks. Overall stability and interface energy density of WT dimeric hGITR and mutants were calculated by ROSETTA relax application.

(B–D) Activities of GITR agonists: IBI37G5 (B), GITRL trimer or GITRL hexamer MED1873 (C), or monovalent (mv) IBI37G5 (D) measured in Jurkat NF-κB reporter cells expressing WT or mutant GITRs. Mean ± SD is presented.

(E) Confocal images showing GITR receptor clustering on Jurkat cells expressing WT or mutant GITRs upon indicated treatments at 10 nM. Scale bar: 10 μm. Quantification of GFP foci number and intensity (cell number = 20–50). Mean fluorescence intensity of foci or diffused cytoplasmic GFP signal was measured. Median (50%) and quartiles (25%, 75%) were shown in violin plots. Experiments were repeated at least twice. p values were calculated using one-way ANOVA. ***p < 0.0001.
Conceivably, at the optimal dose, an antibody-receptor stoichiometric balance is established when two Fab arms of the same antibody simultaneously occupy two different GITR receptors in a 2:2 stoichiometry to facilitate higher order receptor cross-linking. By contrast, at higher concentrations, the existing balance is disrupted when antibodies self-compete for receptor binding. In this case, only one Fab of each antibody is able to engage GITR, while the other arm remains unoccupied and ineffective and somewhat mirrors the binding mode of mvl-BI37G5, thereby resulting in insufficient receptor activation. To test this hypothesis, we designed an experiment to detect the dynamic changes of RO and antibody self-competition by measuring the abundance of antibodies bound to GITR+ T cells and the availability of free Fabs of GITR-bound antibodies on the cell surface, respectively. We found that the binding signal of BI37G5 on activated CD4+ T cells reached saturation at concentrations exceeding 12 nM (Figure 5B, bottom), which was in line with the RO assay that showed near-zero GITR availability on human T cells after pre-incubation with BI37G5 at 22 nM or higher, indicating 100% RO beyond this concentration point (Figure S7C). By using AF647-labeled GITR-ECD protein to measure the free Fabs of GITR-bound BI37G5, we detected only baseline signals on T cells pre-treated with BI37G5 at concentrations below the receptor-saturating point, suggesting that no detectable Fabs were exposed due to the avid, bivalent binding of BI37G5 to GITR.

However, at higher concentrations of saturating GITR occupancy, a marked increase of the GITR-ECD-AF647 signal was detected, indicating the exposure of free Fabs due to self-competition and monovalent binding of IBI37G5 to GITR receptors (Figure 5B, top). Similar results were also obtained in the experiments analyzing different T cell subsets of PBMCs (Figure S7D).

To examine whether different levels of antibody-RO would affect GITR oligomerization on the cell membrane, we used confocal imaging to visualize the receptor cluster formation on Jurkat-GITR-GFP cells. The strongest receptor cross-linking (shown as GFP foci) was observed at the concentration of optimal RO (~10 nM), while at higher concentrations, both the GFP foci numbers and fluorescent signals decreased significantly, consistently with above mentioned results in reporter assays (Figures 5C and 5D). To further investigate the RO-function relationships in primary human T cells, we assessed the immunomodulatory activities of BI37G5 in human CD4+ T cells. In all four donors, the T cell activation markers (CD25 and CD69) and the release of effector cytokines (interleukin-2 [IL-2] and IFN-γ) were all upregulated after IBI37G5 treatment and peaked within the concentration range of 11–33 nM but declined at 100 nM, echoing the previous findings (Figure 5E). Furthermore, in the multiplex human PBMC cytokine release assay, a similar trend of dose response was also observed (Figure S7E).

Besides IBI37G5, we also tested other clinical-stage TNFRSF agonist antibodies, TRX518 (anti-GITR), pogaizumab (anti-OX40), and urelumab (anti-4-1BB), in their respective Jurkat reporter assays. All of these antibodies exhibited bell-shaped dose responses (Figure S7F), suggesting the universality of this phenomenon among TNFRSF agonists.

The bell-shaped anti-tumor response of IBI37G5 is associated with the level of GITR receptor saturation in vivo

To study whether the in vitro-defined RO-function relationship also prevails in animals, we tested the anti-tumor efficacy of IBI37G5 as single agent or in combination with anti-PD1 antibody in different tumor models. In the MC38 tumors, IBI37G5 alone only showed weak activities, while the combination group exhibited marked tumor-growth delays as well as tumor regressions in animals (Figures 6A and S8A). Notably, IBI37G5 at 1 mg/kg combined with anti-PD1 antibody showed best efficacy, including 25% (3/12) tumor free in treated mice. By contrast, at higher doses, the incremental benefit of drug combination was diminished at 3 mg/kg or near abrogated at 10 mg/kg compared with anti-PD1 monotherapy, suggesting the loss of IBI37G5 activities at high exposure levels in vivo (Figures 6A–6C). Next, we validated these findings in another anti-PD1-resistant B16F10 tumor model. Despite no tumor regressions, the most dramatic tumor-growth inhibition was found in 1 mg/kg IBI37G5-treated mice (Figures 6D, 6E, and S8B).

To establish the in vivo PK/PD relationship, we performed a PK study of IBI37G5 in hGITR knockin mice. Although there was a higher clearance rate at low dose (0.3 mg/kg) due to the antigen-sink effect, IBI37G5 exhibited a trend of linear exposure at doses above 1 mg/kg (Figures 6F and S8C). Interestingly, we found that the exposure level of IBI37G5 at 1 mg/kg covered the range of optimal activities (10–100 nM, blue shade) in the majority of the weekly dosing cycles. By contrast, due to the fast clearance at 0.3 mg/kg, the systemic exposure rapidly fell outside the optimal range after the first 24 h. Nevertheless, the systemic exposures of IBI37G5 at 3 and 10 mg/kg exceeded that of optimal range, leading to complete saturation of GITR RO throughout the dosing cycle (Figure S8D). We speculate that, analogous to our in vitro findings, constant (over-)saturation of GITR by high IBI37G5 concentrations in vivo could also lead to antibody self-competition and insufficient engagement with GITR and thereby affect the anti-tumor efficacy. By analyzing the RO- efficacy relation, we saw that the near-saturated RO of GITR at 1 mg/kg conferred best anti-tumor activities in both models, recapitulating the bell-shaped dose responses in vivo (Figure 6G).

Together, these results indicate that the optimal target saturation during the dosing cycle is a key determinant factor for allowing IBI37G5 to fully engage and activate GITR in a proper antibody-receptor stoichiometry, which is more beneficial for achieving maximal anti-tumor efficacy than insufficient or over saturation of GITR receptors.

PK/PD and toxicology studies of IBI37G5 in NHPs

To better predict human responses, we carried out the good laboratory practice (GLP) PK/PD and toxicology studies in NHPs. IBI37G5 showed a typical monoclonal antibody PK profile with long terminal half-life and dose-dependent exposures to support at least bieweekly dosing in humans (Figures S9A and S9B). After repeated IBI37G5 administrations, the percentages of different T cell subsets in the peripheral blood did not change compared with the control group (Figure S9C). Consistent with the findings in mice, no meaningful reductions of Foxp3 expressions or total Treg cell numbers were observed after drug treatment, further...
Figure 5. IBI37G5 induces a bell-shaped dose response in vitro

(A) Bell-shaped response of IBI37G5 in human CD4+ T cells. SEB-primed human CD4+ T cells were incubated with IBI37G5 for 5 min, and NF-κB p65 phosphorylation was detected by flow cytometry.

(B) Detection of freely exposed Fabs of IBI37G5 at different levels of target saturation.

(C) GITR receptor clustering upon IgG or IBI37G5 treatment at different concentrations. Scale bar: 10 μm.

(D) Quantification of foci number and intensity by GFP florescence (cell number = 23–66). Mean florescence intensity of foci (cells with foci formation) or diffused cytoplasmic GFP signal (cells without foci) was measured. Median (50%) and quartiles (25%, 75%) were shown in violin plots. Experiments were performed in duplicate.

(E) Bell-shaped response induced by IBI37G5 in human CD4+ T cell activation and functional analyses. Experiments were performed in triplicate using T cells from 4 healthy donors. Mean ± SEM is presented, and p values were calculated using one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
substantiating the Treg-independent mechanisms of IBI37G5. More importantly, IBI37G5 was well tolerated after repeated doses in monkeys, and the maximal tolerated dose (MTD) was not defined in this study. No drug-related serious clinical symptoms were recorded, and the hematology and blood biochemistry parameters were all within the normal range despite minor fluctuations (data not shown). Most cytokines were maintained at their pre-treated baselines, except the evident increase of IL-6 (although one to two orders of magnitude lower than that in cytokine release syndrome [CRS]) in monkey plasma (Figure S9D). Few significant toxic effects were seen in the major organs at postmortem examinations except for some abnormalities in thymus and adrenal gland in the highest-dose group (Figure S9E).

**DISCUSSION**

Co-stimulatory antibodies that enhance anti-tumor immunity have great potential in cancer therapy. But until now, successful translation of these therapies into patients still remains a big challenge, due to either insufficient efficacy or severe immune-related toxicities. In-depth research on the MOAs of immune agonists is of great significance to better guide their clinical development. In this study, we characterized an anti-GITR agonist antibody IBI37G5 and studied its molecular mechanisms using primary human cells and human gene knockin animals. Unlike many TNFRSF agonist antibodies that non-competitively stabilize the pre-formed receptor-ligand complex and/or require Fc-mediated secondary cross-linking for efficient signal transduction, IBI37G5 is unique in that the high-affinity ligand-competitive antibody is able to directly induce robust GITR clustering and downstream signaling through bivalent Fab-receptor engagement, independent of ligand co-binding or Fc-related functions. In many cases, antibodies that fully block the receptor-ligand interactions exhibit antagonistic, rather than agonistic, activities, so how IBI37G5 induces strong GITR agonism is an intriguing recent question. Recent studies have shown that by simply switching the IgG isotypes (hlgG1→hlgG2), the CD40 antagonists can be converted into strong agonists, possibly due to the hlgG2-mediated higher binding avidity and strong self-association tendency upon antigen binding. Although such self-association has been previously described for other human IgG isotypes, it is less likely that IBI37G5 exerts its agonistic activity through similar mechanistic changes due to the much weaker self-association capability of hlgG1. In addition, the assembly of IgG1 oligomers is dependent on Fc-Fc interactions, but the Fc-agonist F(ab')2 of IBI37G5 is fully functional, therefore excluding the effects of IgG self-assembly on agonist activity. Rather, these results suggest that the intrinsic binding properties of IBI37G5, such as kinetics, epitope, and stoichiometry, could be the key determinants for the unique MOAs that distinguish it from other TNFRSF agonists.

Through epitope mapping and in silico antibody-receptor docking, we show that IBI37G5 recognizes GITR at an epitope largely overlapping with GITRL. It is able to maintain proper spacing between receptors on the cell membrane to form complexes that resemble the basic signaling units reported in other TNFRSFs. The high affinity of IBI37G5 enables avid, bivalent engagement of GITR homodimers, which may further increase the chance of free IBI37G5 to successfully link and stabilize two individual antibody-receptor complexes and drive higher-order receptor clustering (Figure 7A). In contrast to other well-studied TNFRSF agonists, this model suggests a different mechanism that the natural ligands are dispensable and that the pre-arranged receptor-ligand complexes are not required for IBI37G5-induced receptor activation. Particularly, IBI37G5 can mimic the function of GITRL to pre-assemble and then cross-link the basic signal units. The correct stoichiometry between IBI37G5 and GITR is essential for efficient receptor oligomerization and signal amplification (Figures 7A–7C). However, despite the similarities between IBI37G5 and GITRL, their differences in valency may greatly impact the stoichiometry of receptor-ligand complexes and the manner of higher-order signaling network formation on the cell membrane. Based on the crystal structures of GITR, Wang et al. proposed that the hGITR-GITRL complex can form a hexagonal “honeycomb-like” grid consisting of six GITR trimers and six GITR trimers. In contrast, the bivalent IBI37G5 is geometrically improbable to gather GITR receptors into a similar branched hexameric structure. Contrary to the trimeric form of hGITR, mouse GITRLs favor the biologically active dimer conformation. The C-terminal three-residue deleted mGITRLs can form activity-enhanced trimers, indicating that both human and mouse GITRLs can utilize various oligomeric forms (including dimer, trimer, and even superclusters) to fine-tune the receptor activations. Likewise, bivalent IBI37G5 serves as a hyperactive surrogate of hGITRL dimer (Figure S6B) and has 2:2 stoichiometry that resembles the murine GITR-GITRL interactions. As to whether the antibody-receptor complexes form a linear chained structure or higher-dimensional complexes still remains a matter of further investigation.
The instances of bell-shaped dose responses have been reported in many drug classes. For immunostimulatory antibodies, many studies have investigated the mechanisms underlying this phenomenon through in vitro experiments, and various explanations were proposed. However, whether these theories can be verified in animal models, or even in clinical studies, remain to be explored. In this work, we have defined the stoichiometry of the antibody-receptor complex and established an RO-function relationship that determines the optimal GITR activations. These findings were validated in different murine syngeneic tumor models. To our best knowledge, it is the first attempt to investigate the bell-shaped anti-tumor responses based on the dynamic changes of RO-activity relations. Potentially, our study proposes a strategy for developing practical biomarkers to predict patient response and guide the clinical dose determination of immune co-stimulators that share similar MOAs. In the GLP toxicology studies, IBI37G5 showed linear PK and excellent safety profiles at exposure levels much higher than the efficacious dose, enabling its clinical translation in cancer patients. The human PK profiles can be projected from the monkey data alongside the clinical results of other GITR agonists using the interspecies allometric-scaling methods. It is reasonable to expect that IBI37G5 is able to achieve high exposures with acceptable tolerability in patients. If the predicted exposure significantly exceeds that of optimal efficacious dose, we should consider the possibility of bell-shaped dose responses.
In conclusion, despite hurdles in clinical translation, the ample pre-clinical evidence and signs of clinical response have demonstrated the promise of GITR agonists in cancer treatment. Given the unique properties of immune agonist antibodies, the dynamic RO-response-based approaches for PK/PD prediction and efficacious dose finding may be essential for the successful development of this drug class. In this respect, strategies described herein will be explored in future clinical studies to help unlock the full therapeutic potential of IBI37G5, and possibly other costimulatory agonists, in cancer patients.

Limitations of the study
First, the correlation between RO and anti-tumor efficacy was established based on the in vitro activity experiments and the retrospective PK/PD analysis in mice. Its prediction accuracy requires further validations in larger-scale animal experiments or even in clinical settings. Second, in contrast to using mouse surrogate antibodies or humanized mice with partially reconstituted immune system, immunocompetent hGITR/hPD1 double knockin mice were utilized in our study to better investigate the functions of human specific antibodies. However, the conclusions derived from these mouse models still need to be interpreted cautiously given the notable differences between human and mouse GITRs in both the expression profiles and functionalities. Finally, this study combined scRNA-seq data mining and multiplex immunofluorescence to analyze the spatiotemporal dynamics of GITR and PD1 in TILs of CRC. It would be interesting to explore whether tumors with increased TILs co-expressing GITR and PD1 benefit more from combination therapy. In addition, whether the dynamic changes of co-expression patterns can be extrapolated to other tumor types, and whether the expression levels of GITR-GITRL axis in the TME can be used as biomarkers to predict therapeutic efficacy or drug resistance are issues worthy of further exploration.

STAR Methods
Detailed methods are provided in the online version of this paper and include the following:

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Supplemental Information
Supplemental information can be found online at [https://doi.org/10.1016/j.xcrm.2022.100660](https://doi.org/10.1016/j.xcrm.2022.100660).

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Author Contributions
Conceptualization, K.H.; in vitro experiments, H.L., X.L., J.G., Z.K., and L.H.; pharmacology & toxicology, W.W., L.C., Y.Y., J.W., J.L., M.W., D.W., and B.C.; protein sciences, F.F., Z. Wu, S.Z., and Z.S.; structural analysis, G.S., W.X., and Z. Wang; bioinformatics, T.C.; writing – original draft, K.H.; writing – review & editing, all authors; supervision, K.H. and Z. Wang.

Declaration of Interests
H.L., W.W., T.C., L.C., X.L., J.G., F.F., Y.Y., Z. Wu, S.Z., J.W., J.L., Z.K., M.W., Z.S., L.H., D.W., B.C., and K.H. are current employees and own stocks of Innovent Biologics (Suzhou) Co., Ltd. F.F. is co-inventor of GITR antibodies used in this study (patent no. WO2019201301A1).

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| APC anti-human IgG Fc Antibody | BioLegend | Cat#409306 |
| PE anti-rat IgG2b Antibody | BioLegend | Cat#408214; RRID: AB_2749893 |
| BV421 Mouse Anti-Human CD279 (PD-1) | BD Biosciences | Cat#565935; RRID: AB_11153482 |
| CD357 (AITR/GITR) Monoclonal Antibody (eBioAITR), PE | eBioscience™ | Cat#12-5875-42; RRID: AB_2572634 |
| PE/Cyanine7 anti-human CD3 Antibody | BioLegend | Cat#300316; RRID: AB_314052 |
| Alexa Fluor® 488 anti-human CD4 Antibody | BioLegend | Cat#300519; RRID: AB_389311 |
| Alexa Fluor® 700 anti-human CD8a Antibody | BioLegend | Cat#300920; RRID: AB_528885 |
| Brilliant Violet 785™ anti-human CD25 Antibody | BioLegend | Cat#356140; RRID: AB_2750205 |
| Alexa Fluor® 647 anti-mouse/rat/human FOXP3 Antibody | BioLegend | Cat#320014; RRID: AB_439750 |
| Purified NA/LE Mouse Anti-Human CD3 | BD Biosciences | Cat#555329; RRID: AB_395736 |
| Purified NA/LE Mouse Anti-Human CD28 | BD Biosciences | Cat#555725; RRID: AB_396068 |
| FITC anti-human CD69 Antibody | BioLegend | Cat#310904; RRID: AB_314839 |
| APC Mouse Anti-Human CD25 | BD Biosciences | Cat#555434; RRID: AB_398598 |
| Perm Buffer III | BD Biosciences | Cat#558050; RRID: AB_2869118 |
| Phospho-NFkB p65 (Ser529) Antibody, PE | eBioscience™ | Cat#12-9863-42; RRID: AB_2572751 |
| Phospho-NF-kB p65 (Ser536) (93H1) Rabbit mAb, PE | Cell Signaling | Cat#5733 |
| Alexa Fluor® 488 anti-mouse CD45 Antibody | BioLegend | Cat#103122; RRID: AB_493531 |
| Alexa Fluor® 700 anti-mouse CD45 Antibody | BioLegend | Cat#103128; RRID: AB_493715 |
| CD8a Monoclonal Antibody (53-6.7), PerCP-Cyanine5.5 | eBioscience™ | Cat#45-4321-80; RRID: AB_1107004 |
| Brilliant Violet 510™ anti-mouse CD8a Antibody | BioLegend | Cat#100752; RRID: AB_2563057 |
| CD4 Monoclonal Antibody (GK1.5), APC | eBioscience™ | Cat#17-0041-81; RRID: AB_469320 |
| BB700 Rat Anti-Mouse CD4 | BD Biosciences | Cat#566407; RRID: AB_2744427 |
| Brilliant Violet 785™ anti-mouse CD3 Antibody | BioLegend | Cat#100232; RRID: AB_2562554 |
| PE anti-mouse CD25 Antibody | BioLegend | Cat#102008; RRID: AB_312857 |
| FOXP3 Monoclonal Antibody (FJK-16s), APC | eBioscience™ | Cat#17-5773-82; RRID: AB_469457 |
| TruStain FcX™ (anti-mouse CD16/32) Antibody | BioLegend | Cat#101320; RRID: AB_1574975 |
| FITC anti-human/mouse Granzyme B Recombinant Antibody | BioLegend | Cat#372206; RRID: AB_2687030 |
| PE anti-mouse IFN-γ Antibody | BioLegend | Cat#505808; RRID: AB_315402 |
| Brilliant Violet 421™ anti-mouse TNF-α Antibody | BioLegend | Cat#506328; RRID: AB_2562902 |
| Alexa Fluor® 700 anti-mouse CD45 Antibody | BioLegend | Cat#103128; RRID: AB_493715 |
| CD8a Antibody, PerCP-Cyanine5.5 | eBioscience™ | Cat#45-0081-82; RRID: AB_1107004 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| BUV395 Rat Anti-Mouse CD4 | BD Biosciences | Cat#740208; RRID: AB_2734761 |
| BUV737 Hamster Anti-Mouse TCR β Chain | BD Biosciences | Cat#612821 |
| PE/Cyanine7 anti-mouse Ly-6G Antibody | BioLegend | Cat#127618; RRID: AB_1877261 |
| V500 Rat Anti-Mouse I-A/I-E | BD Biosciences | Cat#562366; RRID: AB_11153488 |
| Brilliant Violet 785™ anti-mouse/human CD11b Antibody | BioLegend | Cat#101243; RRID: AB_2561373 |
| Brilliant Violet 421™ anti-mouse CD11c Antibody | BioLegend | Cat#117343; RRID: AB_2563099 |
| BV766 Hamster Anti-Mouse CD183 | BD Biosciences | Cat#741032; RRID: AB_2740650 |
| PE anti-mouse/human CD45R/B220 Antibody | BioLegend | Cat#103208; RRID: AB_312993 |
| APC anti-mouse CD64 (FcyRI) Antibody | BioLegend | Cat#139306; RRID: AB_11219391 |
| FITC anti-mouse CD16 Antibody | BioLegend | Cat#158008; RRID: AB_2860740 |
| CD32b Monoclonal Antibody (AT130-2), APC | eBioscience™ | Cat#75-5875-42; RRID: AB_2573485 |
| Alexa Fluor® 488 anti-mouse CD16.2 (FcyRII) Antibody | BioLegend | Cat#149524; RRID: AB_2687088 |
| Alexa Fluor® 700 anti-mouse CD45 Antibody | BioLegend | Cat#103128; RRID: AB_493715 |
| Alexa Fluor® 488 anti-mouse CD4 Antibody | BioLegend | Cat#100529; RRID: AB_389303 |
| BV421 Rat Anti-Mouse CD25 | BD Biosciences | Cat#564571; RRID: AB_2738849 |
| CD357 (AITR/GITR) Monoclonal Antibody (eBioAITR) | eBioscience™ | Cat#17-0321-82; RRID: AB_2573142 |
| Brilliant Violet 785™ anti-mouse CD45 Antibody | BioLegend | Cat#103149; RRID: AB_2564590 |
| BUV395 Rat Anti-Mouse CD4 | BD Biosciences | Cat#740208; RRID: AB_2734761 |
| InVivoMab anti-mouse CD4 | Bioxcell | Cat#BE0003-1; RRID: AB_1107636 |
| InVivoMab anti-mouse CD8β (Lyt 3.2) | Bioxcell | Cat#BE0223; RRID: AB_2687706 |
| Liberase™ TM Research Grade | Roche | Cat#05401127001 |
| DNase I | Sigma-Aldrich | Cat#D5025-375KU |
| foxp3 fixation/permeabilization kit | eBioscience™ | Cat#00-5523-00 |

**Chemicals, peptides, and recombinant proteins**

- **Human GITR**
  - ACROBiosystems
  - Cat#GIR-H5228
- **Puromycin**
  - Gibco
  - Cat#A11138-02
- **Staphylococcal enterotoxin B**
  - TOXIN TECHNOLOGY
  - Cat#B7202
- **Brefeldin A Solution**
  - BioLegend
  - Cat#420601
- **Alexa Fluor™ 488 Antibody Labeling Kit**
  - Thermo Fisher
  - Cat#A20181
- **Alexa Fluor™ 647 Antibody Labeling Kit**
  - Thermo Fisher
  - Cat#A20186
- **Bio-Glo™ Luciferase Assay System**
  - Promega
  - Cat#G7940
- **Human IFN gamma kit HTRF**
  - Cisbio
  - Cat#62HIFNPEG
- **MILLIPLEX MAP Human High Sensitivity T cell Panel**
  - Millipore
  - Cat#HSTCMAG-28SK
- **EasySep™ Human CD4+ T cell Enrichment Kit**
  - STEMCELL
  - Cat#19052
- **Human IL2 kit HTRF**
  - Cisbio
  - Cat#62HIL02PEG
- **LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit**
  - Thermo Fisher
  - Cat#L34968
- **LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit**
  - Thermo Fisher
  - Cat#L34976
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact Kaijie He (kaijie.he@innoventbio.com).

Materials availability
All unique/stable reagents generated in this study are available from the Lead contact with a completed Materials Transfer Agreement.

Data and code availability
CRC scRNA-seq data used in this study are downloaded from the ArrayExpress database at EMBL-EBI: E-MTAB-8107. Annotated BCC scRNAseq data were downloaded from the Gene Expression Omnibus (GEO) : GSE123814.

The code supporting for modeling, molecular dynamics and in-silico mutagenesis and analysis of this study are available within the article and supplemental information. Other data are available from the corresponding authors upon reasonable request.

Any additional information required to reanalyze the data reported in this paper is available from the Lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
HEK293T cells (ATCC) were cultured in DMEM supplemented with 10% FBS. Jurkat cells (genomeditech), Raji cells (COBIOER), and CTLL-2 cells (ATCC) were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS. MC38 (HYC3401, Obiosh) and B16F10 (ATCC, CRL-6475) cells were cultured in DMEM supplemented with 10% FBS. CT26 (ATCC, CRL-2638) cells were cultured...
in RPMI1640 supplemented with 10% FBS. CHO-S cells (Invitrogen) were electroporated with vectors containing human GITR (NCBI ID: NP_004186.1) or cynomolgus GITR (NCBI ID: XP_005545180.1) according to the manufacturer’s instructions. All cell lines were maintained at 37 °C in a humidified incubator at 5% CO2.

**Animals**

4–6 weeks old female hGITR/hPD1 double knocked-in C57BL6 mice (Biocytogen) or hGITR/hPD1 double knocked-in Balb/c mice (Gempharmatech) were maintained under standardized conditions with a 12 h/day light cycle and controlled temperature (20–22 °C) and humidity (40–60%). All mice studies were performed according to Regulations for Care and Use of Laboratory Animals at Innovent Biologics and were approved by Innovent’s Institutional Animal Care and Use Committee (IACUC). For cynomolgus monkeys studies, same number of male and female monkeys were included into each group. All monkey experiments were approved by IACUC and performed by WestChina-Frontier PharmaTech, according to the regulations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

**Human PBMCs**

Human PBMCs were purchased from Miao Tong Biological Technology and cultured in AIM V Medium CTS. Miao Tong Biological Technology represents and warrants that it has obtained ownership rights with respect to products and that such products were provided to Miao Tong Biological Technology with every donor’s informed consent and in compliance with all applicable laws and regulations.

| Donor | Gender | Blood type | Age | Height (cm) | Body weight (kg) |
|-------|--------|------------|-----|-------------|-----------------|
| 1     | male   | A          | 26  | 175         | 63              |
| 2     | male   | A          | 19  | 173         | 62              |
| 3     | male   | B          | 32  | 160         | 77              |
| 4     | male   | O          | 28  | 185         | 76              |
| 5     | male   | B          | 18  | 172         | 56              |
| 6     | male   | O          | 27  | 175         | 60              |
| 7     | male   | O          | 30  | 178         | 85              |
| 8     | male   | O          | 33  | 180         | 72              |
| 9     | female | O          | 24  | 171         | 59              |
| 10    | male   | O          | 29  | 169         | 62              |
| 11    | male   | A          | 34  | 175         | 62              |

**METHOD DETAILS**

**In vitro assays**

**Protein expression**

For GITRL trimer protein, the coding sequences of a his-tag, a coronin trimeric domain, and the GITRL ECD were cloned into pcDNA3.1 expression vector. For GITRL hexamer protein, the coding sequences of a coronin trimeric domain, a Fc domain, and the GITRL ECD were cloned into pcDNA3.1. Proteins were expressed using Expi293 expression system (Thermo Fisher). Cell supernatants were collected at 5–7 days post-transfection, and proteins were purified using Ni-NTA (Qiagen) or protein A affinity chromatography.

**Affinity measurement**

Affinity of IBI37G5 to human GITR and cynomolgus GITR were measured on a Biacore T200 using HBS-EP+ (Cytiva, BR-1006-69) as the running buffer. Firstly, anti-human Fc IgG (Abcam, Ab97221) was immobilized on the activated flow cells of a CM5 sensor chip (Cytiva, 29-1496-03) at around 10,000 RU. Then, 2 μg/mL IBI37G5 was captured onto the immobilized sensor chip for 30 s. 2-fold serial dilutions (1.25–40 nM) of human GITR (Acro biosystems, GIR-H5228) and cynomolgus GITR (Sino Biological, 90871-C08H) as well as blank running buffer were injected onto the sensor surface for 180 s, and followed a 600-s phase of dissociation. At the end of each cycle, the sensor was regenerated by a 30-s injection of 10 mM glycine pH 1.5 (Cytiva, BR-1003-54). Raw data were processed using a 1:1 binding model using the Biacore T200 evaluation software version 3.1. For IBI37G5’s affinity to FcγR, anti-his antibody (Cytiva, 28995056) was immobilized on the CM5 sensor chip at a density of 6,000–8,000 RU. 1 μg/mL poly-histine FcγRs were added onto the immobilized sensor chip for 60s, and IBI37G5 of different IgG subtypes were injected onto the sensor surface. Cross-reactivity of IBI37G5 were analyzed using Octet Red96e (ForteBio). Briefly, IBI37G5 was loaded onto AHC biosensors (ForteBio) at a density of around 1 nm, and 2-fold serial dilutions of GITR from rhesus, canine, mouse and rat were loaded on the biosensors.
After a 180-s association phase, the biosensors were transferred into SD buffer (1x PBS, 0.1% BSA, 0.05% tween-20) for 600 s for dissociation. All the dilutions were prepared in SD buffer, and experiments were performed at 30°C. Binding affinity was calculated using the Octet Data Analysis software (Version 11.0) using 1:1 binding model.

**Competitive binding assay**

For ligand competition assay using flow cytometry, Jurkat cells overexpressing human GITR were incubated with varying concentrations of IBI37G5 and mixed with 3 nM GITRL-mFc (Sino Biological, 16080-H38H) in 1% BSA/PBS buffer at 4°C for 30 min. After three PBS washes, competition of IBI37G5 was determined by measuring the binding of GITRL-mFc using APC-labeled goat anti-mouse Fc antibody (Biolegend, 405308). To measure the blocking ability of IBI37G5 to GITR/GITRL by SPR, poly-histidine-tagged GITR (ACRO biosystems, GITR-H5228) at 100 nM was loaded onto HIS1K biosensors (18–50, Fortebio) and the loaded biosensors were saturated by 100 nM IBI37G5. SD buffer was used as unsaturated control. 100 nM GITRL trimer protein (manufactured in house) was loaded onto the biosensors for competition and the lack of GITRL binding signal after IBI37G5 saturation indicates a blocking effect.

**Epitope mapping**

Ala scanning assay was used to map the binding epitope of IBI37G5 to GITR. Briefly, wild-type GITR and mutant GITR were cloned into pcDNA3.1-EGFP and transiently expressed in HEK293T. Cell binding was measured by flow cytometry 48-h post-transfection.

**Receptor occupancy assay**

PBMC derived CD4⁺ T cells were activated by Dynabeads CD3/CD28 (Gibco, 11131D) for 4 days. 2 × 10⁵ activated CD4⁺ T cells were seeded onto 96-well U-bottom plates and incubated with serially-diluted antibodies for 30 min at 4°C. After several rounds of washes with PBS, samples were stained with Alexa Fluor 488 labeled IBI37G5 (IBI37G5 was conjugated using Alexa Fluor™ 488 antibody labeling kit (Thermo Fisher, A20181)). The intensity of the AF488 fluorescence signal was detected to measure GITR receptor availability on CD4⁺ T cells by flow cytometry. GITR occupancy was calculated as the mean fluorescent intensity (MFI) ratio of test samples in relative to the cells with over-saturated IBI37G5.

**Detection of freely exposed Fabs of IBI37G5**

3 × 10⁵ PBMCs or Jurkat-GITR reporter cells were seeded onto 96-well plates and incubated with different concentration of IBI37G5 at 4°C for 1 h. Cells were then fixed with 4% fixative solution (Solarbio, P1110). Recombinant GITR protein (Acrobiosystems, GIR-H5228) conjugated with Alexa Fluor 647 (Thermo Fisher, A20173) and anti-human IgG-PE (Southern biotech, 201109) were used to stain respectively on cell surface and cell surface-bound IBI37G5, respectively. Fluorescent signals were detected by flow cytometry.

**Antibody internalization assay**

1 × 10⁵ Jurkat-GITR reporter cells were incubated with 100 nM Alexa Fluor 488 labeled IBI37G5 or control IgG at 4°C for 1 h to saturate the cell surface expressed GITR. Unbound IBI37G5 was then washed away and cells were incubated at 37°C for 0, 1, 2, 3, 4, 5, or 6 h. APC anti-human IgG Fc (BioLegend, 409306) was then used to detect remaining IBI37G5 on cell surface. The internalization assay of anti-mouse antibody DTA-1 was performed using the same method with CTLL-2 cells and PE anti-rat IgG antibody (BioLegend, 408248) to detect remaining DTA-1 on cell surface. The internalization rate was calculated by the following formula: ([MFI_A488/MFI_APO_Sample] - [MFI_A488/MFI_APO_Control])/([MFI_A488/MFI_APO_Control] - [MFI_A488/MFI_APO_Control]).

**Lentivirus production and Jurkat reporter cell line generation**

Variants of GITR-EGFP were cloned into pLenti-IRES-puro (Shanghai Generay Biotech) to generate stable cell lines. Briefly, lentiviral plasmid and associated helper plasmids were co-transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen). Virus-containing medium was collected at 48 and 72 h post-transfection, and the virus was concentrated by Lenti-X™ Concentrator (Clontech, 631231). GITR-AA, GITR-DD, GITR-RR mutant plasmids were constructed as previously described (Wang et al., 2021). Briefly, two phenylalanine residues in hGITR CRD3, F137 and F139, were mutated to alanine (GITR-AA), aspartic acid (GITR-DD), or arginine (GITR-RR), to abolish the hydrophobic receptor-receptor membrane-proximal interface. To construct the chimeric receptor (GITR-RR), the wild-type GITR receptor was cut after the hydrophobic interface. To construct the chimeric GITR-EGFP reporter Jurkat cell line and selected by EGFP cell sorting and 1 μg/mL puromycin (Gibco, A11138-02).

**NF-κB Luciferase reporter assay**

GITR luciferase reporter Jurkat cells were incubated with anti-GITR Ab or GITRL, together with 3 × 10⁶ Raji cells as the Fc cross-linker at 2:1 ratio, for 6 h at 37°C with 5% CO₂. Luciferase signal was measured using Bio-Glo™ Luciferase Assay System (Promega, G7940) on the SpectraMax i3x plate reader (Molecular Devices).

**Expression profile of PD1 and GITR in human T cells**

To measure PD1 and GITR expression in activated human T cells, PBMCs (PB100C-W, Miao Tong Biological Technology) were activated with Staphylococcal enterotoxin B (SEB) and flow cytometry was performed at different time points. The antibodies used were anti-PD1 (BD, 562516), GITR (ebioscience, 12-5875-42), CD3 (Biolegend, 300316), CD4 (Biolegend, 300519), CD25 (Biolegend, 356140), CD8 (Biolegend, 300920) and FoxP3 (Biolegend, 320014).

**PBMC cytokine release assay**

To measure the synergistic effect between PD1 antibody and IBI37G5, 1 × 10⁵ PBMCs were cultured with Staphylococcal enterotoxin B (SEB) in the absence or presence of IBI37G5 or IBI308 for 72 h. Supernatant IFN-γ levels were measured by Human IFN gamma HTRF kit (Cisbio, 62HIFNGPEG). For multiplex PBMC cytokine release assay, a 96-well plate was coated overnight with...
were calculated starting from the 200th cells. For sliding windows analysis, cells were sorted in ascending order according to pseudotime, and simple moving averages (N = 200) was chosen as the root cell and pseudotime was computed using the first 3 diffusion components and a minimum group size of 10.

Human CD4+ T cells were isolated from PBMCs using EasySep™ Human CD4+ T Cell Isolation Kit (STEMCELL, 19052). CD4+ T cell activation assay (Millipore, HSTCMAG-28SK) and cultured for 24 h. Cytokines from supernatant were measured using MILLIPLEX MAP Human High Sensitivity T Cell Panel (Millipore, HSTCMAG-28SK).

Computational modeling method. Seven pairs of disulfide bonds were used as additional constraints during the comparison modeling. 10,000 models were generated, and the optimal model was picked with lowest total energy and disulfide geometry potential.

Confocal imaging analysis
Jurkat cells expressing wild type hGITR-GFP or mutant hGITR-GFP (AA/DD/RR) were cultured in RPMI1640 medium with 10% FBS. To detect receptor clustering, 1 x 10^6 of Jurkat cells were aliquoted into 96-well plates, and incubated on ice with IgG, IBI37G5 or mVIB37G5 antibodies at various concentrations for 1 h. Cells were then fixed in 4% paraformaldehyde for 15 min at room temperature. After that, cells were washed with PBS twice and stained with DAPI for 6 min. After staining, cells were washed with PBS and mounted on slides with Pro-Long Gold Antifade Mountant (Thermo Fisher, P36961). 3D images were acquired using Leica SP8 confocal microscope and presented as max projection. ImageJ software (NIH) was used to quantitate staining intensity.

Multiplex immunohistochemical staining
Tissue microarray containing tumoral and peritumoral specimens from colon adenocarcinoma patients was stained using the TSA 7-color kit (Yuanxibio, D110071-50T) according to manufacturer’s instruction. Antibodies used include anti-human panCK (Genetech, GM351507), anti-human CD8 (Biolyx, BX50036), anti-human FoxP3 (Abcam, ab20034), anti-human PD-1 (Sinobiological, 10377-MM23), anti-human GTR (Cell Signaling, 68014), anti-human CD4 (Abcam, ab133616) and anti-human DAPI (Thermo Fisher, GM351507), anti-human CD8 (Biolynx, BX50036), anti-human FoxP3 (Abcam, ab20034), anti-human PD-1 (Sinobiological, 10377-MM23), anti-human GTR (Cell Signaling, 68014), anti-human CD4 (Abcam, ab133616) and anti-human DAPI (Thermo Fisher, D1306). The stained TMA slide was scanned using Pannoramic MIDI imaging system (3D HISTECH). Number of target cells were counted by HALO Software (Indica Labs).

In silico analysis
scRNAseq analysis
For CRC scRNA-seq, data were obtained from the ArrayExpress database at EMBL-EBI: E-MTAB-8107. All analyses were performed using Seurat 4.0.1 and Scanpy 1.5.2. Matrices were filtered by removing cells with <201 expressed genes, >6,000 expressed genes or >25% mitochondrial RNA content. Raw UMI counts were log-normalized. TCR and immunoglobulin genes were removed to avoid clustering based on variable V(D)J transcripts. Confounding factors were minimized by regressing out the number of UMLs, mitochondrial percentage, S phase score, G2/M phase score and heat-shock score. Single z-scores were calculated using the ScaleData function. Variable genes were selected using the FindVariableFeatures function, and were used to construct principle components (PCs). PCs were selected based on elbow and Jackstraw plots, and clusters were calculated using the FindClusters function with a resolution of 0.5. Differentially expressed genes in each clusters were calculated using the Wilcoxon Rank Sum test. z-scores for Immune cell markers in each cluster were calculated using SciPy and presented as heatmaps.

For analyzing tumor infiltrating T cells, cells from normal adjacent tissue (N = 1,497) were removed. Clusters were annotated based on known markers as indicated in the heat maps in Figures 1D and S1D. “CD8+ Pre-effector” cluster was annotated based on expression of early activation markers (CST7, DUSP2, GZMK). For diffusion map and pseudotime analysis, CD3+ T cells matrices were exported to Scanpy. A neighborhood graph was computed using 20 neighbors and the first 50 PCs, and the first three diffusion components were calculated. A randomly selected naïve T cell was chosen as the root cell and pseudotime was computed using the first 3 diffusion components and a minimum group size of 10. For sliding windows analysis, cells were sorted in ascending order according to pseudotime, and simple moving averages (N = 200) were calculated starting from the 200th cells.

For basal cell carcinoma (BCC) scRNA-seq, annotated data were downloaded from the GEO database: GSE132814 and reanalyzed for GTR, PD1 and CD39 expression, and TCR clonality. Diffusion map was computed in Scanpy using 40 neighbors and the first 20 PCs. Violin plots and diffusion maps were plotted using the standard functions in Seurat.

Human GITR and Fv (IBI37G5) modeling
The complete structure of human GITR protein was modeled based on the crystal structure (PDB: 7KHD) by ROSETTA comparative modeling method. Seven pairs of disulfide bonds were used as additional constraints during the comparison modeling. 10,000 models were generated, and the optimal model was picked with lowest total energy and disulfide geometry potential.
The Fv (IBI37G5) structure was modeled using ROSETTA antibody homology modeling application and further optimized conformations of CDR3 by ROSETTA antibody_H3 application. The detailed scripts are provided in the supplementary materials.

**Modeling of the hGITR/Fv (HZ37G5) complex**

Global docking of human GITR and Fv (IBI37G5) was implemented by ClusPro antibody docking server with four residues constraints. The four residues (R90, K105, F106, S107) were assumed at the epitope of GITR based on our experimental evidence (Figures 2F, S2E). 27 global docking results were generated and 15 reasonable docking results were manually picked as the initial complexes based on their cluster size and conformational properties. 15 previously picked docking results were locally refined by ROSETTA SnugDock application. Five hundred refined models were generated for each picked docking model. Among the 7,500 locally refined docking results, the top 2,000 docking models were picked up based on their interface energy. The top 2,000 models were clustered to 10 clusters based on interface RMSD by ROSETTA cluster application. 5 complexes with relatively low energy from 5 different clusters were considered as candidates for further validation by analyzing molecular dynamics.

The 5 candidates of GITR and Fv complexes were solvated in a periodic TIP3P dodecahedron water box with 0.15 M NaCl and 15 Å buffer between the protein and box edge. All simulations were performed in 2019-3 version of Gromacs. The system was modeled with Charmm36 force field. The constructed system was first energy minimized for 50,000 steps using the steepest decent methods, and then heated to 310K with a constant box volume (NVT). Restraints were applied to Cz atoms of the protein with a force constant of 1,000 kJ-mol-1-nm-2 for 1 ns. Subsequently, the heated system was coupled accordingly using isotropic Berendsen control with a time constant of 2 ps for 1 ns pressure regulation (NPT). A non-bonded interaction cut-off of 12 Å was employed. Long-range electrostatics were treated with the particle-mesh Ewald (PME) method. Covalent bonds involving hydrogen atoms were constrained with the LINCS algorithm. Eventually the production MD simulations was running under the random initial speed for a total time of 100 ns, with a time step dt = 2 fs and no restraints applied.

**In silico mutagenesis (constraint at C-terminal)**

To investigate the roles played by the two critical phenylalanine residues (F137 and F139) in GITR homo-dimerization, in silico mutagenesis was applied to F137 and F139. These two phenylalanine residues were mutated into alanine (A), arginine (R) and aspartic acid (D) simultaneously by ROSETTA relax application. Additionally, a 6 Å distance constraint was applied to the N termini of two GITRs in a homo-dimeric complex to maintain a reasonable conformation on cell membrane. One thousand models were generated for each mutant. ROSETTA interface analyzer was used to calculate the interface binding energy. The detailed scripts are provided in the supplementary materials.

**In vivo studies**

**In vivo tumor microenvironment profiling**

Tumor dissected from mice were digested with Liberase (Roche, 05401127001) and DNase I (Sigma, D5025-375KU) for 30 min and washed with cold PBS before filtering through a 70 μm cell strainer (Biologix Group). The following antibodies were used for flow cytometry: anti-mouse CD45 (Biolegend, 103122), anti-mouse CD45 (Biolegend, 103128), anti-mouse CD8 (Invitrogen, 45-0081-80), anti-mouse CD8 (Biolegend, 100752), anti-mouse CD4 (Invitrogen, 17-0041-81), anti-mouse CD4 (BD, 566407), anti-mouse CD3 (Biolegend, 100232), anti-mouse CD25 (Biolegend, 102008), anti-mouse FoxP3 (Invitrogen, 17-5773-82), anti-mouse CD16/32 (Biolegend, 149524) and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher, L34976). To evaluate the expression of human GITR on tumors and spleen after antibody treatment, the single-cell suspensions from tumors, flow cytometry analysis of single-cell suspensions from blood, spleen, tumor-draining lymph node and tumor was performed using anti-mouse CD45 (Biolegend, 103128), anti-mouse CD8 (eBioscience, 45-0081-82), anti-mouse CD4 (BD, 740208), anti-mouse TCR χchain (BD, 612821), anti-mouse Ly-6G (Biolegend, 127618), anti-mouse I-A/I-E (BD, 562366), anti-mouse CD11b (Biolegend, 101243), anti-mouse CD11c (Biolegend, 117343), anti-mouse NK-1.1 (BD, 741032), anti-mouse CD11b (Biolegend, 158008), anti-mouse CD12b (Thermo Fisher, 17-0321-80), anti-mouse CD16.2 (Biolegend, 505807) and anti-mouse TNF-α (Biolegend, 506328). For intracellular TNF-α and IFN-γ staining, tumor-infiltrating lymphocytes were incubated with IFN-γ-stimulated (50 IU/mL; 48 h) MC38 cells for 6 h at 37°C in the presence of brefeldin A (Biolegend, 420601), and then TNF-α and IFN-γ were stained with foxp3 fixation/permeabilization kit according to the manufacturers’ instructions (eBioscience, 00-5523-00) and analyzed by flow cytometry. For detecting FcγR expression in tumor infiltrating immune cells, flow cytometry analysis of single-cell suspensions from blood, spleen, tumor-draining lymph node and tumor was performed using anti-mouse CD45 (Biolegend, 103128), anti-mouse CD8 (eBioscience, 45-0081-82), anti-mouse CD4 (BD, 740208), anti-mouse TCR jchain (BD, 612821), anti-mouse Ly-6G (Biolegend, 127618), anti-mouse I-A/I-E (BD, 562366), anti-mouse CD11b (Biolegend, 101243), anti-mouse CD11c (Biolegend, 117343), anti-mouse NK-1.1 (BD, 741032), anti-mouse CD220 (Biolegend, 103208), anti-mouse CD64 (Biolegend, 139306), anti-mouse CD16 (Biolegend, 139306), anti-mouse CD12b (Thermo Fisher, 17-0321-80), anti-mouse CD16.2 (Biolegend, 149524) and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher, L34976). To verify the depletion efficiency in CD4 and CD8 immune cells depletion experiment, tumor infiltrated lymphocytes were stained with anti-mouse CD45 (Biolegend, 103149), anti-mouse CD4 (BD, 740208), anti-mouse CD8 (eBioscience, 45-0081-80), anti-mouse CD25 (Biolegend, 102008), anti-mouse FoxP3 (eBioscience, 17-5773-82) and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher, L34976).

To evaluate the expression of human GITR on tumors and spleen after antibody treatment, the single-cell suspensions from tumors and spleen were stained with the following antibodies: anti-mouse CD45 (Biolegend, 103128), anti-mouse TCR jchain (BD, 612821), anti-mouse CD8 (Invitrogen, 45-0081-82), anti-mouse CD4 (Biolegend, 100529), anti-mouse CD25 (BD, 564571), anti-human GITR (Invitrogen, 25-5875-42), anti-mouse FoxP3 (Biolegend, 320014), LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen, L34976).

**In vivo efficacy and pharmacokinetics of IBI37G5 in mice**

To evaluate the anti-tumor activity of IBI37G5 in vivo, MC38 (HYC3401, Obiosh), CT26 (CRL-2638, ATCC) and B16F10 (CRL-6475, ATCC) were implanted in hGITR/hPD1 double knocked-in C57BL6 mice (Biocytogen) or hGITR/hPD1 double knocked-in Balb/c mice (Gempharmatech). When tumor volume reached 60–100 mm³, mice were randomly grouped and intraperitoneally injected with
indicated drugs twice per week for 2 weeks. For CD4 and CD8 immune cells depletion experiment, MC38 syngeneic model was used here and when tumor volume reached 60–100 mm³, mice were randomly grouped and CD4 depletion antibody (Bioxcell, BE0003-1) and CD8 depletion antibody (Bioxcell, AB_2687706) were intraperitoneally injected twice per week for 2 weeks. Anti-PD1 antibody (IBI308) and anti-GITR antibody (IBI37G5) were injected 1 day after the depletion antibody injection. Body weight, maximum length of the major axis (L), and maximum length of the minor axis (W) of tumors were measured twice a week. The tumor volume was calculated using the formula: (width)² x length/2. Mice were euthanized either when the tumor volume reached 2000 mm³, or the percentage of body weight loss exceeded 20%. To measure the pharmacokinetics (PK) profiles of IBI37G5, mice were injected intravenously with a single dose of 0.3, 1, 3, or 10 mg/kg of IBI37G5. Blood samples for PK analysis were collected at pre-treatment, and 0.083, 0.5, 2, 6, 24, 48, 96 and 168 h post-treatment for PK analysis. The plasma concentration of IBI37G5 was determined by ELISA.

**Pharmacokinetics and toxicity study of IBI37G5 in cynomolgus macaques**

In a single-dose pharmacokinetic (PK) experiment, 18 cynomolgus monkeys (three per sex per group) received a single dose of IBI37G5 intravenously at 1, 3, or 10 mg/kg. Blood samples for PK analysis were collected pre-treatment, and 0.017, 1, 2, 4, 8, 24, 72, 120, 168, 240, 336, 504, 672, 840, and 1008 h post-dose. The plasma concentration of IBI37G5 was determined by ELISA.

For toxicity study, 40 cynomolgus monkeys (five per sex per group) were intravenously injected with 8 doses of IBI37G5 at 0, 10, 30 or 100 mg/kg once every fortnight for a total of 14 weeks. Lymphocytes subsets were analyzed using flow cytometry at pre-treatment, 1 day after the 3rd dose, 1 day after the 8th dose, and week 25 (the recovery phase). Cytokines (IFN-γ, TNF-α, IL-2, IL-6, IL-8, IL-10) in plasma were measured at pre-treatment, 2 and 24 h after 1st, the 3rd, and the 8th dose. Organs, including thymus and adrenal gland, were collected for hematoxylin and eosin staining after euthanasia.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Linear regression module was used for correlation analysis. Ordinary one-way ANOVA or unpaired Student’s t-tests were used for comparisons between groups. Two-way ANOVA and Turkey’s multiple comparison tests were used to assess continuous variables.