



Published in final edited form as:

Nature. 2019 March ; 567(7746): 56–60. doi:10.1038/s41586-019-0988-7.

Structure of the IFN γ receptor complex guides design of biased agonists

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Author contributions J.L.M. and K.C.G. conceived and designed the project and wrote the manuscript. J.L.M. and T.M.H. designed and performed preliminary yeast display experiments. J.L.M. engineered IFN γ R1, designed and prepared all recombinant proteins for the study, crystallized the complexes, solved the structure, and carried out SPR and cytokine secretion assay. J.L.M. and K.M.J. refined the structures. J.L.M. engineered signalling ligands and measured affinities by SPR. N.T. cloned and expressed receptors for SPR and helped J.L.M. with SEC experiments characterizing receptor binding properties of the GIFNs. J.S.B. performed single-molecule TIRF measurements. J.L.M. and L.S. performed cytokine secretion assays and screened cancer cell lines for MHC I:PD-L1 bias. N.K.E. performed PD-L1 and MHC class I upregulation assays on A549 cells, monocytes, macrophages and dendritic cells by FACS. J.L.M. measured gene expression by qPCR and prepared samples for next-generation sequencing of a human gene expression AmpliSeq panel. S.J.B. performed mass spectrometry analysis of IFN γ R2(T168N) neo *N*-glycosylation site. R.S.H., J.P., E.G.E. and K.C.G. supervised the research.

Data availability

Structure factors and coordinates have been deposited in the Protein Data Bank with identification numbers 6E3K and 6E3L.

Diffraction images have been deposited in the SGrid Data Bank with dataset ID 591 and 592. Next-generation sequencing data files from the human transcriptome study were deposited to the NCBI Gene Expression Omnibus (GEO) data repository with accession number GSE122672. Other data and materials are available upon request from the corresponding author.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41586-019-0988-7>.

Extended data is available for this paper at <https://doi.org/10.1038/s41586-019-0988-7>.

Supplementary information is available for this paper at <https://doi.org/10.1038/s41586-019-0988-7>.

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Reviewer information *Nature* thanks Michael Parker, Antoni Ribas and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41586-019-0988-7>.

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Abstract

The cytokine interferon- γ (IFN γ) is a central coordinator of innate and adaptive immunity, but its highly pleiotropic actions have diminished its prospects for use as an immunotherapeutic agent. Here, we took a structure-based approach to decoupling IFN γ pleiotropy. We engineered an affinity-enhanced variant of the ligand-binding chain of the IFN γ receptor IFN γ R1, which enabled us to determine the crystal structure of the complete hexameric (2:2:2) IFN γ -IFN γ R1-IFN γ R2 signalling complex at 3.25 Å resolution. The structure reveals the mechanism underlying deficits in IFN γ responsiveness in mycobacterial disease syndrome resulting from a T168N mutation in IFN γ R2, which impairs assembly of the full signalling complex. The topology of the hexameric complex offers a blueprint for engineering IFN γ variants to tune IFN γ receptor signalling output. Unexpectedly, we found that several partial IFN γ agonists exhibited biased gene-expression profiles. These biased agonists retained the ability to induce upregulation of major histocompatibility complex class I antigen expression, but exhibited impaired induction of programmed death-ligand 1 expression in a wide range of human cancer cell lines, offering a route to decoupling immunostimulatory and immunosuppressive functions of IFN γ for therapeutic applications.

The type II interferon IFN γ is a potent immunomodulatory cytokine with many pleiotropic effects on the innate and adaptive immune systems due to the broad expression of its receptors on immune cells¹. IFN γ exhibits an array of immunostimulatory, immunosuppressive, anti-proliferative and antiviral activities that are vital to normal immune homeostasis, and has a key role in tumour surveillance². Among the most important actions of IFN γ are activating macrophages and dendritic cells, and inducing upregulation of major histocompatibility complex (MHC) molecules to enhance presentation of bacterial, viral and tumour antigens³. However, despite its central role in many important functions related to disease, IFN γ has not achieved therapeutic utility owing to its pleiotropy and counterbalancing immunostimulatory and immunomodulatory activities⁴.

IFN γ is a homodimer⁵; it engages two α -receptor chains, IFN γ R1, which are constitutively expressed on all nucleated cells, and two β -receptor chains, IFN γ R2, the expression of which is tightly regulated⁶⁻⁸. A structure of IFN γ in complex with IFN γ R1⁹ revealed the mode of binding of the high-affinity receptor subunit. However, the structure of the complete extracellular hexameric (2:2:2 IFN γ -IFN γ R1-IFN γ R2) signalling complex has not been solved, principally because of the extremely low affinity of the IFN γ R2 subunit within the complex. Determination of the structure of the complete signalling complex is important for understanding IFN γ signalling and the mechanism of receptor-complex assembly, and for providing a blueprint for cytokine engineering to access the full therapeutic potential of IFN γ in cancer and immune diseases. Here, we have taken a receptor engineering approach to stabilize the complete IFN γ receptor complex, which has enabled structure determination and subsequent design of biased agonists.

Stabilization of the IFN γ receptor complex

When considering the assembly of the complete complex, it was important to know whether IFN γ drives the association of the receptors to form the signalling complex, or whether IFN γ R1 and IFN γ R2 are pre-dimerized, as some studies have suggested^{10,11}. We used two-colour single-molecule co-tracking to quantify binding of IFN γ R1 and IFN γ R2 in the plasma membrane^{12–14}. By monitoring dimerization of individual receptors on a cell, we found that IFN γ R1 and IFN γ R2 co-track only on addition of IFN γ (Fig. 1a, b). Similarly when monitoring either IFN γ R1 or IFN γ R2 binding steps, receptor dimerization was demonstrated to be a ligand-driven event (Extended Data Fig. 1a–c).

We expressed IFN γ R1 on the surface of yeast cells and showed that IFN γ R2 only binds to the preformed IFN γ R1–IFN γ complex, but not IFN γ alone. This implied that a composite binding surface is formed between IFN γ R1 and IFN γ , which subsequently engages IFN γ R2 (Extended Data Fig. 1d). We sought to use this readout of cooperativity to engineer and select for a stabilized interaction with IFN γ R2 (Fig. 1c). First, we generated an IFN γ R1 library using a non-biased error-prone approach with approximately three mutations created in each gene copy, to select for IFN γ R1 variants with improved affinity. Second, we used gene shuffling of the first-generation IFN γ R1 variants to further select for higher affinity (Fig. 1d and Extended Data Fig. 1e). After a single round of selection, the highest-affinity IFN γ R1 variant was IFN γ R1 F05, which contains six mutations (Fig. 1e and Extended Data Fig. 1e). The two most common mutations among the selected clones were located in the D2 FNIII domain of IFN γ R1 in an area commonly observed forming receptor–receptor, or ‘stem’ contacts in other dimeric cytokine–receptor complexes¹⁵ (Fig. 1f).

Structure of the IFN γ receptor complex

We obtained crystals of the deglycosylated and fully glycosylated IFN γ receptor complexes (Extended Data Fig. 2a), which diffracted to 3.25 Å and 3.8 Å resolution, respectively, and determined the structures by molecular replacement using previously determined structures of the 2:2 IFN γ –IFN γ R1 intermediate complex (Protein Data Bank (PDB): 1FG9)¹⁶ and IFN γ R2 (PDB: 5EH1)¹⁷ (Extended Data Table 1). The complete 2:2:2 IFN γ receptor complex is star-shaped with a two-fold symmetry imposed by the IFN γ homodimer (Fig. 2a). The structure reveals six total interaction sites: two site 1 interfaces shared between IFN γ and IFN γ R1, two site 2 interfaces shared between IFN γ and IFN γ R2, and two site 3 interfaces shared between IFN γ R1 and IFN γ R2. IFN γ R2 binds to the composite interface formed by the high affinity IFN γ –IFN γ R1 interaction, which enables IFN γ R2 to contact the open face of IFN γ site 2, as well as make extensive stem contacts with IFN γ R1 site 3 (Fig. 2a). Each of the two site 2 interfaces of IFN γ presents a concave surface that buries a total area of 1,243 Å² formed by helices A, D and E, and the N terminus of the cytokine (Fig. 2a, 3a, b). In contrast to the site 1 interfaces, in which both chains of IFN γ form the IFN γ R1 binding interfaces, only one IFN γ chain is needed to form each IFN γ R2 binding site in the site 2 interface. IFN γ R2 binds to IFN γ principally through a cluster of aromatic residues in loop 3 (F67, Y69 and F75) and through F109 in loop 4 of IFN γ R2, which insert into a small pocket formed by helices A and D of IFN γ (Fig. 3b, left, and Extended Data Fig. 2b). The site 3 stem interfaces (1,469 Å² of total buried surface area) consist of

primarily flat surfaces between IFN γ R1 and IFN γ R2 that interact through extensive van der Waals interactions (Figs. 2a, 3a, b). IFN γ R1 F05 contains two mutations, M161K and Q167K, located at the site 3 interfaces (Fig. 3b, right). IFN γ R1(M161K) shares a hydrogen bond with T149 of IFN γ R2, and IFN γ R1(Q167K) forms a salt bridge with D164 of IFN γ R2; both interactions are likely to contribute to the stabilization of the site 3 interfaces (Fig. 3b, right, and Extended Data Fig. 2b).

Although the IFN γ signalling complex is ‘doubled’ into a 2:2:2 hexamer (compared to typical 1:1:1 trimeric cytokine-receptor complexes¹⁵) because of the homodimeric nature of the ligand, each 1:1:1 half of the hexamer shares structural similarities with the type III IFN trimeric (1:1:1) IFN λ receptor complex (PDB: 5T5W) (Fig. 2b), including the relative binding modes of the high (IFN λ R1) and low (IL-10R β) affinity receptors binding to one end of the helical bundle of the respective cytokines¹⁵ (Fig. 2b). By contrast, the IFN γ complex uses a structural paradigm that is distinct from the trimeric (1:1:1) type I IFN complex in both the relative geometries of the ligand–receptor complexes and the mode of binding to each receptor (Fig. 2c).

Role of the neoglycan in IFN γ R2(T168N) to MSMD

Lack of IFN γ responsiveness in mycobacterial disease syndrome (MSMD) has been attributed to a homozygous T168N mutation in IFN γ R2¹⁸, which results in a life-threatening predisposition to mycobacterial infections¹⁰. The structure of the complete complex provides further insight into the molecular basis of this disease-associated mutation in IFN γ R2. The structure places T168N (IFN γ R2) directly at the site 3 interface (Fig. 3c). The additional steric bulk that would result from the addition of an *N*-linked glycan at this site would introduce steric clashes, and is thereby likely to prevent IFN γ R2 from docking to the high affinity 2:2 IFN γ –IFN γ R1 intermediate complex. We used two approaches to test the hypothesis that glycosylation at the T168N position of IFN γ R2 prevents docking with the 2:2 IFN γ –IFN γ R1 intermediate complex. We produced a recombinant form of the neoglycan mutant IFN γ R2(T168N) extracellular domain and verified that it was glycosylated at the T168N position with almost quantitative occupancy (Fig. 3d and Extended Data Fig. 3). Using surface plasmon resonance (SPR), we measured the affinity of either wild-type IFN γ R2 (Fig. 3e, left) or the neoglycan mutant IFN γ R2(T168N) (Fig. 3e, right) for IFN γ –IFN γ R1. We detected a loss of binding between IFN γ R2(T168N) for IFN γ –IFN γ R1. In a second approach, we used single-molecule dimerization assays to quantify binding of the IFN γ receptors in the plasma membrane on addition of the ligand (Fig. 3f). Binding of IFN γ R1 is maintained, whereas IFN γ R2(T168N) is not recruited to the complex following addition of IFN γ (Fig. 3f). Thus, we propose that the molecular basis for the effect of the T168N mutation in mycobacterial disease syndrome is principally that the neoglycan sterically prevents IFN γ R2(T168N) from engaging the IFN γ –IFN γ R1 complex to complete the signalling complex.

Structure-guided design of partial agonists

The structure of the IFN γ signalling complex provides a topological blueprint for probing the signalling properties of intermediates in the assembly pathway to the hexameric complex

(Fig. 4a). We designed partial agonists by first engineering a version of IFN γ that retains binding to IFN γ R1 but abrogates binding to IFN γ R2 (Extended Data Fig. 4a, b). On the basis of our structure, we rationally designed the IFN γ (K74A/E75Y/N83R) triple mutant and confirmed loss of measurable binding to IFN γ R2 by SPR (Extended Data Fig. 4a, b). Using our knowledge of the site 2-specific mutations, we engineered ‘asymmetric’ single-chain mutants to selectively control receptor occupancy at either one or both IFN γ R2 binding sites of IFN γ by expressing the molecules as single-chain fusions, in which, for example, only one chain of IFN γ contained a mutated binding site, and the other was wild-type IFN γ (Extended Data Fig. 5). Using this linker strategy, together with different combinations of site 1^{19,20} and site 2 mutations, we created a panel of partial agonists that control both the number and location of the receptors in the complex (Extended Data Fig. 5b), and characterized receptor-binding stoichiometry and oligomerization of the asymmetric variants by size-exclusion chromatography (SEC) (Extended Data Fig. 5c–e), and measured receptor dimerization and phosphorylated STAT1 (pSTAT1) signalling (Fig. 4b, c and Extended Data Fig. 4c, d).

We observed that there was a relationship between the number and location of receptors bound and the maximal pSTAT1 signal, E_{\max} . The 2:2 IFN γ –IFN γ R1 complex (using IFN γ variant 3, termed GIFN3) exhibits a 25% pSTAT1 E_{\max} , whereas addition of only 1 copy of IFN γ R2, to create a 2:2:1 IFN γ –IFN γ R1–IFN γ R2 intermediate complex (using IFN γ variant 1, termed GIFN1), results in 100% pSTAT1 E_{\max} compared to the complete hexameric complex (Fig. 4a, c). The second copy of IFN γ R2 therefore appears to be functionally redundant; this also demonstrates the extreme sensitivity of IFN γ responsive cells to expression levels of IFN γ R2. Of note, the 2:1:1 complex (using IFN γ variant 2, termed GIFN2) of IFN γ –IFN γ R1–IFN γ R2 exhibits a 50% E_{\max} for pSTAT1, and appears to be ‘capped’ until a third receptor subunit binds (using IFN γ variant GIFN1) (Fig. 4a, c).

Biased agonists decouple IFN γ gene expression

We carried out gene expression studies of wild-type IFN γ and GIFN variants. We treated A549 cells, a lung carcinoma cell line, with either wild-type IFN γ or GIFN variants, and measured gene expression by next-generation sequencing using an AmpliSeq panel of more than 20,000 genes. Overall, we observe a general trend of the partial agonists inducing lower levels of gene expression in accordance with their pSTAT1 E_{\max} potencies (Fig. 4d, e). However, we find that a subset of genes exhibit discordant, biased expression patterns (Fig. 4f, g). For example, CD274, more commonly known as programmed death ligand 1 (PD-L1), is one of a subset of tunable genes, the expression of which is greatly reduced in response to the partial agonists (Fig. 4g, top), whereas MHC class I remains highly expressed (Fig. 4g, bottom panel).

We measured induction of surface expression of MHC class I and PD-L1, and cytokine secretion in response to wild-type IFN γ or GIFN variants (Fig. 5a, b and Extended Data Fig. 4e, i). The partial agonists retained nearly wild-type levels of activity in inducing upregulation of MHC class I in A549 cells and purified human dendritic cells, but induction of PD-L1 expression by the partial agonists was greatly reduced (Fig. 5a, b and Extended Data Fig. 4f). The GIFNs exhibited bias, with up to approximately 50-fold difference

between induction of MHC I and PD-L1 in A549 cells, and similarly for dendritic cells, monocytes, and macrophages (Extended Data Fig. 4g, h). We screened an additional six cancer cell lines, including Hap1, MeWo, HT-29, Hep G2, HeLa and Panc-1 cell lines, finding that the partial agonists consistently decouple MHC I:PD-L1 expression to different degrees depending on the cell line (Fig. 5c, d).

The uncoupling of MHC I and PD-L1 expression shows that the partial agonists are also biased agonists that have uncoupled an important pleiotropic activity of IFN γ and, more broadly, that different genes downstream of IFN γ may exhibit different thresholds of activation that can be exploited by structure-based partial and/or biased agonists. This uncoupling of PD-L1 and MHC I expression is potentially of interest in the context of immunotherapy, as these IFN γ variants could enhance presentation of tumour antigens without the concomitant immuno-suppression through checkpoint expression that occurs in response to wild-type IFN γ .

METHODS

Evolution of IFN γ R1 on yeast.

IFN γ R1 was displayed on yeast as previously described^{15,22}. Staining and selection were performed using streptavidin–Alexa Fluor 647-labelled IFN γ R2 with separation of the receptor-yeast population with anti-Alexa Fluor 647 antibody labelled with paramagnetic microparticles. Unlabelled IFN γ (750 nM) was present as a saturating condition during all selections. Expression on the yeast surface was assayed by staining with a Myc-tagged antibody conjugated to Alexa Fluor 647 (Cell Signaling). Progression of the enrichment was monitored by staining the receptor on yeast and analysis by flow cytometry (BD Accuri). Error-prone PCR and DNA shuffling^{15,23}, and 96-well screening were used for engineering IFN γ R1 as previously described¹⁵.

Protein expression, purification, and structural determination.

IFN γ and signalling variants were expressed in the Hi5 insect expression system (Invitrogen BTI-TN-5B1–4), and purified as previously described²¹. For crystallization, IFN γ was expressed in the presence of kifunensine. IFN γ DN (K74A, E75Y, and N83R) and related variants were synthesized (Operon) and cloned into the insect expression plasmid. Asymmetric variants were expressed with three tags, allowing for their expression and purification. An 8 \times His tag at the C terminus was used at the first step of purification from the insect secreted medium. In the second step, rhinovirus 3C protease was used to cleave the encoded 3C linker between the two hetero subunits of the asymmetric IFN γ proteins. After cleavage, the proteins were further purified using a protein C tag encoded at the N terminus and a second 8 \times His purification to isolate the heterodimeric IFNs. Their receptor binding properties were validated by SEC by injecting 200 μ g of each IFN γ alone, in combination with equimolar quantities of IFN γ R1, or with IFN γ R1 and IFN γ R2. IFN γ R1 and IFN γ R2 were expressed in HEK293S GnTI⁻ cells (ATCC CRL-3022) that were transduced by lentivirus²⁴ encoding each receptor. For glycan-shaved complexes, IFN γ , IFN γ R1 F05, and IFN γ R2 were deglycosylated by treatment with EndoF and EndoH overnight at 4 °C. Both the deglycosylated and the glycosylated proteins were mixed in

equimolar ratios and digested with carboxypeptidase A and B before co-purification by SEC on a Superdex 200 column (GE). The final protein concentration of the glycosylated and deglycosylated complex used in the crystallization screen was 10 mg/ml. Crystals of the deglycosylated complex were obtained within 24 h at 20 °C from the MCSG3 (Anatrace) in the screen condition containing 0.1 M bis-tris propane:HCl, pH 7, 2 M sodium formate. Crystals were cryoprotected by the addition of ethylene glycol to 25%. Diffraction data were collected at 100 K at the Stanford Synchrotron Radiation Lightsource beamline 12-2 to 3.25 Å resolution, using X-rays of wavelength 0.97946 Å. For the glycosylated complex, crystals were grown at 20 °C from the MCSG2 screen in 1.1 M ammonium tartrate dibasic, pH 7.0. Crystals were cryoprotected with 25% ethylene glycol, and diffraction data were acquired at 100 K at the Advanced Light Source beamline 8.2.2 to 3.8 Å resolution using X-rays of wavelength 0.999989 Å.

Data for both structures were processed in XDS²⁵. The structure of the shaved complex was solved by molecular replacement in Phaser²⁶ using models of IFN γ and IFN γ R1 from PDB ID 1FG9 and IFN γ R2 from PDB ID 5EH1. Iterative cycles of rebuilding and refinement were performed using Coot²⁷, Phenix^{28,29}, and Buster³⁰ using individual atomic *B*-factors, torsional NCS restraints, and automatically determined TLS groups³¹. Ligand atoms in the deglycosylated structure include ethylene glycol that was present from the cryoprotectant, glycan residues that remained after digestion of the protein with EndoH, and peaks adjacent to Cys174 on the surface of IFN γ R2 that were modelled as disulfide-bound cysteines. Mindful of the low resolution of our data, all heteroatoms were built into *mFo* – *DFc* peaks $>3\sigma$ and assessed after refinement for appropriate hydrogen-bond or disulfide-bond geometry, lack of clashes, and density in the resulting *2mFo* – *DFc* map. The high resolution limit for the final round of refinement was 3.25 Å, chosen by performing paired refinement tests with d_{\min} of 3.1, 3.25 and 3.4 Å resolution as described³². The final model has 97.47% of residues in favoured regions of the Ramachandran plot with zero outliers. The structure of the glycosylated complex was solved by molecular replacement using the refined shaved complex as a search model. Iterative cycles of rebuilding and refinement were performed using Coot and Phenix using torsional NCS restraints, grouped *B*-factors, and per-chain TLS. To assess our choice of resolution cut-off, we performed paired refinements as above using d_{\min} values of 3.6, 3.8, 4.0 and 4.2 Å, and we selected a 3.8 Å resolution limit for our final refinement. The final structure has 97.39% of residues in favoured regions of the Ramachandran plot with zero outliers. In both structures, partial disorder of one copy of IFN γ R2 required the use of NCS-averaged maps for rebuilding. Structure quality was assessed using Molprobability³³.

Surface plasmon resonance.

A GE Biacore T100 was used to measure the *KD* by equilibrium methods. Approximately 100 response units (RU) of IFN γ R1 was captured on a SA-chip (GE Healthcare), including a reference channel of an unrelated cytokine receptor (IL-2R β). The saturating concentration for both wild-type IFN γ or IFN γ (K74A/E75Y/N83R) was 50 nM and was present in all dilutions of IFN γ R2 or IFN γ R2(T168N).

Mass spectrometry analysis of IFN γ R2(T168N) neo N-glycosylation site.

Approximately 1 μ g of purified, recombinant human IFN γ R2(T168N) expressed in HEK293S GnTI $^{-}$ (ATCC CRL-3022) cells was denatured and reduced in 8 M urea containing 20% ammonium bicarbonate and 10 mM tris(2-carboxyethyl) phosphine (Thermo Fisher Scientific), then subsequently alkylated with iodoacetamide. After fourfold dilution, the protein was enzymatically digested with chymotrypsin overnight at 37 $^{\circ}$ C. The resulting digest was then subjected to a C18 Zip-Tip filter clean-up and eluted using 50% acetonitrile, 0.1% formic acid, and filtered through a 0.2- μ m nylon spin filter for high-quality peptide purification. A portion of the purified peptides were diluted to 20% acetonitrile and 0.1% formic acid, and 5 μ l of \sim 2ng/ μ l digests was injected into a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an Easy nano-LC HPLC system with a C18 EasySpray PepMap RSLC C18 column (50 μ m \times 15 cm, Thermo Fisher Scientific). Separation of (glyco)peptides was performed with a 30-min binary gradient consisting of solvent A (0.1% formic acid in water) and solvent B (90% acetonitrile and 0.1% formic acid in water) with a constant flow rate of 300 nl/min. Spectra were recorded with a resolution of 35,000 in the positive polarity mode over the range of m/z 350–2,000 and an automatic gain control target value of 1×10^6 . The 10 most prominent precursor ions in each full scan were isolated for higher energy collisional dissociation–tandem mass spectrometry (HCD–MS/MS) fragmentation with normalized collision energy of 27%, an automatic gain control target of 2×10^5 , an isolation window of m/z 3.0, dynamic exclusion enabled, and fragment resolution of 17,500. Raw data files were analysed using Proteome Discoverer v.2.1.0.81 (Thermo Fisher Scientific) with Byonic v.2.10.5 (Protein Metrics) as a module for automated identification of (glyco)peptides. EICs of all identified (glyco)peptides were generated using Xcalibur v.4.0.27.19 (Thermo Fisher Scientific).

Decoupling of MHC I:PD-L1 expression in cancer and immune cells.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors, who provided written informed consent for research protocols approved by the Stanford Institutional Review Board. Human blood dendritic cells and monocytes were enriched from blood in leukoreduction system chambers by Ficoll-Hypaque density gradient centrifugation. For monocyte enrichment, blood was preincubated with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies). White blood cells were removed and monocytes were isolated with EasySep Human CD14 Positive Selection Kit II (StemCell Technologies). For macrophage differentiation, monocytes were cultured for 6 days in chRPMI (RPMI 1640 (Thermo Fisher Scientific), 10% human serum and 100 U/ml penicillin–streptomycin (GIBCO) and 50 ng/ml recombinant human M-CSF (Peprotech). Dendritic cells were enriched using the EasySep Human Myeloid DC Enrichment kit (19061; StemCell Technologies). The dendritic-cell-enriched samples were stained with DAPI and lineage markers CD19–PE–Cy5 (Beckman Coulter); CD56–FITC, CD3–Alexa700 (Biolegend); CD11c–PE–Cy7, HLA–DR v500, CD14–APC–H7 (BD); and CD304–PE (MACs Miltenyi Biotech). Dendritic cells were sorted on a BD FACsAria II as HLA-DR $^{+}$ CD11c $^{+}$ cells, which were negative for all other lineage markers. Monocytes, macrophages and dendritic cells were stimulated in chRMPI for 18 or 48 (dendritic cells) hours and harvested using PBS with 5 mM EDTA or Accutase (Fisher Scientific). A549

cells (ATCC CCL-185) were cultured at 37 °C in 5% CO₂ and RPMI 1640 (Thermo Fisher Scientific) containing 10% FBS and 100 U/ml penicillin–streptomycin (GIBCO). Cells were plated into 48-well plates, and stimulated for 48 h with various concentrations of protein and harvested using 0.25% trypsin-EDTA (GIBCO). Cells were analysed by flow cytometry using an LSR II (BD). Dead cells were discriminated using the Live/Dead Aqua Fixable Dead Cell Stain Kit (Invitrogen), non-specific antibody binding was minimized using Human FC Block (BD) and surface staining was performed with PE–Dazzle-conjugated anti-PD-L1 (clone 29E.2A3, Biolegend) and v450-conjugated anti-HLA–ABC (clone G46–2.6, BD). The MFI change was calculated by subtracting the MFI of non-stimulated controls from the MFI of stimulated samples, relative to wild-type IFN γ . The relative MHC I:PD-L1 ratio was calculated by dividing the MFI ratios for MHC I that for PD-L1. For screening of cancer cell lines other than A549 cells (ATCC CCL-185), Hap1 (NKI-AVL), MeWo (ATCC HTB-65), Hep G2 (ATCC HB-8065), HT-29 (ATCC HTB-38), Panc-1 (ATCC CRL-1469) or HeLa (ATCC CCL-2) cells were plated in 96-well format, treated with different concentrations of wild-type IFN γ or GIFNs for 24 h, and MHC I:PD-L1 ratios were quantified as previously detailed. For quantification of gene expression by RT–qPCR and next-generation sequencing, 600,000 cells were plated in a 6-well format and treated with proteins for 48 h. RNA was extracted (RNeasy Micro Kit, Qiagen), 1.5 μ g of this RNA was then used for RT–qPCR (High Capacity RNA-to-cDNA Kit, Applied Biosystems), and measured by quantitative PCR (qPCR) (PowerSYBR Green PCR Master Mix, Applied Biosystems) on a QuantStudio 3 instrument (Applied Biosystems) according to the manufacturer's instructions. Primers were purchased from (Operon) for 18S (fwd 5'GTAACCCGTTGAACC CCATT3', rev 5'CCATCAATCGGTAGTAGCG3'), HLA-A (fwd 5'CCAGGTAGG CTCTCAACTG3', rev 5'CCAGGTAGGCTCTCAACTG3'), HLA-B (fwd 5'AACCGTCCCTCCTGCTGCTCTC3', rev 5'CTGTGTGTTCCGGTCCCA ATAC3'), PD-L1 (fwd 5'TGGCATTGCTGAACGCATTT3', rev 5'TGCAGCCAG GTCTAATTGTTTT3'). Expression of over 20,000 human genes was measured by next-generation sequencing using the Ion AmpliSeq Human Gene Expression kit (Thermo Fisher). Samples were loaded on a 550 chip and sequenced on an Ion S5 XL sequencer (Thermo Fisher). RNA samples from two biological qPCR experiments were used for sequencing. Samples were barcoded following manufacturer protocols and were as follows: untreated-A (fwd 5'CTAAGGTAAC3'), wild-type-A (fwd 5'TAAGGAGAAC3'), GIFN2-A (fwd 5'AAGAGGATTC3'), GIFN3-A (fwd 5'TACCAAGATC3'), GIFN4-A (fwd 5'CAGAAGGAAC3'), untreated-B (fwd 5'CTGCAAGTTC3'), wild-type-B (fwd 5'TTCGTGATTC3'), GIFN2-B (fwd 5'TTCCGATAAC3'), GIFN3-B (fwd 5'TGAGCGGAAC3') and GIFN4-B (fwd 5'CTGACCGAAC3'). Gene mapping and analysis was performed using Ion Torrent Suite v.5.10.0 (Thermo Fisher). Heat maps and figures showing PCA of gene expression were generated in MATLAB v.R2018b (MathWorks).

On-cell receptor dimerization.

Receptor homo- and heterodimerization was quantified by two-colour single-molecule co-tracking as described previously^{34,35}. For quantifying receptor heterodimerization, IFN γ R1 and IFN γ R2 N-terminally fused to variants of monomeric GFP were co-expressed in HeLa cells and labelled using anti-GFP nanobodies Enhancer and Minimizer, respectively. For

quantifying homodimerization, GFP-tagged IFN γ R1 or IFN γ R2 were expressed and labelled with two different colours. Time-lapse dual-colour imaging of individual IFN γ R1 and IFN γ R2 in the plasma membrane was carried out by total internal reflection fluorescence microscopy with excitation at 561 nm and 640 nm and detection with a single EMCCD camera using an image splitter. Molecules were localized using the multiple-target tracing (MTT) algorithm³⁶. Receptor dimers were identified as molecules that co-localized within a distance threshold of 100 nm for at least 10 consecutive frames.

pSTAT1 signalling and bead-based immunoassay cytokine secretion.

Hap1 cells (NKI-AVL) were plated in a 96-well format and treated with either wildtype IFN γ or partial agonists at varying concentrations for 15 min at 37 °C. The medium was removed and cells were detached with Trypsin (Gibco) for 5 min at 37 °C. Cells were transferred to a deep-well 96-well block containing 10% paraformaldehyde (PFA) by volume and incubated for 15 min at room temperature, washed three times with phosphate-buffered saline containing 0.5% (w/v) BSA (PBSA), resuspended with 100% methanol overnight, and stained with Alexa Fluor 488 conjugated anti-pSTAT1 antibody (Cell Signaling). The half-maximal response concentration (EC50) and E_{\max} of signalling was determined by fitting the data to a sigmoidal dose–response curve (GraphPad Prism v.7). The bead-based immunoassay cytokine secretion assay was performed at the Human Immune Monitoring Center at Stanford University as previously described³⁷ except the experiment was performed on PBMCs isolated from two different donors and measured in triplicate.

Eukaryotic cell lines.

Authentication of cell lines used in this study is guaranteed by the sources. Original validation of Hap1 cells was by whole-genome sequencing, A549 cells by Sanger Sequencing, EBV100 yeast cells by genotyping and sequencing, HeLa cells by the ATCC Cell Line Authentication Service. Invitrogen does not indicate an authentication method for Hi5 cells. ATCC does not provide authentication information for HEK293S GnTI-ATCC (CRL-3022), SF9 (ATCC CTL-1711), MeWo (ATCC HTB-65), Hep G2 (ATCC HB-8065), HT-29 (ATCC HTB-38) or Panc-1 (ATCC CRL-1469) cells. None of the cell lines tested positive for mycoplasma contamination.

Statistical analyses.

No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment. *P* values were determined for the difference between wild-type and PHA control in the cytokine secretion experiments using the Student's *t*-test with a two-tail distribution of a two-sample heteroscedastic test. For the mass-spectrometry experiment, sequence coverage was determined by dividing the number of amino acids identified in the proteomic analysis (179 residues, highlighted in Extended Data Fig. 3b) by the total number of amino acids in the protein (233). The confidence for identification of the peptides in the highlighted region is based on the Byonic algorithm as previously described³⁸.

Reporting summary.

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Extended Data

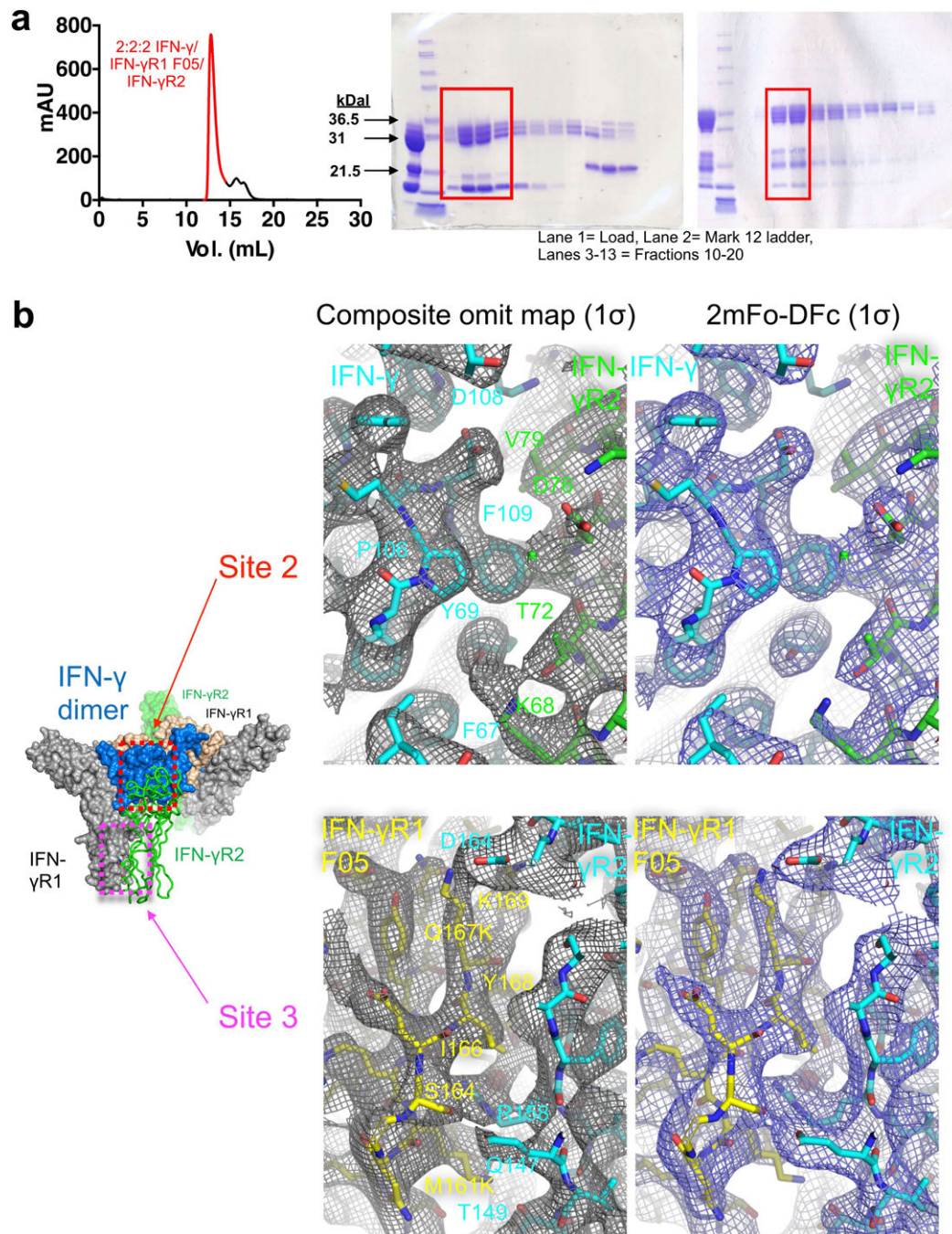
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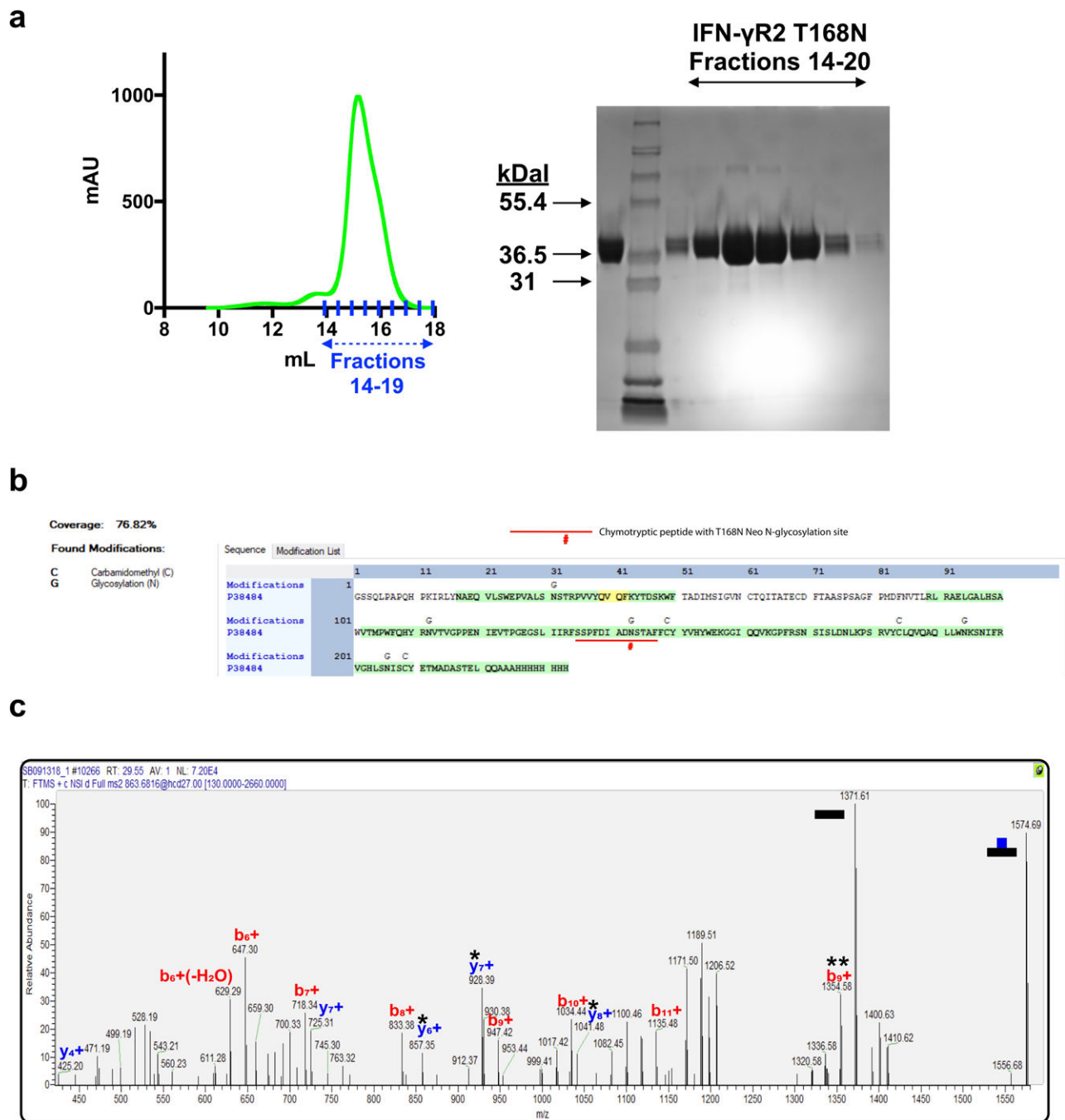
left, streptavidin–Alexa Fluor 647. The high avidity form of IFN γ R2 only binds to IFN γ R1 in the presence of IFN γ (far right) and does not bind IFN γ R1 alone (second from right). Data are representative of at least 3 biologically independent experiments. **e**, Sequence alignment of IFN γ R1 genes including 13 first-generation variants and the shuffled IFN γ R1 F05 variant relative to wild-type. IFN γ R1 F05 combines six mutations including Q167K and M161K. The combination of Q167K and M161K is not seen in any single first-generation mutant.



Extended Data Fig. 2. Purification and electron density maps of the IFN γ hexameric signalling complex.

a. SEC (Superdex S200 column) of the 2:2:2 IFN γ -IFN γ R1-IFN γ R2 complex and SDS-PAGE gels of the deglycosylated (top, left gel) and fully glycosylated (top, right gel) forms. Data shown are representative of at least 3 biologically independent experiments. mAU, milli absorbance units. **b.** Electron density maps showing interactions at site 2 (top) and site 3 (bottom) in the deglycosylated complex. For each pair of site 2 or site 3 panels, the left panel shows a simulated annealing composite omit map (grey) contoured at 1σ , and the right

panel shows a $2mF_0 - DF_c$ map (blue) calculated using phases from the final refined model and contoured at 1σ . IFN γ (green) engages IFN $\gamma R2$ (cyan) at site 2, whereas the stems of IFN $\gamma R1$ F05 (yellow) and IFN $\gamma R2$ interact at site 3.



Extended Data Fig. 3. Quantification of IFN γ R2(T168N) glycoforms by mass spectroscopy.

a, The mutant IFN γ R2(T168N) protein was expressed in HEK293S GnTI⁻ cells and purified by SEC. The SEC profile is shown (left) with the corresponding fractions on SDS-PAGE (right). Lane 1 shows the sample loaded on the SEC column, lane 2 shows the Mark 12 protein ladder, and lanes 3–9 are fractions 14–20. Data are representative of at least 3 biologically independent experiments. **b**, The protein coverage map shows sequence coverage of 76.82% for the entire IFN γ R2(T168N) protein including the peptide of interest, containing N168, which is underlined. This peptide was detected as a glycopeptide with

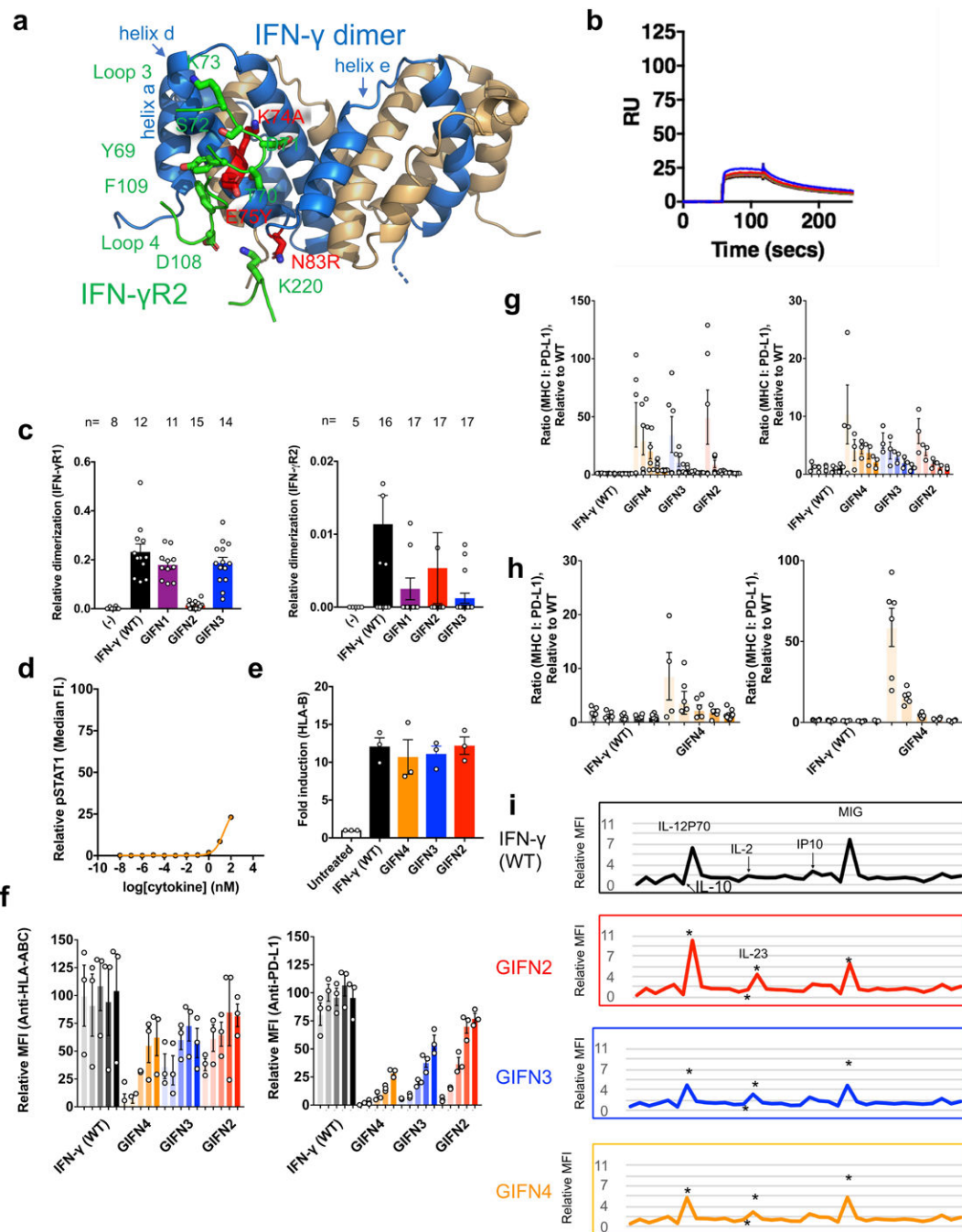
several glycoforms as quantified in Fig. 3d. Mappings highlighted in green indicate high confidence with a false discovery rate (FDR) below 1% and yellow indicates a FDR of 1–5%. Carbamidomethyl (C) and glycosylation (G) sites are indicated above the site of modification. Confidence levels were determined as previously described³⁸. **c.** MS2 spectra confirming that the ion used for the EICs shown in Fig. 3d is the peptide SSPFDIADNSTAF from IFN γ R2(T168N) modified with a HexNAc2Hex5 glycan attached to N168. The data shown are for a single experiment.

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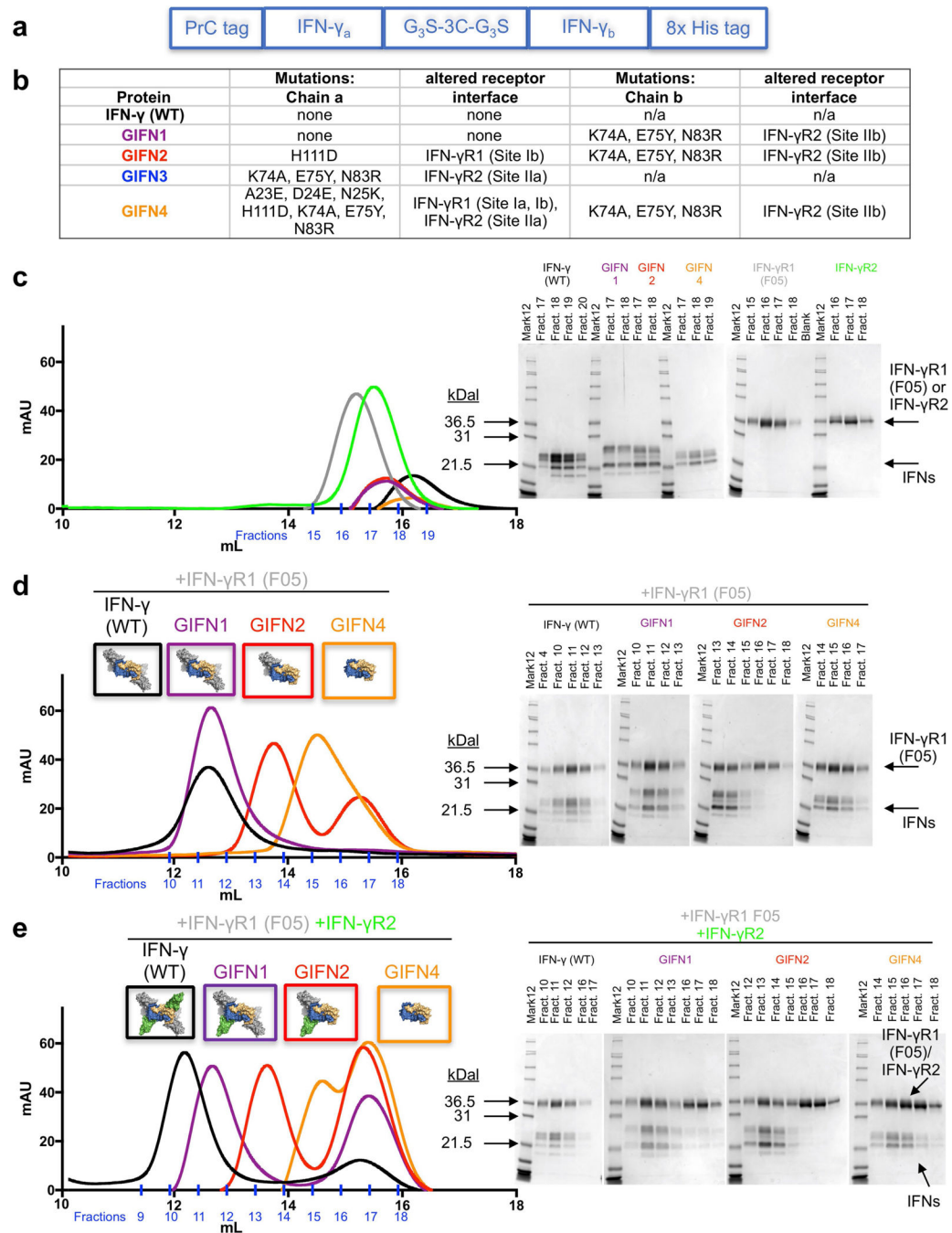
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Extended Data Fig. 4. Disrupting IFN γ R2 binding and characterization of IFN γ partial agonists.

a, The structure of IFN γ (blue and tan cartoon) binding site for IFN γ R2 (interacting loops are shown in green). Based on the hexameric complex, positions in IFN γ at the IFN γ R2 binding interface were identified to be important for binding to IFN γ R2. The location of IFN γ mutations K74A, E75Y, and N83R are shown as sticks coloured in red. **b**, When complexed with IFN γ R1, the IFN γ triple mutant (K74A/E75Y/N83R) results in the loss of detectable binding to IFN γ R2 (up to 100 μ M) as determined by SPR. The titration data are from a single experiment. **c**, Relative co-tracking of binding of IFN γ R1 (left panel) and

IFN γ R2 (right panel) for wild-type IFN γ and variants. GIFN2, one IFN γ R1 and one IFN γ R2 binding in *cis*; GIFN3, two IFN γ R1 molecules bound; GIFN4, reduced affinity for both IFN γ R1 and IFN γ R2 (see Extended Data Fig. 5). Data are mean \pm s.e.m.; *n* is indicated over each bar; *n* refers to the number of biologically independent samples. **d**, STAT1 activation of GIFN4 in Hap1 cells. Curve was fit to a first-order logistic model. Data are mean; *n* = 2 biologically independent samples. **e**, Quantification of MHC class I expression by qPCR using primers against HLA chain b in A549 treated cells. Data are mean \pm s.e.m.; *n* = 3 biologically independent experiments. **f**, Dendritic cells purified from whole blood were treated for 48 h with either wild-type IFN γ or the partial agonists for 48 h; these agonists upregulate MHC class I antigen expression as quantified by fluorescently labelled antibody (left) or PD-L1 (right). Data shown are for ligand concentrations of 0.1, 0.5, 2.5, 12.5 and 62.5 nM (left to right, respectively, for each agonist). Data are mean \pm s.e.m.; *n* = 3 biologically independent experiments. **g**, Using the MHC class I and PD-L1 expression data (**f** and Fig. 5b), the ratio of MHC I:PD-L1 induction was determined for each protein concentration relative to wild-type. Left, dendritic cells; right, A549 cells. Data are mean \pm s.e.m.; *n* = 3 biologically independent experiments. **h**, Biased MHC I:PD-L1 expression for monocytes (right) and macrophages (left) isolated from PBMCs. Data as shown are for protein concentrations of 0.1, 0.5, 2.5, 12.5 and 62.5 nM (left to right, respectively, for each agonist). Data are mean \pm s.e.m.; *n* = 6 independent samples. **i**, Cytokine secretion profile of IFN γ and partial agonists for PBMCs treated with 100 nM of each protein for 24 h. Shown are the mean expression of 36 secreted cytokines that are significantly different ($P < 0.05$) between the wild-type and PFA-treated control. Expression of IL-10, IL-12P70, IL-2, IP-10, MIG and IL-23 are indicated in text or asterisks aligned below the text. Data shown are for *n* = 2 biologically independent samples, each assayed in triplicate. *P* values were determined using the Student's *t*-test with a two-tailed distribution of a two-sample heteroscedastic test.



Extended Data Fig. 5. Design and biochemical characterization of GIFNs.

a, Diagram showing the strategy for expression and purification of heterodimeric IFN γ variants. The asymmetric variants containing three tags were expressed as single polypeptides in Hi5 insect cells. The proteins were first harvested from the secreted medium with a C-terminal 8 \times His tag. Proteins eluted from nickel resin were then treated with 1:100 (by mass) human rhinovirus 3C protease for 24 h at 4 $^{\circ}$ C to cleave the 3C protease tag. The 3C tag is flanked by Gly-Gly-Gly-Ser motifs at both ends (G3S-3C-G3S) to ensure accessibility of the protease site between the two chains of IFN γ . The cleaved proteins were

then purified using the N-terminal protein C-tag and a final 8× His tag purification to ensure isolation of the correctly paired heterodimeric IFN γ proteins. **b**, Table of mutations for each of the GIFN proteins, indicating the affected receptor binding sites. GIFN2, one IFN γ R1 and one IFN γ R2 binding in *cis*; GIFN3, two IFN γ R1 molecules bound; GIFN4, reduced affinity for both IFN γ R1 and IFN γ R2. **c**, SEC profiles and SDS–PAGE gel fractions for 200 μ g wild-type IFN γ (black) or equal molar quantities of GIFN1 (purple), GIFN2 (red), GIFN4 (orange), IFN γ R1 F05 (grey), and IFN γ R2 (green). Individual proteins have been purified and analysed by SEC at least three times. **d**, **e**, To determine the receptor-binding properties of the GIFN proteins, the shifts in the SEC profiles and gels were compared relative to the wild-type protein as described for **c** except IFNs were mixed with equimolar quantities of IFN γ R1 F05 (**d**) or equimolar quantities of both IFN γ R1 F05 and IFN γ R2 (**e**). These data are from single experiments except the wild-type experiments which were performed at least three times.

Extended Data Table 1 |

Data collection and refinement statistics (molecular replacement)

	Shaved IFN- γ :IFN- γ R1:IFN- γ R2 complex ^a	Glycosylated IFN- γ :IFN- γ R1:IFN- γ R2 complex ^b
Data collection		
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions		
<i>a, b, c</i> (Å)	78.284 151.464	78.694 150.212
α, β, γ (°)	211.30	212.668
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	37.19–3.25 (3.366–3.25)*	48.38–3.8 (3.936–3.8)
<i>R</i> _{merge}	0.09555 (1.646)	0.2724 (3.243)
<i>I</i> / σ <i>I</i>	12.66(1.43)	9.98 (0.95)
Completeness (%)	97.32 (99.85)	97.32(99.85)
Redundancy	6.5 (6.8)	14.6(14.9)
CC _{1/2}	0.999 (0.685)	0.999(0.461)
Refinement		
Resolution (Å)	37.19–3.25 (3.366–3.25)*	48.38–3.8 (3.936 3.8)
No. reflections	39329 (3965)	25569 (2543)
<i>R</i> _{work} / <i>R</i> _{free}	0.1906/0.2336	0.2487/0.2695
No. atoms	8966	8984
Protein	8677	8641
Ligand/ion	281	343
Water	8	0
<i>B</i> -factors	164.82	215.78
Protein	163.67	214.02
Ligand/ion	202.05	260.34
Water	108.67	–
R.m.s. deviations		
Bond lengths (Å)	0.003	0.004

Shaved IFN- γ :IFN- γ R 1 :IFN- γ R2 complex ^a		Glycosylated IFN- γ :IFN- γ R 1 :IFN- γ R2 complex
Bond angles (°)	0.59	.80

^{a,b} One crystal was used for structure determination.

* Values in parentheses are for highest-resolution shell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank W. Schneider, H.-H. Hoffman and C. Rice for assistance with antiviral experiments; S. Bendall and L. Borges for assistance with CyTOF experiments; and J.-L. Casanova, J. Bustamante and C. Oleaga for assistance with experiments with IFNGR2 T168N cell lines. This work was supported by NIH grants 1U19AI109662, 5R01CA177684 and NIH RO1-AI51321 (to K.C.G.), by the DFG grants SFB 944 and PI 405/10-1 (to J.P.), by NIH HD090156 (to R.S.H.), and by NIH U54 CA209971 and DoD BC140436 (to E.G.E.). K.C.G. is an investigator of the Howard Hughes Medical Institute and is supported by the Ludwig Institute and the Younger Family Chair. J.L.M. is supported by NIH award K01CA175127. We thank the staff at Stanford Synchrotron Radiation Lightsource and Advanced Light Source for their assistance. The Advanced Light Source is a Department of Energy Office of Science User Facility under Contract No. DE-AC02-05CH11231. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393).

Competing interests K.C.G. and J.L.M. are co-inventors on provisional patent application 62/712,128, which includes discoveries described in this manuscript. K.C.G. is the founder of Synthekine Therapeutics.

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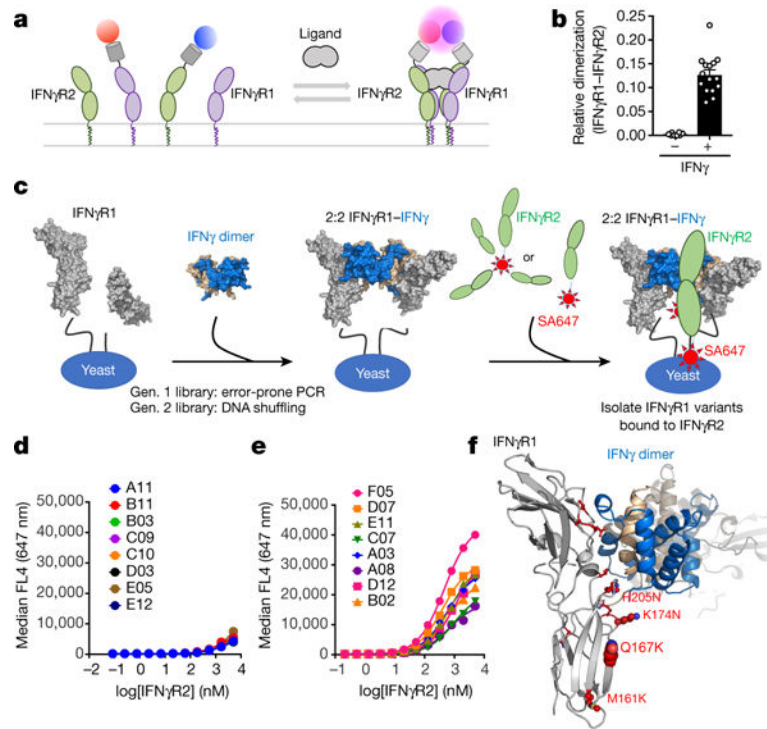


Fig. 1. Assembly and engineered stabilization of the IFN γ receptor complex.

a, Cell-surface labelling of IFN γ R1 (purple) and IFN γ R2 (green) using Rho-11 (red circle) and DY647-labelled (blue circle) anti-GFP nanobodies is used to determine receptor dimerization. b, Relative co-tracking of Rho-11-IFN γ R1 and DY647-IFN γ R2 in the absence and presence of ligand. Data are mean \pm s.e.m.; $n = 8$ (-IFN γ), $n = 15$ (+IFN γ); n is the number of biologically independent samples. c, Experimental design for engineering higher-affinity IFN γ R1 variants. IFN γ R1 (grey) is displayed on yeast and, in the presence of unlabelled IFN γ dimer (blue and tan), forms the intermediate 2:2 IFN γ -IFN γ R1 complex (middle), enabling detection of variants binding to either tetrameric or monomeric IFN γ R2 (green; labelled with streptavidin-Alexa Fluor 647 (SA647)). d, Using this platform, a first-generation library was generated using non-biased error-prone PCR followed by DNA shuffling. e, After a single round of selection, eight clones were titrated to estimate their relative binding to IFN γ R2. f, Sites of mutations on IFN γ R1 F05 (see Extended Data Fig. 1e).

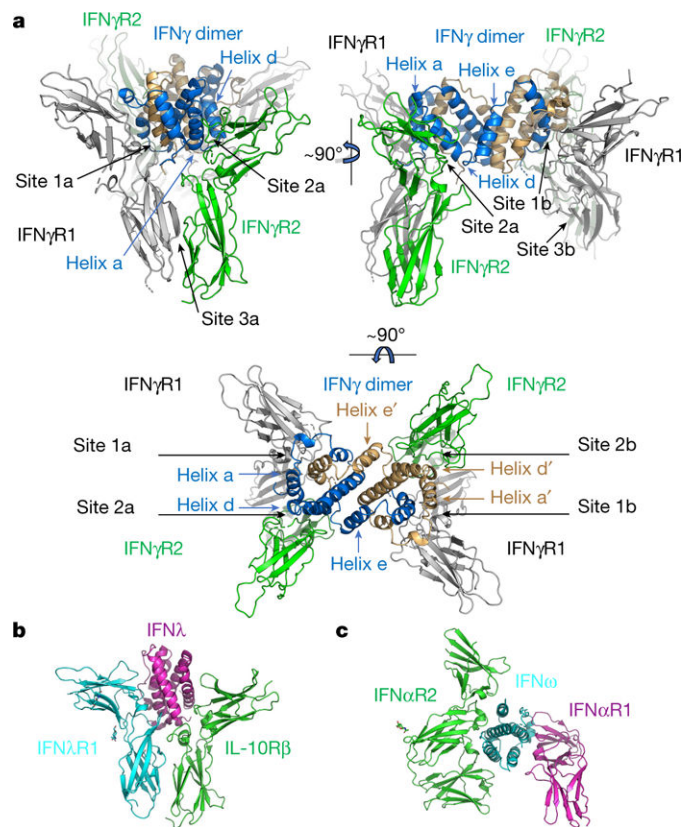


Fig. 2. Structure of the IFN γ hexameric complex.

a, The structure of the IFN γ hexameric complex reveals the mechanism of IFN γ R2 (green) recognition of IFN γ (blue and tan) and IFN γ R1 (grey). IFN γ R2 receptors make extensive contacts with the IFN γ dimer at sites 2a and 2b, and site 3a and 3b makes stem-stem contacts with IFN γ R1. **b**, Structure of the IFN λ -IFN λ R1-IL-10R β signalling complex (PDB: 5T5W)¹⁵ shares a similar geometry with the IFN γ signalling complex. The binding mode of IFN γ R2 is nearly identical to that of IL-10R β . **c**, Structure of a type I IFN receptor complex (PDB: 3SE4)²¹ with distinct ligand-receptor geometries compared to either type II or III IFNs.

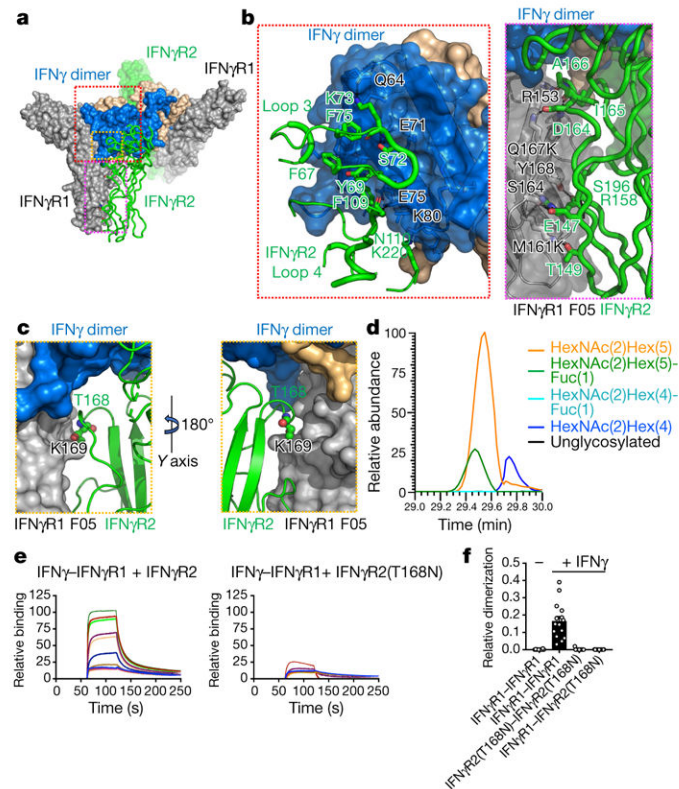


Fig. 3. Interactions within the IFN γ receptor complex and mechanism of disease mutation.

a, Overview of the IFN γ -IFN γ R1 F05-IFN γ R2 ternary complex. IFN γ , blue and tan; IFN γ R1 F05, grey; IFN γ R2, green. **b**, View of the site 2a (identical to site 2b) contacts between IFN γ and IFN γ R2 (left). Detailed view of site 3a (identical to site 3b) between IFN γ R1 F05 (grey) and IFN γ R2 (green) (right). **c**, The complex structure places the neoglycosylation site of IFN γ R2(T168N) at the site 3a (identical to site 3b) interface. **d**, IFN γ R2(T168N) was expressed in HEK293S GnTI⁻ cells and analysed by nano-liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). The peptide containing N168 was identified (see Extended Data Fig. 3), and relative amounts of various glycoforms were determined by extracted ion chromatograms (EICs). The data shown are for a single experiment. **e**, The affinity (K_D) of IFN γ R2 to the 2:2 IFN γ -IFN γ R1 intermediate complex was determined to be $\sim 5 \mu\text{M}$ by SPR (left), whereas the mutant IFN γ R2(T168N) results in a loss of binding (right). The titration data are from a single experiment. **f**, Single-molecule dimerization experiments in cells co-expressing IFN γ R1 and IFN γ R2(T168N). IFN γ retains binding to IFN γ R1 but fails to recruit IFN γ R2(T168N). Data are mean \pm s.e.m.; $n = 14, 15, 13,$ and 17 (left to right); n is the number of independent experiments.

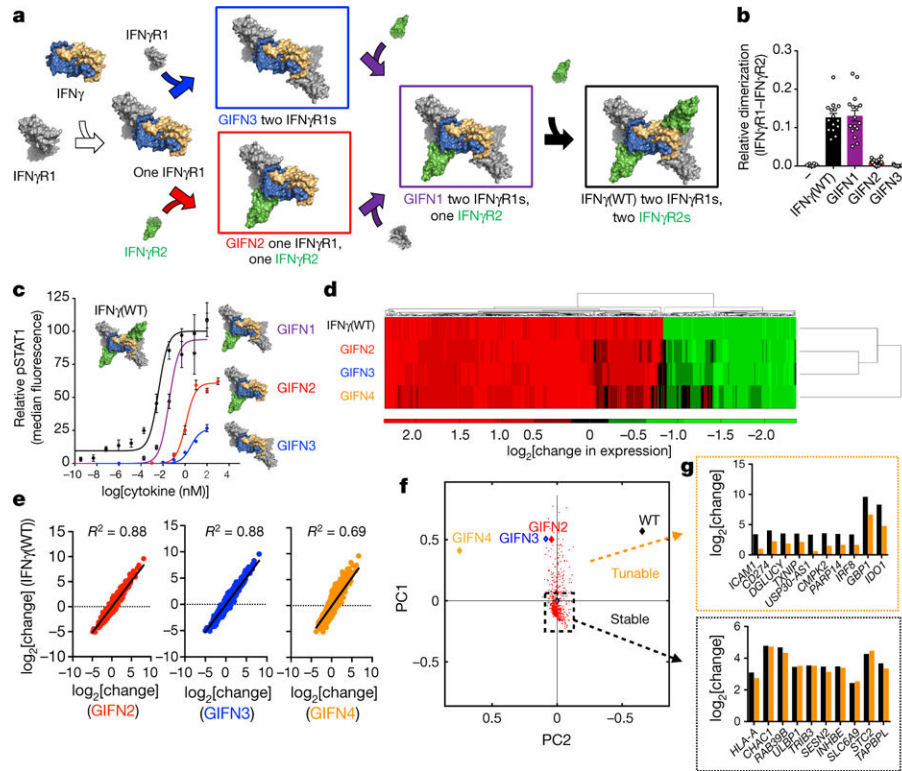


Fig. 4. Structure-based design of IFN γ partial agonists with biased signalling outputs.

a, Assembly of the hexameric IFN γ signalling complex can proceed through multiple intermediates. **b**, Co-tracking of IFN γ R1 and IFN γ R2 for wild-type and variants of IFN γ . Data are mean \pm s.e.m.; $n = 8, 15, 16, 16,$ and 15 independent experiments (left to right). **c**, IFN γ mutants that stabilized intermediate complexes were assayed for STAT1 activation in Hap1 cells. GIFN2, one IFN γ R1 and one IFN γ R2 binding in *cis*; GIFN3, two IFN γ R1 bound; GIFN4, reduced affinity for both IFN γ R1s and IFN γ R2s (see Extended Data Fig. 5). Curves were fitted to a first-order logistic model. Data are mean \pm s.e.m.; $n = 3$ biologically independent experiments. **d**, Heat map depicting the relative expression (\log_2 [change in expression]) of 1,000 genes from the human transcriptome. **e**, Correlations in relative expression of wild-type IFN γ and GIFNs for all genes. $n = 2$ independent biological experiments. **f**, Principal component analysis (PCA) of the top 600 genes induced by IFN γ , were compared to GIFN2–4. $n = 2$ biologically independent experiments. **g**, Genes, including *CD274* (which encodes PD-L1), that have significantly lowered expression with GIFN4 relative to wild-type IFN γ (top). Genes, including *HLA-A*, that are robustly expressed with both wild-type IFN γ and GIFN4 (bottom panel).

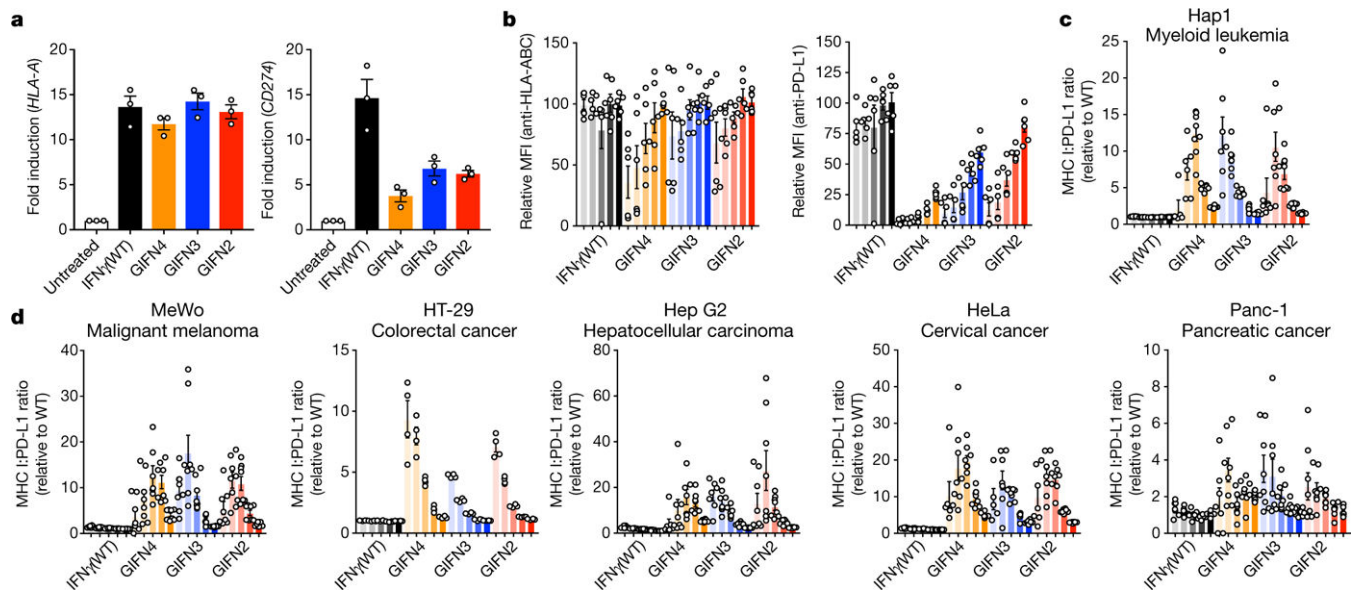


Fig. 5. Decoupled expression of MHC I versus PD-L1 in response to IFN γ or partial agonists.

a, b, A549 cells were treated with either IFN γ (wild-type) or partial agonists for 48 h.

Upregulation of MHC class I antigen and PD-L1 expression were quantified by reverse transcription with quantitative PCR (RT-qPCR) (**a**; ligand concentration, 62.5 nM; mean \pm s.e.m., $n = 3$ biologically independent experiments), by using fluorescently labelled HLA-ABC antibody (**b**, left; ligand concentrations 0.1, 0.5, 2.5, 12.5 and 62.5 nM (left to right, respectively, for each agonist); mean \pm s.e.m.; $n = 6$ independent samples), and using fluorescently labelled PD-L1 antibody (**b**, right). **c, d,** Six additional cancer cell lines (Hap1, MeWo, HT-29, Hep G2, HeLa, and Panc-1) were screened for MHC I:PD-L1 bias as in **b**, but after 24 h treatment (ligand concentrations 0.1, 0.5, 2.5, 12.5 and 62.5 nM (left to right, respectively, for each agonist); mean \pm s.e.m.; $n = 8$ independent samples).