Assembly and structural properties of glucocorticoid-induced TNF receptor ligand: Implications for function

Kausik Chattopadhyay*, Udupi A. Ramagopal†, Arunika Mukhopadhyaya*, Vladimir N. Malashkevich†, Teresa P. DiLorenzo**, Michael Brenowitz†, Stanley G. Nathenson*§¶, and Steven C. Almo†¶

Departments of *Microbiology and Immunology, †Cell Biology, ‡Biochemistry, §Physiology and Biophysics, ¶Medicine (Division of Endocrinology), Albert Einstein College of Medicine, Bronx, NY 10461

Contributed by Stanley G. Nathenson, September 28, 2007 (sent for review August 3, 2007)

Glucocorticoid-induced TNF receptor ligand (GITRL), a recently identified member of the TNF family, binds to its receptor GTR on both effector and regulatory T cells and generates positive costimulatory signals implicated in a wide range of T cell functions. Structural analysis reveals that the human GITRL (hGITRL) ectodomain self-assembles into an atypical expanded homotrimer with sparse monomer–monomer interfaces. Consistent with the small intersubunit interfaces, hGITRL exhibits a relatively weak tendency to trimerize in solution and displays a monomer–trimer equilibrium not reported for other TNF family members. This unique assembly behavior has direct implications for hGITRL–GTR signaling, because enforced trimerization of soluble hGITRL ectodomain results in an ≈ 100 -fold increase in its receptor binding affinity and also in enhanced costimulatory activity. The apparent reduction in affinity that is the consequence of this dynamic equilibrium may represent a mechanism to realize the biologically optimal level of signaling through the hGITRL–GTR pathway, as opposed to the maximal achievable level.

crystal structure | T cell costimulation

Glucocorticoid-induced TNF receptor (GTR) and its ligand GITRL are recently described members of the TNF receptor/ligand families and are implicated in a wide range of immune functions involving both effector T cells and regulatory T cells (Tregs) (1–3). GTR exhibits low levels of expression on resting mouse and human T cells and is up-regulated on activation of CD4⁺ and CD8⁺ T cells. A substantial level of GTR is constitutively expressed on CD4⁺CD25⁺ Tregs. GTR is activated by its ligand GITRL, which is expressed on the surface of various antigen-presenting cells (APCs), including macrophages, B cells, and dendritic cells. In the context of suboptimal T cell receptor (TCR) stimulation, GTR engagement generates a positive costimulatory signal leading to increased T cell proliferation and cytokine production (4–6). Notably, GTR stimulation on effector T cells has been shown to reverse the suppressive effects of Tregs (4, 7, 8). The GITRL–GTR pathway thus represents a potential target for manipulating T cell responsiveness to clear infectious pathogens and tumors and to reverse globally suppressed immune responses resulting from chronic infections.

GITRL is a ≈ 20 -kDa type II transmembrane protein that belongs to the TNF family, and the extracellular domain shares $\approx 20\%$ sequence identity with other TNF ligands (9). GTR is a ≈ 26 -kDa type I transmembrane protein that displays 14–28% sequence identity to other members of the TNF receptor (TNFR) family (9). Known structures of TNF/TNFR molecules display a common organization in which trimeric TNF ligands engage three receptor molecules (characterized by pseudorepeats of one-to-four cysteine-rich domains) resulting in a threefold symmetry complex with 3:3 receptor–ligand stoichiometry (10). These geometric constraints are also imposed on the receptor cytoplasmic tails and are thought to promote the

recruitment of the signaling/adaptor proteins, including TNF receptor-associated factors (TRAFs), resulting in the activation of downstream signaling pathways (11). However, the generalization of this model requires additional structural information because of the low sequence identity shared by the members of the TNF/TNFR families. Currently, no data are available on the structural and biophysical properties that might contribute to signaling in the GITRL–GTR pathway.

In the present study we have determined the crystal structure of human GITRL (hGITRL), and used a structure-based mutagenesis approach to map its receptor binding surface. Similar to other TNF molecules, the hGITRL ectodomain adopts a classic β -sandwich “jelly-roll” topology and self-assembles into a noncovalently associated homotrimer, in which solvent-exposed loops near the intersubunit clefts form the receptor-recognition surface. Notably, the hGITRL trimer differs from conventional TNF family members by adopting an atypical expanded assembly that more closely resembles a “blooming flower,” instead of the truncated pyramidal shape observed in most TNF trimeric assemblies. The structure also reveals that the hGITRL trimer interfaces are remarkably small and lack the tightly packed aromatic/hydrophobic residues commonly observed in traditional TNF trimer interfaces. An approximately similar expanded organization has been recently reported for OX40L (12), and sequence analysis suggests that OX40L, GITRL, and CD30L may represent a distinct subfamily within the TNF family that is characterized by a short (≈ 120 – 130 residues) TNF homology domain (THD) compared with the traditional ≈ 150 -residue THDs. Consistent with the relatively small intersubunit interface observed in the crystalline state, hGITRL also displays considerably weaker tendency to trimerize in solution compared with other TNF ligands. The propensity of hGITRL to display a reversible monomer–trimer equilibrium may have implications for GTR signaling, because enforced trimerization of soluble hGITRL results in a ≈ 100 -fold increase in its apparent receptor binding affinity, as well as enhanced signaling capabilities. This dynamic oligomerization behavior may serve as a mechanism to modulate the ligand–receptor engagement at the T cell–APC interface and ensure the biolog-

Author contributions: K.C., A.M., T.P.D., M.B., S.G.N., and S.C.A. designed research; K.C., U.A.R., A.M., and M.B. performed research; K.C. and V.N.M. contributed new reagents/analytic tools; K.C., U.A.R., A.M., and M.B. analyzed data; K.C., S.G.N., and S.C.A. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The structure factors and coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2Q1M, 2R30, and 2R32).

¶To whom correspondence may be addressed. E-mail: nathenso@aeom.yu.edu or almo@aeom.yu.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0709264104/DC1.

© 2007 by The National Academy of Sciences of the USA

ically optimal level of signaling through the hGITRL–GITR pathway, as opposed to the maximal achievable level.

Results

Crystal Structure of hGITRL Exhibits an Atypical TNF Trimeric Assembly. The crystal structure of the biologically active ectodomain of hGITRL expressed in *Escherichia coli* was determined at 2.3 Å [supporting information (SI) Table 1]. Consistent with the modest sequence identity (SI Fig. 5), the hGITRL monomer resembles other TNF family members, displaying a two-layer β -sandwich jelly-roll topology with inner and outer sheets composed of the A'AHCF and B'BGDE strands, respectively (Fig. 1A). However, detailed structural comparisons of the hGITRL–THD monomer with other TNF ligand monomers revealed rms deviations in the range of ≈ 2.0 – 3.0 Å (SI Table 2). This contrasts with other TNF ligands, which show considerably greater structural similarity (i.e., smaller rms deviations of 1–2 Å) when compared across the family. For example, rms deviations calculated for the hTNF- α monomer are shown in SI Table 3. Notably, the hGITRL–THD is strikingly shorter (≈ 119 aa) than most other TNF molecules (≈ 150 aa) and contains a single disulfide bridge (formed between Cys-58 and Cys-78) linking strands A and B', whereas most other TNFs possess disulfide bonds that connect either the CD and EF loops (TNF and CD40L), or the E and F strands (BAFF, APRIL, and EDA) (10). The only other structurally characterized TNF ligand that possesses noncanonical intrachain disulfide bonds is OX40L (12); one (conserved in human and mouse) connecting the C-terminal extension and the BC loop, and another (only in mouse) connecting the AA' and GH loops. The reduced size of the hGITRL–THD is apparent from its superposition with hTNF- α (Fig. 1B), which highlights the shortening of two β -strand pairs: the C and F strands in the inner face and the D and E strands in the outer face. The AA', DE, and EF loops are the most divergent in length and conformation, a feature commonly observed with other TNF ligands. The only other known TNF family members possessing a similarly short THD are OX40L (≈ 126 aa) and CD30L (≈ 117 aa predicted) (SI Fig. 5).

The hGITRL monomers self-assemble into a threefold symmetric homotrimer that resembles a blooming flower-like assembly (Fig. 1C, SI Fig. 6A). This expanded organization differs from the more compact truncated pyramidal arrangement observed in conventional TNF molecules (Fig. 1D, SI Fig. 6B). Superposition of the hGITRL and hTNF- α trimers results in a rms deviation of 4.7 Å, which is considerably larger than that observed between other TNF trimers (superposition of TNF- α with RANKL, TALL, and EDA-1 yields rms deviations 1.67, 1.74, and 1.82 Å, respectively; data not shown). In the hGITRL trimer, the subunits are oriented at an angle of $\approx 45^\circ$ with respect to the trimer axis, whereas for most of the TNFs, the monomers are displaced by only ≈ 20 – 30° . This difference in orientation results in a greatly reduced height of ≈ 40 Å for the hGITRL trimer, compared with most other TNF trimers (≈ 60 Å). At the top of the hGITRL trimer, the distance between the adjacent subunits is much greater (≈ 40 – 45 Å) than that observed in other TNF ligands (≈ 20 – 25 Å) (Fig. 1C and D, SI Fig. 6). These structural features result in a deep cleft that runs downward from the top of the hGITRL trimer (Fig. 1C, SI Fig. 6A) and contrasts with the more compact assembly of conventional TNF trimers. The only other TNF ligand that has been demonstrated to possess an approximately comparable organization is OX40L (12) for which the crystal structure has been solved recently (SI Fig. 6C and D). The expanded assembly of the hGITRL trimer is consistent with the potential *N*-glycosylation site present at N129, positioned at the top rim of the trimeric assembly. Modeling suggests that the substantial opening at the upper half of hGITRL trimer would accommodate a carbohydrate moiety at this site. In contrast, glycosylation at this site is inconsistent with the typical

Fig. 1. Structure of the hGITRL ectodomain shows an atypical expanded architecture not observed in conventional TNF family members. (A) Ribbon diagram of hGITRL monomer showing the classic jelly-roll fold of the THD. The β -strands are labeled, and the N and C termini are marked. (B) Superposition of hGITRL (red) and hTNF- α (blue; 1TNF-A) monomers shows significant differences in the strand and loop lengths. hGITRL trimer (C) and hTNF- α (1TNF) trimer (D) are shown in side view as ribbon diagrams. C and D clearly demonstrate that the hGITRL trimer possesses an atypical expanded assembly and lacks the typical compact architecture of the conventional THDs.

compact TNF organization, because this site is buried in the core of the classical TNF trimers (SI Fig. 6E and F).

Owing to the greater excursion of its monomers from the trimer axis, the hGITRL trimer possesses a substantially smaller intersubunit interface, resulting in the burial of 3,172 Å² of solvent-accessible surface area for the entire assembly, which is at least ≈ 2 -fold lower than that associated with other TNF ligands (7,236 Å² for TNF- α , 5,958 for TNF- β , and 6,151 Å² for RANKL trimers). The conventional TNF trimers are assembled such that one edge of each subunit is packed against the inner sheet of its neighbor, forming a large and mostly hydrophobic interface (10, 13). Such interfacial organization involves a large number of residues and results in a very tight trimeric assembly of the typical TNF ligands. For example, in hTNF- α approximately 40 residues contribute side chains to the monomer–monomer interface (13). In contrast, the monomer–monomer interface in the hGITRL trimer is formed by approximately 10 residues, including several containing hydrophobic and/or aromatic side chains, which are located at the interior of the lower half of the trimer (SI Fig. 7). The most prominent hydrophobic interaction occurs between Y98 (C strand) and L94 (C strand) (≈ 4 Å apart) from the two adjacent subunits. Other hGITRL residues involved in intersubunit contacts are K61 (A strand), L96 (C strand), N135 (EF loop), and Y164 (H strand) from one monomer, and T139 (F strand), Y140 (F strand), E141 (F strand), and L170 (H strand) of the neighboring monomer (SI Fig. 7). Remarkably, unlike other TNF ligands, the hGITRL

Fig. 3. Enforced trimerization of hGITRL. (A) Molecular surface representation of the CC-hGITRL crystal structure shows that the fusion of the coiled-coil peptide to the N terminus of hGITRL does not induce any significant alterations in the global/local conformation of the protein. (B) Enforced trimerization of hGITRL augmented the *in vitro* T cell proliferative response. CD4⁺ T cells were stimulated with hGITRL variants (5 μ g/ml) in the presence of 30 ng/ml anti-CD3. Data shown are the mean ($\pm$ SEM) of quadruplicate cultures and are representative of at least three independent assays.

slightly lower than the molecular weight of the trimeric CC-hGITRL calculated from the sequence (56,684 Da), the concentration distributions were not better fit by self-association models compared with a stable trimer. The crystal structure of CC-hGITRL (Fig. 3A, SI Table 1) revealed that fusion of the coiled-coil peptide did not cause any gross alteration in the hGITRL-THD monomer or in its trimeric assembly (wild type and CC-hGITRL trimers superimpose with an rms deviation of 0.69 Å based on 321 C α).

Very tight binding was observed when hGITRL was titrated with CC-hGITRL in SPR experiments conducted under the same conditions used for wild-type hGITRL (SI Fig. 10B). Responses approaching saturation were all that could be measured by the steady-state analysis even at the lowest concentration of 112 nM, suggesting that receptor binding by CC-hGITRL is very tight, with a $K_d < 10$ nM (data not shown). The absence of data over most of the transition except for the saturation plateau and zero ligand concentration preclude precise determination of the binding affinity. Because the association progress curves measured for CC-hGITRL-GITR interaction were consistent with the simple single-site binding model, the K_d was calculated from the ratio of k_{on}/k_{off} yielding a value of 3.84 nM. The value of $k_{off} = 6 \times 10^{-4} \text{ s}^{-1}$ measured for CC-hGITRL is comparable with that measured for hGITRL. These results are consistent with the conclusion that the low apparent affinity of hGITRL is the result of the coupled monomer-trimer equilibrium with the trimer binding the receptor. The difference between the receptor binding affinities calculated for the hGITRL trimer (K_d of ≈ 60 nM) and that experimentally determined for CC-hGITRL (K_d of ≈ 4 nM) may reflect either subtle conformational changes or complexities in the self-assembly reaction that are not resolved by our analysis.

The functional consequence of high receptor binding affinity of the constitutively trimeric CC-hGITRL was demonstrated in an *in vitro* T cell costimulation assay. Human peripheral blood CD4⁺ T cells were treated with soluble hGITRL proteins (5 μ g/ml), wild-type or the coiled-coil variant, in the presence of a suboptimal anti-CD3 stimulation (30 ng/ml), and T cell proliferation was measured by [³H]thymidine incorporation. The coiled-coil variant induced a significantly enhanced proliferative response compared with the wild-type protein (Fig. 3B). Altogether, these data suggest a mechanism in which the dynamic equilibrium of hGITRL modulates its receptor binding activity and contributes to the generation of the biologically optimal signal for effective T cell costimulation.

Fig. 4. Receptor recognition surface of hGITRL. (A) Receptor binding activities of hGITRL mutants (125 nM) to immobilized hGITR. (B) hGITRL residues whose mutation affected receptor binding are mapped onto the hGITRL trimer and are colored accordingly. Orange and blue represent $>90\%$ and $\approx 50\%$ reduction in binding, respectively. Alanine substitution of N106 (red) resulted in a ≈ 2 - to 3-fold increase in receptor binding. (C) Surface representation of hGITRL-trimer, docked with a model of the hGITR monomer (shown in magenta). Areas on the surface of hGITRL that contribute to receptor binding are colored as described in B. (D) Alterations of the hGITRL-GITR interaction modulated T cell costimulation. The N106A mutation augmented the *in vitro* T cell proliferative response relative to the wild-type protein. The L65A-P66A-K68A triple mutant induced T cell proliferation similar to the PBS control. Data shown are the mean ($\pm$ SEM) of quadruplicate cultures and are representative of at least three independent assays.

Mapping Receptor Binding Sites on hGITRL. Based on known structures of TNF-TNFR family complexes, the receptor binding sites are formed by the solvent-accessible loops located at the three identical clefts between neighboring monomers in the trimeric ligand (10). A series of structure-guided mutations were generated in these loops, and the mutants were tested for their impact on the biochemical function of hGITRL. The mutants are divided into four categories based on the location of the residues involved: (i) the L65A-P66A-K68A triple mutant in the AA' loop, (ii) N106A in the CD loop, (iii) N120A, K121A, and D122A single mutants in the DE loop, and (iv) the L159A-N161A double mutant in the GH loop. The abilities of the wild-type and the mutant forms of hGITRL to bind hGITR were determined by SPR at a ligand concentration of 125 nM (Fig. 4A). The L65A-P66A-K68A, N120A and L159A-N161A mutations led to undetectable or extremely weak ($>90\%$ reduction) binding activity compared with the wild-type hGITRL. The D122A mutation caused $\approx 50\%$ reduction in binding, whereas the K121A mutation had no impact on receptor binding. Notably, the N106A mutation resulted in an ≈ 2 - to 3-fold increase over the wild-type binding activity. All of the mutations described above are located distal to the trimerization interface of hGITRL, and therefore the effects of these mutations are likely due to direct influence on receptor binding. Our results indicate that the AA', CD, DE and GH loops of hGITRL contribute to receptor binding and recognition (Fig. 4B) and are consistent with a homology model of the hGITRL-GITR complex (Fig. 4C) that was generated by using the recently reported hOX40L-OX40 complex structure (12) as the template. The model suggests that solvent-accessible residues from the AA', CD, DE,

and GH loops of hGITRL lie in close proximity to hGTR and thus are poised to contribute to the hGTR recognition surface.

To further characterize the high-affinity N106A mutant, we determined its crystal structure (SI Table 1 and SI Fig. 11). Superimposition of the hGITRL-N106A and wild-type trimers resulted in a rms deviation of 0.37 Å (based on 348 Cα), suggesting that the N106A mutation did not induce any global alteration in the protein structure or assembly properties. Therefore, the enhanced receptor binding activity of the N106A mutant can be best explained by better steric and/or polar interactions on the receptor engagement. Further structural analysis of the hGITRL–GTR complex will be necessary to detail the ligand–receptor interface.

Increased Receptor Binding Ability of the hGITRL-N106A Mutant Results in Increased T Cell Proliferation Response. hGITRL mutants with altered hGTR binding properties were further analyzed for T cell costimulatory activity. In particular, the hGITRL-N106A mutant was tested to determine whether its enhanced binding activity correlates with an increased ability to costimulate T cell proliferation. As shown in Fig. 4D, the hGITRL-N106A mutant significantly enhanced the T cell proliferative response, compared with the wild-type protein, demonstrating that the alanine substitution of N106 residue in hGITRL not only enhanced receptor binding, but could also effectively augment GTR-mediated T cell costimulation. For comparison, the mutant L65A-P66A-K68A, which did not show any detectable binding to hGTR, was found to elicit T cell proliferation similar to the PBS control. The behavior of the N106A mutant also points to the evolution of biologically optimal affinity and costimulatory activity, as opposed to maximal signaling activity.

Discussion

This study demonstrated that recombinant hGITRL possesses unusual structural and biochemical properties, including an expanded trimeric organization, a dynamic monomer–trimer equilibrium, and apparent receptor binding behavior that is considerably weaker than typical TNF family members. A concern is that these properties are the consequence of the protocol used to refold hGITRL from inclusion bodies. Although the majority of the hGITRL expressed in *E. coli* goes to inclusion bodies, there is a small soluble fraction that can be isolated directly on cell lysis. Importantly, this soluble fraction exhibits chromatographic behavior (i.e., two dynamically interconverting species) that is comparable to the refolded material. The OX40L and GITRL genes are immediately adjacent to one another in all species sequenced and are likely related by a gene duplication process. Based on this evolutionary relationship, it is significant that the structures of human and murine OX40L determined from material expressed in insect cells exhibit an expanded quaternary structure (12) similar to that observed in the hGITRL structure. Furthermore, the N-linked glycosylation site at N129 in hGITRL is consistent with the expanded structure and cannot be accommodated by the more typical TNF family trimer architecture. The apparent receptor binding affinity of refolded hGITRL (K_d of 560 nM) is considerably weaker than typical TNF family members (K_d in the 0.1–10 nM range). It is notable that the experimentally measured K_d for the human OX40L–OX40 interaction (both expressed in insect cells) is ≈ 140 nM, which is remarkably similar to the apparent affinity of the hGITRL–GTR interaction. These observations suggest that the hGITRL behavior reported in the present work reflects the true properties of hGITRL and are not the consequence of refolding.

In general, signaling through the members of the TNFR family appears to rely on the ability of the intrinsically trimeric TNF ligands to drive the “trimerization” of the membrane-associated receptor ectodomains. In all cocrystal structures determined to date, a complex of 3:3 stoichiometry is formed in which each

receptor molecule binds at the interface formed by two adjacent ligand molecules (10, 11, 18). In the majority of cases, the compact truncated pyramidal organization of the conventional TNF trimer causes the membrane-proximal C-terminal ends of three TNFRs to be positioned at the vertices of an equilateral triangle with an edge length of ≈ 35 Å. This organization presumably generates specific constraints on the spatial organization of the receptor cytoplasmic domains, which in turn promotes optimal recruitment of adaptor and signaling molecules to initiate further downstream signaling events. Specifically, the recruitment of the trimeric signal adaptor molecules, TRAFs, depends on the presence of a recruitment motif on the cytoplasmic domains of the TNFRs as well as on the particular trimeric geometry of the receptor cytoplasmic tails (19, 20). Structures of the TRAFs in complex with peptides corresponding to the TRAF-recruiting regions of TNFR cytoplasmic tails suggest an assembly with 3:3 stoichiometry, in which peptides are separated from each other by a distance of ≈ 55 Å. The hGITRL structure shows an atypical, expanded trimeric assembly that would be predicted to bind three hGTR molecules such that the cytoplasmic tails are positioned at a distance considerably greater than that expected for most conventional TNFR family members. It is notable that the only other TNF ligand exhibiting such an unusual trimeric organization is OX40L (SI Fig. 6 C and D), and the structure of the hOX40L–OX40 complex shows that the receptor C termini are separated by a distance of ≈ 70 Å (12). The overall organization of each ligand controls the relative placement of the associated receptor cytoplasmic domains, which in combination with the length and composition of the receptor cytoplasmic tail, may contribute to the energetics of the different receptor–TRAF interactions.

Although hGITRL forms noncovalently associated homotrimers in the crystalline state, the solution behavior of this protein is well described by a reversible monomer–trimer equilibrium, a phenomenon that has not been reported for any other member of the TNF family. This behavior has a profound influence on the receptor binding properties of hGITRL. In our studies, soluble hGITRL displayed a considerably weaker apparent affinity ($K_d \approx 560$ nM) for its receptor than other TNF members in the family ($K_d \approx 0.1$ –10 nM) (11); however, the forced trimerization of hGITRL before receptor binding markedly enhanced its receptor binding activity ($K_d \approx 4$ nM). Notably, our mutagenesis data show that the hGITRL–GTR binding interface is similar to those present in all TNF–TNFR pairs examined to date, that is, the receptor binding surface is formed by loops contributed from two adjacent ligand monomers. Consistent with previous structural and biochemical data with conventional TNF/TNFR molecules (10), these observations support the notion that ligand trimerization is also a prerequisite for receptor binding by hGITRL. Extrapolation to the cell surface suggests that the dynamic monomer–trimer equilibrium may represent a unique mechanism that directly contributes to hGITRL–GTR signaling. Owing to the fact that only hGITRL trimers are competent to bind hGTR, on the basis of thermodynamic principles, the existence of the monomer–trimer equilibrium reduces the effective affinity between receptor and ligand because an energetic penalty must be paid to drive hGITRL trimerization. Consequently, not all of the interaction energy goes to the hGITRL–GTR binding interaction, but some of it must be used for the formation of the intermolecular interfaces in hGITRL trimer. This finding is supported by the observation of the significantly enhanced affinity (≈ 100 -fold) of the CC-hGITRL trimer relative to the wild-type hGITRL. The *in vivo* contribution of this dynamic equilibrium to function will depend on the cell surface expression levels of hGITRL and the specific effects of the plasma membrane environment. We speculate that this feature of hGITRL assembly may represent a mechanism to ensure that the ligand–receptor interaction is not too tight, but is tuned to

give the biologically optimal signal. Indeed, under conditions typically used to study GITR costimulation, the CC-hGITRL trimer results in a significant enhancement of the T cell proliferation response.

Apart from influencing the outcome of the ligand–receptor engagement with respect to the T cell costimulation, dynamic oligomeric assembly of hGITRL could potentially effect “reverse signaling” into the ligand-bearing cells. An increasing body of evidence suggests that different members of the TNF family, including GITRL, can transmit positive and negative feedback signals into the APCs (21). For example, GITRL stimulation induces the production of proinflammatory cytokines by macrophages (22, 23). It has also been reported that in several tumor cell lines reverse signaling through GITRL mediates a marked down-regulation of the immunoregulatory surface molecules (CD40 and CD54) and adhesion molecules (EpCAM), and up-regulation of the immunosuppressive cytokine TGF- β (24). More recently, it has been shown that reverse signaling through GITRL acts on plasmacytoid DCs to activate indoleamine 2,3-dioxygenase (IDO) by means of the noncanonical NF- κ B-dependent signaling pathway (25). These observations clearly implicate GITRL-mediated reverse signaling as a means of balancing the immunomodulatory effects of GITRL–GITR pathway. However, little is known about the molecular basis of GITRL-mediated costimulation through reverse signaling. The cytoplasmic domains of the TNF ligands, in general, do not possess any conserved signaling motifs and therefore seem to use signal transduction mechanisms that are distinct from those operating in canonical pathway (21). It is notable that many TNF ligands feature phosphorylation sites in their cytoplasmic tails, and it has been proposed that clustering of the TNF ligands on the cell surface may serve as the first step toward signal initiation (21). Analysis of the hGITRL cytoplasmic region also suggests the presence of potential serine phosphorylation sites (NetPhos 2.0 server at www.cbs.dtu.dk/services/NetPhosK/) (26). Furthermore, in the case of hGITRL, the monomer–trimer equilibrium may provide a unique mechanism to support the reverse signaling processes. Specifically, as each hGITR molecule recognizes a binding surface composed of residues contributed from two adjacent hGITRL monomers, it is possible that receptor engagement will modulate the extent of trimerization by driving the

hGITRL equilibrium toward the trimeric state, with potential consequences on signaling into the ligand-expressing cells.

In summary, our structural and biochemical characterizations of hGITRL suggest that its unique trimeric organization results in assembly behavior that may contribute to bidirectional signaling through the hGITRL–GITR pathway. The behavior of the hGITRL system emphasizes that mechanisms must evolve to support the biologically optimal output rather than to achieve maximal output.

Experimental Procedures

Cloning, expression and purification of the extracellular domains of hGITR and hGITRL. The ectodomains of hGITRL (residues 52–177), hGITRL variants, and hGITR (residues 30–155) were expressed and purified as described in *SI Experimental Procedures*.

Structure Determination and Analysis. The crystal structures of wild-type hGITRL and hGITRL variants (CC-hGITRL and hGITRL-N106) were solved and analyzed as described in *SI Experimental Procedures*.

Analytical Ultracentrifugation Analysis. The analytical ultracentrifugation studies were conducted as described in *SI Experimental Procedures*.

SPR Binding Assays. SPR binding assays were performed by using a BIAcore X optical biosensor at 25°C as described in *SI Experimental Procedures*.

In Vitro T Cell Proliferation Assay. *In vitro* T cell proliferation assays were performed as described in *SI Experimental Procedures*.

We thank the staff of the X4A and X29 beam lines at the National Synchrotron Light Source: Y. Wang and Dr. C. Rubin for help with BIACORE studies; Dr. S. Khrapunov for assistance with SPR data analysis; R. Toro for assistance with protein crystallization; Drs. H. Wu and C. Terhorst for critical reading of the manuscript and insightful comments. This work was supported by National Institutes of Health Grants AI07289 and DK065247 (to S.G.N. and S.C.A.); DK64315, DK52956, and DK77500 (to T.P.D.); and DK20541 (to Albert Einstein College of Medicine, Diabetes Research and Training Center); and grants from the Juvenile Diabetes Research Foundation (to T.P.D.) and a postdoctoral fellowship from the Cancer Research Institute (to K.C.).

- Shevach EM, Stephens GL (2006) *Nat Rev Immunol* 6:613–618.
- Nocentini G, Ronchetti S, Cuzzocrea S, Riccardi C (2007) *Eur J Immunol* 37:1165–1169.
- Watts TH (2005) *Annu Rev Immunol* 23:23–68.
- Stephens GL, McHugh RS, Whitters MJ, Young DA, Luxenberg D, Carreno BM, Collins M, Shevach EM (2004) *J Immunol* 173:5008–5020.
- Ronchetti S, Zollo O, Bruscoli S, Agostini M, Bianchini R, Nocentini G, Ayroldi E, Riccardi C (2004) *Eur J Immunol* 34:613–622.
- Tone M, Tone Y, Adams E, Yates SF, Frewin MR, Cobbold SP, Waldmann H (2003) *Proc Natl Acad Sci USA* 100:15059–15064.
- McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC (2002) *Immunity* 16:311–323.
- Ji HB, Liao G, Faubion WA, Abadia-Molina AC, Cozzo C, Laroux FS, Caton A, Terhorst C (2004) *J Immunol* 172:5823–5827.
- Gurney AL, Marsters SA, Huang RM, Pitti RM, Mark DT, Baldwin DT, Gray AM, Dowd AD, Brush AD, Heldens AD, et al. (1999) *Curr Biol* 9:215–218.
- Bodmer JL, Schneider P, Tschopp J (2002) *Trends Biochem Sci* 27:19–26.
- Locksley RM, Killeen N, Lenardo MJ (2001) *Cell* 104:487–501.
- Compaan DM, Hymowitz SG (2006) *Structure (London)* 14:1321–1330.
- Eck MJ, Sprang SR (1989) *J Biol Chem* 264:17595–17605.
- Wingfield P, Pain RH, Craig S (1987) *FEBS Lett* 211:179–184.
- Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, Ambrose C, Tschopp J, Schneider P (2006) *J Biol Chem* 281:13964–13971.
- Harbury PB, Zhang T, Kim PS, Alber T (1993) *Science* 262:1401–1407.
- Harbury PB, Kim PS, Alber T (1994) *Nature* 371:80–83.
- Banner DW, D’Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, Loetscher H, Lesslauer W (1993) *Cell* 73:431–445.
- Ye H, Park YC, Kreishman M, Kieff E, Wu H (1999) *Mol Cell* 4:321–330.
- Park YC, Burkitt V, Villa AR, Tong L, Wu H (1999) *Nature* 398:533–538.
- Eissner G, Kolch W, Scheurich P (2004) *Cytokine Growth Factor Rev* 15:353–366.
- Shin HH, Kim SJ, Lee HS, Choi HS (2004) *Biochem Biophys Res Commun* 316:24–32.
- Shin HH, Lee MH, Kim SG, Lee YH, Kwon BS, Choi HS (2002) *FEBS Lett* 514:275–280.
- Baltz KM, Krusch M, Bringmann A, Brossart P, Mayer F, Kloss M, Baessler T, Kumbier I, Peterfi A, Kupka S, et al. (2007) *FASEB J* 21:2442–2454.
- Grohmann U, Volpi C, Fallarino F, Bozza S, Bianchi R, Vacca C, Orabona C, Belladonna ML, Ayroldi E, Nocentini G, et al. (2007) *Nat Med* 13:579–586.
- Blom N, Gammeltoft S, Brunak S (1999) *J Mol Biol* 294:1351–1362.