NON-CORE REGION MODULATES INTERLEUKIN-11 SIGNALING ACTIVITY:
GENERATION OF AGONIST AND ANTAGONIST VARIANTS

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Human interleukin-11 (hIL-11) is a pleiotropic cytokine administered to patients with low platelet counts. From a structural point of view, hIL-11 belongs to the long-helix cytokine superfamily, which is characterized by a conserved core motif consisting of four α-helices. We have investigated the region of hIL-11 that does not belong to the α-helical bundle motif, and that for the purpose of brevity we have termed “non-core region”. The primary sequence of the interleukin was altered at various locations within the non-core region by introducing glycosylation sites. Functional consequences of these modifications were examined in cell-based as well as biophysical assays. Overall, the data indicated that the non-core region modulates the function of hIL-11 in two ways. First, the majority of muteins displayed enhanced cell-stimulatory properties (super-agonist behavior) in a glycosylation-dependent manner, suggesting that the non-core region is biologically designed to limit the full potential of hIL-11. Second, specific modification of a predicted mini α-helix led to cytokine inactivation, demonstrating that this putative structural element belongs to site-III engaging a second copy of cell-receptor gp130. These findings have unveiled new and unexpected elements modulating the biological activity of hIL-11, which may be exploited to develop more versatile medications based on this important cytokine.

Human Interleukin-11 (hIL-11) is a secreted cytokine inducing a wide variety of biological effects in vivo (1-3). For example, hIL-11 is a potent hematopoietic growth factor (2), possesses anti-inflammatory activity against chronic inflammatory diseases (4), protects intestinal epithelial cells from tissue damage (5), and attenuates cardiac fibrosis after myocardial infarction (6). Moreover, hIL-11 is a medically relevant protein of clinical potential. In particular, recombinant hIL-11 (oprelvekin) has been authorized by FDA (Food and Drug Administration) to treat adult patients following myelosuppressive chemotherapy to prevent severe thrombocytopenia (7-9).

Interleukin-11 belongs to the superfamily of long helical cytokines. Invariably, this class of ligands display a four-helix bundle motif consisting of two pairs of anti-parallel α-helices connected by three loops of variable length (10-17). Signaling is initiated by cytokine binding to cell-surface receptors, leading to oligomeric complexes that subsequently induce activation of cytoplasmic factors of the STAT (signal transducers and activators of transcription) pathway, or MAPK (mitogen-activated protein kinase) cascade (13,18).

Specifically, binding of interleukin-11 to cell-surface receptors comprises three mechanistically separate events. First, secreted interleukin binds with low affinity to a membrane-anchored specific receptor hIL-11R (also described as α-chain receptor) (19,20). In the second step, the hIL-11•hIL-11R heterodimer engages with high-affinity to shared receptor gp130 forming a stable heterotrimer. In the third stage, the heterotrimer further homo-dimerizes yielding a hexameric complex that elicits the cell-stimulatory signals (Supplemental Figure 1) (21,22). This general mechanism is analogous to that of other gp-130 cytokines (reviewed in (12,13,23,24)).

Although hIL-11 was first described 20 years ago (1), no high-resolution structural data has been yet reported. The only available experimental model of hIL-11 corresponds to...
cryoelectron microscopy density maps at 30 Å resolution of the hexameric complex with cognate receptor hIL-11R and shared receptor gp130 (22). Importantly, this study indicates that the relative position of interleukin and receptors in the complex is roughly similar to that described in the high-resolution crystal structure of the hexameric complex of interleukin 6 (hIL-6) with receptors (17,25). Together with previous mutational studies, these data unveiled up to three epitopes within the “core” α-helical region that engage cell-surface receptors: Site I (to cognate α-receptor), Site II (to shared receptor gp130) and Site III (to a second receptor gp130) (26).

Several hypotheses have been proposed to explain how the relatively unsophisticated four-helix bundle motif can lead to such a vast array of specific and overlapping biological responses. For example, it has been suggested that pleiotropy and redundancy are regulated by characteristic cell-dependent distribution of cognate receptors, or that cytokines have the ability to signal through more than one receptor (reviewed in (27)). More recently, an elegant mechanism based on the novel concept of “thermodynamic plasticity” has been developed to explain cross-reactivity in shared receptor gp130 (28).

Herein, we have turned our attention to the region of hIL-11 that does not belong to the core α-helical motif, and that for the purpose of brevity we have termed “non-core region”. Our mutational analysis demonstrates that modifications in the non-core region can also modulate the biological function of hIL-11. The majority of muteins generated in this study showed enhanced signaling activity compared with wild type interleukin. In contrast, altering the sequence of a putative mini α-helix strategically located in the vicinity of site III can specifically inactivate the interleukin. Overall, our study has generated two interesting interleukin variants, a superagonist and a strong antagonist, that may expand the pharmacological repertoire of this medically relevant cytokine.

Experimental Procedures

Expression and Purification of hIL-11. Expression vector pSRt-hIL-11 containing the sequence of hIL-11 (SwissProt Accession Number P20809) or its variants was co-transfected together with vector pAdD26SVA#3 at a ratio 10:1 into dihydrofolate reductase deficient Chinese hamster ovary cells (CHOdhfr−) using Lipofectamine-2000 (Invitrogen, CA) (29). To select the clones of interest, transfectants were initially cultured for two weeks in α-MEM(-) medium (Gibco, CA) supplemented with 10% fetal calf serum (FCS). Methotrexate (MTX) (Sigma, MO) was added to CHOdhfr(+) cells cultured in α-MEM(-) medium to increase the yield of protein expression. The concentration of MTX was gradually increased from 0.01 to 1 μM. Cells resistant to 1 μM MTX were initially cultured in 75 cm² flasks and subsequently transferred to 250-ml spinner flasks (Iwaki, Japan) containing 0.3% Cytodex 3 microcarrier particles (GE Healthcare, NJ) (30). Cultured CHOdhfr(+) cells containing hIL-11 vector (or the desired variant) were grown in α-MEM medium supplemented with adenosine, deoxyadenosine, thymidine, and 10% FCS. Culture supernatants containing secreted protein were filtered to eliminate unwanted cell debris and subjected to a first chromatography step using a hydroxyapatite Bio-Gel column (Bio-Rad, CA). Protein fractions in the flow-through were collected and subjected to a second chromatographic step using a silica column. Bound protein was washed with 80 ml of each of the following solutions: 20 mM phosphate (pH = 6.8); 20 mM phosphate, 40% ethylene glycol (pH = 6.8); 50 mM acetate, 50% ethylene glycol (pH = 4.0); 15 mM HCl, 20% ethylene glycol. Finally the interleukin was eluted with a solution containing 15 mM HCl and 50% ethylene glycol. Eluted fractions containing interleukin-11 were neutralized with 1 ml of a solution containing 1 M Tris-HCl (pH = 8.0). Protein concentration was determined with an ELISA kit specific for hIL-11 (R&D Systems, MN), and spectrophotometrically at 280 nm.

Expression and purification of hIL-11R. Cognate receptor hIL-11R was expressed in a baculovirus expression system as described previously (31). The receptor was fused to an Fc fragment to improve its solubility without affecting binding of the interleukin (32,33). Supernatant containing the secreted receptor was diluted with Fab binding solution and loaded into a Protein A chromatography column following the manufacturer’s instructions (Thermo Fisher Scientific, MA). The column was washed with 10 ml of Fab binding solution to remove unwanted
protein contaminants. Receiver was recovered from the column with 10 ml of Fab elution solution. Fractions containing hIL-11R-Fc were subjected to an additional size-exclusion chromatographic step to remove aggregated material. Fractions containing the receptor were concentrated and stored at -80 °C.

**Cell proliferation assay.** T1165 cells from a murine plasmacytoma line were cultured in RPM1640 medium supplemented with 10% FCS and 10 ng/ml wild-type hIL-11 at 37 °C. Prior to each assay, cells were washed three times with medium containing no-interleukin, applied to 24 well plates at a concentration of 1×10⁶ cells per well, and cultured in RPM1640 medium supplemented with 10% FCS at 37 °C. Wild-type hIL-11 or its variants were added at a concentration of 0.001, 0.01, 0.1, 1, 10, or 100 ng ml⁻¹. Cell-proliferation was determined after 4 days of incubation using a Coulter particle counter (Beckman, CA).

**Surface Plasmon Resonance (SPR).** Binding of hIL-11 to cognate receptor hIL-11R was analyzed by SPR in a Biacore T-100 instrument (GE Healthcare, NJ) in an analogous manner to that described in Sakamoto et al. (34). Briefly, a CM5 Biacore chip decorated with hIL-11R was injected with solutions containing increasing amounts of wild-type hIL-11 or its variants. Association rate constants (kon) and dissociation rate constants (koff) were obtained by a global fitting procedure using the BIAevaluation software (GE Healthcare, NJ). The apparent equilibrium dissociation constant was calculated from the ratio of the kinetic rate constants:

\[
K_D = \frac{k_{off}}{k_{on}}
\]

**Differential Scanning Calorimetry (DSC).** Thermal unfolding of interleukin-11 was examined in a VP-DSC instrument (MicroCal, MA). Protein samples were heated from 10 to 120 °C at a rate of 1 °C min⁻¹. Thermograms were analyzed using the Origin software package supplied by the manufacturer of the calorimeter. Each thermogram was subtracted with the buffer baseline, and the resulting data normalized by protein concentration.

**Serum clearance experiment.** Wild-type hIL-11 and N₇-3N mutein were subjected to in vivo serum clearance experiment. A total of 20 mice were each injected intravenously with a solution containing 5 µg of interleukin. Mice were sacrificed at several time points, their blood collected, and the concentration of interleukin-11 in serum determined with a hIL-11 specific ELISA kit (R&D Systems, MN).

**Quantification of monosaccharides.** Neutral, basic and sialic-acid sugars covalently attached to interleukin variants were determined in an LC-9A HPLC instrument (Shimazu, Japan). In a typical assay, 50-µl samples containing glycoprotein were first evaporated, and subsequently hydrolyzed in acid solution at 100 °C for 6 hr, except those samples employed to determine sialic acid content where milder hydrolysis conditions were employed (80 °C for 1 hr). Following hydrolysis, sugar content was determined in an HPLC instrument using a TSK-gel AXG column (neutral sugars), a TSK-gel SCX column (basic sugars), or a Gelpack GL-C-620-10 column (sialic acid). Each column was calibrated with commercial samples of each of the monosaccharides analyzed. Protein content in the samples was determined with a hIL-11 specific ELISA kit (R&D Systems, MN).

**Mass Spectrometry.** Equal volumes of purified L₆₃₃₀-O at a concentration of 0.1 mg/ml and sinapinic acid cocktail were mixed, deposited on a MALDI plate, briefly dried, and the mass spectrum collected in a MALDI-TOF-MASS Voyager instrument (Shimadzu, Japan).

**Preparation of deglycosylated N₇-3N interleukin.** Variant N₇-3N was deglycosylated with N-glycosidase A enzyme (Roche, Germany) following the manufacture’s instructions. Briefly, the appropriate amount of N-glycosylated interleukin was mixed with 5 mU of N-glycosidase A, diluted with 150 µl PBS buffer (pH 5.0), and incubated for 24 hours at 37 °C. Proliferation of T1165 cells in the presence of glycosylated or deglycosylated N₇-3N mutein was monitored as described above.

**RESULTS**

**Homology Model of hIL-11.** The homology model of hIL-11 depicted in Figure 1 was obtained from the ModBase database (35). This model is based on the crystallographic coordinates of human granulocyte colony-stimulating factor (GCSF), which also belongs to the long helix cytokine superfamily (16). GCSF was selected over other suitable candidates such as hIL-6 because of a higher sequence identity to hIL-11 (24% and 19%, respectively; Figure 1C) and a
more complete coverage of the primary sequence in the final model. Only first five and last three residues of the mature hIL-11 protein are absent in the model depicted in Figure 1A. Overall, the quality of this model is very acceptable as demonstrated during the analysis of its geometry with the program MolProbity (36), which indicated that only 3% of residues fall outside the allowed regions in the Ramachandran plot (not shown). As a comparison, 4.9% of residues of hIL-6 in the crystal structure in complex with gp130 and α-receptor fall in non-allowed regions of the Ramachandran plot (PDB accession code 1P9M).

The homology model shown in Figure 1 exhibits the main signature element of this family of cytokines, i.e. a four-helix bundle arranged in a top-top/down-down topology (12,23). Residues not belonging to the core α-helical motif – that for the purpose of brevity we termed non-core region – are arranged in loops of variable length connecting the α-helical elements. Interestingly, the model predicts the presence of a mini α-helix comprising residues 48-51 of LoopA/B (Figure 1) that we have modified in the course of this study (see below).

**Cell-proliferation activity of wild-type hIL-11 and muteins.** To examine the role of non-core regions in the signaling activity of hIL-11, we prepared engineered variants of the interleukin containing N- or O-glycosylation consensus sequences at different locations of the non-core region: N-terminus; C-terminus; LoopA/B connecting helix A to helix B; LoopBC connecting helix B to helix C; and LoopCD connecting helix C to helix D (Figure 1; Supplemental Table 1). Thus each mutein contained between three and nine more residues than wild type-interleukin, and a carbohydrate moiety (see below). This mutational approach modifies significantly the primary structure of each targeted position, which may reveal specific roles of non-core elements in the activity of hIL-11.

Wild-type interleukin and muteins were expressed in CHO cells, purified to homogeneity and their cell-proliferation activity assessed in cultured T1165-cells. Figure 2 shows that wild type hIL-11 and all the muteins tested, except L_{B,O}, induced robust proliferation of T1165 cells at interleukin concentrations ranging from 0.1 to 100 ng ml^{-1}. Remarkably, most muteins exhibited higher cell-proliferation activities than wild-type interleukin, i.e. super-agonist behavior. For example, C_{T,O}, N_{T,O} and N_{T,3N} are ~10-fold more active than wild-type interleukin in a protein concentration basis. We could not observe significant differences between N- and O-glycosylated variants. The highest proliferation rates occurred in the presence of muteins carrying modifications at the terminal ends.

**Incorporation of glycan moieties.** N_{T,3N} and L_{A,B,O} variants, showing opposite effects in the proliferation activity assay above, were chosen to examine the extent of glycan incorporation in the muteins. Monosaccharide fragments obtained after hydrolysis of purified protein samples were quantified by HPLC (Table 1). The data indicated that both muteins, N_{T,3N} and L_{A,B,O}, contained several types of saccharides attached to them. In N_{T,3N} the relative molar ratios among the major sugars found fucose, GlcNAc, mannose, galactose, and Neu5Ac were 1:5:6:2:5:2:6:3:1. These values compared favorably with the composition of triantennary N-glycans 1:5:3:3:3 (Supplemental Figure 2) (37,38). Moreover, the molar ratio between N-glycan and N_{T,3N} interleukin determined in the assay was 0.9 (taking fucose as reference), which demonstrated that in average each molecule of interleukin had incorporated c.a. one N-glycan moiety.

In L_{A,B,O}, the molar ratios among the major sugars GalNAc, galactose and Neu5Ac were 1:0.9:1.3. These values contrasted with relative molar ratios of standard O-glycans (molar ratios = 1:1:2; Supplemental Figure 2) (37,38). In particular, the relative concentration of sialic acid Neu5Ac was lower than expected. We collected MALDI-TOF mass spectrum of L_{A,B,O} to examine the origin of these differences (Figure 3). The mass spectrum showed two major peaks appearing at m/z values of 20,840 Da and 20,546 Da that we assigned to interleukin modified with full size O-glycan (calculated mass = 20,799 Da), and O-glycan lacking one molecule of sialic acid (calculated mass = 20,508 Da), respectively. The presence of two forms of L_{A,B,O} differing in one molecule of sialic acid qualitatively explained the sugar ratios observed in the analytical HPLC assay. Moreover, the complete absence of m/z peaks around 19,850 Da (which corresponds to the theoretical mass of non-glycosylated interleukin) demonstrated that L_{A,B,O} variant was quantitatively glycosylated. In summary, these
data indicated that N- and O-glycans were incorporated in N T-3N and L A/B-O variants, respectively.

Binding to α-receptor. Surface Plasmon Resonance (SPR) was employed to evaluate the affinity of wild-type interleukin, variant N T-3N, and variant L A/B-O, for cognate α-receptor (hIL-11R). Samples containing the cytokine were injected into a sensor chip in which hIL-11R had been previously immobilized. Representative real-time response curves (sensorgrams) are shown in Figure 4. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were determined from the sensorgrams by applying a global-fitting regression assuming a stoichiometry of 1:1 (Table 2). Binding of wild-type hIL-11 to receptor is characterized by a rapid association phase, followed by an also fast dissociation phase. In contrast, binding kinetics of mutein N T-3N displayed slower association and dissociation steps, whereas the kinetic profile of L A/B-O was intermediate between those of wild-type and N-glycosylated interleukins. The dissociation constant (K_D) calculated from the kinetic constants conveys the overall affinity of the ligand for the receptor, where higher values of K_D denote lower affinity. K_D values determined for wild-type, N T-3N, and L A/B-O interleukins were 8.1, 0.91, and 0.76 µM, respectively (Table 2). Variant L A/B-O, which presented the lowest signaling activity in Figure 2, was nonetheless the variant showing the strongest affinity for hIL-11R.

We note that the fast binding kinetics of wild-type interleukin to α-receptor resulted in poorer fitting profiles than those of the muteins. To corroborate the robustness of the analysis above, the binding response in equilibrium was used to determine K_D (scatchard plot, supplementary Figure 3). The K_D value obtained by this alternative methodology (9.0 ± 1 µM) was essentially identical, within experimental error, to that estimated from Figure 4A (8.1 ± 0.5 µM).

Variant L A/B-O is a strong antagonist of wild-type hIL-11. SPR data above suggested that L A/B-O is an antagonist of wild-type hIL-11, i.e., competes with wild-type interleukin for α-receptor without eliciting cell proliferation (Figures 2 and 4). To test this hypothesis, T1165 cells were simultaneously incubated with a fixed amount of wild-type interleukin (100 ng ml⁻¹) and varying concentrations of L A/B-O (Figure 5A). In the absence of L A/B-O mutein, wild-type interleukin promoted robust cell proliferation comparable to that shown in Figure 2. However, addition of L A/B-O in the cell culture rapidly reduced cell-growth, even in the presence of a large excess of wild-type interleukin. When the concentration of L A/B-O was equal or higher than 10 ng ml⁻¹ we ceased to observe cell stimulation. Interestingly, the concentration ratio at which the glycosylated variant inactivates wild-type interleukin ([wild-type] / [L A/B-O] = 10) matches the relative affinities of the interleukins for α-receptor shown above (K_D(wild-type) / K_D(L A/B-O) = 10).

An additional experiment was performed to investigate the specificity of the modification in LoopA/B. Two new muteins engineered at the same positions than L A/B-O were prepared: the first one contained a N-glycosylation site (L A/B-N), whereas the second one displayed an octaglycine sequence not bearing glycosylation signal (L A/B-8G). Two key observations were made. First, these two new variants showed a reduced ability to induce cell proliferation, supporting the idea that the amino acidic sequence of this region is important for the proper activity of the interleukin (Figure 5B). Second, the use of variant L A/B-8G demonstrated that N- or O-glycans are not necessarily involved in the mechanism of inactivation.

Effect of modifications in the stability of interleukin. Differential scanning calorimetry (DSC) was employed to assess the thermal stability of the interleukins (Figure 6). On the one hand, wild type and N T-3N proteins showed a complex thermal unfolding behavior characterized by three transitions centered around 28, 62 and 76 °C (Table 3). The enthalpies of each transition were very similar, ranging between 53 and 68 kcal mol⁻¹. On the other hand, L A/B-O exhibited a rather different thermal profile compared with that of the other two proteins: First, L A/B-O showed a single and broad unfolding transition centered at 68 °C. Second, the transition enthalpy of L A/B-O was considerably higher than that of any single transition of the other two variants.

To determine whether the dissimilar thermal behavior between L A/B-O and wild-type interleukin (and N T-3N variant) was caused by major changes of secondary structure, we acquired their circular dichroism (CD) spectra in the far UV-region (200-250 nm) (Supplemental Figure 4). We could not observe significant differences

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among the spectra, indicating that the secondary structure of the cytokines remained relatively unchanged upon mutation. We should point out that CD data presented in Supplemental Figure 4 could not be used to estimate minor structural perturbations caused by the introduction of the glycosylation sites.

We further explored whether differences in stability (other than thermal stability) between wild-type interleukin and super-agonist NT-3N could explain the enhanced cell-proliferation activity of the glycosylated variant. Two types of experiments were conducted: In the first one, cell proliferation activity of freshly purified protein was compared to that of samples stored four months in stabilizing buffer at 26 °C (Figure 7A). Under both conditions, mutein NT-3N showed higher cell-growth stimulation than wild-type interleukin. More importantly, wild-type interleukin activity decayed faster than that of N1-3N. The EC50NT-3N/EC50 WT ratio, which indicates the relative proliferation strength of each protein, declined from a value of 0.2 when samples were prepared fresh, to about 0.03 after their long-term storage period. In other words, cell proliferation activity induced by wild-type interleukin decayed ~7-fold faster than that of mutein NT-3N.

In a second experiment, we monitored the in vivo clearance rate of wild-type and N1-3N interleukins in serum of mice injected with a fixed amount of interleukin (5 µg). Differences between wild-type hIL-11 and N1-3N were dramatic (Figure 7B). Five minutes after injection the concentration of wild-type hIL-11 in serum had dropped below 1 ng ml⁻¹, whereas that of NT-3N remained much higher (19 ng ml⁻¹). The levels of N1-3N decreased steadily to 2 ng ml⁻¹ during the first 40 min after the injection, and after that more slowly up to 75 minutes where this variant was no longer detected under our assay conditions.

The role of N-glycan moiety in the stimulatory properties of N1-3N was also investigated. Cell-proliferation activity of N1-3N treated with N-glycosidase A (deglycosylated) was compared with that of untreated samples. Figure 8 shows that both types of samples led to similar levels of cell growth for a wide range of concentrations, except at the highest concentrations of interleukin tested where the glycosylated interleukin N1-N3 displayed significantly higher activity. These data suggests a critical role (but not an exclusive one) of the N-glycan in the super-agonistic behavior of the muteins.

Overall, this set of experiments demonstrated that engineering a N-glycosylation site at the N-terminus of hIL-11 improved the cell stimulatory potential of the cytokine, and strengthened its long-term and in vivo stabilities. These enhanced properties occurred in a glycosylation dependent manner without disrupting the secondary structure of the protein or its thermal denaturation profile.

**DISCUSSION**

**Non-core region modulates hIL-11 signaling activity.** Several mutational studies had established that certain regions within the four-helix “core” motif of hIL-11 (termed site I, site II and site III) are essential for binding of the cytokine to cell-surface receptors (27,32,39-41) (Supplemental Figure 1). This “core” motif is well conserved among all helical cytokines, raising the interesting question of how this family of regulators can exert their specific stimulatory effect based on such a simple and widespread molecular design. This question is particularly relevant for those cytokines that share common cell-receptor gp130 (24,26,28). In this study we focused our efforts to the less structurally conserved region of hIL-11 – termed non-core region – to determine whether it could also play a significant role in the interleukin’s normal functioning.

A previous site-directed mutagenesis study had identified Met59 (located in LoopA/B of the non-core region) as a key residue for the normal activity of hIL-11 (41). Our strategy consisted in altering, more substantially, selected locations within the non-core region of hIL-11 by introducing between three and nine residues containing glycosylation sites. The initial screening revealed that LoopA/B (modified between His49 and Asn50) is critical for the stimulatory properties of hIL-11. In contrast, all other muteins exhibited comparable or even higher cell proliferation activities (up to 10-fold higher) than that of wild-type protein (Figure 2). This observation indicated that the non-core region is very versatile at adapting to modifications in its primary structure and also to the attachment of carbohydrates. Interestingly, cytokine GSGF...
(whose 3-D structure was used to build the hIL-11 model depicted in Figure 1) also tolerates large rearrangements of its primary sequence while keeping a biologically active conformation (42).

The high propensity (>50%) of hIL-11 muteins exhibiting super-agonist behavior suggests that biological evolution has not optimized this interleukin to achieve maximum cell stimulatory strength. Perhaps an “over-optimized” interleukin with, for example, much higher affinity for receptor hIL-11R (as observed in N7-3N variant) could limit the ability of other cytokines to signal through shared receptor gp130. An attractive hypothesis resulting from these observations is that the non-core region of hIL-11 has been biologically designed to restrain the full stimulatory potential of hIL-11.

Modification of a putative mini α-helix at LoopA/B generates a potent antagonist. L_{A/B}-O is a powerful antagonist of hIL-11 that exhibits very low cell-proliferation activity (Figures 2 and 5). In particular, Figure 5A demonstrates that a large excess of wild-type interleukin (~10-fold) is required to restore cell growth when L_{A/B}-O variant is also present in the medium.

Superposition of the three-dimensional model of hIL-11 onto the crystal structure of the hexameric complex of hIL-6 with receptors (17,22), showed that the putative mini α-helix modified in L_{A/B}-O is located in the immediate vicinity of the second copy of receptor gp130 (Supplemental Figure 1). We have demonstrated that this predicted element is important for the stimulatory properties of hIL-11. First, modification of the mini-helix led to glycan-independent inactivation (Figures 2 and 5B). Second, inactivation rates were sensitive to the number and size of residues incorporated in the mutein (Figure 5). And third, changes in the putative helix did not diminish the ability of L_{A/B}-O to bind to cognate receptor hIL-11R (Figure 4). Well on the contrary, the affinity of L_{A/B}-O for α-receptor was higher than that of wild-type interleukin, which explained why this variant displays antagonistic properties.

In addition, previous studies had shown that Trp147 (located ~12 Å from the mini-helix modified in our study) is a key component of site-III (27,32). Replacement of this key Trp residue with Ala (W147A mutein) generates a strong antagonist with little proliferation activity because of a defective binding to receptor gp130 (32). Together with these previous reports, our data supports the idea that the mini helical element modified in L_{A/B}-O forms part of an expanded site-III.

In summary, we have established that L_{A/B}-O is a new and strong antagonist of hIL-11, and thus a compound of potentially interesting pharmacological properties. This variant of hIL-11 (and other analogous variants with L_{A/B}-O-like properties) could be employed, for example, to ameliorate pathologies that involve endogenously hyper-active interleukin-11 such as in certain classes of inflammation (43), or in conditions where receptor hIL-11R is over-expressed such as in bone metastasis (44). However, further experimentation will be needed before these novel medications can be put into practice.

Generation of super-agonist variant N7-3N. N7-N3 is a super-agonist variant exhibiting ~10-fold higher cell-proliferation activity than wild-type interleukin (Figure 2). Our data revealed several overlapping mechanisms contributing to the enhanced activity of N7-3N. First, SPR binding data indicated that N7-N3 forms a tight complex with α-receptor (10-fold stronger) that is characterized by slower dissociation rates ($k_{off}$) than those observed in wild-type interleukin. In other words, N7-N3 remained attached to α-receptor for longer period of time than wild-type interleukin, which in the biological environment of the cell could increase the likelihood of forming functional complexes through subsequent binding of receptor gp130. Second, although the modifications introduced in N7-3N did not alter thermal stability or secondary structure of the mutein, inactivation rates upon long-term storage and serum clearance rates were greatly slowed down in this variant (Figure 7). And third, the presence of carbohydrate favored the biological activity as illustrated in Figure 8. Interleukin 11, contrary to other cytokines such as hIL-6 or GCSF (45,46), is not glycosylated in its native form (47). Dissimilar glycosylation patterns among interleukins could contribute to the fascinating diversity of signaling events elicited by this structurally homologous family of cytokines.

Overall, our data are consistent with previous studies showing that protein glycosylation may result in enhanced stability but not necessarily higher thermostability (37,48-50).
Indeed, a similar mechanism has been proposed for a PEGylated form of hIL-11, although the unspecific nature of this chemical modification resulted in a cytokine with diminished ability to bind to receptors (51). The specificity of our mutational scheme (as demonstrated in N1-3N) has overcome this limitation, leading to a modified interleukin with enhanced biological activity. In principle, the favorable properties of this new variant would seem suitable to replace the currently administered recombinant (non-glycosylated) hIL-11 in the treatment of severe thrombocytopenia (7-9).

Several alternative routes to produce hIL-11 super-agonists have been described in the literature, such as the design of soluble hIL-11•hIL-11R complexes (52), or the preparation of single-point mutations in the core α-helical region of the cytokine (40,53). Herein we have shown that modification of the non-core region of hIL-11 is a plausible route to generate more potent bioactive variants of this pharmacologically valuable cytokine.

Conclusions
In this study we have shown that modifications of the primary sequence within the non-core region may modulate the function of hIL-11 in two opposite directions depending on the nature of the mutation. First, in the majority of muteins tested the signaling activity of the interleukin was augmented in a relatively non-specific manner. And second, modification of a predicted mini helix in the vicinity of site-III disrupted the cytokine’s cell-stimulatory properties. The generation of these novel super-agonist and antagonist variants of hIL-11 opens new avenues for the development of more versatile medications of this clinically relevant cytokine.

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Footnotes
Abbreviations used are: hIL-11, human interleukin-11; hIL-11R, human interleukin receptor or α-receptor; GCSF, granulocyte colony-stimulating factor; hIL-6, human interleukin 6; CHO, chinese hamster ovary cell; CHOdhfr-, dihydrofolate reductase deficient chinese hamster ovary cell; MTX, methotrexate; FCS, fetal calf serum; SPR, surface plasmon resonance; DSC, differential scanning calorimetry; CD, circular dichroism.

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FIGURE LEGENDS
Figure 1. Modeled three-dimensional structure of hIL-11. (A) Cartoon representation, (B) topology model, and (C) sequence alignment. The α-helices are named in alphabetical order as they appear in the primary sequence of the interleukin: Helix-A (magenta), helix-B (yellow), helix-C (blue) and helix-D (green). Non-core regions are shown in blue, except for a predicted mini α-helix in Loop_{AB} that is highlighted in orange. Residues belonging to non-core regions (depicted in red) indicate the positions at which glycosylation sites were introduced. In panel B, the approximate location of these residues is indicated with red stars. The three-dimensional structure of hIL-11 was modeled from the crystal structure of GCSF in complex with its cognate receptor (16). Atomic coordinates were obtained from the ModBase and depicted without further modification (35). Panel C shows the sequence alignment of human interleukin-11, human granulocyte-colony stimulating factor, and human interleukin-6. The colored boxes indicate the location of α-helices. The predicted mini-helix in Loop_{AB} of hIL-11 (orange), and other small helical elements (gray) are also highlighted. Arrows indicate the positions at which modifications were introduced. Molecular graphics and sequence alignment figures were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (54).
Figure 2. Cell proliferation assay. T1165 cells derived from murine plasmacytoma line were incubated with wild-type interleukin (black), or the following variants: NT-O (light green), NT-2N (light gray), NT-3N (light pink), LA/B-O (purple), LB/C-O (light blue), LB/C-N (light brown), LC/D-O (orange), LC/D-N (blue), and CT-O (brown). Cell growth was monitored after four days. Data corresponds to the average of four assays.

Figure 3. MALDI-TOF spectrum of variant LA/B-O. The position of the two major m/z peaks are indicated by arrows. The putative structure of O-glycans attached to LA/B-O corresponding to each major m/z peak are shown in the panel. The symbols used for the sugar moieties are: GalNAc (open square), Galactose (open circle), and sialic acid Neu5Ac (filled diamond). The symbols used to represent the sugar moieties are based on the recommendations of Varki et al. (55).

Figure 4. Association kinetics of interleukin-11 to cognate receptor hIL-11R. SPR sensorgrams are shown as red traces. Black curves correspond to the global fitting of the sensorgrams with the BIAevaluation software package (GE Healthcare, NJ). An arrow pointing downwards indicates injection of running buffer containing (A) wild-type hIL-11, (B) NT-3N, or (C) LA/B-O variants. An arrow pointing upwards indicates injection of running buffer containing no interleukin. The response signal is proportional to the amount of interleukin that binds to a chip surface decorated with immobilized receptor.

Figure 5. Effect of modifications introduced in Loop A/B in cell proliferation. (A) Cell-proliferation of T1165 cells in the presence of both, wild-type hIL-11 at a fixed concentration of 100 ng ml⁻¹, and variable concentrations of LA/B-O. Data correspond to the average of four assays. (B) Cell proliferation assay of T1165 cells in the presence of wild-type hIL-11 (solid), variant LA/B-O (dashed and dotted), variant LA/B-N (dashed), or variant LA/B-8Gly (dotted). Data corresponds to the average of four assays.

Figure 6. Thermal unfolding of interleukin-11 monitored by differential scanning calorimetry (DSC). Samples of wild-type hIL-11 at 0.79 mg ml⁻¹ (solid line), NT-3N variant at 0.97 mg ml⁻¹ (dashed line), and LA/B-O variant at 0.43 mg ml⁻¹ (dashed and dotted line) in PBS buffer at pH = 7.0 were heated from 10 to 120 °C at a rate of 1 °C min⁻¹. Heat capacity values (Cp) were obtaining after subtraction of the buffer baseline and normalized by protein concentration using the software package Origin.

Figure 7. Stability and serum clearance of wild-type and NT-3N variant. (A) Cell proliferation induced by NT-3N variant or wild-type hIL-11 were compared when fresh samples (black) or samples stored for four-months at 26 °C (light gray) were employed. EC₅₀ indicates the protein concentration at which the interleukin induces 50% maximum proliferative effect. Bars correspond to the average of two measurements. (B) Time course of interleukin clearance from mouse serum after intravenous injection of 5 µg of either wild-type (solid line) or NT-3N variant (dashed line). Protein concentration was determined with an hIL-11 specific ELISA assay kit.

Figure 8. Treatment of NT-3N with N-glycosidase A limits cell stimulation. Solid squares / solid line correspond to untreated NT-3N protein, and empty squares / dotted line correspond to NT-3N treated with N-Glycosidase A. Cell growth was monitored after four days. Data corresponds to the average of four assays.
Table 1

Table 1. Sugar content of hIL-11 variants.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>N\textsubscript{T}-3N\textsuperscript{b}</th>
<th>L\textsubscript{A/B}-O\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sugar Molar Ratio \textit{(Fucose = 1)}</td>
<td>Sugar / Protein Molar Ratio</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.6</td>
<td>2.3</td>
</tr>
<tr>
<td>GlcNAc\textsuperscript{c}</td>
<td>5.6</td>
<td>4.9</td>
</tr>
<tr>
<td>GalNAc\textsuperscript{d}</td>
<td>N.A.\textsuperscript{e}</td>
<td>N.A.</td>
</tr>
<tr>
<td>Neu5Ac\textsuperscript{f}</td>
<td>3.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} N.A. indicates that the value is not applicable.
\textsuperscript{b} Concentration of monosaccharides Rhamnose, Ribose, Xylose, Glucose, N-Acetylmannose, and N-Glycolylneuraminic acid was below 5% that of other sugars, or not detected in the assay.
\textsuperscript{c} GlcNAc is an abbreviation for N-Acetylglucosamine.
\textsuperscript{d} GalNAc is an abbreviation for N-Acetylgalactosamine.
\textsuperscript{e} Although GalNAc is not incorporated in standard N-glycans, we found minor quantities of this sugar during analysis of N\textsubscript{T}-3N (GalNAc to GlcNAc ratio = 0.15).
\textsuperscript{f} Neu5Ac is an abbreviation for N-Acetylneuraminic acid.
Table 2

Table 2: Kinetic parameters of the association of interleukin-11 to receptor hIL-11R.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$) x 10$^{-3}$</th>
<th>$k_{off}$ (s$^{-1}$) x 10$^{3}$</th>
<th>$K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>4.7 ± 0.1</td>
<td>38 ± 0.5</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>NT-3N</td>
<td>1.1 ± 0.05</td>
<td>1.0 ± 0.01</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>LA/B-O</td>
<td>5.3 ± 0.05</td>
<td>4.0 ± 0.03</td>
<td>0.76 ± 0.06</td>
</tr>
</tbody>
</table>
Table 3: Thermal Stability of wild-type interleukin-11 and muteins determined by DSC.\(^a\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>(T_{m1}) (°C)</th>
<th>(T_{m2}) (°C)</th>
<th>(T_{m3}) (°C)</th>
<th>(\Delta H_1) (kcal mol(^{-1}))</th>
<th>(\Delta H_2) (kcal mol(^{-1}))</th>
<th>(\Delta H_3) (kcal mol(^{-1}))</th>
<th>(\Delta H_{\text{Total}}) (kcal mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>27.5 ± 0.05</td>
<td>61.5 ± 0.1</td>
<td>76.3 ± 0.1</td>
<td>68.0 ± 0.3</td>
<td>53.6 ± 0.4</td>
<td>58.0 ± 0.4</td>
<td>179 ± 1</td>
</tr>
<tr>
<td>N(_T)-3N</td>
<td>28.7 ± 0.05</td>
<td>63.6 ± 0.1</td>
<td>75.5 ± 0.1</td>
<td>64.8 ± 0.3</td>
<td>53.0 ± 0.4</td>
<td>65.5 ± 0.04</td>
<td>183 ± 1</td>
</tr>
<tr>
<td>L(_A/B)-O</td>
<td>68 ± 0.5</td>
<td>N.A.</td>
<td>N.A.</td>
<td>245 ± 2</td>
<td>N.A.</td>
<td>N.A.</td>
<td>245 ± 2</td>
</tr>
</tbody>
</table>

\(^a\) \(T_m\) denotes the transition mid-point temperature, and \(\Delta H\) the enthalpy of the transition.
Figure 1 (page 1 of 2)

(A) (B)

Yanaka et al. Non-core region modulates IL-11.
Figure 1 (page 2 of 2)

Non-core region modulates IL-11.
Figure 2

Yanaka et al. Non-core region modulates IL-11.
Figure 3

Intensity (%) vs. m/z × 10^{-3}

- 20,840 Da
- 20,546 Da
- L_{A/B-O}
- L_{A/B-O}

Yanaka et al. Non-core region modulates IL-11.
Figure 4

Non-core region modulates IL-11.

Yanaka et al.
Yanaka et al.

Non-core region modulates IL-11.

Figure 5

(A)

(B)

Cell Count (x 10^4)

Cell Count (x 10^4)

L_{A/B-O} (ng ml^{-1})

Interleukin (ng ml^{-1})
Figure 6

Yanaka et al.  
Non-core region modulates IL-11.
Figure 7

(A) EC_{50} (ng ml^{-1})

Time (min)

N_{T}-3N  WT

(B) Interleukin (ng ml^{-1})

WT  N_{T}-3N

Interleukin modulates IL-11.
Yanaka et al.  Non-core region modulates IL-11.

Figure 8

Cell count (x 10^-4)

Interleukin (ng ml^-1)

- Not treated
- Deglycosylated
Non-core region modulates interleukin-11 signaling activity: Generation of agonist and antagonist variants
Saeko Yanaka, Emiko Sano, Norio Naruse, Kin-ichiro Miura, Mutsumi Futatsumori-Sugai, Jose M. M. Caaveiro and Kouhei Tsumoto

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