Ligand/Receptor Signaling Threshold (LIST) Model Accounts for gp130-Mediated Embryonic Stem Cell Self-Renewal Responses to LIF and HIL-6

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ABSTRACT

We previously demonstrated that embryonic stem (ES) cell self-renewal required sustained signaling by leukemia inhibitory factor (LIF) in a concentration-dependent manner, allowing us to hypothesize that thresholds in ligand-receptor signaling modulate stem cell differentiation control. To test this hypothesis, we have experimentally and computationally compared the abilities of two gp130-signaling cytokines (LIF and Hyper-interleukin-6 [HIL-6]) to sustain ES cell self-renewal. Quantitative measurements of ES cell phenotypic markers (stage-specific embryonic antigen-1 and E-cadherin), functional assays (alkaline phosphatase activity and embryoid body formation efficiency), and transcription factor (Oct-4) expression over a range of LIF and HIL-6 concentrations demonstrated a superior ability of LIF to maintain ES cell pluripotentiality at higher concentrations (≥500 pM). Additionally, we observed distinct qualitative differences in the ES cell self-renewal dose response profiles between the two cytokines. A computational model permitted calculation of the number of signaling complexes as a function of receptor expression, ligand concentration, and ligand/receptor-binding properties, generating predictions for the degree of self-renewal as a function of cytokine concentration by comparison of these calculated complex numbers to experimentally determined threshold cytokine concentrations. Model predictions, consistent with experimental data, indicated that differences in the potencies of these two cytokines were based primarily on differences in receptor-binding stoichiometries and properties. These results support a ligand/receptor signaling threshold model of ES cell fate modulation through appropriate types and levels of cytokine stimulation. Insights from these results may be more generally applicable to tissue-specific stem cells and could aid in the development of stem cell-based technologies. Stem Cells 2002;20:119-138

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INTRODUCTION

Human embryonic stem (ES) cells [1-4] have the potential to serve as renewable sources of functional tissue-specific cells in clinical tissue-replacement therapies. A prerequisite for the use of these and other stem cells in a clinical context is a thorough understanding of the mechanisms that regulate their decision to either self-renew and remain in the stem cell compartment or differentiate and exit the stem cell compartment. Recent evidence from hematopoietic stem cells (HSC) [5-8] and murine ES cells [9-11] suggests that stem cell fate may be amenable to exogenous control by appropriate types and levels of ligand-mediated receptor stimulation.

We recently proposed a ligand/receptor signaling threshold (LIST) model for stem cell fate determination where parametric values of the appropriate cell-surface signaling cytokine-receptor complexes determined whether a gene program associated with a particular cell fate was activated or not [12]. According to this conceptual model, a threshold number of cell-surface cytokine-receptor complexes determines whether or not a stem cell undergoes a self-renewal or differentiation response. Although this model is consistent with a large body of data, its applicability to stem cell differentiation control has not been rigorously examined.

Murine ES cells [13, 14] respond to cytokines from the interleukin-6 (IL-6) family, which maintain them in an undifferentiated state by signaling through the gp130 signal-transducing receptor component (a molecule that is also important in regulating other systems such as HSC [5, 8, 15-17] and primordial germ cells [18-21]). Members of the IL-6 family of cytokines, including leukemia inhibitory factor (LIF) [9, 10], IL-6 in combination with membrane-bound IL-6 receptor 2 (CT-1) [23], ciliary neurotrophic factor (CNTF) [24] and oncostatin M (OSM) [25, 26], can all maintain ES cells in an undifferentiated state for prolonged periods in vitro, albeit their efficacy varies [23, 25, 26]. Members of the IL-6 family of cytokines signal via multimeric receptor complexes usually comprised of a ligand-specific α-receptor subunit (IL-6R, IL-11R, and CNTFR) and gp130, the common signal-transducing subunit [27, 28]. IL-6 induces gp130 homodimerization [29], whereas CNTF [30] and LIF [30-32] signal via heterodimers of gp130 and LIF receptors (LIFR). Membrane-bound α-receptors can be functionally replaced by soluble forms that lack the cytoplasmic domain, but may still function agonistically, as in the case of sIL-6R [27, 33, 34].

We had previously developed quantitative phenotypic (stage-specific embryonic antigen-1 [SSEA-1] [35, 36]) and functional (alkaline phosphatase [ALP] activity [37-39] and the capacity of ES cells to form complex cell aggregates called embryoid bodies (EBs) [40-43]) assays to identify ES cells and their immediately differentiated progeny [11]. These assays, along with measurements of E-cadherin (a cell adhesion molecule important in early development [44, 45]) and Oct-4 (a transcription factor critical for maintenance of ES cell pluripotentiality [46, 47]) expression described herein, provide the quantitative tools necessary to conduct mechanistic investigations of factors that govern early ES cell fate decisions.

We have taken advantage of differences in the signaling mechanisms of the two IL-6-type cytokines, LIF and Hyper-IL-6 (HIL-6), to examine the validity of our LIST model in regulating stem cell fate. HIL-6, a fusion protein formed by covalently linking human IL-6 to a truncated human sIL-6R by a flexible glycine linker [48], is active at 100-1,000-fold lower concentrations than unlinked IL-6 and IL-6R on responsive cells because it has increased stability and may be more resistant to internalization [5, 48-50]. Our results suggest that cell-surface interactions between signaling cytokines and their corresponding receptors provide a mechanistic explanation of the dose dependency of ES cell self-renewal responses to exogenous cytokine stimuli. Differences in the abilities of LIF and HIL-6 to sustain ES cell pluripotentiality can be attributed to the distinct stoichiometries involved in forming functional cell-surface signaling ligand-receptor complexes. Our LIST model explains the ability of LIF to maintain ES cells in an undifferentiated state over a relatively wide range of concentrations, while HIL-6 is able to act equivalently over a relatively narrow range of doses, a result also recently shown to be true for HSC [5]. Our results also confirm that the actions of these cytokines cannot be attributed to differential mitogenic requirements of these cells [11], and that these cytokines may be acting, at least in part, by modulating the differential survival of particular subset(s) of cells. Our ability to correlate differences in cell-specific and measurable parameters (for example, receptor occupancy) to ES cell responses has provided us a mechanistic insight into the regulation of stem cell fate, a critical first step in being able to exploit these cells for clinical gains.

MATERIALS AND METHODS

Cells and Cytokines

Murine ES cells (CCE) [42] were maintained at 37°C in humidified air with 5% CO2 as previously described [11]. All cells were used within 15 passages of the initial thawing. HIL-6 [48] was a generous gift from Stefan Rose-John (Christian-Albrechts-Universität; Kiel, Germany; http://www.
HIL-6 concentration was independently established by a human IL-6 enzyme-linked immunosorbent assay kit (CLB; Amsterdam, The Netherlands: http://www.clb.de). STO embryonic fibroblast cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen Life Technologies; Rockville, MD; http://www.lifetech.com), 10% cosmic calf serum (HyClone; Logan, UT; http://www.hyclone.com), and 2 mM L-glutamine (Life Technologies).

Analysis of ES Cell Viability and Proliferation

Single cell suspensions of ES cells were initiated in triplicate in three gelatin-coated 24-well tissue culture plates at 7,500 cells/well and supplemented with indicated LIF and HIL-6 concentrations. On day 1, adherent cells were washed in phosphate-buffered saline (PBS), lysed in 0.1% Triton 100x, and incubated with 0.5 µM of SYTOX® Green (Molecular Probes; Eugene, OR; http://www.probes.com) for 10 minutes at room temperature. SYTOX® Green exhibited strong fluorescent enhancement when bound to cellular nucleic acid, a standard curve related cell number to fluorescent intensity (data not shown). Fluorescence emission was measured on a SPECTRAmax® spectrofluorometer (490 nm excitation, 524 nm emission with a cut-off filter at 515 nm) with SOFTmax® PRO software. A blank well containing all reagents except the cells served as a background control. Cell numbers were measured on days 2 and 3 in a similar manner (Fig. 1A). ES cells were also cultured in 75 cm² flasks for 5 days at indicated doses of LIF and HIL-6 and then replated in 24-well tissue culture plates (10,000 cells/well) at the corresponding ligand concentrations on day 5 in order to measure adherent cell numbers on days 6-8 as before (Fig. 1B).

Flow Cytometric Analysis of SSEA-1, E-Cadherin, and Intracellular Oct-4 Expression

ES cells were seeded at 2 × 10⁶ cells/25 cm² flask or at 6 × 10⁶ cells/75 cm² flask at indicated LIF and HIL-6 concentrations and subcultured every other day for 7 days using 0.25% trypsin-EDTA (Sigma; St. Louis, MO; http://www.sigmaaldrich.com) to generate single-cell suspensions. Cultures were reinited on alternate days with 1 × 10⁶ cells/25 cm² flask or 3 × 10⁶ cells/75 cm² flask at different doses of LIF and HIL-6 to control for cell density (Fig. 1C). An aliquot of 1 × 10⁶ cells was washed in ice cold Hanks’ HEPES-buffered saline solution (Life Technologies) containing 2% fetal calf serum (FCS) and incubated with α-mouse CD16/CD32 receptor monoclonal antibody at 1 µg/100 µl.

Figure 1. Experimental protocol. Single ES cell suspensions were either (A) directly seeded in 24-well gelatin-coated plates and cell numbers were determined consecutively on d 1-3 or (B) cultured in 75 cm² dishes for d 1-5 and then seeded in 24-well gelatin-coated plates on d 5 and analyzed subsequently on d 6-8. (C) Cells were also seeded at equivalent densities on d 1, 3, 5, and 7 in 25 cm² or 75 cm² dishes at different doses of LIF and HIL-6 and harvested to assess differentiation status.
with 100 µl of IntraPrep™ Permeabilization Reagent 1 (ImmunoTech; Westbrook, ME) for 15 minutes at room temperature, washed 1× with HF, permeabilized with IntraPrep™ Permeabilization Reagent 2 and incubated with a 1:100 dilution mouse α-mouse Oct3/4 monoclonal antibody (Transduction Laboratories; Lexington, KY) for 15 minutes at room temperature. Cells were washed 2× with 1 ml of HF, followed by staining with 1:100 dilution of fluorescein isothiocyanate-conjugated (FITC-conjugated) goat anti-mouse IgG antibody (Sigma). Finally, the cells were washed 2× with HF, and resuspended in HF for analysis on a flow cytometer (XL; Beckman-Coulter; Miami, FL; http://www.coulter.com) using the ExpoADCXL 4 software (Beckman-Coulter). Cells analyzed for SSEA-1 and E-cadherin were incubated first with a 1:10 dilution of monoclonal anti-SSEA-1 (Developmental Studies Hybridoma Bank; Iowa City, IA; http://www.uiowa.edu/~dshbwww) for 40 minutes at 4°C, washed 2× with ice-cold HF and incubated with a 1:500 dilution of phycoerythrin-conjugated goat anti-rat IgG antibody (Sigma). Stained cells were washed 2× with HF, stained with 1 mg/ml 7-AAD (Molecular Probes) and resuspended in HF for analysis on a flow cytometer. Positive staining was defined as the emission of a level of fluorescence that exceeded levels obtained by >99.5% of cells from the control population (cultured at 500 cells (ES nsc)) was not possible to analyze SSEA-1 and Oct-4 expression simultaneously as SSEA-1 expression was reduced when ES cells were permeabilized to stain for intracellular Oct-4. Oct-4 protein expression of the two fractions was also independently analyzed by Western analysis.

**Functional Assays**

The EB formation capacity assay and the ALP activity assay were performed as previously described [11]. We had previously demonstrated that EB formation capacity of ES cells correlated with other differentiation assays including SSEA-1 expression and ALP activity [11].

**Oct-4 Protein Detection**

ES cells were cultured as described above. Aliquots of 2 × 10⁶ cells were washed 3× in PBS, followed by lysis in Solution A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) plus protease (aprotinin and leupeptin [BioShop; Toronto, ON; http://www.bioshopcanada.com]) and phosphatase inhibitors (sodium fluoride, sodium orthovanadate, sodium pyrophosphate [Sigma]) for 10 minutes at 4°C. Cytoplasmic proteins were removed after centrifugation at 13,000 g for 10 seconds. Nuclear proteins were eluted by resuspending in Solution B (20 mM HEPES, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, with protease and phosphatase inhibitors) for 30 minutes at 4°C, followed by centrifugation at 13,000 g for 30 seconds. Quantification of protein levels was performed using Plus Protein Assay Reagent (Pierce; Rockford, IL; http://www.piercenet.com). Equal amounts of protein (28 µg) were separated on SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. Determination of Oct-3/4 levels was performed by incubation of membranes with mouse anti-mouse Oct-3 antibody (Transduction Laboratories in buffer containing 5% milk in tris buffered saline solution containing 0.1% Tween 20 (TBST), followed by incubation with goat anti-mouse IgG-HRP (Sigma). Proteins were visualized by enhanced chemiluminescence (ECL) using a Fluorchem™ Imaging System (Alpha Innotech; San Leandro, CA; http://www.alphainnotech.com).

**Statistical Analysis**

All data are reported as mean ± standard deviation. Experiments measuring changes in ES cell marker expressions...
were repeated two to five times, and statistical significance was assessed using paired Student’s t-test. Experiments measuring ES cell survival and proliferation were conducted over five times; a representative experiment using SYTOX® Green is shown.

**RESULTS**

**ES Cell Survival and Proliferation**

There were no significant differences in ES cell growth rates when cultured at different concentrations of LIF and HIL-6 over a period of 3 days (Fig. 2A). Survival efficiencies (percentage of cells relative to input) were comparable at all doses of LIF and HIL-6 after 1 day in culture, when most cells were predominantly undifferentiated (cells had high SSEA-1 and E-cadherin expression, data not shown) (Fig. 2B). Cell density limitations prevented us from extending this assay to 7 days when downregulation of ES cell markers is known to occur and cells could not be subcultured as we were interested in measuring growth rates. To circumvent this problem, we cultured ES cells at different doses of LIF and HIL-6 in 75 cm² tissue-culture flasks for 5 days and replated cells at low densities in 24-well plates (Fig. 1B) to measure their growth and survival from day 6 to 8. Even after 6 to 8 days of differentiation, growth rates at different ligand concentrations were not statistically different (Fig. 2C), however, there were significant survival differences ($p < 0.05$) between replated cells exposed to low ($\leq 5$ pM) and high ($\geq 500$ pM) ligand concentrations (Fig. 2D). The first day

**Figure 2.** ES cell proliferation and survival at different LIF and HIL-6 concentrations. Doubling times (h) of ES cells at indicated doses of (■) LIF and (□) HIL-6 for (A) 1-3 days and (C) 5-8 days. No significant differences in ES cell growth rates at any ligand concentration were observed ($p > 0.05$). Percent surviving cells relative to input, 24 hours after replating was measured after 1 day (B) and after 5 days in bulk culture (D). Significant differences between higher ($\geq 500$ pM) and lower ($\leq 5$ pM) doses of LIF and HIL-6 were observed ($p < 0.05$) after 5 days in bulk cultures. Data are expressed as mean of triplicate measurements ± standard deviation.
after replating, only 13% of the cells survived in the absence of LIF or HIL-6, whereas survival rates were up to 65% and 40% at 500 pM LIF and HIL-6 respectively ($p < 0.05$). Cell survival efficiencies were greater in the presence of higher LIF concentrations ($\geq 50$ pM) than at equivalent HIL-6 concentrations ($p < 0.05$) suggesting that LIF was more potent than HIL-6 in favoring survival of replated cells.

**ES Cell Differentiation**

There were dramatic changes in ES cell morphology at different doses of LIF and HIL-6 over a 7-day period (Fig. 3) when subcultured on alternate days (to ensure that seeding cell density numbers were equivalent at different ligand concentrations [Fig. 1C]). Undifferentiated ES cells formed homogeneous compact, spherical colonies, while differentiated cells were flattened and expressed a heterogeneous morphology. Differentiation in response to different concentrations of LIF and HIL-6 was quantitatively measured by several, independent assays of ES cell differentiation and, as expected, undifferentiated ES cells maintained above 50 pM LIF concentrations had significantly higher SSEA-1 and E-cadherin expression, ALP activity, and EB

![Figure 3. Morphology of ES cells over 7 days in culture at indicated concentrations of LIF and HIL-6.](image-url)
formation capacity than those maintained at lower LIF concentrations (≤5 pM) \( (p < 0.05) \) (Figs. 4A-4D). Surprisingly, ES cells did not show a similar monophasic dose dependency on different concentrations of HIL-6 (Figs. 4A-4D). ES cells maintained at 50 pM HIL-6 had significantly higher ALP activity and EB formation capacity than those maintained in

![Figure 4. Analysis of ES cell differentiation.](image)

**Figure 4. Analysis of ES cell differentiation.** (A) Dot plots of ES cells stained for α-SSEA-1-PE and α-E-cadherin-FITC after 7 days of culture at: (1) 500 pM of LIF stained with only the secondary antibodies (negative control); (2) in the absence of any ligand; (3) at 50 pM of HIL-6; (4) at 500 pM of HIL-6; (5) at 50 pM of LIF; and (6) at 500 pM of LIF. The percentages in the upper-right quadrant indicate percentage of cells doubly positive for SSEA-1 and E-cadherin. (B) Percentage of cells doubly positive for both SSEA-1 fluorescence-PE and E-cadherin fluorescence-FITC after 7 days in culture at indicated doses of [□] LIF and [■] HIL-6. Data are expressed as mean of three independent experiments ± SD. (C) Mean ALP activity normalized for cell numbers after 7 days in culture at indicated doses of [□] LIF and [■] HIL-6. Data are expressed as mean of two independent experiments ± SD. (D) Mean percent EB formation after 7 days in bulk cultures at indicated doses of [□] LIF and [■] HIL-6. Data are expressed as the mean of three independent experiments ± standard deviation.
the absence of any ligand or in the presence of 500 pM of HIL-6 \((p < 0.10)\). Further, there were no significant differences \((p > 0.10)\) in the SSEA-1 and E-cadherin expression, ALP activity, and EB formation capacity between cells maintained in the presence of 500 pM HIL-6 and in the absence of any ligand, suggesting that HIL-6 exhibited a relatively narrow range of concentration at which it was most efficient at maintaining ES cell pluripotentiality. There were, however, significant differences between the abilities of LIF and HIL-6, at higher ligand concentrations \((50-500\ pM)\), to maintain ES cell pluripotentiality \((p < 0.10)\) with LIF being more potent than HIL-6 (Figs. 4A-4D). Taken together, these results suggested that ES cell self-renewal varied in a dose-dependent manner for both LIF and HIL-6, a monophasic dose dependency for LIF and a bell-shaped, biphasic dose dependency for HIL-6 (though due to variability in the assays it was not possible to definitively conclude this) with LIF being more potent than HIL-6 at higher concentrations \((\sim 500\ pM)\). It was noteworthy that there was a substantial background level of self-renewal obtained in the absence of exogenous cytokine supplementation indicative of either the selective survival of cells with a particular phenotype (i.e., SSEA-1 and E-cadherin positive) or perhaps, of endogenous production of cytokines capable of signaling through gp130, as we \([11]\) and others \([51]\) have previously shown. To minimize the effects of autocrine LIF production (which has been shown to be lower than the levels required for ES cell self-renewal \([52]\)), cell density was carefully monitored and cultures were reinitiated on alternate days.

To confirm and extend the above analysis, ES cell differentiation was also measured through the analysis of Oct-4 protein expression at different doses of LIF and HIL-6 over a 7-day period. To verify that flow cytometric measurements of intranuclear Oct-4 protein expression correlated with other established ES cell markers (for example, SSEA-1 and Western analysis of Oct-4), we conducted an SSEA-1 selection experiment using petri dishes coated with an anti-SSEA-1 antibody. Fluorescent-activated sorting, based on differences in mean SSEA-1 fluorescence, was initially explored as the primary means of correlating Oct-4 intranuclear expression with SSEA-1 expression, however, poor ES cell viability after cell sorting prevented us from forming reliable conclusions. Using an alternative approach, ES cells or a control population of embryonic fibroblasts (STO cells, which do not express SSEA-1 or Oct-4) were incubated in the anti-SSEA-1 antibody-coated plates and the adherent (i.e., SSEA-1\(^{hi}\)) and nonadherent (i.e., SSEA-1\(^{lo}\)) fractions were separated as described for further analysis. Both the adherent (data not shown) and nonadherent fractions (\(\sim 75\%\) of the population) of STO cells were negative for both SSEA-1 and Oct-4 (Figs. 5A, 5B). As expected, the adherent ES cell fractions (\(\sim 52\%\) of the total population) had higher levels of SSEA-1 expression (65.9\% of ES cells were SSEA-1\(^{hi}\)) than the nonadherent fractions (\(\sim 20\%\) of the population), which had lower levels of SSEA-1 expression (23.5\% were SSEA-1\(^{lo}\)) (Fig. 5A). In fact, after the selection, the SSEA-1\(^{hi}\) population was almost completely removed from the nonadherent populations (Fig. 5A). Importantly, the ES adherent fractions also had higher levels of Oct-4 expression (72.2\% of ES cells were Oct-4\(^{hi}\) relative to STO cells) than the nonadherent fractions (22.9\% were Oct-4\(^{lo}\)) (Fig. 5B). A separate experiment confirmed that the adherent cells had higher Oct-4 protein expression by Western

![Figure 5. Correlation of Oct-4 protein expression (flow cytometry) with SSEA-1 and Oct-4 protein expression (Western blot). Dot plots of ES cells stained for: (A) α-SSEA-1-PE and (B) α-Oct-4-FITC in parallel: (1) before panning, (2) the adherent fractions after panning, and (3) the nonadherent fractions after panning. STO cells stained for: (A) SSEA-1 and (B) Oct-4: (4) before panning, and (5) the nonadherent fractions after panning. (C) Western analysis of Oct-4 protein expression of the: (1) adherent, and (2) nonadherent ES fractions after panning from an independent experiment.](https://example.com)
analysis than the nonadherent fraction (Fig. 5C). This suggested that flow cytometric measurements of intracellular Oct-4 protein levels correlated with other established assays of ES cell differentiation status.

Using this novel, flow cytometry-based assay, our results showed that nuclear Oct-4 protein expression was downregulated after 7 days in culture in a dose-dependent manner for both LIF and HIL-6 (Figs. 6A-6C). Cells cultured with LIF

Figure 6. Analysis of ES cell differentiation by Oct-4 protein expression. (A) Dot plots of ES cells stained for α-Oct-4-FITC (intracellular) after culture for 7 days at: (1) 500 pM of LIF stained with only the secondary antibody (negative control); (2) in the absence of any ligand; (3) at 500 pM of LIF, and (4) at 500 pM of HIL-6. The percentages in the lower-left quadrant indicate the percentage of cells negative for Oct-4. (B) Percentage of cells negative for Oct-4 fluorescence-FITC (relative to the negative control) after 7 days in culture at indicated concentrations of (■) LIF and (□) HIL-6 shown on a reversed axis. Data are expressed as a mean of two independent experiments ±SD. (C) A representative Western analysis of intranuclear Oct-4 protein expression on day 1 [(1), (3)] and day 7 [(2), (4)] for LIF [(1), (2)] and HIL-6 [(3), (4)] at indicated doses: (1) no ligand; (2) 5 pM; (3) 50 pM; and (4) 500 pM. (D) A representative plot of the kinetics Oct-4 expression (percentage of cells negative for Oct-4 expression) at indicated doses of: (1) LIF and (2) HIL-6: (■) no ligand; (□) 5 pM; (●) 50 pM, and (○) 500 pM.
had increased Oct-4 protein expression (as analyzed both by Western blots and flow cytometry) at higher concentrations (50-500 pM) than at lower concentrations (0-5 pM) \( (p < 0.05) \). As before, at 500 pM, LIF was more potent than HIL-6 at sustaining Oct-4 protein levels after 7 days in culture \( (p < 0.05) \). Oct-4 expression exhibited a moderately biphasic dose dependency on HIL-6 concentrations, although, in this series of experiments, no significant differences between cells cultured at 50 pM and 500 pM of HIL-6 \( (p > 0.10) \) were observed.

Our experiments have repeatedly shown, albeit to different extents, that the maintenance of high levels of SSEA-1, E-cadherin, and Oct-4 expression, the capacity to form EBs, and high ALP activity (all associated with undifferentiated ES cells) were more efficient when cultures were supplemented with high doses of LIF than with equivalent doses of HIL-6 (Figs. 4 and 6) \( (p < 0.10) \), and this was not due to differential depletion of ligands, as experiments were conducted with frequent medium exchanges. Additionally, while there were significant differences on day 7 between 50 and 500 pM HIL-6 in supporting EB formation efficiency and ALP activity \( (p < 0.10) \) suggestive of a biphasic dose dependency, smaller, nonsignificant differences were observed between 50 and 500 pM HIL-6-dependent SSEA-1/E-cadherin \( (p > 0.10) \) and Oct-4 expression \( (p > 0.10) \). These differences may have been masked by differential kinetics exhibited by ES cells in the loss of SSEA-1, E-cadherin (data not shown), and Oct-4 expression (Fig. 6D) when exposed to a range of HIL-6 and LIF doses. Due to variability within and between assays, it was difficult to definitively conclude that ES cell self-renewal responses had a biphasic dose dependency in response to HIL-6 concentrations. This led us to examine whether further insight into the mechanisms by which HIL-6 and LIF were acting could not be better elucidated using a computational modeling approach.

**Ligand/Receptor Signaling Threshold Model for ES Cell Fate**

A mechanistic model, based on signaling complex formation (LIF-LIFR-gp130 for LIF and gp130-HIL-6-gp130 [trimer] or gp130-HIL-6-gp130-HIL-6 [tetramer] for HIL-6), parametric values of which may be critical in determining ES cell fate, was developed. Mutagenesis studies have revealed that IL-6 and LIF are trivalent ligands [53-57]. LIF is thought to bind two LIFR molecules and one gp130 molecule, but a functional LIF complex is thought to be trimeric consisting of LIF, LIFR, and gp130 in a stoichiometric ratio of 1:1:1 [30] (Fig. 7A). There is some debate as to the composition of a functional IL-6 signaling unit; it may be a tetramer composed of IL-6, IL-6R, and gp130 in a stoichiometric ratio of 1:1:2 [58-60] or, alternatively, it may be a hexamer composed of two molecules of each component in a stoichiometric ratio of 2:2:2 [58, 61, 62]. Since the IL-6R binding site is unavailable in HIL-6 [48], the functional unit may be a trimer composed of HIL-6 and
gp130 in a 1:2 stoichiometric ratio (Fig. 7B) or a tetramer consisting of HIL-6 and gp130 in a stoichiometric ratio of 2:2 (Fig. 7C). A combination model for the competitive formation of both the HIL-6 trimers and HIL-6 trimers was also proposed (Fig. 7D).

As an initial approach in the development of our LIST model, we calculated cell-surface signaling complex numbers at steady state without taking into account variations in signaling complex numbers due to ligand/receptor trafficking. This assumed that initial binding and trafficking events, which may occur on the order of seconds to hours, did not impact cell fate decisions (typically occurring on the order of hours to days). The numbers of LIF (C_{LIF}) and HIL-6 signaling complexes formed in the trimer (C_{HIL-6-trimer}), tetramer (C_{HIL-6-tetramer}), or combination (C_{combination}) modes were calculated on a per cell basis using mass action kinetics equations and were a function of gp130 and LIFR numbers (R1 and R2 respectively), ligand concentration (L), binding affinity constants (k_f and k_r), and cross-linking affinity constants (k_c and k_u) (Appendix 1 and Table 1 [50, 56, 63-65]). Some values in our calculations could not be found for this system in the published literature and, thus, were estimated based on published ranges for similar processes (Table 1). Signaling complex numbers for individual cells were summed over the entire population to determine bulk concentration of signaling complexes and plotted at different ligand concentrations (Fig. 8A); the dose

### Table 1. Typical values of equilibrium binding constants (K_D), dimerization constants (K_C), and receptor numbers

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<th>Values used in simulation</th>
<th>Values used in threshold calculation</th>
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<td>[64]</td>
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SD = standard deviation

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**Figure 8. Model simulation results.** (A) Bulk LIF and HIL-6 signaling complexes at steady state at different ligand concentrations for typical values of binding and dimerization constants and receptor numbers (see Table 1). (—) represents C_{LIF}, (——) represents C_{HIL-6-trimer}, (——) represents C_{HIL-6-tetramer}, and (——) represents C_{combination}. Shifts in signaling complex number formation due to variations in receptor numbers (black arrow), variations in binding affinity constants (K_D) (white arrow), and variations in dimerization affinity constants (gray arrow) are indicated. (B) Fractional occupancy of gp130 (R_{12}) by HIL-6-trimer signaling complexes (C_{HIL-6-trimer}) as a function of lumped product, K_{C2R_{12}} (γ).
dependency of this simulation closely correlated with our experimental data of day 7 ES cell responses to log-fold changes in exogenous cytokine concentrations. Model-predicted bulk concentrations of LIF signaling complexes followed a sigmoidal dose-response curve; model-predicted bulk concentrations of HIL-6-trimer-type signaling complexes followed a bell-shaped, biphasic dose-response curve (Fig. 8A). Maximal HIL-6-trimer-type complex formation (25% receptor occupancy) occurred around 1,000 pM of HIL-6 (equivalent to the low affinity binding constant estimated for HIL-6; similar to the published value of the low affinity equilibrium binding constant for LIF [64]) and the curve was symmetric about this ligand concentration as predicted previously for interactions of bivalent ligands with receptors [66]. Model-predicted bulk concentrations of HIL-6-tetramer-type signaling complexes exhibited a monophasic dependency on HIL-6 concentration, similar to LIF (Fig. 8A), however, maximal HIL-6-tetramer-type complex formation (~25% receptor occupancy) occurred only at very high ligand concentrations (~1 µM). A combination model of HIL-6 signaling competitively between the trimer and tetramer modes followed the trimer curve until 1,000 pM (equivalent to the low affinity binding constant estimated for HIL-6), and then switched to the tetramer mode, which out-competed the trimer mode of signaling at higher HIL-6 concentrations (Fig. 8A). Our model correlated with experimentally observed data that showed that 500 pM LIF was significantly more potent than 500 pM HIL-6 in forming signaling complex numbers, and hence in sustaining ES cell pluripotentiality. Further, as we have shown experimentally, the model predicted a monophasic dependency of signal complex number formation on LIF concentrations, and a bi/multiphasic dependency on HIL-6 depending on which mode of signaling was predominant (trimer, tetramer, or a combination of the two).

An advantage of our computational approach was that it allowed us (by lumping and varying parameters over a range of values in model simulations [66]) to understand how variations in these parameters could effect cell-surface signaling complex formation (and potentially ES cell responses—see below). For example, we showed that by varying cell-surface receptor numbers (such as may occur due to changes in receptor internalization or synthesis rates), we affected the maximal number of signaling complexes formed (at steady-state) at a given ligand concentration (Fig. 8A) (consequently affecting the percentage of cells in a given population above or below a signaling threshold). Varying binding affinity (K_b) constants caused horizontal shifts in the curve (Fig. 8A) suggesting that greater ligand concentrations were needed to achieve maximal receptor occupancy. A normalized curve of HIL-6-trimer complexes/gp130 receptor numbers (reflecting receptor occupancy) attained a maximal value when the ligand concentration (L) was equivalent to the binding affinity constant (K_b), and was a function of the product of ligand cross-linking constant (K_C2) and gp130 receptor numbers (R_T2) [66] (Fig. 8B). Similar normalization for LIF complexes, HIL-6-tetramer, and HIL-6 combination complexes revealed that maximum complex numbers formed, and the ligand concentration at which these occurred depended on the lumped parameters (Appendix 1).

Calculated signaling complex numbers (Table 1) were also used to predict whether or not an individual cell had exceeded a specified threshold for self-renewal. Although our model thresholds were based upon experimental observations that ES cells self-renewed at LIF concentrations greater than 100 pM while cells differentiated at LIF concentrations less than 0.5 pM (4-10 pM LIF was previously shown to be the half-maximally effective concentration to prevent differentiation [26]), our computational approach allowed us to effectively investigate this choice of threshold and other ligand specific parameters. The mean LIF-equivalent complexes formed at the selected concentrations were designated as the “high signal threshold” and the “low signal threshold,” respectively. If the cell-surface complex number exceeded the “high signal threshold,” it was given an individual probability of self-renewal (P_i) value of 1. If the cell-surface complex number on an individual cell fell below the “low signal threshold,” the cell was assigned a P_i value of 0, i.e., it had zero probability of self-renewing. Cells were assigned a P_i value of 0.5 when their cell-surface complex numbers were between the high and low signal thresholds, signifying that they had a 50% chance of self-renewing and a 50% chance of differentiating upon cell division (alternatively, they could divide asymmetrically). Model results were computed with and without the P_i = 0.5 option (Fig. 9A). The individual cell probability values were summed over a theoretical population of 10^6 cells to yield a population probability of ES cell self-renewal and plotted as a function of bulk ligand-receptor signaling complexes for LIF and HIL-6 (Fig. 9B). The most noteworthy feature of the computational plots was the illustration that thresholds in signaling complex numbers were the important determinants of ES cell self-renewal responses; the model-computed threshold for 50% or more of the population to self-renew (calculated using values from Table 1) was between 0.5 and 26 pM of LIF-equivalent signaling complex numbers. On replotting our experimental data from multiple assays as a function of signaling complex numbers (only data for SSEA-1/E-cadherin-positive cells are shown), we observed a similar threshold-dependent quantal effect (Fig. 9C): the threshold for LIF-mediated self-renewal of 50% of the population was between 0.4 and 4 pM of signaling complexes; the threshold for HIL-6-trimer mediated self-renewal
of 50% of the population was between 3 and 22 pM signaling complexes. The threshold-dependence of ES cell markers on cell-surface signaling complex numbers calculated by our computational model, at steady-state, supported our LIST model premise that signaling complex numbers are important determinants of stem cell fate decisions.

**DISCUSSION**

Parametric values of ligand-receptor interactions have previously been shown to be relevant in determining cell fate for hematopoietic stem cells [7, 8], neural progenitors [67], T lymphocytes [68, 69], and even in determining cell fate during development in nonvertebrates [70]. While different stem cell systems may utilize different combinations of signaling ligands and receptors, critical levels of their interactions may ultimately determine the nature of the response that is elicited. A generalized model that illustrates how quantitative variations in ligand-receptor interactions, arising from interactions of the cell with its microenvironment, can result in alteration in cell fate choices would thus be extremely useful (Fig. 10). Importantly, the number of signaling ligand-receptor complexes is not a static property of the cell; it is dynamically modulated by extrinsic factors including soluble factors and cell-cell and cell-matrix interactions,
which can influence receptor up- or downregulation, autocrine secretion, ligand depletion, negative feedback signaling, etc.

Herein, we have obtained results indicating that thresholds in cell-surface ligand-receptor complexes can affect ES cell fate by examining ES cell responses to different doses of IL-6-type cytokines. ES cells are an ideal model system to study stem cell fate as their self-renewal status in vitro correlates with numerous quantitative assays, and decisions made by ES cells to self-renew or differentiate may be similar to those made by other stem cell systems such as HSC [5, 7, 8]. We found correlation between levels of molecular markers (Oct-4 protein expression), cell-surface phenotype markers (SSEA-1 and E-cadherin expression), and cytokine stimulation, implying that thresholds in levels of ligand-receptor activation at the cell surface were directly translated to levels of downstream transcription factors that were activated and were responsible for triggering different gene programs. This correlation might prove useful in designing protocols aimed at enriching stem cell population(s) based on specific cell-surface antigen(s) expression.

We used two cytokines from the IL-6 family of cytokines, LIF, which signals through gp130-LIFR heterodimers, and HIL-6, which signals through gp130-gp130 homodimers, to demonstrate how variations in receptor-binding properties of the signaling cytokines can influence cell fate as predicted by our LIST model. Importantly, differences in LIF and HIL-6 signaling may be additionally influenced by the differential signaling potencies of heterodimers and homodimers of gp130, which, though not shown conclusively, may explain some of the discrepancies between model predictions (Fig. 9B) and experimental data (Fig. 9C). There is some evidence to suggest that heterodimer signaling may be more potent in activating and amplifying janus kinase/signal transducers and activators of transcription (Jak/STAT) and Ras/MAPK (mitogen-activated protein kinase) pathways than homodimer signaling [26], and this may be linked to their differential sorting on internalization, as has been shown for ErbB receptor-tyrosine kinases [71]. However, LIFR is considered to be functionally similar to the gp130 receptor (although not identical [72-74]) suggesting that the signaling potencies of gp130-gp130 homodimers and gp130-LIFR heterodimers may be equivalent, and other explanations, such as we have provided herein, likely play a role in the control of stem cell responses.

We demonstrated that attenuation of cell-surface complex numbers can be achieved by signaling through HIL-6, which required a higher stoichiometric ratio of signaling receptors to ligand than the simple 1:1 ratio required for LIF, and, accordingly, resulted in the formation of lower signaling complex numbers at ligand concentrations that far exceeded available receptor numbers. That is, for a hypothetical cell with “X” number of gp130 receptors, stimulation by LIF resulted in the formation of “X” number of signaling complexes at saturation (providing that LIFR numbers were not limiting), while stimulation by equimolar concentrations of HIL-6 resulted in the formation of X/2 signaling complexes at saturation. If the postulated threshold for self-renewal lies between X/2 and X signaling complexes, LIF would have been more efficient than HIL-6 in maintaining ES cell pluripotentiality. In fact, equimolar concentrations of LIF and HIL-6, although similar, were not equivalent in maintaining marker expression associated with undifferentiated cells in the various assays used to gauge ES cell differentiation status. This correlated with our LIST model predictions, which were based on simple receptor-ligand binding and dimerization mass action equations. The correlation between the LIST model predictions and experimental data was all the more remarkable since the model simulations were confined to cell-surface ligand-receptor interactions and had to contend with estimates of receptor distributions and binding/dimerization constants, and with steady-state approximations of dynamically regulated processes. This suggested that the number of cell-surface signaling complexes at steady-state may be critical determinants of downstream processes that were responsible for turning on different genetic programs associated with ES cell self-renewal or differentiation. Discrepancies between the model and experimental data also arose because we had to contend with correlating discrete probability
values of self-renewal (0, 0.5, 1) assigned to individual cells and iterated for a given population, to experimental data that represented a continuous range of possible values of cell-specific phenotypic and molecular marker expression. Inherent variability within the assays diminished the correlation; however, when data from multiple assays were taken together, the quantal relationship between cells positive for ES cell self-renewal markers and the number of cell-surface signaling complex numbers was apparent, thus validating our core hypothesis that this parameter had an important (governing) effect on the decision of cells to self-renew. Our computational model, which explained cell fate specifications at the population level by taking individual cell properties (i.e., signaling ligand-receptor complex numbers) into account, is a first step toward developing a more comprehensive deterministic model that includes multiple interactions among individual cells within a population, as well as the dynamics and interplay between cell-signaling networks.

Our approach was distinct from stochastic models of stem cell differentiation control that typically assume that cell fate processes are random and are best described by statistical probability distributions [75-81]. These models argue that cytokines support an overall probability of progenitor cell survival and/or proliferation by selecting for cells that have already made the decision to self-renew or differentiate by some unknown random process. Our deterministic model, on the other hand, proposed a specific mechanistic basis (i.e., signaling through the ligand-receptor complexes) to account for the self-renewal of individual mouse ES cells in vitro based on cell-surface-receptor occupancy. Given our data that LIF and HIL-6 also mediate the survival of a subset of cells independent of any effects on ES cell proliferation [7, 8, 11] (possibly through the differential engagement of STAT3, known to be important in both cell survival [82-84] and maintenance of ES cell pluripotentiality [85, 86]), we cannot eliminate the possibility that IL-6 type cytokines also influence ES cell fate by enhancing cell survival in addition to promoting their self-renewal. Ongoing studies should reveal the relative contribution of these two mechanisms on ES cell fate control.

Our model predictions correlated with ES cell marker’s dependence on LIF concentration. For HIL-6, on the other hand, certain ES cell markers (EB formation efficiency and ALP activity) had a biphasic dependency on HIL-6 doses, while other markers (SSEA-1/E-cadherin expression and Oct-4 protein expression) did not exhibit a strong biphasic dose dependency. These results suggested that the kinetics of these endpoint assays used showed variability in response to LIF versus HIL-6 stimulation. The observed biphasic dependency of EB formation efficiency and ALP activity was consistent with model predictions for HIL-6 signaling in the trimer mode and with other experimental data [5]. The self-antagonism of HIL-6 at higher concentrations, in agreement with data obtained for other bivalent cross-linking ligands, such as growth hormone [87, 88] and IL-6 [89] and with model predictions for other bivalent ligands [66], resulted from competition for gp130 receptors. The dose dependence of SSEA-1/E-cadherin and Oct-4 protein expression on HIL-6 concentrations could not be used to distinguish among the proposed trimer, tetramer, or combination modes of HIL-6 signaling. Signaling in the trimer mode may not have been apparent at the HIL-6 concentrations tested, which were not sufficiently high to observe significant antagonistic behavior. Alternatively, HIL-6 may have been signaling in the tetramer mode but, according to our model, signaling would only have been effective at very high ligand concentrations (in the µM range). It may also have been possible that HIL-6 signaled competitively in both the trimer and tetramer mode, oscillating from one mode to another with time and ligand concentration. ES cell responses would then demonstrate a partially biphasic (following the trimer mode of signaling up to 1,000 pM and then following the tetramer mode) dependency on HIL-6 concentration according to the combination model we developed, with the axis of symmetry lying at K_{D,2}, the equilibrium binding constant associated with HIL-6 binding to gp130. A different kind of combination model was previously suggested, which required the preformation of gp130 homodimers [59], however, there is recent evidence for the kind of sequential binding of HIL-6 to two gp130 receptors [60, 90] that can be interpreted in favor of all three models suggested here.

Our LIST model can be adapted to describe critical ligand-receptor interactions in different cell systems; for example, to explain the observed increase in osteoclast formation upon tetracycline-regulated upregulation of gp130 levels in a stromal/osteoblastic cell line upon stimulation with IL-6/sIL-6R, but not with OSM [91].

**Summary**

We have proposed a mechanistically based model for stem cell fate control where the level of cytokine signaling results in the activation of gene programs responsible for determining cell fate. We have shown that gp130-mediated signaling influences self-renewal and possible survival outcomes in a threshold-dependent manner that was independent of any effects on proliferation. We demonstrated that there were differences at higher ligand concentrations in the abilities of LIF and HIL-6 to sustain ES cell pluripotentiality, likely due to different receptor-ligand binding stoichiometries. An
important message from this study is that outcomes of stem cell fate choices are amenable to exogenous control by appropriate types and levels of cytokine stimulation. Insights from our ES cell model should provide deterministic clues to the numerous, seemingly stochastic parameters that affect stem cell fate and, thus, help in developing clinically relevant protocols to either expand stem cell populations or direct their differentiation into appropriate tissues in vitro.

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**REFERENCES**


30 Gearing DP, Thut CJ, VandeBos T et al. Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J 1991;10:2839-2848.


61 Ward LD, Howlett GI, Discolo G et al. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. J Biol Chem 1994;269:23286-23289.


84 Hirano T, Ishihara K, Hibi M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 2000;19:2548-2556.

85 Matsuda T, Nakamura T, Nakao K et al. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J 1999;18:4261-4269.


Appendix I. Formation of signaling complexes at steady-state

The number of LIF signaling complexes was obtained from equilibrium solutions (same as steady-state solutions in this case) of the binding of LIF (L) to LIFR (R₁) and cross-linking of the intermediate complex (C₁) to gp130 receptor numbers (R₂) along with mass balances on total receptor numbers (R𫭟 and R☒):

\[ K_{D1} \quad L + R₁ \rightleftharpoons C₁ \]  
\[ K_{C1} \quad C₁ + R₂ \rightleftharpoons C_{LIF} \]  
\[ R_{T1} = R₁ + C₁ + C_{LIF} \]  
\[ R_{T2} = R₂ + C_{LIF} \]

Accordingly, at equilibrium:

\[ K_{D1} = \frac{C₁}{R₁L} \]  
\[ K_{C1} = \frac{C_{LIF}}{R₂C₁} \]

which, substituted in the mass balances (A3) and (A4) yields:

\[ C_{LIF}[L] - C_{LIF} \left( \frac{1}{K_{D1}K_{C1}} + \frac{L}{K_{D1}K_{C1}} + LR_{T2} + LR_{T1} \right) + LR_{T2}R_{T1} = 0 \]  

where: \( K_{D1} \) was the equilibrium association constant (cell volume/mole) for LIF; \( K_{C1} \) was the equilibrium cross-linking constant (cell volume/mole); \( R₁ \) was the number of unbound LIF receptors; \( R_{T1} \) was the total number of LIFR receptors; \( R₂ \) was the number of unbound gp130 receptors; \( R_{T2} \) was the total number of gp130 receptors; and \( C₁ \) was the number of intermediate LIF-LIFR nonsignaling complexes. Receptor numbers for individual cells were generated using a function that outputted random numbers from a normally distributed population of receptor numbers with mean and standard deviations that were estimated (Table 1).

To understand how variability in receptor numbers influenced the number of cell-surface LIF signaling complexes formed, we lumped parameters and defined \( \alpha_{LIF} \) as the product of \( K_{D1}L \), \( \chi \) as the product of \( K_{C1} (R_{T1} + R_{T2}) \), and \( \theta \) as the ratio of \( R_{T2}/R_{T1} \). Receptor occupancy, “M” defined as the ratio of \( C_{LIF}/R_{T1} \) (number of LIF signaling complexes/the limiting LIFR numbers), was a function of the lumped products:

\[ M_{LIF} = \frac{1}{2LR_{T1}} \left[ \left( \frac{1 + \alpha_{LIF} + \alpha_{LIF}\chi}{K_{D1}K_{C1}} \right) + \sqrt{\left( \frac{1 + \alpha_{LIF} + \alpha_{LIF}\chi}{K_{D1}K_{C1}} \right)^2 - 4(\frac{L}{K_{D1}K_{C1}})^2} \right] \]  

Similarly, \( C_{HIL-6-trimer} \), the number of HIL-6-trimer signaling complexes, \( C_{HIL-6-tetramer} \), the number of HIL-6-tetramer signaling complexes, and \( C_{HIL-6-combination} \) the competitive sum of \( C_{HIL-6-trimer} \) and \( C_{HIL-6-tetramer} \) signaling complexes, were obtained from equilibrium solutions of the binding of HIL-6 (L) to gp130 (R₂) and the cross-linking of the nonsignaling intermediate (C₁) to gp130 (R₂) (A10) or to another nonsignaling intermediate (A11) along with mass balances on total receptor numbers (R_{T2}).

\[ R₂ + L \rightleftharpoons C₁ \]  
\[ C₁ + R₂ \rightleftharpoons C_{HIL-6-trimer} \]  
\[ C₁ + C₁ \rightleftharpoons C_{HIL-6-tetramer} \]  
\[ C_{HIL-combination} = C_{HIL-6-trimer} + C_{HIL-6-tetramer} \]  
\[ R_{T2} = R₂ + C₁ + 2C_{HIL-6-trimer} + 2C_{HIL-6-tetramer} \]
Here, $K_{D2}$ was defined as the equilibrium binding constant of HIL-6 to gp130, $K_{C2}$ (the equilibrium cross-linking constant within the HIL-6-trimer), and $K_{C3}$ (the cross-linking constant within the HIL-6-tetramer) was defined as before. Accordingly:

$$K_{D2} = \frac{C_L}{LR_2}$$  \hspace{1cm} (A14)

$$K_{C2} = \frac{C_{HIL-6trimer}}{C_{R_2}}$$  \hspace{1cm} (A15)

$$K_{C3} = \frac{C_{HIL-6tetramer}}{C_{C_1}}$$  \hspace{1cm} (A16)

Lumping and normalizing the parameters as before: $\alpha_{HIL-6}$ was defined as the product of $K_{D2}L$; $\gamma$ as the product of $K_{C2}R_2$; $\phi$ as the product of $K_{C3}R_2$; and $\sigma$ was defined as $\gamma + \alpha\phi$. Receptor occupancy, “$M$,” defined as the ratio of HIL-6-signaling complexes (trimer [A17], tetramer [A18], or combination [A19]) to gp130 receptor numbers, was expressed in terms of these parameters:

$$M_{HIL-6trimer} = \frac{(1 + \alpha) - \sqrt{(1 + \alpha)^2 + 8\alpha\gamma}}{2 \times \gamma}$$ \hspace{1cm} (A17)

and this reached a maximum value when $\alpha_{HIL-6} \rightarrow 1$

$$M_{HIL-6tetramer} = \frac{(1 + \alpha) - \sqrt{(1 + \alpha)^2 + 8\alpha^2\phi}}{2 \times \phi}$$ \hspace{1cm} (A18)

and this reached a maximum value when $\alpha_{HIL-6} \rightarrow \infty$

$$M_{combination} = \frac{(1 + \alpha) - \sqrt{(1 + \alpha)^2 + 8\alpha\sigma}}{2 \times \sigma}$$ \hspace{1cm} (A19)

and this reached a maximum value when $\alpha_{HIL-6} \rightarrow \infty$

Unit Conversions

Rate constants were reported as cell volume per mole, while receptor numbers, cell-surface complex numbers, and ligand concentrations were reported as moles per cell volume. We were interested in cell-specific values not bulk molar concentrations. To obtain these, all bulk parameters were converted to an equivalent cell-specific molar concentration by dividing them by typical cell volumes. It was assumed that receptors were homogeneously distributed in the medium, cell density was not taken into account, and the cell was assumed to be a perfect sphere. Values for cell-surface complex numbers were converted back to molar concentrations when reporting bulk concentrations for an entire population. Conversion of molar ligand concentration to moles per cell volume is shown here as an example:

$$[L] \equiv \frac{1}{4,188 \times 10^{-9}} \times \frac{1}{1,000 \text{ ml}} \times \frac{1}{\text{ cell volume}} \equiv \frac{1}{\text{ cell volume}}$$

$$\text{cell volume} = \frac{4}{3} \times \pi \times r^3, r = 10 \mu\text{m}$$

$$\therefore \text{cell volume} = 4.188 \times 10^{-9} \text{ ml}$$