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Cellular Level Models as Tools for Cytokine Design

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Abstract

Cytokines and growth factors are critical regulators that connect intracellular and extracellular environments through binding to specific cell-surface receptors. They regulate a wide variety of immunological, growth, and inflammatory response processes. The overall signal initiated by a population of cytokine molecules over long time periods is controlled by the subtle interplay of binding, signaling, and trafficking kinetics. Building on the work of others, we abstract a simple kinetic model that captures relevant features from cytokine systems as well as related growth factor systems. We explore a large range of potential biochemical behaviors, through systematic examination of the model's parameter space. Different rates for the same reaction topology lead to a dramatic range of biochemical network properties and outcomes. Evolution might productively explore varied and different portions of parameter space to create beneficial behaviors, and effective human therapeutic intervention might be achieved through altering network kinetic properties. Quantitative analysis of the results reveals the basis for tensions among a number of different network characteristics. For example, strong binding of cytokine to receptor can increase short-term receptor activation and signal initiation but decrease long-term signaling due to internalization and degradation. Further analysis reveals the role of specific biochemical processes in modulating such tensions. For instance, the kinetics of cytokine binding and receptor activation modulate whether ligand–receptor dissociation can generally occur prior to signal initiation or receptor internalization. Beyond analysis, the same models and model behaviors provide an important basis for the design of more potent cytokine therapeutics by providing insight into how binding kinetics affect ligand potency.

Keywords

systems biology; network modeling; computational biology; ordinary different equation model; cellular trafficking; signal transduction; growth factor; optimal potency

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1 Introduction

Cytokines are small, secreted proteins that bind to specific cell-surface receptors to regulate the differentiation and proliferation of various cell types [1–3]. Erythropoietin (Epo), for example, binds to the erythropoietin receptor (EpoR) to initiate the process of red cell maturation, or hematopoiesis. Granulocyte-colony stimulating factor (GCSF) binds to its receptor to stimulate neutrophil production. Many cytokines such as epidermal growth factor (EGF), insulin-like growth factor, interleukin-1, interleukin-2 (IL-2), and tumor necrosis factor α regulate cell growth and activity, the latter four doing so as part of immunological and inflammatory responses [1] (In this paper we will use “cytokine” as a general term to include growth factors as well). Improper cytokine levels may lead to serious illnesses. Patients with Epo deficiency as a result of renal failure, for example, can experience anemia [4]. The direct result of disease or other treatments, such as chemotherapy, may cause conditions that can be ameliorated by cytokine therapy. This has produced a market for natural cytokines and engineered versions with improved properties. Currently, exogenously introduced cytokines generally enter into the patient as a bolus of ligand and are removed by a variety of processes, including clearance by the liver and filtration by the kidneys, leading to excretion, as well as intracellular trafficking processes, leading to degradation [5]. The overall potency, or response to a cytokine therapeutic, may be at least partially related to the concentration of exogenous cytokine over time. In this paper we focus solely on the intracellular degradation mechanism that contributes to cytokine potency, noting that it is but one mechanism through which ligand is depleted.

Most cytokines undergo a core set of cellular-mediated processes over the course of their effective lifetimes — they bind to cell-surface receptors to cause a downstream signal, they may become internalized (brought into the cell via endocytosis), and, once internalized, they may either be re-secreted or undergo lysosomal degradation. While the specifics governing a cytokine’s regulation and efficacy vary greatly from system to system, there is a common set of core, shared processes. Useful models accounting for these processes (as well as processes specific to the system being studied) can be abstracted from the details of many important cytokine and growth factor systems [6–16].

Fig. 1 shows a simple, abstracted, kinetic model of the core set of processes in a generalized cytokine signaling and trafficking network. First, ligands reversibly bind to surface receptors. The mechanism of ligand–receptor interaction varies among systems. In certain systems, such as the human growth hormone (hGH) system, binding causes sequential oligomerization of surface receptors [17]. In many cases, such as EpoR and perhaps EGF receptor (EGFR), receptors are thought to be pre-associated in an “off” state prior to ligand binding [18, 19]. The stoichiometry of binding also varies. In the Epo and hGH systems, the resulting complex has one ligand molecule and two receptor monomers; for GCSF, the ratio is 2:2 (see reference [18] for a review).

After binding ligand, receptor activation becomes far more likely. Activation occurs through mechanisms that vary across systems. For example, Epo is thought to induce a conformational change in the receptor dimer to which it binds, causing JAK2 kinase domains that are pre-associated to the EpoR cytoplasmic regions to phosphorylate each other, thereby becoming activated [20, 21]. In the case of hGH, kinases bind to receptors only after ligand is bound [22], and they phosphorylate each other, leading to activation [18]. Once the kinases are active in these systems, they may phosphorylate critical residues on the receptor monomers, they may phosphorylate other signaling molecules, or they may form docking sites for yet other molecules [18, 23].

After a receptor has been activated, downstream signaling can take place until deactivation occurs via phosphatases or other mechanisms. For example, the phosphatase SH-PTP1 is believed to dephosphorylate the EpoR receptor, returning it to an inactive state [24].

In addition, receptors or complexes can be internalized through receptor-mediated endocytosis. Once endocytosed, there is evidence to suggest that a receptor may still become or remain activated (see reference [25] for a review), as topologically, a surface receptor and an internalized receptor have the same relationship to the cytoplasm. Eventually, through sorting mechanisms, the endocytosed species can either be recycled back to the cell surface or remain in the intracellular compartment through its progression to a lysosome for degradation. Again, the specifics governing how species are recycled or degraded varies from system to system. For example, EGFR (and bound EGF ligand) is targeted to degradation by a sorting nexin, SNX-1 [26, 27], while free EGF ligand appears to recycle. In the fibroblast growth factor (FGF) system, the extent to which different FGF receptors are ubiquitinated upon internalization appears to affect their sorting fate into recycling compartments or to degradation [28]. As a final example, the interleukin-2 receptor consists of three subunits, termed α , β , and γ ; the α subunit (and complexed ligand) is constitutively recycled [29], and the β and γ subunits (and any complexed ligand) are trafficked to degradation upon recognition of specific amino-acid components [30, 31].

The binding, internalization, and recycling processes described above affect cytokine signaling, as they all directly affect the number of ligand–receptor complexes that could potentially become or remain activated. A cytokine’s overall potency therefore depends critically on the effect of interactions among these mechanistic steps. As shown in recent work, an understanding of the link between individual, potentially competing reaction rates and cytokine potency in specific systems, including EGF, IL-2, GCSF, and others, can lead to improved therapeutics [7, 32–36]. While models that are parameterized for specific systems are important for design, exploring the parameter space of a general cytokine model can provide valuable insight into how each of the different components in any system may affect cytokine potency. The focus of the current work is a thorough exploration of such a general model.

In this report, we systematically explore the parameter space of a model that captures the common features of cytokine binding, signaling, and trafficking. Specifically, we are interested in how the kinetics of cytokine–receptor binding affect overall ligand potency, in both the short- and the long-term, and how these effects vary with other properties of the system. The properties considered include the on and off rate constants for binding between ligand and receptor, the number of receptors per cell, the starting ligand concentration, the internalization and recycling rate constants, and others. However, these properties vary from system to system, as exemplified in this work by a comparison of GCSF and Epo. Knowledge of how cytokine potency depends on parameters and the corresponding biochemical kinetic properties can provide guidance in the design of improved therapeutics.

We have found that opposing effects are at play in the model, consistent with the results of others [7, 27]. First, the need for a cytokine to remain bound to a receptor long enough to activate it favors tighter binders. However, the benefits of “falling” off a receptor quickly, either to avoid internalization and degradation or to activate multiple receptors, favors weaker binders. Here we build on the initial findings of others by analyzing, in detail, how these competing factors are qualitatively and quantitatively affected by where the system lies in property space and also by the time scale over which adequate potency is desired. By analyzing the results of thousands of simulations, each representing a different point in the cytokine property space, we provide a comprehensive framework for understanding how the potency of a cytokine can be explained by the kinetic parameters that characterize the

system in which it operates. We characterize general classes of systems, termed “activation-limited” and “ligand-limited” systems, showing that the former favor tightly-binding ligands while the latter favor more weakly-binding ligands. We also isolate three control points of the system — initial activation, internalization, and recycling — that each contribute to determining the dynamics of competing factors over time. We explore the contributions of these three control points as a function of where the system lies in property space. From a design perspective, we explore the use of mathematical optimization to improve cytokine potency. Interestingly, optimization analysis via discrete sampling frequently revealed a particular sensitivity of potency to both the cytokine association rate constant and, especially, the dissociation rate constant.

2 Methods

Cytokine potency was simulated at the cellular level with an ordinary differential equation (ODE) model. Processes accounted for in the model included surface and endosomal association and dissociation between receptors and ligand, receptor and complex internalization into an endosomal compartment, ligand and receptor recycling and degradation, receptor activation and deactivation, receptor synthesis, and exponential growth of cellular mass in a fixed volume. Fig. 1 shows a pictorial depiction of the model. The base model is available in MATLAB format (The Mathworks, Inc., Natick, MA) as supplementary material.

Equation 1 is a sample ODE from the model, representing the change in concentration over time of inactive, unliganded surface receptors.

$$D\left(\frac{d(R)}{dt}\right) = D(-k_{\text{on}}(R)[L] + k_{\text{off}}(RL) - k_{\text{bsigf}}(R) + k_{\text{sigr}}(R^*) - k_{\text{bintf}}(R) + k_{\text{recrf}}(R_i) + R_s) - (R)\left(\frac{dD}{dt}\right) \quad (1)$$

where (R) , $[L]$, (RL) , (R^*) , and (R_i) are surface receptor, extracellular ligand, surface complex, active surface receptor, and internalized receptor concentrations, respectively. D is the cell density, which changes throughout the simulation. k_{on} , k_{off} , k_{bsigf} , k_{sigr} , k_{bintf} , k_{recrf} , and R_s are kinetic rate constants whose base values and physical interpretations are given in Table 1. Surface concentrations are denoted with parentheses, and bulk concentrations with square brackets. The first and second terms within the parentheses on the right-hand side of Eq. 1 represent binding and dissociating from ligand. The third and fourth terms represent unliganded activation and deactivation. The fifth, sixth, and seventh terms represent internalization, recycling, and receptor synthesis, respectively. The final term on the right hand side $-(R)\left(\frac{dD}{dt}\right)$ accounts for dilution due to cell growth.

Experimentally there is an observed lag between initial ligand addition and ultimate ligand and receptor degradation; in the Epo system, degraded ligand was observed only 15–20 minutes after addition [37]. A feature of the model implemented here was to use a chain of nine first-order “lag steps” through which ligand and receptor slated for degradation effectively passed before actually being degraded. Using only one effective first-order step with a time constant on the order of tens of minutes would allow for degraded ligand to be instantaneously produced upon sorting. However, a series of n first-order steps, each with a time constant n times faster than the observed lag, would generate effectively no degraded ligand instantaneously, therefore better modeling the observed lag within the degradation pathway. A similar approach was taken and explained in reference [37].

The model assumed a single ligand molecule binding to a single receptor unit (either a monomer or a pre-associated dimer). Moreover, the model assumed that deactivation (by

phosphatases, etc.) does not permanently inactivate receptors; rather, deactivated receptors may reactivate if ligand rebinds or remains bound. Variants of this model (in which receptors could be permanently desensitized, for example), were also simulated and are discussed as well.

Simulations of the model and its variants were made by integrating the ODE's with the ABACUSSII package [38]. MATLAB (The Mathworks, Inc., Natick, MA) was used to plot results and generate the figures shown and to create the base model available as supplementary material. To check the robustness of results to simulated experimental conditions, several sets of simulations were run in parallel, with varying initial cell densities and growth rates. The simulations also modeled multiple experimental techniques that may be used to keep cell density from becoming unreasonably large over time, such as diluting the cells with fresh media at a certain cell density value or diluting cells with fresh media containing the current media's ligand concentration (such maintenance of ligand concentration in the media could also be experimentally achieved by removing a particular number of cells from the media). While the quantitative predictions were sensitive to the variation between the parallel simulations, the overall trends were robust. We opted to present results from runs using the second method of dilution, essentially modeling either the dilution with media containing the current media's ligand concentration or the removal of cells to restore initial cell density. While these may be less likely procedures for maintaining reasonable cell density because of the experimental need to keep media fresh enough to support growth over the desired time period or the potential difficulties of measuring ligand concentrations at each dilution, we chose this dilution scheme because ligand degradation can be easily quantified, and ligand depletion can almost entirely be attributed to cellular mechanisms rather than a combination of cellular mechanisms and dilution effects. In the results presented here, therefore, the initial cell density was 5×10^8 cells L^{-1} , and cells were grown until they reached a density of 2.0×10^9 cells L^{-1} , at which point a dilution with media was modeled to reduce to the initial density of 5×10^8 cells L^{-1} . As explained, the new media contained ligand at the same concentration as the old media at the time of replacement. Therefore, the intact ligand media concentration was unchanged at the time of dilution, so no intact ligand in the media was considered "lost" upon dilution. Note that during this process, the number of receptor-bound and internalized free ligand molecules per liter of cell culture is reduced by the dilution factor in the context of the model, as it would be in a real-world experiment. This very small amount ($< 1\%$ of total ligand) of ligand was "lost," from the simulation, and as a check for conservation of matter, accounting for this lost ligand as well as intact and degraded ligand maintained the constant, initial concentration of ligand (Fig. 2b).

The behavior of the model was examined across a range of discrete parameter values. Holding all other parameters fixed at their base values, the value of k_{on} or k_{off} was systematically and discretely sampled over a wide range of values. k_{on} , in $\mu M^{-1} min^{-1}$, was uniformly varied on a $\log_{10}$ scale between -4 and 5 , in increments of $\frac{1}{8}$ (base value $100 \mu M^{-1} min^{-1}$). k_{off} , in min^{-1} , was varied uniformly on a $\log_{10}$ scale between -5 and 4 , also in increments of $\frac{1}{8}$ (base value $0.01 min^{-1}$). Changing the sampling frequency did not change the results appreciably. The sampling used to generate results shown in Fig. 8 was done with a coarser spacing, in increments of $\frac{1}{4}$ on a $\log_{10}$ scale, due to the large number of simulations required for these analyses.

Two objective functions were used to represent cytokine potency — integrated activity over a predefined period of time, and the absolute activity measured at a given time after the simulation began. Activity was defined as the total concentration of active receptors (in $\mu mol m^{-2}$). Other objective functions considered were the activity per degraded ligand and

the peak activity, which capture the extreme long- and short-term signaling characteristics (results not shown).

Choice of Base Parameters—A base set of parameters was chosen, informed by experimentally determined values for Epo [37] and GCSF [10]. Table 1 shows all base parameters used in the model. Where parameters were not experimentally available, we selected base parameters to lie in the range of physically and biologically reasonable values for these systems; the average surface area of cells was based on a cell radius of $5 \sqrt{10} \mu\text{m}$ (A. Gross, personal communication), and value of k_{sigf} was chosen to produce fast initial activation upon addition of ligand (with activity peaking on the order of tens of minutes). Guided by experimental observations in other cytokine systems (Epo and IL-2), for which it was found that non-endocytic trafficking mechanisms may result in loss of activation on a time scale on the order of tens of minutes [24, 39], we chose a base value of $k_{\text{sigr}} = 0.1 \text{ min}^{-1}$ for the deactivating rate constant. Because the purpose of this study was to examine how cytokine potency changes as a function of parameters values, and because values were systematically varied to explore the parameter space of the model, the main results depend only weakly on the particular set of base parameter values selected.

Receptor concentration was expressed as the number of receptors per cell surface area (in units of $\mu\text{mol m}^{-2}$). The surface area per cell was assumed to remain constant in these simulations, thereby allowing for a direct correspondence between receptor concentration and number of receptors per cell. Using the GCSF surface receptor count from reference [10], the base initial receptor concentration ($8.3 \times 10^{-6} \mu\text{mol m}^{-2}$) and average cell surface area ($1 \times 10^{-9} \text{ m}^2$) were chosen correspond to approximately 5,000 surface receptors per cell ($8.3 \times 10^{-6} \mu\text{mol m}^{-2} \times (1 \times 10^{-9} \text{ m}^2 \text{ cell}^{-1}) \times (6.022 \times 10^{17} \text{ receptors } (\mu\text{mol})^{-1}) \approx 5000 \text{ receptors/cell}$).

The receptor synthesis rate and initial internalized receptor concentration were found by solving the steady-state equations for surface and internalized receptor, ignoring the negligible amount of basally active receptor, the degradation lags, and the growth rate of the cell density. To check the robustness of the results to neglecting the growth of cell density, we also ran a subset of parallel simulations where we accounted for the growth of the cell density; this did not change the overall qualitative trends and results, and thus we do not present those particular results here. Internalized receptor and complex surface concentrations were expressed in units of $\mu\text{mol } (\text{m}^2 \text{ cell surface})^{-1}$, for simplicity.

GCSF-Specific Model—A qualitative analysis of GCSF trafficking and signaling was carried out using some of the experimentally-fit and cited parameter values provided by Sarkar and Lauffenburger [10] and [40], including the initial cell density and growth rate. A full parameter listing is available as Supplementary Material. Parameters not explicitly given/explained in reference [10] included signal activation (k_{sigf}) and deactivation (k_{sigr}) rate constants. Based on the same experimental evidence noted above in choosing base parameters, we chose a value of $k_{\text{sigr}} = 0.1 \text{ min}^{-1}$ for the deactivating rate constant. Here, however, we have varied the activation rate constant. A base value of 100 pM was used for the initial ligand concentration. With small model modifications, signal generation could be selectively turned off at the membrane surface or from endosomes, although the base model provided for signal activation generation and deactivation in both locations.

Epo-Specific Model—The qualitative analysis of Epo binding, trafficking, and signaling was carried out using model parameters measured experimentally by Gross and Lodish [37]. The initial cell density, growth rate, and dilution parameters used were from the base model, as these reflect the long-term behavior of the cell line used in the experiments. Because it is unknown whether Epo receptor is preferentially degraded or recycled relative to ligand, the

ligand, receptor, and complex recycling fractions were all set to the experimentally observed ligand recycling fraction of 61% [37]. As in the GCSF base model, k_{sigr} was set to 0.1 min^{-1} , and the ligand concentration was 100 pM. A full parameter listing is provided as Supplementary Material.

3 Results

3.1 Simulations with Base Parameters

Simulations were run using the ODE cellular-level model of cytokine binding, receptor activation, and trafficking (Fig. 1) with the base parameters given in Table 1. In the simulations, model cells participated in exponential growth from an initial density of $5 \times 10^8 \text{ cells L}^{-1}$ until a critical density was reached ($2 \times 10^9 \text{ cells L}^{-1}$; over the course of 2–3 days of simulated time), at which point dilution with media was modeled to return the cell density to its original value, and growth was continued. In the version of the model presented here, the new media contained ligand at the same concentration as the old media at the time of replacement, to maintain intact ligand concentration at the time of dilution. Typical simulations were carried out for a 15,000-minute trajectory (approximately 10 days).

The black curves in Fig. 2 represent time courses for some quantities of interest from a single simulation. The cell density (Fig. 2a) grew exponentially to its critical (maximal) value, returned to its starting value, and repeated for a total of just over four dilution cycles, as intended. In the version of the model presented here, the rate of cell growth did not depend on the activity of the receptors, but such an extension for a specific system would be straightforward and could be useful [10].

Ligand conservation is shown in Fig. 2b. The Figure shows the sum total concentration of all ligand-containing species (free, bound, and internalized) expressed in μM , including degraded ligand, assuming a fixed volume of 1 L. With appropriate accounting (described in the Methods section), the sum total of all ligand concentrations remained constant over the course of the simulation, equaling the initial input ligand concentration, as one should expect.

To assess the potency of the ligand, two measures are displayed — instantaneous receptor activity as a function of time and integrated (cumulative) activity from time $t = 0$ (when ligand was added). The first measure may be especially useful to capture the notion of a “binary” system, one for which signal will be generated as long as the level of activity is above a certain threshold. This may be the case with Epo, as the ED_{50} for Epo activation is low enough to suggest that only approximately 60 receptors per cell — a small fraction of the total cell-surface receptors — would need to be active to achieve a 50% response [18,41]. The second measure may be more applicable to systems for which the amount of downstream signal increases as the number of active receptors increases.

Instantaneous activity was defined as the concentration of active, intact receptor — either at the surface or internalized — at a given time. The instantaneous activity (Fig. 2c, black trace) spiked initially, when approximately 13% of surface-bound receptors were active. Because bound surface receptors were more rapidly internalized (and thus more rapidly degraded) than unbound receptors and receptor synthesis was constant, the number of overall intact receptors dropped rapidly from its value at time zero, causing the signal to decrease to about one-third of its peak value by approximately 200–300 minutes. The signal declined steadily, but more slowly thereafter, as ligand was slowly degraded. The integrated activity (Fig. 2d, black trace) rose quickly initially and then slowly began to level off as the activity decreased at later times.

Intact ligand concentration (Fig. 2e, black trace) steadily declined over the course of the simulation, as more ligand became endocytosed and degraded. The total internalized ligand (Fig. 2f, black trace), including receptor-bound ligand, is shown in terms of an endosomal concentration, in $\mu\text{mol} \cdot (\text{endosomal volume})^{-1}$, using an endosomal volume of 1×10^{-14} L per cell [10]. Note that the internalized ligand concentration time course closely followed the activity time course (panel c), as one would expect, because activation expedites the internalization process.

Surface receptor concentration (Fig. 2g, black trace), initially dropped quickly because active receptors were internalized more rapidly than inactive ones. The total number of receptors in the system decreased because receptor synthesis was too slow to replenish degraded receptor. As mentioned above, this loss of receptors appeared to be the primary cause of the rapid decrease in initial activity (data not shown). Even though internalized receptor could activate in our model, there were simply fewer receptors in the entire system, and so the overall activity decreased accordingly.

As Fig. 2c, e, f, and g show, there were cusps in several time courses that coincided with each culture dilution. The sudden decrease in cell density meant that there were fewer cells in the 1-L volume to take up ligand, so the rate of ligand degradation slowed discontinuously, resulting in a cusp in the intact ligand concentration. As the cell density increased, so did the rate of ligand depletion. The cusps in other time courses can be rationalized similarly. Moreover, because of the choice of units used to show each time course, the only time course that changed discontinuously with each dilution was the cell density (Fig. 2a). All surface concentrations and the endosomal ligand concentration were unaffected by dilution of the media, accounting for the continuity of the time courses of active receptor (and therefore the smoothness of the integrated activity), internalized ligand, and total surface receptor. The time course for intact ligand actually does have small discontinuities at each dilution time, as the small amount of bound or internalized ligand at that time was reduced by the dilution factor. However, this amount was quite small ($< 1\%$ of total ligand), and therefore the time course essentially looks continuous at the sampling of time points used to generate the plots.

In this simulation, very little activity remained in the system after 10,000 minutes (approximately one week). Whereas the short-term activity induced by this cytokine was strong early in the simulation, the cytokine did not achieve long-term activation. Fig. 2e suggests that ligand degradation was the reason for the lack of long-term potency — very little intact ligand remained at 10,000 minutes. A ligand that is able to avoid degradation yet still signal effectively might be more potent than the base ligand presented here; this idea is explored in the next section.

3.2 Altering the Binding Affinity Affects Short- and Long-Term Activity Differently

As a first step of using the model to explore how changes in molecular biochemistry affect signaling activity, we examined the effect of ligand affinity and bound-state lifetime by changing the off rate constant for binding, k_{off} , from its base value to two new values — one higher and one lower, each by two orders of magnitude. The red and blue traces in Fig. 2 show how these altered-affinity ligands compare to the ligand with the base binding kinetics. Fig. 2c shows that the tighter binder's total active receptor time course (shown in red) closely followed that of the base simulation (in black). However, the weaker binder's time course for total active receptor looks substantially different. In the short term, the weaker binder generated less activity than its more tightly-binding counterparts. But as time progressed, this trend reversed, with the weakest binder maintaining almost constant activity throughout the simulation (after the initial burst), while the activity generated by the tightly binding ligands decreased substantially over time. Fig. 2d is a plot of integrated activity

generated by the three ligands. Eventually, the weakest binder generated the highest integrated activity. The reason for this somewhat counterintuitive idea is suggested by Fig. 2e and f — far less ligand was degraded in the simulation with the weakest binder, partly because far less ligand was even internalized. These results stress an idea, also noted by others [7,27]; namely, there are two competing factors that contribute to a ligand's efficacy — its ability to signal through binding and activation, and its ability to avoid internalization by *not* remaining bound to its receptor. The first factor favors tighter binders while the second favors weaker binders. The effects are not linear, in that a 100-fold decrease in the off rate constant had a much smaller effect than a 100-fold increase.

To further understand how cytokine potency is altered as its binding kinetics change, we varied k_{off} , in min^{-1} , on a log scale from -5 to 4 and plotted both the resulting activities and integrated activities after a 15,000-minute trajectory (Fig. 3a and b). Interestingly, activity at 15,000 minutes had a pronounced maximum at intermediate values of k_{off} . Integrated activity over 15,000 minutes showed a maximum near $k_{\text{off}} = 1 \text{ min}^{-1}$.

To probe the competing factors responsible for these maxima, we analyzed how various short-term and long-term quantities were affected by varying k_{off} . Fig. 3c is a plot of both the fraction of initial ligand that was intact after 15,000 minutes and the peak activity achieved in that time, as a function of the ligand's k_{off} . The fraction of intact ligand increased with increasing k_{off} . The peak signal, however, decreased with increasing k_{off} . These data suggest that tightly binding ligands with small k_{off} 's were degraded very quickly and therefore were not potent in the long term. However, extremely weak binders did not generate enough signal, even at their initial concentrations (when the activity peaks), to achieve sufficient potency. Therefore, intermediate values of k_{off} were most potent.

Intuitively, the ability of a system to preserve ligand becomes increasingly important at longer time scales, while the initial peak activity becomes less important. One might therefore expect the relative importance of these two competing factors (signaling efficiently and degradation avoidance) to be governed by the time scale over which one wishes to achieve high potency. Stronger binders (slower off rates) should be favored if short-term potency is desired, while weaker binders (faster off rates) should be favored if long-term potency is desired. To test this hypothesis, we varied the length of time over which our model was simulated and determined at which sampled k_{off} value the instantaneous and integrated activities achieved their maxima as a function of the time simulated. Fig. 3d is a plot of these optimal k_{off} values as a function of assay time. As expected, the k_{off} that generated the maximal activities was an increasing function of the length of time over which potency is desired.

Taken together, the data in Fig. 3 show that in a 10-day simulation, there was an optimal value of k_{off} , for both instantaneous and integrated long-term activity, that was in the middle of the biochemically reasonable range. The tension between remaining bound long enough to generate reasonable signal in the short term and binding briefly enough to avoid internalization and degradation in the long term led to these intermediate optimal values. Values too small led to too much degraded ligand, and values too large did not produce enough signal per ligand molecule, even though nearly all the ligand was intact.

3.3 Exploring Optimal Binding Kinetics in Parameter Space

Thus far we have kept parameters fixed at their base values and have varied only k_{off} . We found that two opposing factors yielded a peak in potency at an intermediate k_{off} value, when the base parameters were used. We now describe how this intermediate, optimal value varied as we explored the parameter space of our model more thoroughly. Doing so provides

insight into how the characteristics of a potent ligand depend on the particular properties of a system, which in turn might allow a ligand for a certain system to be optimized for potency.

As a first step, we performed a series of simulations in which the sampled k_{off} that yielded maximum instantaneous and integrated activities at 15,000 minutes was found as a function of one other varying parameter, with all other parameters fixed at their base values. For ease of presentation, we refer to the sampled k_{off} 's that yielded maximal potencies as “optimal” k_{off} 's, even though they were not found by rigorous optimization procedures. The results are shown in Fig. 4.

Fig. 4a shows that the optimal k_{off} steadily increased as the activation rate constant for complexed receptor, k_{sigf} , was increased. When activation of a ligand-bound receptor occurred quickly, maximal potency was achieved with a worse-binding ligand than when activation was slow. The opposite trend was observed as the deactivation rate constant k_{sigr} was varied; systems with a faster deactivation rate required tighter binding (slower dissociation) for maximal potency, as shown in Fig. 4b. Taken together, these data suggest that systems where activation is fast and easy to maintain once ligand is bound prefer weak binders; in these systems, the weak binders will still be able to signal effectively, but they have the added advantage of escaping internalization and degradation as well.

Fig. 4c shows that optimal k_{off} decreased almost sigmoidally as the initial ligand concentration L_0 was increased. The opposite trend was seen as the surface receptor concentration R_0 was increased (Fig. 4d). These two results suggest that systems in which ligand is scarce with respect to receptor will prefer weaker binders because maintaining intact ligand is more important for such systems than maximizing the short-term activity generated by each ligand.

As the association rate constant k_{on} was increased, so did the optimal k_{off} (Fig. 4e), although the correlation was far from linear, corroborating the idea that individual kinetic rate constants play an important role in determining potency, and modulating potency by considering only the thermodynamics of binding is a less effective strategy. In fact, the dependence is almost sigmoidal, with a region in which the optimal binding affinity worsened quickly (between $\log_{10}(k_{\text{on}}) = 0.5$ and $\log_{10}(k_{\text{on}}) = 2$), followed by one in which optimal binding affinities increased (the slope of the dependence was less than 1). This result suggests that once the off rate is “fast enough” in relation to other parameters in the system, such that ligand degradation is adequately small, tightly-binding ligands begin to be favored.

Fig. 4f shows that the optimal k_{off} decreased as the receptor and complex recycling rate constant k_{recrf} was increased. When bound ligand had a greater probability of being recycled, a tighter binder — although it might get internalized more frequently — would likely be recycled. Therefore, tighter binders were preferred in this regime. Finally, as the internalization rate constant k_{intf} was increased, the optimal k_{off} increased as well (Fig. 4g). This is consistent with a picture in which, when internalization occurs quickly upon activation, potency is limited by the system's ability to preserve ligand, so a weaker binder would be preferred.

From the trends seen above, we can infer some general conclusions about how binding kinetics affect potency for systems located in different regions of parameter space. Any system in which ligand has the potential to be very scarce — e.g., one with rapid complex internalization, a low complex recycling rate, or a low initial ligand concentration — favors weaker (rapidly dissociating) binders, so one would expect an optimal off-rate constant that is high in the biologically reasonable range. We will refer to these systems as “ligand-limited.” On the other hand, a system that has poor signaling activity, where the ability to

bind well matters — e.g., one with a slow activation rate, a fast deactivation rate, or a low surface receptor concentration — tends to favor the tighter (slowly dissociating) binders in the space. We will refer to such systems as “activation-limited”. The problem of determining preferred binding properties of a system qualitatively reduces to one of determining whether a system is “ligand-limited” or “activation-limited.”

In our simulations, the parameter that had the most drastic effect on the ability for a weaker binder to improve cytokine potency was the surface receptor concentration (data not shown). In principle, with a high receptor concentration, even a very small ligand concentration can achieve high potency if an individual ligand molecule can transiently bind to and activate multiple receptors, in a sense hopping across a set of surface receptors. This idea is explored further below.

Moreover, as shown in each of the plots in Fig. 4, the optimal k_{off} 's for maximizing integrated activity up until a given time were always less than or equal to those for maximizing instantaneous activity at that time. Strong instantaneous activity at later times requires less degradation, which can be achieved with a faster off rate constant.

In addition to analyzing how the optimal k_{off} varied in the parameter space of our model, we also performed the same analyses for k_{on} , the on rate constant. The range over which k_{on} was varied was the same order of magnitude as that over which k_{off} was varied, and yielded the same range of binding affinities (For data presented here, k_{off} was varied from 10^{-5} to 10^4 min^{-1} while keeping k_{on} fixed at $10^2 \mu\text{M}^{-1}\text{min}^{-1}$, and k_{on} was varied from 10^{-4} to $10^5 \mu\text{M}^{-1}\text{min}^{-1}$ while keeping k_{off} fixed at 10^{-2} min^{-1} ; this yielded a binding affinity range from $100 \mu\text{M}$ to 100 fM for both sets).

Generalities from these simulations will be described first, and then specific data supporting the most important points will be presented. Indeed, for our base parameters, there was an optimal k_{on} in the middle of the biologically reasonable range, but it increased integrated activity by only 3% over the tightest binder in our search space and yielded lower potency than the ligand found through optimizing k_{off} (data not shown). In exploring parameter space, the trends for the optimal k_{on} were often nonexistent or not as strong as those for the optimal k_{off} in general, although when there was a definitive trend, it served to alter the binding affinity in the same way as the corresponding trend for k_{off} . However, in nearly all cases, the optimal affinity when varying k_{on} was higher than that when varying k_{off} , as Fig. 5 shows; weaker binders were generally not as potent when affinities were lost via k_{on} . The very few exceptions will be explored further below. Overall, we found that altering affinity via k_{off} as opposed to k_{on} yielded more potent ligands most of the time, especially in ligand-limited regimes, when the optimal affinity for a given set of parameters was weak.

Fig. 6 shows one sample result from our k_{on} analysis — the optimal k_{on} as a function of varying k_{sigf} . When signaling was slow, a tighter binder was favored, and when signaling was fast, a slightly weaker binder was favored. Fig. 6b and c plot the log ratio of the optimal instantaneous and integrated activities when k_{off} was varied to that when k_{on} was varied as parameter space was explored. In most of the explored space, varying k_{off} had the potential to generate greater activity than varying k_{on} . The few exceptions to this occurred in regimes in which the system was far more activation-limited than ligand-limited, including low $[R_0]$, high k_{recrf} , and low k_{kintf} . In most cases, these were the regimes in which the very tightest binder in the space was chosen, regardless of whether k_{off} or k_{on} was being varied. The tightest binder in the search space for the optimal k_{on} , while it has the same affinity as that in the k_{off} search space, comes to binding equilibrium much more rapidly. Perhaps the extremely rapid equilibrium is responsible for the slightly larger potencies achieved by varying k_{on} in these small regions of parameter space. This suggests that additional modest

gains might be achieved by varying both k_{on} and k_{off} simultaneously when trying to optimize a particular ligand, as the rate at which equilibrium is achieved plays a role in modulating cytokine potency. The fast overall equilibrium may also explain the handful of points in explored parameter space where optimizing k_{on} for integrated potency actually yielded a weaker binder than optimizing k_{off} (see Fig. 5). These four points lie adjacent to regions in which the tightest binder was chosen in both sets of simulations, in regimes where a somewhat weaker binder that comes to equilibrium more quickly might be more potent than a stronger binder that reaches equilibrium more slowly, so the k_{on} simulations, by picking a somewhat weaker binder in the space, were still able to achieve slightly higher potency than the k_{off} simulations.

Taken together, these results suggest that generally, when maximal activity is achieved through a weakly-binding ligand (i.e., in “ligand-limited” regimes), affinity should be altered by increasing the off rate. Conversely, when maximal activity is achieved through a tightly-binding ligand (i.e., in “activity-limited” regimes), affinity should be altered by increasing the on rate (though the advantage here appears to be smaller). In our study, high potencies could be obtained by optimizing k_{on} , especially in regions where tighter binders are favored, but even higher potencies were often achieved by changing k_{off} . We hypothesized that the major reason for this is that the deactivation rate in the model — unlike the activation rate — did not depend on whether the ligand was bound or not. Physically, this can be realized if activation is caused by the phosphorylation of a critical receptor residue; while binding is necessary for the phosphorylation to initially occur, the receptor remains phosphorylated even if the ligand unbinds. Therefore, once the ligand falls off its receptor, the signal can persist in its absence, and its persistence is inversely proportional to k_{sigr} , which in our model could represent the actions of phosphatases. In this model, therefore, a ligand whose binding affinity is weakened via a faster k_{off} can still achieve high amounts of signal — it binds to the receptors quickly, and as long as it activates them before it falls off, they will remain active for as long as k_{sigr} permits. A ligand whose binding affinity is weakened via a slower k_{on} , however, merely binds more slowly and begins signaling later, but it cannot take advantage of the persistent process of activation, because that only occurs after the binding event has taken place.

To explore the idea that persistent receptor activation is crucial for weak binders to achieve high potency via faster k_{off} 's, we created a parallel model in which the processes of activation and deactivation were thermodynamically connected to binding. One example of this is a system in which a receptor was active only if it was in a certain conformation, and binding ligand greatly stabilized this conformation. Because the free energy of the active, unliganded receptor state is so high, there would not be any active, unliganded receptors. Therefore, signal would essentially never persist when ligand falls off. This idea contrasts with the case in which phosphorylation initiates activation.

Operationally, the parallel model was constructed by deleting the unliganded, active receptor state and the liganded, inactive receptor state from the original model. We also deleted the basal activation rate constant (k_{bsigf}), the complex activation rate constant (k_{sigf}), and the deactivation rate constant (k_{sigr}). When ligand bound to a receptor, activation would automatically occur, and when ligand dissociated from receptor, deactivation would automatically occur. We ran the same analyses on this parallel system as we did on the original system. In contrast to the original system, the optimal k_{off} for integrated activity as a function of every parameter was always the slowest one in the space. In other words, the tightest binder was always the most potent. Moreover, the optimal k_{off} for instantaneous activity was consistently less than or equal to the corresponding optimal value in the original system. Fig. 6d shows a sample result, where initial ligand concentration was varied. The

data demonstrate that the ability for a signal to persist after a ligand unbinds is an important factor in allowing a weak-binding ligand to be potent.

Also, in the parallel model, the optimal values achievable for either instantaneous or integrated activities were less than those from the original model in regions of parameter space that were “ligand-limited”, such as low $[L_0]$, high R_0 , low k_{recf} , and high k_{intf} (Fig. 6e and f), as it is in these regimes that a weaker binder might gain the greatest benefit if signal persists once it unbinds. In regions of parameter space where the tightest binder was chosen — the “activation-limited” regions — the importance of signaling once ligand unbinds is lessened, because ligand either needs to remain bound to generate signal or need not fall off to avoid degradation. Hence, in these regimes, there was no significant difference in the optimal activities attained from the two models (Fig. 6e and f). Finally, we also varied k_{on} in the parallel model to optimize for integrated and instantaneous activity. Interestingly, the optimal activities attained from varying k_{off} were no longer generally higher than those obtained through varying k_{on} (data not shown). In fact, optimal activities attained from varying k_{on} were often higher. In other words, in a system that does not have persistent activation, there is no advantage to altering binding affinity via k_{off} . This result also suggests that the ability for a signal to persist after a ligand unbinds is the reason why modulating affinity via the off rate rather than via the on rate can often give higher potencies in “ligand-limited” regimes.

3.4 Three Control Points in the Model Help Determine Optimal Binding Kinetics

The results of our parameter space exploration are qualitatively summarized in Fig. 7. These results show the dominance of two opposing strategies — directly increasing the amount of signaling-competent complex versus avoiding ligand internalization and degradation. Together this trade-off helps to determine the binding kinetics that yield maximal potency, and the relative importance of these strategies depends strongly on the context of the relative costs and benefits, which is determined by where the particular cytokine–receptor system lies in parameter space. Activation-limited systems prefer more strongly-binding ligands, while ligand-limited systems prefer more weakly-binding ligands. To explore these opposing effects in terms of specific biological processes, we examined three control points in the model — initial activation at the surface, internalization through receptor-mediated endocytosis, and recycling from endosomes back to the medium just outside the cell. Our goal was to develop a deeper sense of how each of these processes affects cytokine potency through individual changes to signaling efficacy or ligand degradation. In the models used here, different receptor systems can be represented by different points in parameter space; therefore, one can imagine classifying a system’s potency by its location in parameter space, as being dominated by one of the three control points.

Control at Initial Activation—There would no longer be opposing factors (signal generation and degradation) if there was no possibility for ligand internalization and degradation — if the only processes in the model were binding, activation, and deactivation. Would the tightest binder now be the most potent in all of parameter space? To find out, we turned off receptor and complex internalization in our model by setting all internalization rate constants to zero. By effectively removing receptor-mediated endocytosis and all downstream processes from the model, we isolated the effects of initial activation on cytokine potency. Note that for these simulations receptor synthesis was set to balance the cell density growth rate (i.e., $R_s \equiv G \times R_0$, where G is the time constant used for growth, defined in Table 1), as this was now the only factor determining surface receptor concentration.

Fig. 8a shows the optimal k_{off} values (for integrated activity) as a function of k_{sigf} , k_{sigr} , and R_0 . Throughout most of this parameter space, the tightest binder was in fact chosen, consistent with expectations. However, at values of high k_{sigf} , low k_{sigr} , and high R_0 , ligands with intermediate values of k_{off} were chosen for maximal potency. Interestingly, intermediate values of the off-rate constant were chosen only when R_0 was at least $10^{-4} \mu\text{mol}/\text{m}^2$. This corresponded to the first sampled point at which the total number of surface receptors could become greater than the total number of ligand molecules, per volume of cell culture, as the density grew. One reason for this result may be that if there are fewer receptor molecules than ligand per volume, then every receptor can be activated by a different ligand, so ligand can theoretically achieve maximal activation by binding as tightly as possible. However, once there is more receptor than ligand, it could be advantageous for ligands to bind more weakly, such that each ligand can hop around and activate multiple receptors. As long as the ligand remains bound long enough to generate signal, and that signal persists after ligand dissociation and rebinding to a new inactive receptor, potency can be increased by such a hopping mechanism. One would therefore expect that the faster the activation rate (or the slower the deactivation rate), the less time a ligand needs to remain bound to generate signal. The results in Fig. 8a corroborate this idea, as the optimal off-rate constant increased with increasing k_{sigf} and with decreasing k_{sigr} in those regimes where R_0 was sufficiently high.

A more complete description of these processes would involve consideration of the spatio-temporal distribution of ligand concentration together with diffusion of ligand and surface-bound receptor (see references [42–44], for example, for a study of this effect in G-protein coupled receptor systems). The description used in the current work is formally appropriate for a well-mixed system, although results from such models generally have a much wider range of applicability. The key notion emerging from the activation control point is that two modes of signal generation are important. When sufficient ligand is available, signal maximization occurs through maximizing the binding of ligand to receptor. With insufficient ligand and sufficient signal persistence, maximum signal results from weak binding and hopping kinetics.

Control at Internalization—The long-term potency of a ligand depends critically on its ability to avoid degradation; reduced internalization is one mechanism that could lead to reduced ligand degradation.

To explore this control point, we ran simulations of our base model and systematically varied k_{sigf} , k_{sigr} , and k_{intf} simultaneously. The receptor concentration was set sufficiently low (to the base value of $8.3 \times 10^{-6} \mu\text{mol}/\text{m}^2$) such that the advantage of hopping (described above) was not dominant; this allowed us to isolate better the effect on the optimal k_{off} that was due to the tension between activating receptors and avoiding internalization.

Fig. 8b shows the optimal k_{off} values (for integrated activity) as a function of k_{sigf} , k_{sigr} , and k_{intf} . For cases in which activation was slow and deactivation was fast, the strongest binder (slowest k_{off}) led to maximal potency. These regions of parameter space may be classified as activation-limited; activity is not persistent in this parameter region, so tight binding is the most effective way to signal.

However, in the region of parameter space where values of k_{intf} and k_{sigf} were high and values of k_{sigr} were low, intermediate values of k_{off} led to maximal potency. This region is more ligand-limited because activity is persistent but internalization occurs quickly enough for ligand degradation to be an important factor in determining ligand potency. The potency of a ligand in a system with fast internalization rate dynamics is strongly affected by control at internalization, especially if the system easily generates and maintains active receptors.

Interestingly, another generalized model [45] showed that while receptor internalization is often viewed as a negative regulatory mechanism (as it is here), it might also be a useful mechanism to improve the response time of the signaling pathway to rapidly varying ligand input. Throughout our work, we are assuming that the desired outcome is significant activity over an extended period of time; if a fast response time is what is desired, the effect of internalization on optimal ligand binding properties may be quite different.

Control at Recycling—Once a receptor or complex is internalized, its fate — whether it is ultimately recycled or degraded — depends on multiple factors. Receptors and complexes might be recycled or degraded at a different rate than ligand, as these degradation processes appear to be regulated by proteins that are specific to particular molecular species (see references [26, 28, 30, 31] for examples). Due to the differing fates of free and bound ligand, this control point can strongly influence ligand potency.

To explore how recycling affects the preferred off-rate constant for ligand binding, our base model was adjusted such that all free ligand was recycled and all free or bound receptors were degraded (previously, all ligand, receptor, and complex species were recycled and degraded at the same rates). We then varied k_{sigf} , k_{sigr} , and k_{intf} exactly as was done previously to generate the plot in Fig. 8b. Fig. 8c is a plot of the resulting optimal k_{off} 's, to be compared to those shown in Fig. 8b. Now, the tightest binder was chosen only in a small corner of explored parameter space and when k_{sigf} was extremely slow (0.01 min^{-1}). The preferred value of k_{off} in parameter space was now generally higher than those found when bound and unbound ligand were recycled at the same rate. These results suggest that a system in which free ligand is preferentially recycled will often prefer more weakly-binding ligands than a system in which all species are recycled and degraded to the same extent. Here, the surface and endosomal binding kinetics were set to be the same. If ligand can preferentially dissociate when internalized in endosomes, tighter surface binding and weaker endosomal binding are more favorable [10]. This idea is further explored below.

3.5 Cellular Models Can Inform the Cytokine Design Process

The purpose of this study was to understand how binding kinetics can affect cytokine potency by systematically exploring the parameter space of a general model. Different cytokine–receptor systems can occupy different regions of parameter space (i.e., be more “activation-limited” or “ligand-limited”), and, as we have begun to map out parameter space, one may use insights gained from this general study to aid in the design of improved therapeutics for specific systems.

To begin to explore specific systems, we chose GCSF and Epo, as they may inhabit somewhat different regions of parameter space for our general model, as explained below, leading to different optimal binding properties.

Using the GCSF-specific parameters described in the Methods section, we ran simulations to determine instantaneous and integrated activity at 15,000 minutes, as a function of k_{off} and k_{sigf} (a currently unknown parameter for these systems), with results shown in Fig. 9a and b. The experimentally measured off-rate constant for GCSF is indicated by the black line in the Figure.

Evidence suggests that internalized receptors and complexes are degraded in the GCSF system [46], and so the control point at recycling plays an extremely important role. Therefore, ligand remaining unbound from receptor in the endosome could have greatly increased potency in the long term. Sarkar and co-workers used this idea to design GCSF mutants that sacrificed endosomal binding while retaining binding affinity at the cell surface in order to increase the potency of GCSF [33]. As the pH in the endosome is lower than that

at the cell surface, they engineered a GCSF mutant that demonstrated greater pH-dependence, such that it bound nearly as well as wild type at the cell surface but worse in the lower-pH endosomal environment. As a result of its poorer endosomal binding, it was recycled 50% more than wild type, yielding a 10-fold increase in ligand half-life and a 50–100% increase in cell proliferation after a week.

By varying only the endosomal k_{off} and keeping the off-rate constant at the cell surface fixed, we simulated the addition of pH-dependent binding. Figs. 9c and d show an analogous simulation to Figs. 9a and b, where now k_{off} was altered only in the endosome. Note that both instantaneous and integrated activity were maximized at higher k_{off} values (lower endosomal binding affinities) than when k_{off} was altered at both the cell surface and in the endosome (Fig. 9a and b). This supports the idea that modulating pH-dependence of GCSF binding can further increase its potency. Because a pH-dependent switch could largely affect the on rate in the endosome, parallel calculations were done in which only endosomal k_{on} was varied. When optimizing for instantaneous activity, weaker binders were consistently preferred (data not shown). When optimizing for integrated activity, however, weaker binders were *not* preferred. Because signaling was allowed to occur from the endosome, greater potency was achieved by modulating k_{off} as opposed to k_{on} perhaps because a faster k_{off} still maintains endosomal activity while a slower k_{on} may not. To test this hypothesis, we disallowed signaling in the endosome and redid the simulations. Now that the priority to signal did not compete with the priority to avoid degradation, the slowest k_{on} in the space — the weakest binder — was chosen in all circumstances (data not shown). This result shows that a ligand's ability to signal from within an endosome can have an important impact on its optimal binding kinetics. Knowing whether endosomal signaling can take place in a specific cytokine system, therefore, is important to quantitative design considerations. Additionally, it has been demonstrated that the endosomal volume in a particular cell system can greatly impact the binding and signaling kinetics in the endosome [47]. Although we did not vary endosomal volume in this study, this is an additional parameter that can impact quantitative predictions.

In addition to GCSF, Epo was also examined for the potential effects of its binding affinity on long-term potency. With Epo-specific parameters described in the Methods section, simulations were run to determine instantaneous and integrated activity at 15,000 minutes as a function of k_{off} and k_{sigf} , with the results shown in Fig. 9e and f. The experimentally measured k_{off} of Epo is indicated by the black line in each plot. Note that even though k_{on} for Epo is higher than k_{on} for GCSF, the optimal Epo k_{off} chosen at every value of k_{sigf} for either objective function was always lower than the corresponding value for GCSF. In other words, tighter binders were consistently preferred in the Epo system. This is in spite of the fact that cells in the Epo model grew at a faster rate than those in the GCSF system, a difference expected to yield a higher cell density on average, leading to faster ligand depletion and a faster optimal off rate. Looking at the two systems, there are three possible reasons for the observed result that our model captures: Epo has a slower measured internalization rate constant, smaller number of receptors per cell, and, in our model, a higher fraction of recycled complexes. These three factors together make the Epo system more activation-limited than ligand-limited, when compared to GCSF. Therefore, an optimal Epo molecule would be a tighter binder than an optimal GCSF molecule.

Nevertheless, Epo may be made more potent by increasing its k_{off} . Data suggest that Epo can support half-maximal growth of cells when less than 10% of cell-surface receptors are activated [18, 20, 41]. This suggests that the instantaneous activity at a given time might be more important than integrated activity for the Epo system — as long as the activity is greater than 10% of the total receptor concentration, Epo could be highly effective, and activating more than 10% of receptors may not necessarily yield additional benefits.

Combining these data with a more specific model for Epo may yield more quantitatively valuable characteristics of an improved cytokine via weaker binding. Indeed, AraNESP [48], a hyperglycosylated form of Epo with reduced binding affinity — a 5-fold slower k_{on} and a nearly 2-fold faster k_{off} [37], was shown to remain intact longer than Epo. Though it was less effective in the short term than Epo, its overall effectiveness was greater than Epo over a 10-day experiment [49]. While it is certainly possible that the long-term potency of NESP may be due to other factors regarding its circulation in the body besides the trafficking processes studied here (Steve Elliott, personal communication), the models suggest a partial role for trafficking.

4 Discussion

We have shown that the insights gained from exploring the parameter space of a simple model can provide a conceptual framework that can aid in the analysis of cytokine binding, activation, and trafficking kinetics and in design of improved cytokines, using GCSF and Epo as illustrative examples. Of course, more detailed and validated models are necessary for high accuracy predictions, but the trends explored here are likely to be robust. Also, it should be noted that our current model will be most valid for understanding outcomes in cell culture experiments, as the base parameters have been obtained from *in vitro* systems. Consequently, this model would need several modifications in order to quantitatively model analogous systems *in vivo*. Nevertheless, we expect that many of the qualitative trends and the insight presented here to be transferable between *in vitro* and *in vivo* systems, as the tensions between activating receptor and maintaining intact ligand at each of the control points will still be of critical importance *in vivo*.

With the exception of our explorations of the three specific control points in the model, we generally examined the optimal kinetic constants as a function of only one varying parameter, with the others fixed at their base values. In fact, by sampling some points in parameter space where multiple parameters were varied, we found that there is often coupling between parameters, and certain trends disappear or even reverse, especially in regions near the extremes of the multidimensional parameter space (see, for example, certain extreme regions of the parameter space plotted in Fig. 8b, such as the slight reversal of the dependence of optimal k_{off} values on k_{intf} when the latter is high, at either very low or very high k_{sigf} and k_{sigr} values). Future work, including more simulations, will be useful to build intuition about the coupling between different parameters in various regimes of parameter space.

As a first step to looking at some of the details that might be more or less specific to particular systems, we have examined the effects of changing certain aspects of the network topology, such as disallowing signaling in the endosome (discussed above) and allowing inactive complexes to be endocytosed at the same rate as active ones. Quantitative results were different with these systems, but the qualitative ideas presented here remained essentially the same. We also tried to prevent receptor depletion at the cell surface by introducing a negative feedback term that increased receptor synthesis if surface receptor concentration dropped below its basal level. This change increased the value of the optimal off-rate constant for our base parameters (results not shown) and was consistent with the observation, shown in the Results section, that optimal k_{off} increased with increasing receptor concentration. It has been shown that receptor depletion at the cell surface is a major regulatory mechanism in the Epo system [50]. Accurately modeling the control on receptor synthesis in Epo will therefore be important for quantitative prediction of more potent binding kinetics.

The importance of pH-dependent binding toward altered endosomal kinetics was explored in our analysis of the GCSF system. Experimental work on the EGF/transforming growth factor α (TGF- α) systems [51, 52] has also shown the strong dependence of cytokine and growth factor potency on both the system-specific determinants of ligand and receptor endosomal sorting and the relative ligand and receptor concentrations. In their system, however, receptor degradation is enhanced by ligand occupancy in the endosome, and ligand degradation can be enhanced by remaining *unbound* in the endosome, due to the steady, non-saturable, fluid-phase mechanism of degradation [51]. These effects would produce a different model than that used for GCSF, one which would suggest that dissociating from the endosome could increase ligand degradation. Indeed, it was shown that TGF- α , which preferentially dissociates from the EGF receptor at endosomal pH, can be less potent than EGF under ligand-limited circumstances, with the opposite being true in receptor-limited circumstances [52]. This suggests that in certain systems, “ligand-limited” regimes may actually favor ligands that remain bound within the endosome, should they be internalized.

In the work presented here, a 1:1 stoichiometry between ligand and receptor was assumed. Preliminary variants of the model were created to treat the binding stoichiometries and mechanisms of specific systems (GCSF and Epo, in particular, in which the binding stoichiometries between ligand and receptor are 1:2 and 2:2, respectively). Quantitative activity depended on the ligand–monomer relative binding affinities and on potential cooperative binding mechanisms within a system, and further study of these dependencies could be the focus of future work. Further fine-tuning of our model to include varied binding stoichiometries, sequential binding of a ligand to two receptor monomers, cooperative binding, ligand dimerization, or all variations should be a fairly straightforward extension involving adding states and reactions into the model to account for all possible initial species, final complexes, and intermediate complexes. As always, the accuracy of such a model would depend on the accuracy of the kinetic rate parameters of each possible reaction in the model. Indeed, previous modeling efforts have accounted for the mechanistic details of specific ligand–receptor interactions. For example, models of the complex ErbB signaling pathways have accounted for the reversible formation of multiple combinations of ligand-dependent dimers between the various members of the ErbB receptor family [12, 16]. Due to the potentially nonlinear dependence of the number of states and parameters on the topology of the model in such complex systems, simplifying assumptions often have to be made as to the mechanisms of activation or the trafficking kinetics of each receptor dimer. As another example, systems with a 1:2 ligand:receptor stoichiometry that have one high-affinity site and one lower-affinity site may show a peaked dose response curve because 1:1 inactive complexes are favored at higher ligand concentrations [53–55]. In a model that explicitly considered the sequential binding of the 1:2 human growth hormone (hGH) – hGH receptor complex [53], modest increased activity in the hGH system could be realized by *increasing* ligand–receptor affinity via the on rate through mutation (no gains could be made by decreasing the off rate). These findings are in agreement with the principles demonstrated here when one recognizes that the model assumed constant ligand concentration and used an off rate that was similar to the internalization rate, such that the system was far more activation-limited than ligand-limited. Therefore tighter-binding ligands would be favored, with the on rate being the primary mechanism to achieve increased potency in such situations, as was also found here. Moreover, activation was not modeled as a separate event from ligand–receptor binding, so that the consequences of persistent receptor activation, as defined in this work, would not be observed.

Receptor desensitization is another regulatory mechanism that is believed to occur in certain systems, including interleukin-5 (IL-5), interleukin-3 (IL-3), and granulocyte macrophage colony stimulating factor (GM-CSF) [56]. Note that desensitization is different from our idea of deactivation, in that ligand rebinding to a desensitized receptor cannot re-activate it

unless the receptor is resensitized, if that is possible. For example, desensitization may involve the phosphorylation of a receptor such that it cannot become active even if ligand binds, as opposed to deactivation, which may involve removal of the activating phosphate groups by phosphatases. Desensitization can also involve the specific degradation of intracellular portions of the receptor responsible for signal transduction [56]. As a first step to exploring the effect of desensitization, we altered our model to allow for active receptors and complexes at the surface to desensitize, after which they could still bind to and dissociate from ligand but not become active until they were sensitized. Preliminary results showed that optimal activity decreased when the desensitization rate increased and also that, like the trend seen for k_{sig} , as the desensitization rate increased, the optimal k_{off} decreased because the system became more activation-limited than ligand-limited (data not shown). The potentially interesting interplay between deactivation and desensitization, both of which are mechanisms to regulate activity, will call for more rigorous future study. Also, in specific systems, mechanisms for either receptor desensitization or dephosphorylation may be location-specific; for example, modeling of the ErbB system suggested that phosphatase activity was localized in the endosomal compartment, assuming that receptor phosphorylation was localized to the surface [12]; such location-specific processes must be accounted for in a model tailored for a specific system.

In the results shown here, the starting and ending cell densities used in the modeled experiments were not explicitly varied. It has been shown elsewhere [57] that cell density can greatly impact the dose responses of ligands, and that the ratio of ligand and surface receptor numbers per cell can control the response. In our work, parallel simulations were run with differing cell densities, and it was found that higher cell densities yield weaker optimal binding affinities. A higher cell density causes ligand to be depleted at a faster rate because more cells can internalize and degrade ligand, even when activity, as measured by active receptors per unit surface area, remains the same. Hence, a system with a higher cell density is more ligand-limited, and weaker binders will tend to result in greater long-term potency. Of course, an *in vivo* or highly-tuned experimental model should account for the dependence of the cell density on the activity of the system, much like the pharmacokinetic model of Sarkar *et al.* [10]. Given that a very high cell density depletes ligand quickly, one interesting question for further study is whether there is an optimal dependence of the growth on activity that will maximize long-term survival of the cells; if the density grows too quickly, the ligand might be depleted too quickly, resulting in poor long-term survival. But if the cell density grows too slowly as a function of activity, the cells might not reach the proper density needed for survival. The current model can be extended to address these questions.

The idea of an optimal binding affinity has been put forth in various systems. For example, the balance between serial triggering (to activate as many TCR's) and kinetic proofreading (to ensure specificity) [58] for endogenous peptide and major histocompatibility complex molecules binding to T-cell receptors has been predicted to give rise to an optimal half-life for such species [59], and internalization and degradation also can effect the dynamic behavior [60]. Goldstein *et al.* provide a review of models of such systems that give rise to optimal binding kinetics [61]. As described above, the idea of optimal cytokine binding has also been proposed for systems such as IL-2 [7] and EGF [27]. Here we have performed a detailed analysis of how the trade-offs leading to maximal potency behave as a function of many relevant parameters in cytokine systems. As more parameters become experimentally determined and the details of specific systems are further understood, abstracted models may soon be quantitative enough to allow for the prediction of truly "optimal" molecules. Even once this accuracy is achieved, it may still be difficult to rationally engineer molecules with specifically altered on or off rate constants. On rates have been rationally altered via changing long-range electrostatic interactions [62, 63], and off rates may be potentially

changed by interfering with close-range van der Waals interactions. While the ability to tailor ligand binding kinetics is still an emerging field, there has been rapid progress. Integrating cellular-level models in the manner described here along with emerging techniques in molecular engineering will prove to be an extremely powerful method to design potent therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Epo	erythropoietin
EpoR	erythropoietin receptor
GCSF	granulocyte-colony stimulating factor
hGH	human growth hormone
IL-2	interleukin-2
ODE	ordinary differential equation
TGF	transforming growth factor

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Figure 1. Pictorial representation of the model used in this work. Ligands are represented as red circles and receptors as blue ovals. The base value for each rate constant is given in Table 1.

Figure 2.

Time courses (in black) of quantities obtained from running the model with base parameters (Table 1). Also, a comparison of time courses using a slower k_{off} (red), and a faster k_{off} (blue) is shown. The base k_{off} was 10^{-2} min^{-1} , and the faster and slower k_{off} 's were two $\log_{10}$ units larger and smaller, respectively. (a) cell density (b) total ligand (including degraded and lost) (c) active receptor (d) integrated activity (e) intact ligand (f) internalized ligand (g) total surface receptor.

Figure 3.

(a) Instantaneous activity, in $\frac{\mu\text{M}}{\text{m}^2}$, at 15,000 minutes, as a function of k_{off} , with other parameters at their base values. There is a very pronounced maximum at an intermediate value of k_{off} . (b) Integrated activity at 15,000 minutes, in $\frac{\mu\text{M}}{\text{m}^2}$, as a function of k_{off} . The value also reaches a maximum, although it is less pronounced. (c) Fraction of ligand intact after 15,000 minutes (blue) and peak signal achieved (green) as a function of k_{off} (To generate these curves, points were sampled at 0.125 $\log_{10}$ units). (d) The sampled k_{off} yielding maximal instantaneous (red) and integrated (blue) activity is plotted as a function of the time simulated.

Figure 4.

Dependence of the optimal k_{off} on various parameters in the model, with all other parameters set to default values. In each plot, the $\log_{10}(\text{optimal } k_{\text{off}})$ is plotted against $\log_{10}(\text{parameter})$. The two lines in each graph correspond to: optimizing for integrated activity at 15,000 minutes (red pluses), and optimizing for instantaneous activity at 15,000 minutes (red circles).

Figure 5.

(a),(b) The log ratio of the optimal K'_D 's for maximal instantaneous (part a) and integrated (part b) activities at 15,000 minutes when k_{off} was varied to that when k_{on} was varied. Red bars are regions of parameter space where varying k_{off} yielded a more weakly-binding optimum than varying k_{on} over the same binding affinity range.

Figure 6.

(a) Optimal on rate as a function of k_{sigf} [blue pluses (circles) correspond to optimizing for instantaneous (integrated) activity]. (b),(c) the log ratio of the optimal achievable instantaneous or integrated activities at 15,000 minutes when k_{off} was varied to that when k_{on} was varied. Red bars are regions of parameter space where varying k_{off} yielded greater potency than varying k_{on} over the same binding affinity range. (d) optimal k_{off} vs. initial ligand concentration in a parallel model where activation was via a reversible, binding-dependent change instead of a persistent process. The data in (a) and (d) are presented in the same manner as that in Fig. 4. (e),(f) the log ratio of the optimal achievable instantaneous/integrated activities at 15,000 minutes between the original model and the parallel model where signal was not allowed to persist once ligand unbinds.

Figure 7.

A qualitative summary of how optimal binding affinities (generally modulated via k_{off}) varied with parameters in our model. Conditions listed under the “low affinity” column are those for which a weaker binder is expected to be more potent, with conditions under the “high affinity” column behaving oppositely. It can be assumed that a low value of any of these parameters also yields the opposite result. Parameters that were tested and discussed but for which direct data are not shown here are marked with an asterisk.

Figure 8.

Representations of portions of parameter space where the optimal off rate is affected at each of three control points in the model: (a) initial activation, (b) internalization, and (c) recycling. The model was modified, if necessary, to partly isolate or highlight each of these control points, (as described Section 3.4), in order to relate the specific functional processes with their potential effects on optimal binding kinetics. The color at each point in the space designates the $\log_{10}(k_{\text{off}})$ that maximized integrated activity as a function of three parameters that are relevant to that functional process within the model. These optimal values are colored according to the color bars shown.

Figure 9.

(a) Activity and (b) Integrated activity at 15,000 minutes as a function of k_{off} and k_{sigf} for GCSF-like parameters. (c) Activity and (d) Integrated activity at 15,000 minutes as a function of endosomal k_{off} and k_{sigf} for GCSF-like parameters. Here, the surface k_{off} was fixed at its experimental value. (e) Activity and (f) Integrated activity at 15,000 minutes vs. k_{off} and k_{sigf} for Epo-like parameters. In all plots, the experimentally measured k_{off} (at cell surface pH) is indicated by the black line.

Table 1

Base parameters used in the model. Parameters were systematically varied around these values.

param.	Description	Units	Base value
L_0	initial ligand concentration	μM	1×10^{-4}
R_0	initial receptor concentration	$\mu\text{mol m}^{-2}$	8.3×10^{-6}
RI_0	initial internalized receptor concentration	$\mu\text{mol m}^{-2}$	$k_{\text{bint}} \frac{(R_0)}{k_{\text{recrf}} + k_{\text{degrf}}}$
R_s	receptor synthesis rate	$\mu\text{mol m}^{-2} \text{min}^{-1}$	$k_{\text{bint}}(R_0) - k_{\text{recrf}}(RI_0)$
k_{on}	surface/endosomal association rate constant	$\mu\text{M}^{-1} \text{min}^{-1}$	100
k_{off}	surface/endosomal dissociation rate constant	min^{-1}	0.01
k_{bsigf}	basal activation rate constant, unliganded receptor	min^{-1}	1×10^{-6}
k_{sigf}	activation rate constant, liganded receptor	min^{-1}	100
k_{sigr}	deactivation rate constant, active receptor	min^{-1}	0.1
k_{bintf}	basal internalization rate constant, inactive receptors/complexes	min^{-1}	0.005
k_{intf}	internalization rate constant, active receptor/complex	min^{-1}	0.1
k_{degrf}	degradation rate constant of receptor/complex	min^{-1}	0.2
k_{recrf}	recycling rate constant of receptor/complex	min^{-1}	0.2
k_{deglf}	degradation rate constant of free ligand	min^{-1}	0.2
k_{reclf}	recycling rate constant of free ligand	min^{-1}	0.2
k_{lagf}	initial degradation pathway sorting rate constant	min^{-1}	10
k_{lagdf}	degradation lag constant (9 steps to create lag)	min^{-1}	0.5
SA	Average surface area of cells	m^2	1×10^{-9}
D_0	initial cell density	cells L^{-1}	5×10^8
G	time constant used for growth	min^{-1}	4×10^{-4}
V_e	volume of endosome	L cell^{-1}	1×10^{-14}

Table 2

Parameters specified in the GCSF-specific model. Most parameters were taken from Refs. [10] and [40] (see text). The on rate constant in the endosome was adjusted to account for the 85-fold weaker affinity in the endosome [10]

param.	Description	Units	Base value
L_0	initial ligand concentration	μM	1×10^{-4}
R_0	initial receptor concentration	$\frac{\mu\text{mol}}{\text{m}^2}$	8.3×10^{-6}
RI_0	initial internalized receptor concentration	$\frac{\mu\text{mol}}{\text{m}^2}$	$k_{\text{bint}} \frac{[R_0]}{k_{\text{recrf}} + k_{\text{degrf}}}$
R_s	receptor synthesis rate	$\frac{\mu\text{mol}}{\text{m}^2 \cdot \text{min}}$	$k_{\text{bint}} - k_{\text{recrf}} [RI_0]$
k_{on}	surface association rate constant	$\mu\text{M}^{-1} \text{min}^{-1}$	111
k_{on}	endosomal association rate constant	$\mu\text{M}^{-1} \text{min}^{-1}$	111/85
k_{off}	surface/endosomal dissociation rate constant	min^{-1}	0.03
k_{bsigf}	basal activation rate constant, unliganded receptor	min^{-1}	1.00×10^{-6}
k_{sigr}	deactivation rate constant, active receptor	min^{-1}	0.1
k_{bintf}	basal internalization rate constant, inactive receptors/complexes	min^{-1}	0.005
k_{intf}	internalization rate constant, active receptor/complex	min^{-1}	0.104
k_{degrf}	degradation rate constant of receptor/complex	min^{-1}	0.059
k_{recrf}	recycling rate constant of receptor/complex	min^{-1}	0.0
k_{deglf}	degradation rate constant of free ligand	min^{-1}	0.0
k_{reclf}	recycling rate constant of free ligand	min^{-1}	0.15
k_{lagf}	initial degradation pathway sorting rate constant	min^{-1}	10
k_{lagdf}	degradation lag constant (9 steps to create lag)	min^{-1}	10
SA	Average surface area of cells	m^2	1.00×10^{-9}
D_0	initial cell density	$\frac{\text{cells}}{\text{L}}$	1.25×10^8
G	time constant used for growth	min^{-1}	1.9×10^{-4}
V_e	volume of endosome	$\frac{\text{L}}{\text{cell}}$	1.00×10^{-14}

Table 3

Parameters specified in the Epo-specific model. Most parameters were taken from Ref. [37] (see text).

param.	Description	Units	Base value
L_0	initial ligand concentration	μM	1×10^{-4}
R_0	initial receptor concentration	$\frac{\mu\text{mol}}{\text{m}^2}$	3.3×10^{-6}
RI_0	initial internalized receptor concentration	$\frac{\mu\text{mol}}{\text{m}^2}$	$k_{\text{bint}} \frac{[R_0]}{k_{\text{recrf}} + k_{\text{degrf}}}$
R_s	receptor synthesis rate	$\frac{\mu\text{mol}}{\text{m}^2 \cdot \text{min}}$	$k_{\text{bint}} - k_{\text{recrf}} [RI_0]$
k_{on}	surface/endosomal association rate constant	$\mu\text{M}^{-1} \text{min}^{-1}$	480
k_{off}	surface/endosomal dissociation rate constant	min^{-1}	0.01
k_{bsigr}	basal activation rate constant, unliganded receptor	min^{-1}	1.00×10^{-6}
k_{sigr}	deactivation rate constant, active receptor	min^{-1}	0.1
k_{bintf}	basal internalization rate constant, inactive receptors/complexes	min^{-1}	0.005
k_{intf}	internalization rate constant, active receptor/complex	min^{-1}	0.06
k_{degrf}	degradation rate constant of receptor/complex	min^{-1}	0.204
k_{recrf}	recycling rate constant of receptor/complex	min^{-1}	0.347
k_{deglf}	degradation rate constant of free ligand	min^{-1}	0.204
k_{reclf}	recycling rate constant of free ligand	min^{-1}	0.347
k_{lagf}	initial degradation pathway sorting rate constant	min^{-1}	10
k_{lagdf}	degradation lag constant (9 steps to create lag)	min^{-1}	0.5
SA	Average surface area of cells	m^2	1.00×10^{-9}
D_0	initial cell density	$\frac{\text{cells}}{\text{L}}$	5×10^8
G	time constant used for growth	min^{-1}	4×10^{-4}
V_e	volume of endosome	$\frac{\text{L}}{\text{cell}}$	1.00×10^{-14}