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Multifarious Determinants of Cytokine Receptor Signaling Specificity

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Abstract

Cytokines play crucial roles in regulating immune homeostasis. Two important characteristics of most cytokines are pleiotropy, defined as the ability of one cytokine to exhibit diverse functionalities, and redundancy, defined as the ability of multiple cytokines to exert overlapping activities. Identifying the determinants for unique cellular responses to cytokines in the face of shared receptor usage, pleiotropy, and redundancy will be essential in order to harness the potential of cytokines as therapeutics. Here, we discuss the biophysical (ligand–receptor geometry and affinity) and cellular (receptor trafficking and intracellular abundance of signaling molecules) parameters that contribute to the specificity of cytokine bioactivities. Whereas the role of extracellular ternary complex geometry in cytokine-induced signaling is still not completely elucidated, cytokine–receptor affinity is known to impact signaling through modulation of the stability and kinetics of ternary complex formation. Receptor trafficking also plays an important and likely underappreciated role in the diversification of cytokine bioactivities but it has been challenging to experimentally probe trafficking effects. We also review recent efforts to quantify levels of intracellular signaling components, as second messenger abundance can affect cytokine-induced bioactivities both quantitatively and qualitatively. We conclude by discussing the application of protein engineering to develop therapeutically relevant cytokines with reduced pleiotropy and redirected biological functionalities.

1. INTRODUCTION

Four-helical cytokines are secreted proteins that regulate most facets of immune function and numerous other aspects of mammalian physiology (Bazan, 1989, 1990). Cytokines exert their biological activities by inducing cell surface receptor dimerization in either homo- or

hetero-oligomeric assemblies (Stroud & Wells, 2004; Wang, Lupardus, Laporte, & Garcia, 2009; Watowich, Hilton, & Lodish, 1994). In the canonical cytokine signaling pathway, assembly of the cytokine-receptor complex activates tyrosine kinases of the Janus Kinase (Jak) and Tyk2 family, which are constitutively bound to receptors (Ihle, Witthuhn, Quelle, Yamamoto, & Silvennoinen, 1995). Jaks, in turn, phosphorylate and activate Signal Transducer and Activator of Transcription (STAT) transcription factors (Levy & Darnell, 2002; Schindler, Hoey, & McKnight, 1996) to modulate gene expression and, ultimately, determine cell fate (Murray, 2007; O'Shea & Plenge, 2012). In addition to their signaling through the Jak/STAT pathway, some cytokines can also activate the Akt and Erk pathways (Platanias, 2005), as well as other signaling networks (Gough, Levy, Johnstone, & Clarke, 2008; Heinrich et al., 2003; Malek, 2008; Schindler, Levy, & Decker, 2007; van Boxel-Dezaire, Rani, & Stark, 2006).

It has been well established that cytokines exhibit two features: (1) pleiotropy, the capacity of one cytokine to elicit a multitude of diverse functional responses; and (2) redundancy, the ability of multiple different cytokines to effect overlapping activities (Ozaki & Leonard, 2002). The properties of pleiotropy and redundancy emanate from the degenerate nature of cytokine complexes. A single cytokine may engage more than one receptor complex to activate distinct sets of Jaks and STATs, leading to diverse functional effects (Zurawski, Vega, Huyghe, & Zurawski, 1993). Receptor subunits may also be shared between several cytokines, and the limited number of Jak (four) and STAT (seven) proteins results in redundant activation of Jak/STAT combinations by distinct cytokine complexes (Pestka, Krause, Sarkar, et al., 2004; Pestka, Krause, & Walter, 2004; Vignali & Kuchroo, 2012). It is, however, remarkable that despite using such a seemingly constrained set of signaling proteins in a finite number of combinations, cytokines are still able to promote a broad range of activities and to regulate a highly complex immune system (Delgoffe, Murray, & Vignali, 2011). Our understanding of the multifarious mechanisms through which cytokines affect such a diverse range of biological activities remains incomplete, and we have yet to illuminate the details of how functional specificity is achieved given the rampant redundancy and pleiotropy of cytokines. What we do know is that there is no clear correlation between the particular signaling molecule that is activated and the bioactivity that results. For instance, although the cytokines Interleukin (IL)-6 and IL-10 both activate STAT3, their roles in immune regulation are diametrically opposed, with IL-10 eliciting an anti-inflammatory and IL-6 eliciting a proinflammatory response (Hunter & Kastelein, 2008; Mosser & Zhang, 2008; Murray, 2007). Another example of divergent functional outcomes being transmitted through a shared signaling molecule is found in the type I interferon (IFN) system in which more than 16 subtypes engage an identical receptor complex yet elicit distinct biological activities (Borden et al., 2007; Piehler, Thomas, Christopher Garcia, & Schreiber, 2012). Gaining insight into the molecular mechanisms that underlie this observed functional specificity will greatly advance our understanding of cytokine biology and immune regulation. In this review, we summarize findings that demonstrate the importance of biophysical (ligand–receptor binding kinetics and complex stability) and cellular (receptor trafficking and abundance or localization of signaling molecules) parameters in the determination and diversification of cytokine activities. We also discuss recent examples of

how these parameters can be used to modulate cytokine activities through implementation of biomolecular engineering techniques.

2. LIGAND–RECEPTOR COMPLEX FORMATION: GEOMETRY AND AFFINITY

Signal activation is initiated when cytokines engage the extracellular domains of their cognate receptors. Many receptor subunits are shared by multiple cytokines but paired with different receptor chains to form unique signaling complexes (Liao, Lin, & Leonard, 2011; Pestka, Krause, & Walter, 2004; Wang et al., 2009). For example, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 share the common γ chain (γ_c) (Rochman, Spolski, & Leonard, 2009); IL-6, IL-11, leukemia-inducible factor, oncostatin M (OSM), and ciliary neurotrophic factor (CNTF) share the gp130 receptor (Boulanger, Bankovich, Kortemme, Baker, & Garcia, 2003; Silver & Hunter, 2010); and granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and IL-3 share the common β chain (β_c) (Bagley, Woodcock, Hercus, Shannon, & Lopez, 1995; Tavernier et al., 1991; Wang et al., 2009). In some instances, identical receptor complexes are shared by multiple cytokines which induce distinct biological activities. This is the case for type I IFNs, as well as for the two cytokines IL-4 and IL-13, which signal through heterodimeric complexes composed of IL-4R α , γ_c , and IL-13R α 1 (Chomarat & Banchereau, 1998; Junttila et al., 2008; Uze, Schreiber, Piehler, & Pellegrini, 2007). Given their shared usage of receptor subunits, it has been proposed that parameters such as the geometry and affinity of the ligand–receptor complex may account for functional diversity of these cytokines (LaPorte et al., 2008). The role of the inter-subunit geometry of the ligand–receptor complex has been extensively studied but there is still no consensus on whether or not modulation of receptor–ligand architectures can influence downstream signal activation (Ballinger & Wells, 1998). On the other hand, the importance of the ligand–receptor binding affinity in quantitative and possibly qualitative aspects of downstream signaling events has been well established (Pang, Qin, & Zhou, 2011; Piehler et al., 2012). Affinity impacts the kinetics of complex formation as well as the stability of the cytokine–receptor complex, thereby tuning both the strength and duration of signal activation.

2.1. Surface receptor complex geometry and signal activation

As more cytokine–receptor ECD complexes have been solved in recent years, it has become apparent that these complexes exhibit a diverse range of molecular architectures, yet all of these structural orientations result in activation of the Jak/STAT pathway (Boulanger, Chow, Brevnova, & Garcia, 2003; de Vos, Ultsch, & Kossiakoff, 1992; Hansen et al., 2008; Jones, Logsdon, & Walter, 2008; LaPorte et al., 2008; Livnah et al., 1996; Livnah et al., 1999; Logsdon, Deshpande, Harris, Rajashankar, & Walter, 2012; McElroy, Dohm, & Walsh, 2009; Rickert, Wang, Boulanger, Goriatcheva, & Garcia, 2005; Ring et al., 2012; Syed et al., 1998; Thomas et al., 2011, 2012; Walter et al., 1995; Wang, Rickert, & Garcia, 2005). The tolerance of signal activation for variable binding topologies raises the question of whether mere dimerization of receptors is sufficient for the activation of downstream signaling (i.e., dimerization serves as a binary “on/off” switch) or whether particular receptor geometries serve instructive roles in determining the degree and nature of receptor activation.

Several early studies suggested that the structural constraints on dimerization are quite relaxed, since grafting of the extracellular domain (ECDs) of one receptor onto the intracellular domain (ICD) of an unrelated receptor resulted in functional signal activation of the chimeric molecule. As one of many such examples, replacing the ECD of the erythropoietin (Epo) receptor (EpoR) with those of epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), or c-Kit produces an active construct that induces STAT5 phosphorylation and Ba/F3 proliferation in a ligand-dependent manner (Ohashi, Maruyama, Liu, & Yoshimura, 1994). Similarly, fusion of the gp130 ICD to EpoR ECD leads to Epo-mediated activation of STAT1 and STAT3, consistent with activation of the full-length gp130 by its native ligands (Kawahara et al., 2006; Schaeffer et al., 2001). Other examples of receptor ectodomain swapping have been reported for the type I IFN (Pattyn et al., 1999) and IL-4/IL-13 (Fujiwara, Hanissian, Tsytyskova, & Geha, 1997; Heller et al., 2012) systems, and in all cases, the chimeric receptors elicit some extent of activation of the expected Jak/STAT signaling molecules. These results suggest that cytokine signal activation is quite permissive with respect to complex dimer geometry. However, the confounding factor in these signaling studies is that they were performed in cell lines expressing high levels of chimeric receptors and conducted with elevated doses of ligand, thereby limiting their physiological significance. *In vivo*, the consequences of cytokine action are tightly regulated by the relative abundance of various cell types whose sensitivities to stimulation are governed by receptor expression levels. Thus, while *in vitro* signaling of a chimeric receptor may appear to be qualitatively similar to that of wild-type (wt) receptor in terms of STAT activation patterns (which is not surprising given that the ICDs used in the chimeric receptors are identical to those of the wild-type receptors), these studies offer an incomplete perspective of cytokine behavior by failing to account for the finely tuned balance of receptor expression levels maintained by the diverse cell types that exists *in vivo*. In order to paint a clearer picture of the activity of these chimeric receptors, quantitative studies must be undertaken in which the EC₅₀ and E_{max} values of induced STAT activation are directly compared to those of wt receptors for a range of cell types. In addition, sophisticated *in vivo* models must be developed to examine the broader functional consequences of chimeric receptor signaling.

In apparent contrast to the architectural promiscuity observed in chimeric receptor studies, other experimental findings have presented evidence that geometry of the surface complex plays a crucial role in determining the nature and extent of the transmitted signal. It appears that certain orientations of the Epo and thrombopoietin (Tpo) complexes are more likely to activate signaling than others. For these studies, the authors substituted the EpoR ECD with the coiled-coil dimerization domain of the yeast transcription factor *Put3* (Swaminathan, Flynn, Reece, & Marmorstein, 1997), inducing constitutive dimerization of the receptor and thus activating downstream signaling in a ligand-independent manner. The rotational orientation of the EpoR ICD was then systematically modulated by inserting alanine residues into the juxtamembrane region, which was presumed to be helical such that each residue insertion would be predicted to rotate the helix by approximately one-third of a turn. Some alanine insertions are not compatible with signal activation, other insertions lead to wt-like signal activation, and yet another group of insertions preferentially activated one of the two downstream signaling pathways (Constantinescu, Huang, Nam, & Lodish, 2001;

Stærk et al., 2011). These findings suggest that there are preferred rotational orientations for signaling, but in the absence of direct structural information these conclusions remain speculative. Nevertheless, the results of these EpoR rotational studies resonate with those obtained from studies investigating the functionality of disulfide-mediated EpoR dimers. Cysteine pairs were introduced in the transmembrane (TM) region of the receptor, forcing constitutive dimerization in the absence of ligand. Among the six disulfide bridges evaluated, only three cysteine–cysteine linkages were capable of promoting robust ligand-independent signal activation, suggesting that specific orientations of the TM domain are required to achieve a productively signaling Epo complex (Constantinescu, Keren, et al., 2001; Kubatzky et al., 2001; Lu, Gross, & Lodish, 2006; Watowich et al., 1992). Moreover, it was reported that the introduction of asparagine mutations to induce changes in the interhelical packing of the EpoR TM segments resulted in complete activation of STAT5, but did not induce the activation of Erk, suggesting that ligand-induced signal diversification could also be regulated by the geometry and packing of the TM dimer interaction (Becker et al., 2008). Here again, in the absence of direct structural information, the precise molecular consequences of these mutations remain unclear, but evidently alteration of the structural context of the receptor dimer impacts the nature and extent of signaling. As such, these studies on the Epo and Tpo systems provide some of the most compelling evidence to date for a role of receptor–ligand structure in signaling beyond serving as a binary on/off switch. This notion that structural effects can modulate signaling has been corroborated in other cytokine systems such as gp130 (Greiser, Stross, Heinrich, Behrmann, & Hermanns, 2002) and growth hormone (Brown et al., 2005). However, other cytokines such as prolactin do not appear to follow this model, as rotational manipulation of the TM domain of the prolactin receptor does not impact signaling behavior in the absence or presence of ligand (Liu & Brooks, 2011). Overall, studies of the effects of complex rotation on functional outcomes suggest that modifications of the binding mode and receptor architecture impact the signaling events induced by at least some cytokines. However, as with the chimeric receptor studies, the experimental evidence is confounded by overexpression of receptor mutants, making it difficult to draw conclusions about receptor behavior under physiological conditions.

As an alternative to manipulating the signaling complex geometry via direct modification of the receptor, attempts have been made to design ligand variants that alter cytokine-receptor complex structure. A series of studies on Epo peptide mimetics provide some of the most convincing data so far that ligand-mediated alteration of receptor dimer geometry can profoundly influence signaling. Livnah and colleagues demonstrated that a 13-residue Epo mimetic peptide was able to symmetrically dimerize two chains of the EpoR and to agonize receptor activity, inducing activation of STAT5 and proliferation of Ba/F3 cells, albeit less efficiently than the Epo cytokine (Livnah et al., 1996; Remy et al., 1999; Wrighton et al., 1996). Modification of this agonist peptide by adding two bromo groups to Tyr 4 (2–5 dibromotyrosine) abolished the induced signal, rendering it antagonistic (Livnah et al., 1998). In a crystal structure of the nonstimulatory peptide in complex with EpoR, the peptide effected dimerization of two receptors, but the rotational orientation of the dimer differed from that of the agonist peptide-mediated dimer by approximately 15°. The lack of receptor activation was thus attributed to difference in dimer geometry. This result was quite

striking, as it implies that a seeming small difference in receptor ECD geometry can be transmitted through the receptor complex and sensed inside the cell. A major caveat to this result, however, is that the binding affinity of the antagonistic peptide for Epo receptor was at least 200-fold lower than that of the agonistic peptide, making it difficult to distinguish between effects resulting from changes in complex geometry and those resulting from changes in binding affinity. In particular, due to the low affinity of the antagonistic peptide, it is unclear whether it was capable of dimerizing EpoR at the concentrations used in cell signaling studies. A subsequent structure of the EPO cytokine complexed with EpoR again showed a dimeric receptor orientation, but one that was asymmetric and quite distinct from those of the agonistic and antagonistic EPO peptide mimetics (Syed et al., 1998). Here the relative “efficiency” of Epo-induced signaling over the peptide was attributed to this altered dimer geometry (Syed et al., 1998). But again, vast differences in the affinities of the agonist peptide, antagonist peptide, and Epo must be considered when evaluating whether divergence in receptor signaling results from structural effects, kinetic effects, or a combination thereof. Clearly, the role of receptor geometry in determining signal behavior requires further study in the EpoR and other cytokine systems to parse the relative contributions of structural and affinity effects on functional outcomes.

2.2. Ligand–receptor affinity: Stability of the ternary complex

Clear correlations have been established between complex stability and signal activation in several cytokine systems. Most cytokines recruit their cognate receptors in a two-step mechanism, first recruiting one receptor chain with high affinity and then forming a ternary complex with a second lower-affinity receptor chain (Cunningham et al., 1991; Junttila et al., 2012; Piehler et al., 2012; Wang et al., 2009). Cytokines form surface complexes with a broad range of stabilities and variations in complex stability can differentially influence the activation of downstream signaling events. The importance of complex stability in determining signal activation is abundantly clear in the type I IFN family, also known as IFN α/β . IFNs are a large family of cytokines comprising more than 16 different subtypes, all of which bind to a heterodimeric surface receptor complex that consists of the IFNAR1 and IFNAR2 chains (Fig. 1.1, left; Pestka, Krause, & Walter, 2004; Piehler et al., 2012). Through this singular receptor complex, type I IFNs can activate all seven STAT molecules that are present in the cell (van Boxel-Dezaire et al., 2006). A large body of work has shown that different IFN subtypes activate STATs to the same extent despite large discrepancies in binding affinities between the subtypes (Abramovich et al., 1994; Ghislain, Sussman, Goelz, Ling, & Fish, 1995; Jaitin et al., 2006; Marijanovic, Ragimbeau, van der Heyden, Uze, & Pellegrini, 2007; Piehler et al., 2012; Subramaniam, Khan, Pontzer, & Johnson, 1995). However, when more complex biological activities such as antiproliferative and antiviral effects are studied, a broad range of potencies are found, indicating a lack of connectivity between early signal activation and late biological outcomes (Jaitin et al., 2006; Marijanovic et al., 2007; Moraga, Harari, Schreiber, Uze, & Pellegrini, 2009; Thomas et al., 2011). Furthermore, there does not seem to be a correlation between the potencies of the antiproliferative and antiviral activities; one IFN subtype can have a strong antiproliferative and a medium-strength antiviral effect, whereas another subtype can have a weak antiproliferative but very strong antiviral effect (Brideau-Andersen et al., 2007; Jaitin et al., 2006). How is it possible that a family of ligands that activates the same extent of

downstream signaling through the same receptor heterodimer can display these dramatic differences in the potency of induced biological activities?

Several models have emerged over the past 20 years to address this question. One idea that has been proposed is that other non-STAT signaling pathways may be activated in an IFN subtype-specific manner, contributing to the fine-tuning of biological outcomes (Platanias, 2005). There are several reports showing that IFN can activate the PI3K (Pfeffer et al., 1997; Uddin et al., 1995, 1997), MAPK (David et al., 1995; Joshi et al., 2009; Verma et al., 2002), and NF κ B pathways (Rani et al., 2001; Wei et al., 2006; Yang et al., 2000) in various cell types, and there is evidence that these pathways play a role in IFN-induced bioactivities (Joshi et al., 2009; Kaur et al., 2008; Rani et al., 2001). However, to date there is no direct and conclusive evidence linking these non-STAT pathways to differences in the intensity of biological activities elicited by different IFN subtypes, and the mechanism through which different IFN subtypes might induce distinct non-STAT signaling events remains unknown. Another proposed explanation for the differential potency of antiviral and antiproliferative effects is that different IFN subtypes could form structurally unique ternary complexes, analogous to the rotationally shifted EpoR complexes formed in the presence of agonistic versus antagonistic peptides (Livnah et al., 1996, 1998). However, this explanation was ruled out by the crystal structures of ternary complexes formed by two different IFN subtypes, an IFN α 2 mutant and IFN ω , which revealed that despite variation in the ligand, the complex architectures are virtually identical (Thomas et al., 2011).

The most widely accepted rationalization of differential activities in the IFN system at present rests on variation in the stability of the ternary complex (Piehler et al., 2012). Mutations that decrease the IFN α 2 binding affinity for either IFNAR1 or IFNAR2 result in a corresponding decrease in STAT activation and antiviral and antiproliferative potency (Jaitin et al., 2006; Kalie, Jaitin, Podoplelova, Piehler, & Schreiber, 2008; Piehler, Roisman, & Schreiber, 2000; Piehler & Schreiber, 1999). However, the inverse does not hold as an increase in binding affinity for either IFNAR1 or IFNAR2 leads to stronger antiproliferative potency but does not enhance STAT activation or antiviral response, both of which plateau at affinities close to those of wild-type ligands (Jaitin & Schreiber, 2007; Kalie, Jaitin, Abramovich, & Schreiber, 2007; Slutski, Jaitin, Yehezkel, & Schreiber, 2006). Moreover, any increase in affinity for either one of the IFNAR chains that is accompanied by a concomitant decrease in affinity for the other chain (such that the overall complex stability is unchanged) will result in the same antiproliferative and antiviral potency as the parent IFN, confirming the connection between ternary complex stability and bioactivity potency (Kalie et al., 2008). Taken together, these results suggest that the key factor in determining functional outcome is the half-life of the full ternary complex rather than the independent affinities that a ligand exhibits toward its two independent receptors chains. This finding was confirmed via analysis of STAT activation induced by various IFNs exhibiting a range of complex stabilities (Thomas et al., 2011). Using phospho-flow, a state-of-the-art technique for the quantitative analysis of phosphorylated proteins in a high-throughput manner, the EC₅₀ values for activation of all seven STATs were measured in each immune cell type isolated from whole blood (Krutzik & Nolan, 2006). A direct correlation was observed between complex stability and signaling kinetics: IFNs forming a longer lived ternary complex induced faster, although not stronger, STAT3 and STAT5 activation. How

this “kinetic proofreading” of signal initiation determines the *in vivo* functional outcomes induced by different IFNs remains to be elucidated, but at this point it seems clear that complex stability allows for a family of ligands sharing the same surface receptor to exhibit differential potencies of biological responses by regulating the kinetics of signal activation. Another case in point is the divergence in the STAT3 activation profiles induced by IL-6 and IL-10. IL-6 induces transient activation of STAT3 whereas IL-10 elicits a more sustained STAT3 response and this difference in activation kinetics was shown to be sufficient to account for the distinct biological outcomes of these two cytokines (Braun, Fribourg, & Sealton, 2013). In summary, multiple cytokine systems utilize differential stability of the ternary complex to discriminate between redundant ligands in order to affect specific functional outcomes.

2.3. Ligand–receptor affinity: Kinetics of complex formation

Another way in which the properties of ligand–receptor binding can modulate cytokine signaling is through differential kinetics of ternary complex formation. An example of this is found in the IL-4/IL-13 system. Both IL-4 and IL-13, which are produced by T helper 2 (Th2) cells, recruit and activate IgE-producing B cells to enhance IgE-mediated responses (Wills-Karp, 2004; Zhu, Yamane, & Paul, 2010). In addition, the IL-4 cytokine plays a critical role in the differentiation of Th2 cells (Wills-Karp, 2004; Zhu et al., 2010). IL-4 and IL-13 exhibit a broad spectrum of common bioactivities, yet there are some pockets of specificity in which the functions of these two cytokines do not overlap (Chomarat & Banchereau, 1998; Junttila et al., 2008; Liang et al., 2011). IL-4 binds to the type I receptor, consisting of the IL-4R α 1 and γ_c subunits, which is found exclusively on hematopoietic cells (Kelly-Welch, Hanson, Boothby, & Keegan, 2003; LaPorte et al., 2008; Wang et al., 2009). IL-4 can alternatively bind the type II receptor, composed of the IL-4R α 1 and IL-13R α 1 chains, which also serves as the receptor for IL-13 (Fig. 1.1, center). Both IL-4 and IL-13 activate STAT6 through the type II receptor, with IL-4 inducing more than 10-fold stronger STAT6 activation than IL-13 (Heller et al., 2008; LaPorte et al., 2008; Zhu, Guo, Watson, Hu-Li, & Paul, 2001). Differential activity between the two cytokines cannot be explained by increased stability of the ternary complex, as IL-4 forms a less stable complex than IL-13 (Andrews, Holloway, Holgate, & Davies, 2006; Andrews, Holloway, Puddicombe, Holgate, & Davies, 2002; LaPorte et al., 2008; Zhang, Buehner, & Sebald, 2002; Zhang, Simeonowa, Wang, & Sebald, 2002). In this case, the differences in STAT6 activation are presumably due to the distinct kinetics of complex formation exhibited by these two cytokines, as well as differences in the amounts of IL-4R α 1 and IL-13R α 1 expressed on the surface of immune cells (Junttila et al., 2008, 2012). In type II receptor complex formation, IL-4 first recruits IL-4R α 1 with very high affinity ($K_d \approx 100$ pM) and IL-13R α 1 is subsequently recruited to the binary complex with low affinity. IL-13, on the other hand, follows the reverse scheme, first binding IL-13R α 1 with moderate affinity ($K_d \approx 4$ nM) and then recruiting IL-4R α 1 to the binary complex with low affinity (Andrews et al., 2002, 2006; LaPorte et al., 2008; Zhang, Buehner, et al., 2002; Zhang, Simeonowa, et al., 2002). Due to its high affinity of interaction, formation of the IL-4-IL-4R α 1 binary complex is acutely sensitive to both ligand concentration and surface expression of IL-4R α 1 and cells are responsive to very low quantities of the IL-4 cytokine (Junttila et al., 2008). The rate-limiting step in this system is engagement of the IL-13R α 1 chain due to the low affinity that

the binary complex exhibits for this chain. IL-13, on the other hand, requires higher levels of ligand to form the moderate-affinity IL-13-IL-13R α 1 binary complex, rendering the cells less sensitive to levels of this cytokine. However, due to the higher affinity of the IL-13-IL-13R α 1 binary complex for IL-4R α 1, this system is more resistant to changes in the expression levels of this latter chain. It can therefore be expected that the abundance of IL-13R α 1 will have a strong impact on the ability of IL-4 and IL-13 to induce STAT6 phosphorylation. This hypothesis was confirmed when it was demonstrated that cells expressing high levels of IL-13R α 1 respond more strongly to IL-13 than to IL-4 (Junttila et al., 2008).

There appears to be an interesting structural origin behind the differential complex assembly kinetics for IL-4 and IL-13. The IL-13R α 1 receptor chain appears to have evolved from γ_c although it differs in that it contains an additional N-terminal D1 domain (LaPorte et al., 2008). In the structures of the IL-14 and IL-13 ternary complexes, the IL-13R α 1 D1 domain has extensive interactions with both IL-4 and IL-13 (LaPorte et al., 2008). However, mutational and domain deletion studies showed that this contact is only energetically required for the IL-13 interaction; the D1 domain can be removed from IL-13R α 1 without impacting the ability of IL-4 to form the ternary complex, whereas D1 deletion ablates the IL-13-IL-13R α 1 interaction (Ito et al., 2009). Thus, the reversal in assembly order for the type II IL-4 and IL-13 complexes is a result of these structural differences, and ties directly into the “mass action” based mechanism through which cells expressing the type II receptor distinguish IL-4 from IL-13. Clearly, evolutionary pressures have altered the binding energetics between the cytokines and the IL-13R α 1 subunit so as to allow a shared receptor to differentiate between the two stimuli.

The impact of complex formation kinetics and receptor abundance on signal activation is also illustrated in the case of cytokines that engage homodimeric receptors, such as for the cytokines Epo, Tpo, and growth hormone (GH). Epo induces activation of both STAT5 and Erk, leading to the differentiation and maturation of erythrocytes (Constantinescu, Ghaffari, & Lodish, 1999). Although the Epo cytokine recruits two identical EpoR molecules (Fig. 1.1, right), ternary complex formation still follows a two-step assembly scheme similar to those utilized by IFNs and IL-4/IL-13. Epo first recruits one EpoR chain with high affinity and then, using a second binding interface on the opposite side of the cytokine, recruits a second EpoR chain with low affinity, leading to the formation of an asymmetric complex (Matthews, Topping, Cass, & Giebel, 1996; Philo, Aoki, Arakawa, Narhi, & Wen, 1996; Syed et al., 1998). Complex formation results in the characteristic biphasic Epo activation profile wherein high doses of Epo activate STAT5/Erk less efficiently than do intermediate levels of Epo, generating a bell-shaped curve of activity versus cytokine concentration (Fuh et al., 1992; Wells & de Vos, 1996). The molecular mechanism underlying this unintuitive phenomenon was first described for the growth hormone receptor (GHR) and is applicable to all homodimeric receptors (Fuh et al., 1992; Wells & de Vos, 1996). At low doses of ligand, few ternary complexes are formed as most receptors are unbound or singly bound. As the Epo concentration rises, the number of ternary complexes rises up to the point that Epo saturates the receptors on the cell (specifically, a 2:1 ratio of EpoR:Epo). At super-saturating doses of Epo, the equilibrium will be shifted to the formation of binary complexes as unbound Epo competes with singly bound Epo for EpoR binding, effectively

antagonizing the formation of active ternary complexes, and leading to decreased signal activation (Fuh et al., 1992; Wells & de Vos, 1996). As in the IL-4/IL-13 system, the kinetics of Epo complex formation enables modulation of signaling extent through tight regulation of both cytokine concentration and surface receptor levels.

3. RECEPTOR DYNAMICS AND ENDOSOMAL TRAFFICKING

Studies of cytokine receptor dynamics and trafficking are complicated by several factors. First, cytokine receptors are expressed at very low levels, generally less than a thousand receptors per cell. IFNAR1 surface levels are estimated to be in the low hundreds (Uze, Lutfalla, & Gresser, 1990) and similar numbers were found for IL-4R α 1 (Lowenthal et al., 1988; Obiri, Debinski, Leonard, & Puri, 1995; Ohara & Paul, 1987). Another complicating factor in examination of receptor trafficking is that surface expression of most cytokine receptors is tightly regulated by their associated Jaks, making it challenging to stably overexpress these receptors at high levels. Nonetheless, there are a few reports suggesting that membrane receptor dynamics and endosomal trafficking play crucial roles in cytokine-induced signal activation and functional diversification. One such report demonstrated the relevance of receptor trafficking in granulocyte colony-stimulating factor (G-CSF) signaling (Avalos et al., 1990; Irandoust et al., 2007). Through STAT5 and Akt pathway signaling, G-CSF orchestrates both the production of granulocytes by the bone marrow and their subsequent release into the bloodstream, and also regulates the survival, proliferation, and differentiation of neutrophils (Hamilton, 2008). It was reported that truncations of the G-CSF receptor (G-CSFR) ICDs prevent its internalization, causing the receptor to persist on the surface, which in turn promotes the development of myeloid leukemia and severe neutropenia due to sustained G-CSFR signaling (Avalos et al., 1990). A later study established that ICD mutants of G-CSFR that cannot traffic to lysosomes become trapped in early endosomes where they continue to activate STAT5 but not Akt, leading to altered biological activities (Irlandoust et al., 2007). This later work not only illustrated the key role of trafficking through the different endosomal compartments in cytokine-induced signal activation but also suggested that regulation of receptor trafficking could impact the extent and diversification of signaling activated by a given cytokine.

The role of receptor trafficking has been extensively studied in the IFN system. As described earlier, IFNs engage a surface receptor composed of the IFNAR1 and IFNAR2 chains. An interesting aspect of this system is that the two chains do not follow parallel paths of downregulation and trafficking (Fig. 1.2). Following activation, IFNAR1 is heavily ubiquitinated and traffics to the lysosomes where it is degraded, regardless of the IFN subtype used for stimulation (Jaitin et al., 2006; Marijanovic et al., 2007). In contrast, the subcellular trafficking of IFNAR2 depends on the binding affinity of the stimulating IFN subtype (Jaitin et al., 2006; Marijanovic et al., 2007; Thomas et al., 2010). While IFN α 2, which binds IFNAR1 with low affinity, promotes the recycling of IFNAR2 due to the short half-life of the complex (Marijanovic et al., 2007), IFN β , which binds IFNAR1 with higher affinity, promotes formation of a more stable ternary complex, leading to the downregulation and lysosomal degradation of both IFNAR1 and IFNAR2 (Jaitin et al., 2006; Marijanovic et al., 2007). Coimmunoprecipitation experiments have revealed that the ternary complex formed by IFN β remains intact long after STATs have been activated inside

the cells. The same is not true, however, for the IFN α 2 ternary complex (Croze et al., 1996). This discrepancy is thought to be due to differential pH sensitivity of the ternary complexes formed by the different IFN subtypes. Whereas complexes formed by weaker binders dissociate under the acidic conditions found in early endosomes (pH 6), more stable complexes formed by tighter binding IFN subtypes endure the acidic conditions and continue routing to the lysosomal compartment, where they are degraded (Fig. 1.2; Lamken, Lata, Gavutis, & Piehler, 2004; Uze, Bandu, Eid, Grutter, & Mogensen, 1988). It has been established that IFN-activated signaling molecules (Tyk2, STAT1, STAT2) can be found in early endosomes after IFN stimulation, indicating that signaling continues to occur inside the cells following receptor internalization (Payelle-Brogard & Pellegrini, 2010). Moreover, when internalization of the ternary complex is prevented by blocking the formation of endocytic vesicles (via clathrin silencing or overexpression of a dominant-negative form of dynamin), IFN-induced STAT activation along with antiviral and antiproliferative activity is diminished (Marchetti et al., 2006). However, when only downregulation but not endocytosis of IFNAR1 is blocked by mutation of the ubiquitination site serine 535, IFN induces a stronger antiproliferative response (Kumar, Krolewski, & Fuchs, 2004). These results clearly point to an important role for endosomal trafficking in the determination of the final biological outcomes induced by IFN and also highlight the need for follow-up studies in the IFN system and others to fully elucidate the contributions of endosomal trafficking to signal activation.

Studies of other cytokine systems such as prolactin further support the notion that pH sensitivity of complexes can affect signaling outcomes, as is speculated for the IFN α 2 and IFN β cytokines (Keeler et al., 2007). It was shown that the binding affinity of prolactin to its native receptor is pH sensitive. At acidic pH (such as that of early endosomes), prolactin dissociates from its ternary complex with prolactin receptor and recycles back to the cell surface (Keeler et al., 2007). Prolactin receptor can alternatively be dimerized by growth hormone to form a ternary complex that is not pH sensitive. In this case, GH is transported to the lysosomes and degraded, leading to a completely different set of biological outcomes compared to those evoked by prolactin (Keeler et al., 2007).

Endosomal trafficking also impacts signal activation in the Epo system. Ligand-induced downregulation of murine EpoR was found to require ubiquitination of the receptor, and inhibition of this ubiquitination via mutation of key lysines in the murine EpoR led to a decrease in the phosphorylation of STAT5, Erk, and Akt (Bulut, Sulahian, Ma, Chi, & Huang, 2011). Conversely, a prior study had reported that inhibition of ubiquitination of the human EpoR led to a more sustained level of EpoR on the surface of responsive cells and thus to stronger activation of downstream signaling pathways (Meyer et al., 2007). The discordance between these two studies may be attributable to the differences between mouse and human systems or to the differential sensitivity of the readouts used to detect EpoR degradation and signal activation. What is clear is that receptor dynamics and trafficking significantly impact signaling outcomes induced by Epo and the importance of strictly regulated ligand and receptor availability is magnified in the case of a homodimeric complex that exhibits a biphasic activation profile. Indeed, a recent study showed that ligand depletion and endocytic removal of surface EpoR are hallmarks of Epo-activated signaling

and that these events facilitate linear information processing for a broad range of ligand concentrations (Becker et al., 2010).

The role of downregulation and trafficking of cytokine receptors in cytokine-induced signal activation and bioactivities seems universal, as examples have been reported in other systems such as IL-2, IL-4, and IL-6 (Friedrich et al., 1999; German, Sauer, & Howe, 2011; Rocca, Lamaze, Subtil, & Dautry-Varsat, 2001; Shah et al., 2006; Subtil, Delepiepierre, & Dautry-Varsat, 1997; Wang & Fuller, 1994). However, we still lack a clear picture of precisely where receptors are localized within cells, which signaling molecules the receptors encounter in various subcellular compartments, and the extent to which signal activation is influenced by receptor trafficking. Further, we know very little about how receptors are organized and distributed on the cell surface, how cytokine-receptor complexes are formed within the cell membrane, and how the mobility of these membrane-associated complexes impacts their availability, trafficking, and ability to activate downstream signaling pathways. Recent developments in super-resolution microscopy and single-particle techniques as well as TIRF microscopy (Huang, Bates, & Zhuang, 2009) have enabled scientists to begin exploring some of these questions by tracking receptors on the surface of living cells.

New methodologies have been employed for multicolor tracking of the IFN receptor complex in living cells. These methodologies are based on the labeling of single proteins with quantum dots (QD) in a 1:1 stoichiometry, thus enabling single particle tracing. To achieve multiplex tracking, proteins of interest are expressed with either an acceptor peptide (AP) tag or a histidine tag. The AP tag is biotinylated by the enzyme BirA and subsequently labeled by streptavidin-coated QDs and the histidine tag is labeled by trisNTA-coated QDs of a different color. This system allows for simultaneous observation of two proteins on the cell surface and analysis of their colocomotion. Using this technique, IFNAR1 and IFNAR2 were found to have two diffusion regimes, one fast and one slow, in the absence of IFN treatment, presumably correlating to the localization of receptors in separate (fast) or common (slow) membrane domains (Roullier et al., 2009). In the presence of IFN α 2, the diffusion constant of both IFNAR1 and IFNAR2 decreased, presumably due to formation of the constrained ternary complex (You et al., 2010). Triple-color super-resolution imaging of live cells has also been used to study the organization of IFNAR1 and IFNAR2 in the context of the cytoskeleton. IFNAR1 and IFNAR2 were often found in the periphery of actin structures, hinting at the existence of an interaction between these chains and actin or actin-bound adaptor proteins, which is in accord with previous reports implicating involvement of actin-associated adaptors in IFN-induced signaling (Pfeffer, Stebbing, & Donner, 1987; Usacheva et al., 2001; Wilmes et al., 2012).

Super-resolution microscopy has also been applied to the study of other cytokine systems. The use of AlexaFluor-647-labeled IL-4 for the study of type I and type II IL-4 receptor complex formation revealed that while the engagement of the type II IL-4 receptor (IL-4R α 1–IL-4–IL-13R α 1) by IL-4 follows the aforementioned canonical ligand-induced receptor dimerization model, the type I receptor complex (IL-4R α 1–IL-4- γ_c) does not (Weidemann et al., 2011). In contrast with the recruitment of the IL-13R α 1 chain to the IL-4–IL-4R α 1 binary complex, recruitment of the γ_c chain could not be detected, suggesting that the affinities of the binary complex for the two coreceptors, IL-13R α 1 and γ_c , differ

dramatically. This result conflicts with biochemical studies, performed in solution, which concluded that the affinities of the binary IL-4–IL-4R α 1 complex to IL-13R α 1 and γ_c were similar (Weidemann et al., 2011). The discrepancy in these findings could be explained by the presence of the cytoplasmic portion of the receptor in conjunction with the membrane environment in live-cell imaging studies compared to the biochemical studies performed with receptor extracellular domains in solution.

The integration of highly sensitive and quantitative microscopy techniques into the study of cytokine-receptor binding and cytokine-induced signal activation will enable us to capture the dynamics of ligand–receptor interaction at the single-molecule level, providing details that are obscured when using more traditional indirect methods. These new technologies will further our understanding of dimerization kinetics, receptor trafficking and mobility, and the role of membrane structures in regulating signal transduction, enabling the fine-tuning of cytokine systems as well as the design of new and more efficient immunological therapies.

4. INTRACELLULAR PROTEIN LEVELS AND SIGNALING ACTIVATION

Studies of signaling pathways activated by cytokines have historically focused on how the binding of the cytokine to its cognate receptor induces activation of a limited number of downstream pathways and how modulation of that binding event impacts signaling events. More recently, scientists have begun to appreciate the critical role that intracellular signaling proteins play in defining the extent of activation and the nature of the bioactivities induced by each cytokine.

4.1. Jaks and receptor expression and affinity

The surface levels of most cytokine receptors are determined by the abundance of Jak proteins present in the cell (Huang, Constantinescu, & Lodish, 2001; Radtke et al., 2002; Ragimbeau et al., 2003). Manipulation of the levels of Jaks will therefore impact the biological outcomes induced by any cytokine that utilizes a receptor associated with that Jak, making it difficult to distinguish between effects resulting from altered Jak levels versus those arising from altered receptor levels. Moreover in some cases, Jaks also play a role in modulating receptor binding affinity. Early studies showed that certain mutations of the Tyk2 protein, while not affecting surface levels of IFNAR1, lead to a decrease in its affinity for IFN α 2 (Gauzzi et al., 1997). It has been shown that binding of the protease USP18 (a negative regulator of IFN signaling) to IFNAR2 ICD also leads to a decrease in its binding affinity for IFN, again without altering the surface levels of the receptor (Francois-Newton et al., 2011). These examples suggest an interplay between the signal-mediating intracellular compartment and the ligand-binding extracellular domains of cytokine receptors in determining the affinity of binding and, therefore, the cellular responses elicited by different cytokines.

4.2. Crosstalk between STATs

It was discovered more than a decade ago that there are networks of communication between the different STATs activated by a cytokine. An example of one such network is observed in the IFN system. All the IFN-induced STATs, with the exception of STAT2,

bind to the same two phospho-tyrosine (P-Tyr) sites on the ICD of IFNAR2 and the mutation of either of those two tyrosines impairs the receptor's ability to promote full STAT activation (Wagner et al., 2002; Zhao et al., 2008). It is therefore possible that there is a competition between different STATs for the binding to these tyrosine residues, as most STATs exhibit similar kinetics of activation.

Using phospho-flow barcoding, we recently showed that the ratios of activated STATs in a given cell differs not only between treatments with different IFN subtypes, but also between different cell types treated with the same IFN, presumably due to their differing amounts of STATs (Thomas et al., 2011). These differences in STAT activation ratios play an important role in signal modulation. The potency of IFN was shown to be determined by STAT1/STAT3 ratio, as activated STAT3 was able to sequester activated STAT1 and in turn decrease the antiviral and antiproliferative potency of IFN (Ho & Ivashkiv, 2006). Thus, the treatment of cells with any cytokine or other agent that upregulates STAT3 levels negatively impacts the potency of a future response to IFN (Ho & Ivashkiv, 2006). Similarly, a treatment that upregulates STAT1 results in a more potent IFN response, clearly demonstrating that the balance between the expression levels of different STATs in a given cell will greatly impact its response to a cytokine stimulation (Tassiulas et al., 2004). Another example of the importance of STAT levels in determining signal activation is found in the Epo system. Erythroblasts modulate the level of STAT5 during erythroid maturation and, consequently, different stages of maturation induce different levels of maximal Epo-induced STAT5 activation (Porpiglia, Hidalgo, Koulis, Tzafriri, & Socolovsky, 2012). These results demonstrate that the dynamics of STAT5 protein levels convey specific information to a cell, allowing that cell to transduce Epo stimuli with high fidelity over its entire physiological and stress range.

Changes in intracellular STAT protein levels can impact the activity of a given cytokine not only quantitatively, but also qualitatively. For example, IL-6 primarily signals via STAT3, but in murine embryonal macrophages that lack expression of STAT3, IL-6 completely alters its signaling behavior by predominantly activating STAT1, evoking biological responses nearly identical to those induced by the STAT1-activating cytokine IFN γ (Costa-Pereira et al., 2002; Fig. 1.3). These data suggest that while IL-6 preferentially activates STAT3, it can activate STAT1 as well, and the intracellular ratio between these two STATs will determine the nature of IL-6 response.

Another example of the crosstalk between STAT molecules can be found in the IL-10 system. IL-10 induces the activation of STAT3, which mediates the anti-inflammatory response induced by this cytokine (Williams, Bradley, Smith, & Foxwell, 2004). However, if macrophages are stimulated with type I or type II IFN prior to IL-10 exposure, STAT1 protein levels become upregulated, and IL-10 will primarily promote the activation of STAT1 to induce proinflammatory behavior. This aberrant IL-10-induced proinflammatory behavior has been implicated in the pathology of immune disorders such as lupus and endotoxemia (Herrero et al., 2003; Sharif et al., 2004).

The exquisite balance that exists between different STATs in order to regulate functional outcomes is not restricted to STAT1 and STAT3; other STAT proteins are also susceptible

to tight regulation to modulate biological activities. For many years it was not known why type I IFN promotes the proliferation of activated T cells, as it potently inhibits the proliferation of all other cell types (Dondi, Rogge, Lutfalla, Uze, & Pellegrini, 2003; Dondi, Roue, Yuste, Susin, & Pellegrini, 2004; Gil, Salomon, Louten, & Biron, 2006; Kolumam, Thomas, Thompson, Sprent, & Murali-Krishna, 2005; Kurche, Haluszczak, McWilliams, Sanchez, & Kedl, 2012). Recently, an elegant study showed that STAT1 is the most abundant STAT molecule in naïve T cells, and, consequently, treatment of these cells with IFN activates STAT1, leading to inhibition of cell proliferation (Gil et al., 2012). Upon activation via T cell receptor (TCR), STAT4 is upregulated within the T cell. In consequence, IFN preferentially activates STAT4 in activated T cells, leading to their proliferative response (Gil et al., 2012). In summary, there is compelling evidence over a range of cytokine systems of the importance of the absolute and relative abundance of STATs in determining signaling behavior and functional outcomes.

5. TUNING CYTOKINE SIGNALING VIA PROTEIN ENGINEERING

Cytokines control virtually every function of the immune response and thus deregulation of these secreted factors can lead to the development of immunological diseases such as cancer, lupus, allergy/asthma, and multiple sclerosis (O'Shea & Plenge, 2012). Although several instances of cytokines being used as therapeutic drugs can be found (e.g., Epo, IL-2, IFN β), their usage in the clinical setting is not as widespread as one might imagine. One of the main impediments to therapeutic use of cytokines is their pleiotropic nature, which results in off-target effects and toxicity. Therapeutics that target signaling pathways activated downstream of cytokines have also experienced only limited success in the clinic. For instance, the inhibition of a hyperactivated Jak can be used to treat diseases (e.g., Jak2 in polycythemia vera), but given that Jaks and STATs are activated by all cytokines, these drugs elicit nonspecific and toxic side effects (O'Shea & Plenge, 2012).

One way to circumvent the complications of pleiotropy is to generate cytokines skewed toward one given bioactivity, so-called cytokine “tuning.” Not only would cytokine tuning help treat immune disorders by enhancing the desired activity while minimizing side effects, it would also help enrich our fundamental understanding of cytokine receptor activation and signaling.

Most attempts to modify cytokine signaling so far have focused on decreasing ligand–receptor affinity for one of the two receptor chains in the ternary complex, thus generating antagonistic cytokine mimics that impede formation of the signaling dimer (Debinski, Gibo, Obiri, Kealiher, & Puri, 1998; Kang, Bondensgaard, Li, Hartmann, & Hjorth, 2010; Kruse, Tony, & Sebald, 1992; Liu, Maier, Hafler, & Wittrup, 2009). The reason for this focus on antagonism is that we can synthesize structural and biochemical information to predict with reasonable accuracy which amino acid substitutions will promote a decrease of binding affinity. In addition to antagonizing binding, decreasing the complex affinity can also result in modulation of receptor trafficking, leading to unexpected effects on signaling (Berndt, Chang, Smith, & Ciardelli, 1994; Reddy, Niyogi, Wells, Wiley, & Lauffenburger, 1996).

Modulation of cytokine signaling through increasing ligand affinity has proven to be a more difficult objective to achieve, as we cannot accurately predict amino acid changes that will increase affinity. Combinatorial mutant libraries provide the most effective and comprehensive means of selecting for high-affinity binders. Genentech pioneered the use of cytokine engineering and phage display in the hGH system to demonstrate the power of this approach (Cunningham et al., 1991; Cunningham & Wells, 1997; Fellouse et al., 2007; Fuh, Colosi, Wood, & Wells, 1993; Fuh et al., 1992; Pal, Kossiakoff, & Sidhu, 2003; Sidhu & Kossiakoff, 2007). We will present examples of the use of combinatorial libraries to increase the affinity of the cytokine for one receptor chain, leading to modifications in biological outcomes. Another strategy for overcoming the complications of pleiotropy is through development of cytokine-based drugs with preferential receptor specificity, for example, engineering cytokines with higher affinity to one and lower affinity to another receptor complex. The use of large combinatorial libraries of random mutants has enabled the engineering of specificity by allowing for sophisticated selection schemes that stringently isolate variants with customized binding properties. We will discuss a few examples in which cytokine engineering has gone beyond the straightforward maturation of cytokine binding affinity and evolved receptor specificity, leading to interesting, informative, and therapeutically relevant results.

5.1. Modulation of trafficking: IL-2 and EGF

Two decades ago, an IL-2 mutant was generated in which leucines 18 and 19 were mutated to methionine and glycine, respectively, resulting in a cytokine variant that exhibited similar binding affinities for its cognate receptor chains (IL-2R α , IL-2R β , and γ_c) compared with the wt protein, yet evoked a fivefold increase in the stimulation of T cell proliferation. The authors showed that the increase in activity was the result of an increase in the recycling rate of the IL-2–IL-2R β complex, which led to a decrease in IL-2 depletion. The authors speculated that this mutant could dissociate from the signaling complex at the acidic pH found in endosomes and thus follow a different endosomal trafficking route than the wt complex, leading to increased recycling to the cell surface. This increased recycling would help to sustain levels of the cytokine in the media in addition to maintaining the levels of IL-2 receptor components on the surface of cells (Berndt et al., 1994).

Another example of complex binding kinetics modulating receptor trafficking can be found in the EGF system. An EGF mutant, EGF Y316G, exhibits a 50-fold decrease in receptor-binding affinity compared to the wt ligand, yet elicits potent signaling activity that surpasses that of wt EGF. As in the case of IL-2, it was shown that this mutant promotes recycling of the ligand–receptor complex, leading to more sustained and stronger activation of downstream signaling (Reddy et al., 1996).

Collectively, these two studies suggest that controlling the endosomal trafficking of cytokine-receptor complexes through affinity modulation can lead to alternative functional outcomes, introducing novel strategies for the manipulation of cytokine signaling. For instance, it is conceivable that designed cytokine mutants with a high receptor affinity at extracellular pH (7) but low receptor affinity at endosomal pH (5–6) could promote recycling and lead to increased signal activation. Additionally, faster ternary complex

dissociation in endosomes could qualitatively alter signaling outcomes by reducing the activation of signaling pathways that are primarily activated in endosomes. Thus, by integrating affinity engineering with knowledge of trafficking dynamics, we can design cytokine variants with new and potentially advantageous signaling properties.

5.2. Modulation of ligand affinity and activities: IFN and IL-3

In addition to modulating the trafficking behavior of cytokine-receptor complexes, altering complex affinity and stability can also directly impact signal responses. Recently, an IFN α 2 mutant was isolated from phage library selection that binds to the IFNAR1 chain more than 100-fold tighter than the wt protein. In agreement with its enhanced binding affinity, this mutant induces more than 100-fold stronger inhibition of cell proliferation than the wt protein. However, the antiviral responses induced by the high-affinity and wt protein are very similar (Jaitin et al., 2006; Kalie et al., 2007). These data suggest that the two activities induced by IFN require different degrees of complex stability and introduce the possibility for development of new IFN subtypes that specifically regulate either of these two bioactivities. There have also been reports of IFN mutants obtained by DNA shuffling that induce stronger antiviral responses but weaker antiproliferative activity than the wt cytokine (Brideau-Andersen et al., 2007). However, it should be noted that these antiproliferative assays were performed on Daudi B cells, a cell line known to be extremely sensitive to IFN and which exhibits atypical behavior with respect to cell growth.

In addition to providing an opportunity to generate activity-specific mutants (antiviral vs. antiproliferative), the IFN system also allows for the potential development of virus-specific cytokine mutants. It was found that IFN may employ distinct strategies to inhibit different types of viruses through activation of unique subsets of genes that target specific viral types (Schoggins et al., 2011).

Selective alteration of particular functionalities through cytokine manipulation has also been achieved in the IL-3 system. IL-3 stimulates the differentiation of multipotent hematopoietic stem cells into lymphoid progenitor cells and promotes the proliferation of cells of the myeloid lineage through engagement of a surface receptor comprised of IL-3R α and the common β chain. An IL-3 mutant was generated that exhibited stronger binding to IL-3R α , which led to a 20-fold stronger proliferation response of hematopoietic cells compared to wt IL-3 stimulation. However, this mutant only marginally increased the release of histamine from peripheral blood leukocytes relative to the wt cytokine, demonstrating yet again that an increase in binding affinity for one of the receptor chains can lead to selective increase of some bioactivities but not others (Thomas et al., 1995).

5.3. Increasing ligand specificity: IL-2 and IL-4

Recently, our laboratory has reported two examples of cytokine tuning in which the objectives went beyond basic receptor affinity maturation to improve specificity of the cytokines for a particular receptor complex. In these studies, we took advantage of yeast surface display-mediated directed evolution, a robust platform for expressing and selecting many binding variants of a protein of interest (Boder & Wittrup, 1997). The principle underlying the yeast display methodology is analogous to that of phage display, but the

advantage of this system is that yeast have a robust secretory apparatus and can therefore express complex proteins such as cytokines, which possess intricate glycosylation patterns and disulfide bonds (Boder & Wittrup, 1997).

The first of our recently reported cytokine tuning efforts focused on the IL-2 system (Levin et al., 2012). IL-2 requires the IL-2R α , IL-2R β , and γ_c chains to form the high-affinity and fully active heterotrimeric surface receptor (Wang et al., 2005), which activates the STAT5, Erk, and Akt pathways (Liao et al., 2011). The high-affinity IL-2R α chain is upregulated in activated T cells and is also constitutively expressed in regulatory T cells (T_{regs}) (Depper et al., 1985; Zheng & Rudensky, 2007). However, naïve T cells lack the expression of this chain and therefore respond very poorly to IL-2. Regulation of IL-2R α expression levels thus determines the IL-2 sensitivity of different T cell subsets and, consequently, the extent and nature of the subsequent immune response. Using yeast surface-displayed combinatorial libraries, our laboratory isolated an IL-2 mutant, termed super-2, that had lost dependency on the IL-2R α chain for formation of the functional IL-2 signaling complex. This was achieved through a series of core cytokine mutations that stabilize the active conformation of the IL-2 C helix, which is stabilized by engagement of the IL-2R α chain in the wt quaternary complex (Wang et al., 2005). Super-2, by activating IL-2-responsive cells independently of their IL-2R α expression levels, induces a stronger proliferation of cytotoxic CD8⁺ T cells but has little effect on the growth of T_{regs}. *In vivo* experiments with super-2 demonstrate that it lowers the occurrence of the IL-2-associated toxic side effect edema compared to the wt cytokine (Levin et al., 2012). Moreover, super-2 is more potent than IL-2 therapy and as potent as IL-2/anti-IL-2 antibody therapy (Boyman, Kovar, Rubinstein, Surh, & Sprent, 2006; Krieg, Letourneau, Pantaleo, & Boyman, 2010; Letourneau et al., 2010) in reducing the growth of B16F10 melanoma, as well as murine colon carcinoma and Lewis lung carcinoma in mouse tumor models. Thus, by modulating receptor chain affinity and the dynamics of complex formation, super-2 appears to enhance the therapeutic properties of IL-2 in cancer while decreasing its toxic side effects (Levin et al., 2012).

Our second recent cytokine engineering report focused on IL-4 signaling (Junttila et al., 2012). Through activation of its type I and type II receptors, IL-4 plays a critical role in the differentiation of Th2 and Th9 T cells as well as in B cell maturation, macrophage activation, and dendritic cell (DC) differentiation. Whereas the type I receptor is mainly expressed on cells of hematopoietic origin, the type II receptor is more abundant in cells of epithelial origin (Wills-Karp, 2004; Zhu et al., 2010). To better define the activities downstream of both IL-4 receptors, we engineered a series of IL-4 mutants with different specificities for the type I and type II receptor complexes. We found one mutant, termed super-4, that exhibits more than a 1000-fold increase in affinity for the γ_c and more than a 10-fold decrease in affinity for IL-13R α 1, rendering this variant highly specific for type I receptor complexes. Another mutant, termed KFR, exhibits higher affinity for IL-13R α 1 and somewhat lower affinity for γ_c , making it selective for type II receptor. With these two mutants in hand, we explored the relative contributions of type I versus type II receptors to different IL-4-induced bioactivities. Super-4 induces stronger activation of STAT6 in naïve CD4⁺ T cells (which express only the type I receptor) compared to wt IL-4 and promotes

their differentiation into Th9 cells. However, Super-4 is unable to support the differentiation of monocytes into DCs despite activating STAT6 in monocytes to the same extent as IL-4, suggesting that type II receptor-induced pathways are required for monocyte differentiation into DCs. Consistent with this hypothesis, IL-4 variant KFR, which preferentially engages the type II receptor, is fully capable of inducing DC differentiation at levels comparable to those achieved by the wt cytokine. Collectively, our results shed light on the biology of the type I and type II IL-4 receptor complexes and introduce a new IL-4 mutant, super-4, that could potentially be a safer alternative to the wt cytokine in the clinic as it increases the anti-inflammatory Th2 response without activating innate immunity (Junttila et al., 2012). In sum, the recent IL-2 and IL-4 tuning examples from our laboratory provide a glimpse into the bountiful opportunities that cytokine engineering offers for enhancing our understanding of basic scientific questions regarding the specificity and diversification of cytokine signaling, and for applying this knowledge to the design of novel therapeutics for the treatment of immune disorders.

6. PERSPECTIVES

The first cytokine (IFN) was discovered in 1957 (Isaacs & Lindenmann, 1957) and cloned in 1980 (Taniguchi, Fujii-Kuriyama, & Muramatsu, 1980; Weissenbach et al., 1980). Twelve years later, the first cytokine-activated Jak kinase, Tyk2, was isolated and demonstrated to be required for type I IFN signaling (Velazquez, Fellous, Stark, & Pellegrini, 1992). Since then, more than 30 cytokines have been identified and the signaling pathways they activate and share have been extensively characterized. We have also acquired deeper insight into the immune processes controlled by cytokines as well as the pathologies that result from deregulation of their activities. However, there are still many fundamental questions in cytokine biology that remain unanswered, hindering our ability to modulate cytokine activities and to develop treatments for cytokine-dependent immune disorders. Why is Jak3 associated only with γ_c ? Is there a dominant Jak in a given pair or can any Jak do the job? Can the geometry of the ternary complex affect not only the quantity, but also the quality of induced signaling? What role does receptor localization within the membrane play in cytokine signaling behavior?

The development of more sophisticated imaging techniques that will allow us to follow in real time the cytokine-induced surface complexes within the cell membrane as well as in subcellular compartments, together with the engineering of new cytokines variants with precisely tuned properties, will enable us to probe cytokine systems in a much more controlled manner. This will in turn provide us with a more profound understanding of how cytokine surface complex formation is transmitted intracellularly to induce signal activation and the upregulation of specific gene expression programs, leading to the broad range of immunological activities that are elicited by cytokine signaling.

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Figure 1.1.

Complex stability and dynamic determination of cytokine-induced bioactivities. Left, extracellular structures of the type I IFN receptor, IFNAR1 and IFNAR2, and four different IFN subtypes are depicted. The four IFN subtypes presented activate STATs to the same extent (as shown by phosphorylated STAT dose–response curves) and exhibit similar antiviral activity (as shown by virus-infected cell abundance, which decreases with increasing IFN doses), but possess starkly different growth arrest potencies (as shown by the number of cells, which decreases with increasing IFN doses). Rather than following STAT activation profiles, the antiproliferative activities of the IFN subtypes closely parallel binding affinities and complex stabilities instead. Center, extracellular structures of IL-4, IL-13, and their shared IL-4 type II receptor comprised of IL-4R α 1 and IL-13R α 1 are shown. IL-4 and IL-13 share a common surface receptor complex, but IL-4 activates STAT6 more efficiently than IL-13 does (as presented in the phosphorylated STAT6 dose–response curve). Greater potency of STAT6 activation by IL-4 is not the result of a more stable complex, since the complex formed by IL-13 is in fact more stable, but rather the result of the differential kinetics of complex formation in response to IL-4 versus IL-13. Right, extracellular structures of Epo and the EpoR homodimer are presented. Epo engages two molecules of EpoR and forms an active surface asymmetric homodimeric complex, leading to STAT5 activation. As is the case for other cytokines that engage homodimeric receptors, Epo-induced STAT5 activation follows a bell-shaped curve, with high doses of Epo activating STAT5 less efficiently than intermediate doses do due to reduced ternary complex formation (our unpublished data).

Figure 1.2.

Role of receptor endosomal trafficking in regulating cytokine bioactivities. A canonical cytokine–receptor complex is represented schematically. Upon ligand stimulation the surface complex is formed and leads to the activation of membrane proximal signaling events (e.g., Jak/STAT pathway activity). The complex then traffics to endosomal compartments and follows different subcellular routes depending on its stability. Short-lived complexes dissociate in the acidic pH found in early endosomes and consequently follow a recycling route back to the plasma membrane. Long-lived complexes, on the other hand, persist in early endosomes and continue trafficking through subsequent endosomal compartments where specific signaling molecules are present that help to fine-tune the functional response to a given cytokine.

Figure 1.3.

The levels of intracellular signaling proteins impact the nature of the bioactivities induced by cytokines. The cytokine environment surrounding a cell contributes critically to the nature of that cell's response to a given stimulus. As an example, the IL-6 cytokine complex signals through both STAT1 and STAT3 activation. If a cell has previously been exposed to IL-23 or TNF α , its levels of STAT3 will be upregulated. In this case, stimulation with IL-6 will promote a strong activation of STAT3 and will lead to a potent anti-inflammatory response (left). Conversely, if a cell is primed with type I or II IFNs, its levels of STAT1 will instead be upregulated and IL-6 stimulation will therefore induce STAT1 activation, leading to a potent proinflammatory response.