Research Article

Mechanistic Pharmacokinetic/Target Engagement/Pharmacodynamic (PK/TE/PD) Modeling in Deciphering Interplay Between a Monoclonal Antibody and Its Soluble Target in Cynomolgus Monkeys

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Abstract. For therapeutic monoclonal antibodies (mAbs) against soluble ligands, the free ligand level can, theoretically, be used as a surrogate for efficacy. However, it can be extremely challenging technically to measure free ligand level in the presence of an excessive amount of antibody–ligand complex. The interplay among such mAbs, ligands, and the downstream pharmacodynamic (PD) effects has not been well defined. Using siltuximab and interleukin-6 (IL-6) as model compounds, a pharmacokinetic (PK)/target engagement (TE) model was established via simultaneous fitting of total siltuximab, total IL-6, and free IL-6 concentration profiles following a low dose of siltuximab in cynomolgus monkeys. The model adequately captured the observed data and provided estimation of model parameters with good precision. The PK/TE model was used to predict free IL-6 profiles at higher siltuximab doses, where the accurate determination of free IL-6 concentration became technically too difficult. The measured free IL-6 levels from the low-dose groups and PK/TE model-predicted free IL-6 levels from the high-dose groups were used to drive an indirect response TE/PD model to describe the concentration–effect relationship between free IL-6 and C-reactive protein (CRP). The TE/PD model adequately captured both CRP elevation and CRP suppression in response to free IL-6 concentration change from baseline with a linear stimulation function, providing direct evidence that the PK/TE model-predicted free IL-6 levels from the high-dose groups were accurate. Overall, the results provided an integrated PK/TE/PD modeling and bioanalytical framework for prediction of efficacious dose levels and duration of action for mAbs against soluble ligands with rapid turnover.

KEY WORDS: mAb; PK/PD modeling; quasi-equilibrium interaction; soluble ligand; target engagement.

INTRODUCTION

Soluble ligands like cytokines or chemokines have been one of the most important classes of targets for therapeutic monoclonal antibodies (mAbs) (1,2). For mAbs against soluble targets (ligands), their therapeutic efficacy theoretically is driven by the magnitude and duration of the lowering of free ligand. However, for soluble ligands with rapid turnover, it can be extremely difficult to assess the lowering of free ligand (3). First, ligands like cytokines and chemokines often have very low baseline levels. Moreover, they typically have short half-lives in the range of minutes, while the mAbs targeting them often have much longer half-lives in the range of weeks. Therefore, binding of a mAb to a ligand with rapid turnover usually results in

significant accumulation of mAb/ligand complex, sometimes up to 100,000-fold above baseline levels of the ligand, which would greatly increase the difficulty of accurately determining free ligand profiles (3–5). The challenge thus facing pharmaceutical scientists is how to provide a reliable dose projection for mAbs against soluble targets with rapid turnover in both preclinical and clinical development under these circumstances?

Mechanism-based pharmacokinetic/pharmacodynamic (PK/PD) modeling has long been used to facilitate understanding of the dose–response relationship of mAbs (6–9). In particular, a target-mediated drug disposition (TMDD) model has been applied broadly for description of the PK/PD relationship of mAbs against their targets as it delineates the impact of target engagement (TE) on downstream pharmacological effects (9,10). For mAbs against soluble targets, a “quasi-equilibrium,” or “rapid binding,” model has been proposed to simplify the TMDD model, which replaces the two hard-to-estimate binding constants (k_{on} and k_{off}) with one equilibrium dissociation constant (K_D) (11,12). It is based on the fact that the mAb/ligand association process (k_{on}) is usually several orders of magnitude faster compared to the *in vivo* study sampling intervals, and thus, the mAb/ligand interaction can be treated as at “quasi-equilibrium” (11). An important conclusion of the

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model is that under the “quasi-equilibrium” conditions, the free ligand level is a function of total mAb level, total ligand level, and *in vivo* K_D (4,5,11,12). This is important because total mAb and total ligand levels are usually more readily measurable than the free ligand levels (3). Although *in vivo* K_D is not necessarily a readily available parameter, it should remain constant as long as the “quasi-equilibrium” condition is maintained. If the validity of the “quasi-equilibrium” interaction can be demonstrated for a mAb against its soluble ligand with rapid turnover and *in vivo* K_D can be determined, it would provide an effective way to determine free ligand profiles from total mAb and total ligand results.

The theoretical interplay of drug PK and target dynamics under “quasi-equilibrium” conditions has been analyzed extensively (4,5). The “quasi-equilibrium” interaction has also been demonstrated for anti-IgE mAbs and IgE *in vivo* (13–15). The turnover of IgE is relatively slow ($t_{1/2}$ =2.7 days in humans), which allowed relatively easy determination of the free IgE profiles following anti-IgE mAb dosing (13). The anti-IgE mAb PK, total IgE, and free IgE profiles together allowed successful estimation of all model parameters (13–15). The “quasi-equilibrium” model has also been applied to multiple mAbs and Fc-fusion proteins (e.g., denosumab, canakinumab, aflibercept) against soluble ligands with more rapid turnover (16–23). However, unlike the case of IgE, most of these examples lacked the free ligand profiles due to technical difficulties in bioanalysis (17–19,21–23). The lack of reliable free ligand profiles brings uncertainty to the validity and predictability of these models, especially if a good correlation between target engagement and downstream PD effect cannot be shown (19,20).

Here, a PK/TE/PD study in cynomolgus monkeys using interleukin-6 (IL-6) and siltuximab as model compounds was conducted to further investigate the validity of the “quasi-equilibrium” model for a mAb against a soluble ligand with rapid turnover. IL-6 is a 26-kDa multifunctional cytokine. It is a mediator of the acute phase response to inflammation and plays important roles in a wide range of biological activities like immune regulation, hematopoiesis, and oncogenesis (24,25). IL-6 is relevant to many human diseases such as rheumatoid arthritis, systemic lupus erythematosus, multiple myeloma, prostate cancer, Castleman’s disease, and diabetes (26–28). There is great interest in developing anti-IL-6 agents for the treatment of many of these diseases (26–28). Among them, siltuximab is a chimeric, anti-human IL-6 mAb currently being developed for the treatment of multicentric Castleman’s disease, among other indications (29–31).

The PK/TE/PD study in cynomolgus monkeys was specially designed to mitigate potential bioanalytical challenges associated with the free IL-6 assay in the presence of excessive amount of siltuximab/IL-6 complex. First, a very low dose of siltuximab was used to control the levels of siltuximab and siltuximab/IL-6 complexes. In addition, IL-6 infusion was used to increase the baseline levels of free IL-6 and facilitate the measurement of free IL-6 lowering. The resulting free IL-6 concentrations helped the establishment of a PK/TE model and estimation of the *in vivo* K_D of siltuximab/IL-6 interaction. The model-derived *in vivo* K_D was subsequently used to predict the free IL-6 concentration profile at higher, clinically relevant siltuximab doses, where accurate determination of free IL-6 may become technically too difficult. Since IL-6 directly stimulates the synthesis of the acute phase C-reactive protein (CRP) (24), CRP was used as a measure of

in vivo IL-6 activity throughout the study. A TE/PD model was developed to link the TE results (free IL-6) with downstream PD effects (CRP).

MATERIALS AND METHODS

Test Articles

Siltuximab (CNTO 328), a chimeric anti-human IL-6 mAb, was generated at Janssen R&D (Spring House, PA, USA) and had been described previously (31). Since no reliable source of recombinant monkey IL-6 protein can be found, recombinant human IL-6 (Humanzyme, Chicago, IL, USA) was used in the current monkey study. Human and cynomolgus monkey IL-6 share 96% homology in amino acid sequences, and siltuximab cross-reacted with monkey IL-6. No effort was made to distinguish exogenous human IL-6 versus endogenous monkey IL-6 in our studies.

Cynomolgus Monkey Study Design and Sample Collection

The cynomolgus monkey study was conducted at WuXi AppTec (Suzhou, China), using biologics-naïve adult male monkeys. All studies were approved by the IACUC of WuXi AppTec. The animals were randomized into five groups with five monkeys per group:

- Group 1 animals received a single intravenous (IV) bolus dose of siltuximab at 0.1 mg/kg on study day 0. A very low dose of siltuximab was used to control the levels of siltuximab and siltuximab/IL-6 complexes to facilitate free IL-6 measurement. One animal in this group (Gp1–3) suffered mechanical injuries during the study, developed infection later, and had to be removed from the study on day 22. Results from this animal were excluded for modeling purpose.
- Group 2 animals first received two 4-h IV infusions of recombinant human IL-6 at 0.7 and 2.1 ng/kg on day 0 and day 14, respectively, then an IV bolus dose of siltuximab at 0.1 mg/kg on day 42, and finally a 4-h IV infusion of recombinant human IL-6 at 70 ng/kg on day 43. The amounts of IL-6 being infused during the first two infusions were designed to give approximately 10-fold increase in IL-6 levels, based on the initial IL-6 elimination rate estimate from literature data (24,26). The results were used to examine the PK characteristics of IL-6 and characterize the stimulatory effect of free IL-6 on CRP in absence of siltuximab. The third IV infusion following siltuximab dosing was used to increase the baseline levels of IL-6 and demonstrate the inhibitory effect of siltuximab on IL-6 activity.
- Groups 3 and 4 animals first received an IV bolus dose of phosphate-buffered saline (PBS) on day 0 and then on day 7, an IV bolus dose of siltuximab at 1 and 10 mg/kg, respectively.

The interplay between siltuximab and total/free IL-6 was examined following these higher, clinically relevant doses of siltuximab. Dosing of PBS was used to examine the IL-6/CRP elevation in response to IV dosing.

There was a fifth group of animals that received a single IV bolus dose of siltuximab/IL-6 complex (0.1 mg/kg siltuximab with 2% IL-6 occupancy) on day 0. However, significant protein aggregation in the dosing solution of this group was later identified (data not shown). Therefore, no result from group 5 was available for PK/TE modeling purpose. Since the relationship between free IL-6 and CRP was not expected to be impacted by this dosing solution issue, free IL-6 and CRP results from this group were still utilized for the development of TE/PD modeling.

Animals in group 2 were followed for 4 weeks following the siltuximab dosing. Animals in all other groups were followed for 8 weeks following siltuximab dosing. Serial blood samples were collected via a cephalic or saphenous vein from un-anesthetized animals at predetermined time points. Serum was derived by centrifugation at $1,800\times g$ for 15 min after allowing blood samples to clot at room temperature. The serum samples were stored frozen at -70°C or below until samples were analyzed.

Bioanalytical Methods

Serum concentrations of total siltuximab were determined by an electrochemiluminescence-based immunoassay (ECLIA) on the Meso Scale Discovery (MSD®) platform (Meso Scale Discovery, Rockville, MD, USA). Briefly, siltuximab was captured by a biotinylated anti-idiotypic antibody (Janssen R&D) onto a streptavidin-coated MSD plate. After washing, bound siltuximab was detected by a second anti-idiotypic antibody labeled with ruthenium. The assay measures total siltuximab, and the presence of IL-6 did not affect the recovery of siltuximab. The assay was qualified for accuracy, precision, spike recovery, dilutional linearity, and stability. The lower limit of quantification (LLOQ) of the assay was 0.135 nM (20 ng/mL).

Serum concentration of IL-6 was also determined by ECLIA methods on the MSD platform. Two anti-human IL-6 antibodies from R&D Systems (Minneapolis, MN, USA) were labeled with biotin and ruthenium for capture and detection, respectively. For the total IL-6 assay, serum samples were first treated with a high pH buffer to dissociate and denature any potential mAb bound to IL-6. For the free IL-6 assay, serum samples were first passed through MabSelect SuRe Protein A resin (GE Healthcare, Piscataway, NJ, USA) to remove all mAb and mAb-bound IL-6. Both assays were qualified for accuracy, precision, spike recovery, dilutional linearity, and stability. *In vitro* spike-recovery experiments showed that presence of up to 100 $\mu\text{g/mL}$ of siltuximab does not affect the recovery of total IL-6 in the total IL-6 assay, and up to 1,000-fold excess of total IL-6 had limited impact on the recovery of free IL-6 in the free IL-6

assay (data not shown). The LLOQ of the total IL-6 assay was 4.5×10^{-4} nM (12.8 pg/mL), and the LLOQ of the free IL-6 assay was 1.7×10^{-5} nM (0.48 pg/mL).

Serum concentrations of CRP were measured by a particle-enhanced immunoturbidimetric kit from DiaSys Diagnostic System (Holzheim, Germany) on a HITACHI 7180 biochemistry analyzer according to the manufacturer's protocol. The assay was qualified for accuracy, precision, recovery, dilutional linearity, and stability. The LLOQ of the CRP assay was 2.1 nM (0.05 mg/L).

PK/TE Model

A “quasi-equilibrium” TMDD model was used to describe the interaction between siltuximab and IL-6 in the systemic circulation of cynomolgus monkeys (11). The model scheme is shown in Fig. 1. Given the bivalent nature of mAbs, the model assumed two independent IL-6 binding sites for each siltuximab molecule, and the model was operated in molar concentrations. The free siltuximab (C) in the central compartment was assumed to undergo first-order elimination (k_{el_mAb}) and be distributed to and from nonspecific peripheral tissue-binding sites (A_T) by first-order rates (k_{pt} and k_{tp}). The free siltuximab (C) in the central compartment was assumed to interact with the free IL-6 (R) via a reversible “quasi-equilibrium” binding process:

$$K_D = R \cdot C / RC \quad (1)$$

where K_D denotes the equilibrium dissociation constant and RC represents the siltuximab/IL-6 complex. The RC complex was assumed to either dissociate or be degraded by a first-order rate process (k_{el_cplx}). The turnover kinetics of the free IL-6 was characterized by k_{syn} , zero-order rate of synthesis, and k_{el_IL-6} , first-order rate constant of degradation. The “quasi-equilibrium” TMDD model was described as:

$$dC_{tot}/dt = -k_{el_cplx} \cdot C_{tot} - (k_{el_mAb} + k_{pt} - k_{tp}) \cdot C + k_{tp} \cdot A_T / V_c \quad (2)$$

$$dA_T/dt = k_{tp} \cdot C \cdot V_c - k_{pt} \cdot A_T \quad (3)$$

$$dR_{tot}/dt = k_{syn} - (k_{el_cplx} - k_{el_IL-6}) \cdot (C_{tot} - C) - k_{el_IL-6} \cdot R_{tot} \quad (4)$$

Fig. 1. Schematic representation of the PK/TE and TE/PD models for description of the interaction between siltuximab and IL-6 and the impact of free IL-6 on CRP. The parameter names are as defined in “MATERIALS AND METHODS”

$$C = \frac{1}{2} \cdot \left\{ (C_{\text{tot}} - R_{\text{tot}} - K_D) + \text{SQRT} \left((C_{\text{tot}} - R_{\text{tot}} - K_D)^2 + 4 \cdot K_D \cdot C_{\text{tot}} \right) \right\} \quad (5)$$

$$R = R_{\text{tot}} \cdot K_D / (K_D + C) \quad (6)$$

where C_{tot} represents the total siltuximab concentration ($C_{\text{tot}} = C + RC$) and R_{tot} represents the total IL-6 concentration ($R_{\text{tot}} = R + RC$). The initial condition for the differential equations, i.e., Eqs. 2–4, is as follows: $C_{\text{tot}}(0) = \text{Dose}/V_c$; $A_T(0) = 0$; $R_{\text{tot}}(0) = k_{\text{syn}}/k_{\text{el_IL-6}}$.

For PK/TE modeling of the low-dose (0.1 mg/kg) groups (groups 1–2), the “quasi-equilibrium” TMDD model was fitted to total siltuximab concentrations (C_{tot}), total IL-6 concentrations (R_{tot}), and free IL-6 (R) concentrations simultaneously. Free IL-6 values below the LLOQ were treated as $\frac{1}{2}$ of LLOQ, as this method is considered superior over other simple methods (i.e., treat them as 0 or missing) (32). Due to the complexity of our structure models and the limited amount of data available, it is not feasible for our data to support any more sophisticated mathematical modeling of BQLs (32). When applying the PK/TE modeling to the high-siltuximab-dose groups (1 and 10 mg/kg, groups 3–4), only total siltuximab (C_{tot}) and total IL-6 concentrations (R_{tot}) were used for model fitting.

To model the endogenous IL-6 elevation in response to IV bolus dosing (“dosing effect”), a first-order IL-6 input from an “IL-6 depot” function was used to approximate the effect of IV bolus injection. For the “dosing effect” associated with 4-h IV infusion of exogenous IL-6, a zero-order infusion input coupled with a first-order input of endogenous IL-6 from a depot was used to approximate the effect. Details of the modeling of the “dosing effect” are provided in [Supplemental Materials](#).

TE/PD Model

The scheme of the TE/PD model is also depicted in Fig. 1. A modified indirect response model was used to describe the relationship between free IL-6 and CRP, i.e., free IL-6 (R) stimulates CRP production with a linear stimulation factor S_{CRP} . The homeostasis of CRP was described by k_{in} , a zero-order input rate, and k_{out} , a first-order output rate constant, with the relationship of $k_{\text{in}} = k_{\text{out}} \cdot I_{\text{CRP}}$ where I_{CRP} is the basal CRP concentration. The modified indirect response model can be described by:

$$d\text{CRP}/dt = k_{\text{in}} \cdot (1 + S_{\text{CRP}} \cdot (R(t) - I_R)) - k_{\text{out}} \cdot \text{CRP} \quad (7)$$

where CRP is CRP concentration, $R(t)$ is free IL-6 concentration at time t , and I_R is the basal free IL-6 concentration at time 0. The initial condition for this differential equation is $\text{CRP}(0) = k_{\text{in}}/k_{\text{out}}$. In addition, I_R was modeled as a covariate for both k_{in} and S_{CRP} using power covariate models:

$$\text{TV}k_{\text{in}} = \theta_1 \cdot (I_R/\text{median})^{\theta_2} \quad (8)$$

$$\text{TV}S_{\text{CRP}} = \theta_3 \cdot (I_R/\text{median})^{\theta_4} \quad (9)$$

where median represents the median basal free IL-6 concentration (I_R) among all animals.

Model Development

Model fitting and simulations were performed with NONMEM version 7.2 (ICON Development Solutions, Ellicott City, MD, USA) with a Gfortran Compiler (Free Software Foundation, Boston, MA, USA), and the first-order approximation method was used. FOCE was the primary estimation method, and FO was employed for the “quasi-equilibrium” TMDD model for the low-dose groups (groups 1 and 2). NONMEM outputs were processed using PDX-Pop 5.0 (ICON Development Solutions, Ellicott City, MD, USA) and Xpose version 4.1.0 (Uppsala University, Uppsala, Sweden). R version 2.15.1 (Free Software Foundation, Boston, MA, USA) was used for plot generation.

For both the “quasi-equilibrium” PK/TE model and the modified indirect response TE/PD model, a “log-transformation of both sides” approach was used, and additive error models after log-transformation were employed for the residual variability. Final models were evaluated by goodness-of-fit plots, bootstrap analysis, and model stability (represented by condition number).

RESULTS

The “Quasi-equilibrium” Interaction Between Siltuximab and IL-6

Under “quasi-equilibrium” condition, free ligand levels can be calculated from total mAb, total ligand, and *in vivo* K_D (Eqs. 5 and 6). Validity of this “quasi-equilibrium” equation for the interaction of siltuximab and IL-6 in systemic circulation was first examined by comparing the calculated free IL-6 levels to the measured free IL-6 levels from the 0.1 mg/kg low-dose groups (groups 1 and 2). The *in vitro* K_D of siltuximab and human IL-6 was 6.25 pM as measured by Biacore (29). When an *in vivo* K_D of 15 pM was used, the calculated free IL-6 levels aligned well with the measured free IL-6 levels for all ten animals across all time points, including animals that underwent significant IL-6 level fluctuations for various reasons (Fig. 2). The consistent agreement between the two independent sets of data strongly suggested that both measured and calculated free IL-6 values are close to the true free IL-6 values under these low-siltuximab-dose conditions. Since the calculation of free IL-6 was based on validity of the “quasi-equilibrium” equation, it provided direct evidence that the interaction between siltuximab and IL-6 in systemic circulation is at “quasi-equilibrium.” In other words, regardless of how the siltuximab and IL-6 levels may have changed, at any given time point, the relationship between siltuximab, IL-6, and siltuximab/IL-6 complex in systemic circulation can be described by the simple equation: $K_D = R \cdot C/RC$.

Following higher doses (1 and 10 mg/kg) of siltuximab (groups 3 and 4), however, no single *in vivo* K_D value can be found that would allow good alignment between calculated free IL-6 and measured values across all time points for even a single individual animal (data not shown). When the same *in vivo* K_D of 15 pM was used, the measured free IL-6 levels were significantly higher than the calculated values, especially for the 10 mg/kg group (data not shown). These results suggested that either the measured or the calculated free IL-6 levels were not accurate following higher doses of siltuximab.

Fig. 2. Comparison of measured and calculated free IL-6 concentration *versus* time profiles in individual animals of groups 1 and 2. The dotted lines with “X”s represent measured free IL-6 profiles, and the solid lines with hollow circles represent calculated free IL-6. The hollow stars (marked at $\frac{1}{2}$ of LLOQ) represent measured free IL-6 levels that are $<$ LLOQ. **a** Group 1. **b** Group 2

Since the validity of “quasi-equilibrium” interaction between siltuximab and IL-6 had been demonstrated with results from the low-dose groups, it is conceivable that the “quasi-equilibrium” status will not change when higher levels of siltuximab are present. The accuracy of measured total siltuximab and total IL-6 is also unlikely to be compromised under higher siltuximab dose conditions. In contrary, measuring free IL-6 levels in higher excess of siltuximab and siltuximab/IL-6 complex is expected to be more challenging (3). Moreover, while the measured free IL-6 levels for groups 3 and 4 at later time points on average were higher than those of group 1 (compare Figs. 3 and 4), more sustained CRP lowering was observed for groups 3 and 4 (see Fig. 5). Together, these results suggested that the accuracy of measured free IL-6 levels for groups 3 and 4 was in question.

Based on these observations, a decision was made to establish a “quasi-equilibrium” PK/TE model using only data from the 0.1 mg/kg siltuximab dose groups and then use the model-derived *in vivo* K_D to predict free IL-6 profiles following 1 and 10 mg/kg siltuximab doses.

Development of a “Quasi-equilibrium” PK/TE Model

Total siltuximab, total IL-6, and free IL-6 results from groups 1 and 2 were fitted simultaneously into a PK/TE model under “quasi-equilibrium” condition as described in “MATERIALS AND METHODS.” As shown in Fig. 3, the model well captured the observed total siltuximab, total IL-6, and free IL-6 profiles. All key model parameters were successfully estimated with good precision. These include elimination rate constants at 0.0914 day^{-1} for siltuximab (elimination half-life=7.6 days), 0.171 day^{-1} for siltuximab/IL-6 complex (elimination half-life=3.8 days), 232 day^{-1} for free IL-6 (elimination half-life=4.3 min), and an *in vivo* K_D of 21 pM. The estimated model parameters along with inter-subject variability (IIV), residual variability (RV), and

bootstrap estimates are shown in Table I. Model diagnostics are provided in [Supplementary Materials](#).

Using the PK/TE Model to Predict Free IL-6 Levels Following Higher Doses of Siltuximab

Since the *in vivo* K_D between siltuximab and IL-6 is expected to be a constant, the PK/TE model was used to predict free IL-6 levels following higher doses (1 or 10 mg/kg) of siltuximab. The exact same structure of the PK/TE model was applied to high-dose groups, but only the total siltuximab and total IL-6 data from groups 3 and 4 were used for fitting. For parameters not expected to change with the dose conditions but difficult to estimate without free IL-6 results, they were fixed to values obtained from the PK/TE model from the low-dose groups: $K_D=21 \text{ (pM)}$; $k_{el_IL-6}=232 \text{ (day}^{-1}\text{)}$; $k_{el_cplx}=0.171 \text{ (day}^{-1}\text{)}$.

The time courses of observed and predicted total siltuximab, total IL-6, and free IL-6 concentrations are shown in Fig. 4. The model well captured the total siltuximab and total IL-6 profiles following both 1 and 10 mg/kg siltuximab doses. As anticipated, the model-predicted free IL-6 levels deviate significantly from the measured values, especially for the 10 mg/kg group (Fig. 4). The estimated model parameters along with IIV, RV, and bootstrap estimates are shown in Table II. Details on modeling of the “dosing effect” as well as model diagnostic plots are also provided in [Supplemental Materials](#).

Development of a TE/PD Model for Free IL-6 and CRP and Validation of PK/TE Model-Predicted Free IL-6

We next examined whether the PK/TE model-predicted free IL-6 levels following higher doses of siltuximab were consistent with observed CRP results using a TE/PD (IL-6/CRP) model. Since IL-6 is known to stimulate CRP production, a modified indirect response model was used to

Fig. 3. Observed and PK/TE model-predicted total siltuximab, total IL-6, and free IL-6 concentrations *versus* time profiles following a low dose (0.1 mg/kg) of siltuximab. **a** Group 1. **b** Group 2. The LLOQ of free IL-6 assay is shown as a dotted line (1.7×10^{-5} nM, or 0.48 pg/mL). Free IL-6 levels < LLOQ was plotted at $\frac{1}{2}$ of LLOQ. Time of the third IL-6 infusion (day 43) was marked with an arrow in **b**.

describe the relationship between free IL-6 level and CRP level as described in “**MATERIALS AND METHODS.**” Measured free IL-6 values for groups 1 and 2 and PK/TE model-predicted free IL-6 values for groups 3 and 4 were used together for model building. In addition, measured free IL-6 and CRP results from group 5 and those prior to siltuximab dosing from groups 2 (following the first two IL-6 infusions), 3, and 4 (following the PBS dosing) were also used for TE/PD model development.

A simple linear stimulation function was found to be able to capture both elevation and suppression of CRP from the baseline in response to free IL-6 level changes in all groups. Baseline IL-6 level was modeled as a covariate for both k_{in} and S_{CRP} because their inclusion significantly decreased the objective function value of the model and reduced the inter-subject variability for both k_{in} and S_{CRP} . The time courses of observed and predicted CRP levels for all individual animals are shown in Fig. 5. The model was able to capture the CRP profiles in all the settings of our study: Both the CRP elevation and the CRP suppression in response to free IL-6 level changes from baseline were described well by a single indirect response model with a linear stimulation function. In particular, the observed CRP results for groups 3 and 4 agreed well with model-predicted CRP based on PK/TE

Fig. 4. Observed and PK/TE model-predicted total siltuximab, total IL-6, and free IL-6 concentrations *versus* time profiles following a high dose of siltuximab. **a** Group 3, 1 mg/kg. **b** Group 4, 10 mg/kg. The LLOQ of free IL-6 assay is shown as a dotted line (1.7×10^{-5} nM, or 0.48 pg/mL). Free IL-6 levels < LLOQ was plotted at $\frac{1}{2}$ of LLOQ.

model-predicted free IL-6 (Fig. 5). These results provided confidence that the PK/TE model-predicted free IL-6 values following higher doses of siltuximab were pharmacologically plausible. Estimated TE/PD model parameters along with IIV, RV, and bootstrap estimates are shown in Table III. The model diagnostics are provided in [Supplementary Materials](#).

DISCUSSION

Using siltuximab and IL-6 as model compounds, a case example was presented to demonstrate the validity of the “quasi-equilibrium” model for mAbs against soluble ligands with rapid turnover and how an integrated bioanalytical and PK/TE/PD modeling approach can be used to understand the therapeutic efficacy for mAbs against this type of targets. Direct measurement of the lowering of free ligand following mAb treatment can be technically challenging, especially in the presence of an excessive amount of mAb and mAb/ligand complex (3). A great deal of discussion surrounds the question of whether the free ligand results obtained from *in vitro* assays actually reflect the *in vivo* situation (3–5,33). The fact that cytokines are typical of very low baseline levels also

Fig. 5. Comparison of measured and TE/PD model-predicted CRP concentration *versus* time profiles in individual animals. The black circles represent measured CRP data, the red lines represent individual fitting, and the blue dotted lines represent population fitting. Asterisk Animal Gp1-3 was excluded for modeling purpose due to the mechanical injury it suffered

adds difficulty to the task. To address these issues, a free IL-6 assay with relatively high total IL-6 tolerance level was developed, and a monkey PK/TE/PD study specially designed to mitigate the bioanalytical challenges associated with the free IL-6 measurement was conducted. For the monkey study, a very low dose of siltuximab (0.1 mg/kg) was used to control the total/free IL-6 ratio, and IL-6 infusion was used in one group to elevate baseline IL-6 levels. The larger-than-expected “dosing effect” also helped to elevate the initial levels of IL-6 in all other groups. Under these conditions, the free IL-6 assay was able to generate adequately reliable free IL-6 results, as demonstrated by the consistent agreement between the measured free IL-6 values and the free IL-6 values calculated from total IL-6, total siltuximab, and an *in*

vivo K_D (Fig. 2). These results effectively demonstrated that the *in vivo* interaction between siltuximab and IL-6 obeys the simple “quasi-equilibrium” equation: $K_D = R \cdot C / RC$. A PK/TE mathematical model based on the “quasi-equilibrium” equation was developed via simultaneous fitting of the total siltuximab, total IL-6, and free IL-6 profiles from the low-dose groups. The model successfully estimated all key model parameters with good precision. The model estimated PK parameters for siltuximab are in good agreement with those anticipated for a typical mAb (6). The model estimated elimination rate constant for siltuximab/IL-6 complex is only 2-fold higher than that of siltuximab, but more than 1,000-fold lower than that of free IL-6, which explained the dramatic increase of total IL-6 following siltuximab dosing. The model

Table I. Estimated Model Parameters for the PK/TE Model from Low-Siltuximab-Dose Groups

Parameter (unit)	Definition	Estimate (%RSE)	Bootstrap estimate (%RSE)	IIV in %CV (%RSE)
k_{el_mAb} (day ⁻¹)	Elimination rate constant of siltuximab	0.0914 (7.9)	0.0925 (8.28)	21.4 (32.5)
k_{pt} (day ⁻¹)	Transfer rate constant from central to peripheral compartment	0.356 (11.3)	0.36 (10.61)	–
k_{tp} (day ⁻¹)	Transfer rate constant from peripheral to central compartment	0.351 (5.95)	0.352 (5.62)	–
V_c (L)	Volume of central compartment	0.126 (4.94)	0.126 (4.92)	19.7 (48.6)
k_{syn} (nM day ⁻¹)	Synthesis rate of IL-6	0.00712 (13.6)	0.0071 (16.41)	15.7 (61.1)
k_{el_IL-6} (day ⁻¹)	Elimination rate constant of IL-6	232 (20.9)	234 (23.88)	–
k_{el_cplx} (day ⁻¹)	Elimination rate constant of siltuximab/IL-6 complex	0.171 (38.7)	0.17 (42.21)	–
K_D (pM)	Equilibrium dissociation constant	21 (8.76)	21 (8.57)	–
$RV_{total\ siltuximab}$ (%CV)	Residual variability for total siltuximab	7.43 (14.5)	7.37 (14.14)	–
$RV_{total\ IL-6}$ (%CV)	Residual variability for total IL-6	25 (16.7)	24.5 (19.22)	–
$RV_{free\ IL-6}$ (%CV)	Residual variability for free IL-6	40.6 (30.4)	40.15 (30.33)	–

%RSE relative standard error as a percentage, %CV coefficient of variation as a percentage

estimated *in vivo* K_D for siltuximab and IL-6 was 21 pM, only about 3-fold higher than the reported *in vitro* K_D value for siltuximab and human IL-6 (29).

Following higher doses of siltuximab, however, even our high-performance free IL-6 assay generated results that deviated significantly from the values calculated from the “quasi-equilibrium” equation. Since the validity of “quasi-equilibrium” interaction between siltuximab and IL-6 had been demonstrated with results from the low-dose groups and the “quasi-equilibrium” nature of siltuximab and IL-6 interaction is not supposed to change following higher doses of siltuximab, it was concluded that the measured free IL-6 values are no longer reliable under these high-dose conditions. Fortunately, as the *in vivo* K_D between siltuximab and IL-6, elimination rates of IL-6 and siltuximab/IL-6 complex are not expected to change with the siltuximab dose, their values derived from the low-dose PK/TE model allowed development of a PK/TE model for the high-dose groups using total siltuximab and total IL-6 data only. The PK/TE model for the high-dose groups was subsequently used to predict free IL-6 levels following higher, clinically relevant doses of siltuximab.

To assess the *in vivo* relevance of free IL-6 results obtained with *in vitro* assays or model prediction, CRP, the well-established downstream biomarker of *in vivo* IL-6 activity, was used. An indirect response TE/PD model with a simple linear stimulation function was developed to describe the relationship between free IL-6 and CRP. The TE/PD model well captured both the elevation and the suppression of CRP in response to free IL-6 level changes. The model estimated elimination rate constant for CRP was 0.894 day⁻¹ (elimination half-life=18 h), which also agreed well with previously reported CRP half-life in humans (31). Importantly, the model-predicted CRP results using the PK/

TE model-predicted free IL-6 levels for the high-siltuximab-dose groups agreed well with the observed CRP results, providing direct evidence that the PK/TE model-predicted free IL-6 levels are physiologically and pharmacologically plausible.

To further examine the factors that affecting the reliability of free IL-6 assay, the measured/predicted free IL-6 ratio was plotted against total/free IL-6 ratio (Fig. 6). A clear trend was seen that higher total/free IL-6 ratios were associated with higher measured/predicted free IL-6 ratios. There also appeared to be a threshold of total/free IL-6 ratio: When the total/free IL-6 ratio was <2,000, the measured free IL-6 values mostly were within a 2-fold range of the predicted free IL-6 values, but as the total/free IL-6 ratio increased beyond 2,000, the measured free IL-6 values increasingly deviated from the model-predicted free IL-6 values (Fig. 6). A possible explanation for this finding is that a small percentage of mAb-bound ligand inevitably will “fall-off” from the complex during serum sample preparation, storage, or, more likely, bioanalysis processes. This *in vitro* “fall-off” differs from the reversible *in vivo* mAb/ligand association/dissociation process in that the mAb and ligand molecules are no longer at equilibrium during the process. Besides assay properties (i.e., percentage of “fall-off”), it is conceivable that the amount of total IL-6 might have “fallen-off” and subsequently shown as free IL-6 in the free assay is proportional to the amount of total IL-6. But whether this “fall-off” would significantly affect the free IL-6 recovery depends on the true free IL-6 levels. Therefore, total/free ligand ratio is a key parameter to gauge the free ligand assay reliability, and it is ultimately important to evaluate the reliability of any free ligand assay across the anticipated range of total/free ligand ratios. As suggested by the plot in Fig. 6, our free IL-6 can

Table II. Estimated Model Parameters for the PK/TE Model from High-Siltuximab-Dose Groups

Parameter (unit)	Estimate (%RSE)	Bootstrap Estimate (%RSE)	IIV in %CV (%RSE)
k_{el_mAb} (day ⁻¹)	0.0957 (6.8)	0.0957 (6.77)	21.2 (31.7)
k_{pt} (day ⁻¹)	0.472 (13.4)	0.475 (12.69)	–
k_{tp} (day ⁻¹)	0.452 (17.5)	0.456 (17.03)	–
V_c (L)	0.134 (3.86)	0.134 (3.89)	12.1 (18)
k_{syn} (nM day ⁻¹)	0.0219 (20.9)	0.0224 (21.61)	59.1 (30.7)
$RV_{total\ siltuximab}$ (%CV)	9.8 (26.4)	9.63 (25.7)	–
$RV_{total\ IL-6}$ (%CV)	45.9 (21.9)	44.61 (22.6)	–

%RSE relative standard error as a percentage, %CV coefficient of variation as a percentage

Table III. Estimated Model Parameters for the TE/PD Indirect-Response Model

Parameter (unit)	Definition	Estimate (%RSE)	Bootstrap estimate (%RSE)	IIV in %CV (%RSE)
k_{in} (nM day ⁻¹)	Production rate of CRP at I_R (initial value of IL-6)	14 (15.1)	14.34 (15.7)	48.9 (36.7)
k_{out} (day ⁻¹)	Elimination rate constant of CRP	0.894 (14.5)	0.912 (15.1)	–
S_{CRP} (nM ⁻¹)	Linear stimulation factor of IL-6 on CRP production	9,930 (10.9)	9,928.7 (11.8)	45.7 (60.3)
I_R on k_{in}	Effect of I_R (baseline IL-6) on S_{CRP}	0.518 (16.0)	0.51 (20.0)	–
I_R on S_{CRP}	Effect of I_R (baseline IL-6) on k_i	–0.947 (10.6)	–0.96 (15.2)	–
RV_{CRP} (%CV)	Residual variability for CRP	46.2 (10.8)	46.0 (10.8)	–
$Cov_{kin-SCRP}$	Covariance between k_{in} and S_{CRP}	–0.152 (64.1)	–0.155 (63.9)	–

%RSE relative standard error as a percentage, %CV coefficient of variation as a percentage

tolerate a total/free ligand ratio of approximately 2,000, which could be the highest among all reported free ligand assays, at least to our knowledge (3–5,20,33). But following higher, clinically relevant doses of siltuximab, the total/free IL-6 ratio can reach 10^6 – 10^7 , where even a 0.001% “fall-off” of IL-6 from the complex would completely distort the true free IL-6 profile; it apparently exceeded the suitable range of our free IL-6 assay.

Our results also clearly demonstrated the importance of understanding the interplay between mAb/ligand and the ligand dynamics for the prediction of mAb efficacy. For small molecule drugs, the target drug trough concentration usually can be extrapolated from *in vitro* results (34). Similar extrapolation from *in vitro* results can be completely misleading for mAbs against soluble ligands with rapid turnover. Following mAb treatment, there usually will be a rapid accumulation of mAb/ligand complex due to the dramatic differences in the elimination rates for a free ligand and a mAb/ligand complex. Dissociation of the accumulated mAb/ligand complex will result in the return of free ligand to baseline while free mAb levels are still orders of magnitude higher than the free ligand levels. Again, the quantitative relationship among free mAb, free ligand, and mAb/ligand complex can be described by the “quasi-equilibrium” equation, and quantitative PK/TE/PD modeling is an invaluable tool to understand the duration of action for mAbs. Though it has been well recognized that the target dynamics can have profound impact on the efficacy of a mAb (4,5), it is usually difficult to experimentally determine the *in vivo* ligand production and elimination rates (35). Our results showed that through careful planning, PK/PD modeling can be an effective means to determine ligand production and

elimination rates, and the information can in turn be used to assess efficacious dose level and duration of action for therapeutics targeting soluble ligands.

It will be of high interest to expand our current findings into clinics, though a couple of confounding factors need to be taken into consideration first. For example, the signaling pathways for IL-6 are complex and involve multiple IL-6 receptors (IL-6Rs) in multiple organs (36). Our current mechanism-based model only focused on the interaction between siltuximab and IL-6 in the systemic circulation, and only systemic IL-6 activity (CRP) was monitored. Since siltuximab binds to IL-6 with affinities in the picomolar range and soluble IL-6Rs are reported to have IL-6 binding affinities in the nanomolar or micromolar range (36,37), the soluble IL-6Rs in the systemic circulation are not expected to have significant impact on the interaction between siltuximab and IL-6 and were not taken into consideration in our models. However, the therapeutic efficacy of anti-IL-6 antibodies for most disease indications is expected to function at tissue sites, where it should be driven by the local concentrations of anti-IL-6 antibodies and IL-6. Additionally, it had been reported that IL-6 may form high affinity hexameric IL-6/receptor complex at disease tissue, with affinities in the picomolar range (38). Under this circumstance, the interaction between IL-6 and IL-6Rs apparently cannot be ignored for appropriate understanding of the therapeutic efficacy of anti-IL-6 antibodies. Another interesting finding of our work is that the model estimated baseline IL-6 production rate of the high-dose groups (groups 3 and 4) is approximately 3-fold higher than that of the low-dose groups (groups 1 and 2), suggesting that sustained IL-6 lowering may lead to an increase in its production. The potential implication of this finding for anti-IL-6 therapeutics needs to be further investigated. Though thorough understanding of the clinical efficacy of anti-IL-6 antibodies will require considerably more work, our current mechanism-based PK/TE/PD model improves our understanding of the mAb/ligand interplay and provides a framework for more sophisticated, disease-related models, which makes it advantageous over empirical models that only attempt to link drug concentration to clinical response.

CONCLUSIONS

A mechanism-based PK/TE/PD model was established for siltuximab, IL-6, and CRP. Its ability to predict free IL-6 levels at higher, therapeutically relevant doses was demonstrated. Soluble ligands with rapid turnover have been one of the most important classes of targets for therapeutic mAbs, and they continue to make up a significant portion of the

Fig. 6. Plot of the measured/predicted free IL-6 ratios against corresponding total/free IL-6 ratios for all individual data points

biotherapeutic pipeline. The integrated PK/PD modeling and bioanalytical strategy for using target engagement data to project dose regimens for such mAbs should have broad application for development of mAbs against this class of targets.

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REFERENCES

- Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol*. 2010;10:301–16.
- Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. *Nat Rev Cancer*. 2012;12:278–87.
- Lee JW, Kelley M, King LE, Yang J, Salimi-Moosavi H, Tang MT, *et al*. Bioanalytical approaches to quantify “total” and “free” therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. *AAPS J*. 2011;13:99–110.
- Davda JP, Hansen RJ. Properties of a general PK/PD model of antibody–ligand interactions for therapeutic antibodies that bind to soluble endogenous targets. *MAbs*. 2010;2:576–88.
- Tang C, Prueksaritanont T. Theoretical analysis of interplay of therapeutic protein drug and circulating soluble target: temporal profiles of ‘free’ and ‘total’ drug and target. *Pharm Res*. 2011;28:2447–57.
- Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin Pharmacol Ther*. 2008;84:548–58.
- Deng R, Jin F, Prabhu S, Iyer S. Monoclonal antibodies: what are the pharmacokinetic and pharmacodynamic considerations for drug development? *Expert Opin Drug Metab Toxicol*. 2012;8:141–60.
- Zhao L, Shang EY, Sahajwalla CG. Application of pharmacokinetics pharmacodynamics/clinical response modeling and simulation for biologics drug development. *J Pharm Sci*. 2012;101:4367–82.
- Hu L, Hansen RJ. Issues, challenges, and opportunities in model-based drug development for monoclonal antibodies. *J Pharm Sci*. 2013;102:2898–908.
- Mager DE, Jusko WJ. General pharmacokinetic model for drugs exhibiting target-mediated drug disposition. *J Pharmacokinet Pharmacodyn*. 2001;28:507–32.
- Mager DE, Krzyzanski W. Quasi-equilibrium pharmacokinetic model for drugs exhibiting target-mediated drug disposition. *Pharm Res*. 2005;10:1589–96.
- Gibiansky L, Gibiansky E. Target-mediated drug disposition model: approximations, identifiability of model parameters and applications to the population pharmacokinetic–pharmacodynamic modeling of biologics. *Expert Opin Drug Metab Toxicol*. 2009;5:803–12.
- Meno-Tetang GM, Lowe PJ. On the prediction of the human response: a recycled mechanistic pharmacokinetic/pharmacodynamic approach. *Basic Clin Pharmacol Toxicol*. 2005;96:182–92.
- Hayashi N, Tsukamoto Y, Sallas WM, Lowe PJ. A mechanism-based binding model for the population pharmacokinetics and pharmacodynamics of omalizumab. *Br J Clin Pharmacol*. 2007;63:548–61.
- Putnam WS, Li J, Haggstrom J, Ng C, Kadkhodayan-Fischer S, Cheu M, *et al*. Use of quantitative pharmacology in the development of HAE1, a high-affinity anti-IgE monoclonal antibody. *AAPS J*. 2008;10:425–30.
- Benincosa LJ, Chow FS, Tobia LP, Kwok DC, Davis CB, Jusko WJ. Pharmacokinetics and pharmacodynamics of a humanized monoclonal antibody to factor IX in cynomolgus monkeys. *J Pharmacol Exp Ther*. 2000;292:810–6.
- Marathe A, Peterson MC, Mager DE. Integrated cellular bone homeostasis model for denosumab pharmacodynamics in multiple myeloma patients. *J Pharmacol Exp Ther*. 2008;326:555–62.
- Lachmann HJ, Lowe P, Felix SD, Rordorf C, Leslie K, Madhoo S, *et al*. In vivo regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. *J Exp Med*. 2009;206:1029–36.
- Vugmeyster Y, Tian X, Szklut P, Kasaian M, Xu X. Pharmacokinetic and pharmacodynamic modeling of a humanized anti-IL-13 antibody in naive and Ascaris-challenged cynomolgus monkeys. *Pharm Res*. 2009;26:306–15.
- Betts AM, Clark TH, Yang J, Treadway JL, Li M, Giovannelli MA, *et al*. The application of target information and preclinical pharmacokinetic/pharmacodynamic modeling in predicting clinical doses of a Dickkopf-1 antibody for osteoporosis. *J Pharmacol Exp Ther*. 2010;333:2–13.
- Thai HT, Veyrat-Follet C, Vivier N, Dubruc C, Sanderink G, Mentré F, *et al*. A mechanism-based model for the population pharmacokinetics of free and bound aflibercept in healthy subjects. *Br J Clin Pharmacol*. 2011;72:402–14.
- Ait-Oudhia S, Lowe PJ, Mager DE. Bridging clinical outcomes of canakinumab treatment in patients with rheumatoid arthritis with a population model of IL-1 β kinetics citation. *CPT Pharmacometrics Syst Pharmacol*. 2012;1:e5.
- Chakraborty A, Tannenbaum S, Rordorf C, Lowe PJ, Floch D, Gram H, *et al*. Pharmacokinetic and pharmacodynamic properties of canakinumab, a human anti-interleukin-1 β monoclonal antibody. *Clin Pharmacokinet*. 2012;51:e1–18.
- Taga T, Kishimoto T. gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol*. 1997;15:797–819.
- Maggio M, Guralnik JM, Longo DL, Ferrucci L. Interleukin-6 in aging and chronic disease: a magnific pathway. *J Gerontol A Biol Sci Med Sci*. 2006;61:575–84.
- Nishimoto N, Kishimoto T. Inhibition of IL-6 for the treatment of inflammatory diseases. *Curr Opin Pharmacol*. 2004;4:386–91.
- Scheller J, Ohnesorge N, Rose-John S. Interleukin-6 transsignalling in chronic inflammation and cancer. *Scand J Immunol*. 2006;63:321–9.
- Barton BE. Interleukin-6 and new strategies for the treatment of cancer, hyperproliferative diseases and paraneoplastic syndromes. *Expert Opin Ther Targets*. 2005;9:737–52.
- van Zaanen HC, Lokhorst HM, Aarden LA, Rensink HJ, Warnaar SO, van der Lelie J, *et al*. Chimeric anti-interleukin 6 monoclonal antibodies in the treatment of advanced multiple myeloma: a phase I dose-escalating study. *Br J Haematol*. 1998;102:783–90.
- van Rhee F, Fayad L, Voorhees P, Furman R, Lonial S, Borghaei H, *et al*. Siltuximab, a novel anti-interleukin-6 monoclonal antibody, for Castleman's disease. *J Clin Oncol*. 2010;28:3701–8.
- Puchalski T, Prabhakar U, Jiao Q, Berns B, Davis HM. Pharmacokinetic and pharmacodynamic modeling of an anti-interleukin-6 chimeric monoclonal antibody (siltuximab) in patients with metastatic renal cell carcinoma. *Clin Cancer Res*. 2010;16:1652–61.
- Beal SL. Ways to fit a PK model with some data below the quantification limit. *J Pharmacokinet Pharmacodyn*. 2001;28:481–504.
- Hansen RJ, Brown RM, Lu J, Wroblewski VJ. Qualification of a free ligand assay in the presence of anti-ligand antibody Fab fragments. *MAbs*. 2013;5:288–96.
- Acosta EP, Limoli KL, Trinh L, Parkin NT, King JR, Weidler JM, *et al*. Novel method to assess antiretroviral target trough concentrations using in vitro susceptibility data. *Antimicrob Agents Chemother*. 2012;56:5938–45.
- Yewdell JW, Lacsina JR, Rechsteiner MC, Nicchitta CV. Out with the old, in with the new? Comparing methods for measuring protein degradation. *Cell Biol Int*. 2011;35:457–62.

36. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)*. 2012;122(143–59).
37. Ward LD, Howlett GJ, Discolo G, Yasukawa K, Hammacher A, Moritz RL, *et al*. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. *J Biol Chem*. 1994;269:23286–9.
38. Ward LD, Hammacher A, Howlett GJ, Matthews JM, Fabri L, Moritz RL, *et al*. Influence of interleukin-6 (IL-6) dimerization on formation of the high affinity hexameric IL-6.receptor complex. *J Biol Chem*. 1996;271:20138–44.