Research Article

Target-Mediated Disposition Model Describing the Dynamics of IL12 and IFN γ after Administration of a Mifepristone-Inducible Adenoviral Vector for IL-12 Expression in Mice

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Abstract. Interleukin-12 (IL12) is a cytokine with potential applications in the treatment of cancer given the potent immune response that it triggers, in part due to its ability to stimulate expression of interferon- γ (IFN γ). To avoid the toxicity associated with systemic exposure to IL12, a high-capacity adenoviral vector carrying a liver-specific, mifepristone-inducible IL12 expression system (HC-Ad/RUmIL12) has been developed. However, the maintenance of IL12 expression at therapeutic levels is compromised by the inhibitory effect of IFN γ on inducible systems. The aim of this work is to develop a semi-mechanistic model to characterize the relationship between IL12 and IFN γ in wild-type and knock-out mice for the IFN γ receptor treated with HC-Ad/RUmIL12 under different dosing regimens in order to better understand the key mechanisms controlling the system. Rapid binding was considered to account for target-mediated disposition exhibited by both cytokines (equilibrium dissociation constant were 18 and 2.28 pM for IL12 and IFN γ , respectively). The final model included: (1) IFN γ receptor turnover, (2) irreversible free cytokine elimination from the serum compartment, (3) internalization of the IL12 receptor complex, (4) IL12 expression upregulated by the co-administration of the adenoviral vector and mifepristone and downregulated by the IFN γ receptor, and (5) synthesis of IFN γ controlled by the relative increments in the bound IL12. In conclusion, a model simultaneously describing the kinetics of IL12 and IFN γ in the context of gene therapy was developed and validated with additional data. The model was applied to design an experimental dosing protocol intended to maintain sustained therapeutic IL12 levels.

KEY WORDS: cytokines; knock-out mice and gene therapy; pharmacokinetic/pharmacodynamic modelling; target-mediated drug disposition.

INTRODUCTION

Interleukin-12 (IL12) is a cytokine primarily produced by haematopoietic phagocytic cells such as monocytes,

macrophages or dendritic cells upon activation by an antigenic stimulus. It is considered that IL12 plays a central role in the orchestration of an efficient cellular immune response because it serves as a bridge between the innate and adaptive systems. Acting on its specific receptor, IL12 stimulates the proliferation of natural killer cells (NK), T lymphocytes, enhances their cytotoxic activity, promotes a helper T cell type 1 differentiation and activates the production of different cytokines, particularly interferon- γ (IFN γ) (1,2). All these properties give IL12 great potential for therapeutic use in tumours and infectious diseases. Despite promising results obtained in different animal models, clinical trials performed so far with the recombinant protein have shown modest efficacy and important adverse events, and new delivery approaches are needed (3,4).

Gene therapy is an evolving discipline consisting of the transfer of genetic material into somatic cells to either correct genetic defects or over-express therapeutic proteins. Local cytokine production achieved with IL12-based gene therapies has appeared as an interesting alternative to reduce toxicity and increase efficacy, and different viral and non-viral vectors have been developed so far (5,6). Nevertheless, the complex biological effects of IL12 may exert a strong influence on the

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ABBREVIATIONS: $-2LL$, $-2 \times \log$ (likelihood); E_{MAX} , Maximum effect; IFN γ , Interferon- γ ; IL12, Interleukin-12; HC-Ad/RUmIL12, Adenoviral vector carrying a liver-specific, mifepristone-inducible IL12 expression system; KO, Knock-out; mIL12, Mouse interleukin-12; NK, Natural killers; REG, Regulator compartment; RU, Mifepristone; RU₅₀, Mifepristone concentration needed to achieve 50% of the maximum effect; TMDD, Target-mediated drug disposition; WT, Wild-type.

activity of promoters used to control its own expression. In particular, IFN γ and tumour necrosis factor- α , produced in response to IL12, can inhibit transgene expression of vectors and limit treatment efficacy *in vivo* (6,7), representing a major hurdle for the clinical application of gene therapy.

Wang *et al.* (8) developed a high-capacity adenovirus vector encoding for mouse IL12 (mIL12) with a mifepristone (RU)-inducible system for controlled and liver-specific expression of IL12 (HC-Ad/RUmIL12). The system is based on two expression cassettes. One of them contains a chimerical trans-activator (GLP65) expressed under the control of the hepatic transthyretin promoter. The second cassette contains the IL12 sequence (p34 and p40 subunits connected by an internal ribosomal entry site) under the control of an inducible promoter. Upon binding to the inducer mifepristone, GLP65 adopts an active conformation that stimulates the activity of the inducible promoter (9). This gene therapy approach allowed for high level, tissue-specific and time-controlled transgene expression, and offers a promising tool for treatment of hepatic malignancies (10). However, when HC-Ad/RUmIL12 was administered and continuous RU-based induction protocols were performed, a progressive loss of IL12 expression was observed (11). The silencing of transgene expression was transient, and IFN γ -mediated inhibition of the transthyretin promoter was described as the major mechanism. Empirical adaptation of the induction regime with step-wise increases of mifepristone dose and resting periods between cycles of induction was able partially to compensate the inhibitory effect.

However, the characteristics of the above-described interaction between IL12, IFN γ , and RU makes the quantitative model-based approach an alternative to evaluate different hypotheses about the mechanism(s) of the interaction, estimate meaningful system parameters and explore different (untested) dosing/experimental scenarios using computer simulations (12,13).

Based on those considerations, the aim of the present work was to develop a semi-mechanistic model able to describe the kinetics and relationship of IL12 and IFN γ cytokines under different experimental conditions. Then, the selected model was used to evaluate, *in silico*, unexplored dosing regimens where the negative feedback induced by IFN γ on IL12 promoters is minimized in order to obtain sustained therapeutic IL12 levels.

MATERIAL AND METHODS

Ethics Statement

Animals were maintained under standard conditions and all procedures were approved by the Institutional Ethical Committee in accordance with the guidelines of the University of Navarra.

Animal Experimentation

Five- to 8-week-old female mice (C57BL/6) were purchased from Harlan Laboratories (Barcelona, Spain). IFN γ receptor-deficient mice (IFN γ R^{-/-}) were kindly provided by Dra. González-Aseguinolaza.

The HC-Ad/RUmIL12 vector (8) was administered by tail vein injection diluted in 200 μ L of saline or by direct intrahepatic injection dissolved in 50 μ L of saline solution, as previously described (10,14). Viral dose adjustments were performed regarding the IL12 concentration obtained after a first 250 μ g/kg dose of RU. Mifepristone (Sigma, St Louis, MO) was administered by intra-peritoneal (i.p.) injection starting 2 weeks after vector administration, at doses ranging from 125 to 16,000 μ g/kg (5,800–742,400 pmol/mouse), dissolved in 60 μ L of sesame oil. Mifepristone molecular weight (429.6 g/mol) and the average mouse body weight of 20 g (in agreement with the observed mean body weight, 20.2 $\pm$ 2.4 g along the different experiments) were considered for dosing adjustments. Blood samples were obtained by retro-orbital bleeding under inhalatory anaesthesia (isoflurane, Forane Abbott Laboratories, Madrid, Spain) 10 h after RU induction except for experiment 6 (Fig. 1), where a more exhaustive sample collection protocol was followed. Serum was recovered by double centrifugation at 10,000 rpm for 5 min and stored at -20°C until its use. Serum concentrations of IL12 (70 kDa) and IFN γ (16 kDa) were determined by OptE1A mouse IL12 (p70) and mouse IFN γ enzyme-linked immunosorbent assay cytokine determinations. The ranges of quantification for the IL12 and IFN γ assays were 62.5–2,000 and 20–4,000 pg/mL, respectively. Dilutions ranging from 1:5 and up to 1:1,000 were used.

Low viral dose was considered as DNA 1 (2×10^8 infectious units of vector), and consequently, high dose was set to DNA 2.5 (5×10^8 infectious units of vector) (BD Bioscience PharMingen, San Diego, CA, USA).

Figure 1 summarizes the experimental design followed including vector (hereafter called “DNA”) and RU dosing schemes, blood sampling times, and a table listing the number of animals and IL12 and IFN γ determinations.

Data Analysis

Model building was performed in three steps: Initially, a pharmacokinetic model was developed to account for the mifepristone levels over time. Once the model was established, mifepristone pharmacokinetic parameters were fixed and a model able to describe IL12 and IFN γ profiles obtained from the wild-type mice (WT) was developed. Finally, that model was expanded and refined to account for the response measures from knock-out (KO) mice. Data obtained from experiments 1–6 (Fig. 1) were used to develop the final model, which was externally evaluated with the observations from experiments 1' and 7–9 (Fig. 1).

The software NONMEM VII v.1 (15) and the first-order estimation method were used during the model building process. Data were logarithmically transformed and at each step, IL12 and IFN γ observations were fitted simultaneously using the pool approach. For IL12 and IFN γ , the model had the following general form: $\log(y_{\text{obs},j}) = \log(y_{\text{pred},j}) + \varepsilon_j$ where $y_{\text{obs},j}$ and $y_{\text{pred},j}$ represent the observation and model prediction corresponding to the j th time, respectively, and ε_j reflects the discrepancy between the logarithm of the observations and predictions, accounting for both the intra-animal variability and residual error. For each response, the set of ε s forms a random variable with mean 0 and variance equal to ω^2 , estimated also as (random) parameters in the model.

Fig. 1. Schematic representation of the experimental setting. Unframed experiments were used to develop the wild-type model. *Dash box* experiments were included to obtain the final full model. External validation of the model was performed using the *dotdash box* studies. Mifepristone was administered daily (*black arrows*) and blood samples were obtained 10 h after mifepristone dose (*grey arrows*). The number of mice and IL12 or IFN γ serum samples corresponding to each experimental condition are indicated in the table on the *right*

Model Development

To develop the pharmacokinetic model of Mifepristone, data obtained after intravenous administration of the drug to mice of a preceding publication (16) were used. Data were extracted with GetData Graph Digitizer software¹, and fitted to a three compartmental model with elimination from the central

¹ <http://getdata-graph-digitizer.com/>

compartment (see “**ELECTRONIC SUPPLEMENTARY MATERIALS**” (ESM))

The process for developing the selected IL12-IFN γ model was partly data driven and partly based on the current knowledge regarding the IL12-IFN γ interaction. The model finally selected and represented schematically in Fig. 2 is described in detail in the following. Intermediate results obtained during model building are presented in the **RESULTS** and **DISCUSSION** sections.

Fig. 2. Schematic representation of the semi-mechanistic model developed. DNA, viral dose administered; RU, dose of mifepristone (IL12 synthesis inducer); k_{syn_IL12} , $k_{syn_IFN\gamma}$, k_{syn_RIL12} , and $k_{syn_RIFN\gamma}$ are zero order rate constants of IL12, IFN γ , R_{IL12}, and R_{IFN γ} synthesis, respectively; k_{deg_IL12} , $k_{deg_IFN\gamma}$, k_{deg_RIL12} , and $k_{deg_RIFN\gamma}$ are first-order degradation rate constants of IL12, IFN γ , R_{IL12} and R_{IFN γ} , respectively; k_{int_RIL12} is the first-order rate constant controlling the elimination and internalization of R_{IL12}IL12 complex; K_{D_IL12} and $K_{D_IFN\gamma}$ are dissociation equilibrium constants of IL12 and IFN γ , respectively. The rest of terms are defined in the text

IL12 and IFN γ showed target-mediated disposition (17), where IL12 and IFN γ observed concentrations represent the concentrations of cytokine that bind reversibly to their corresponding membrane receptors. Due to data limitation to estimate precisely the association and dissociation rate constants (k_{on} , and k_{off} , respectively) which characterize the binding of IL12 and IFN γ to their respective receptors to form the R_{IL12}IL12 and R_{IFN γ} IFN γ complexes, a rapid binding or quasi-equilibrium between the free compounds and receptors was assumed (18). The parameter K_D represented by the ratio between k_{off} and k_{on} , and defined as the equilibrium dissociation constant was estimated. Expressions 1 and 2 show the relationship between K_D , k_{on} , k_{off} , and free

and bound concentrations of IL12 and IFN γ , respectively.

$$K_{D_IL12} = \frac{k_{off_IL12}}{k_{on_IL12}} = \frac{R_{IL12} \times IL12}{R_{IL12}IL12} \quad (1)$$

$$K_{D_IFN\gamma} = \frac{k_{off_IFN\gamma}}{k_{on_IFN\gamma}} = \frac{R_{IFN\gamma} \times IFN\gamma}{R_{IFN\gamma}IFN\gamma} \quad (2)$$

The expression of IL12, governed by the zero order rate constant k_{syn_IL12} , is regulated by the co-administration of the viral vector and the mifepristone, together with the levels of the R_{IFN γ} IFN γ as it is described by Eq. 3.

$$k_{syn_IL12} = k_{syn_IL12}(0) \times \left[1 + \theta_{DNA/RU} \times \frac{RU}{RU + RU_{50}} \times DNA \times \frac{R_{IFN\gamma}IFN\gamma_{50}}{R_{IFN\gamma}IFN\gamma_{50} + R_{IFN\gamma}IFN\gamma} \right] \quad (3)$$

Where RU are the model predicted concentrations of mifepristone at any time, $\theta_{DNA/RU}$ is the parameter representing the maximum synthesis rate given per unit of viral dose injected (DNA) and considered to remain constant along the experiment, RU_{50} are the predicted levels of RU that exerted half of maximum increase in k_{syn_IL12} , and $R_{IFN\gamma}IFN\gamma_{50}$, is a parameter scaling the inhibitory effects of R_{IFN γ} IFN γ complex on $k_{syn_IL12}(0)$. In the absence of DNA or mifepristone administration, $k_{syn_IL12} = k_{syn_IL12}(0)$.

The synthesis of IFN γ , quantified by the zero-order rate constant, $k_{syn_IFN\gamma}$ was only controlled by the relative

increments in the R_{IL12}IL12 complex as it is represented in Eq. 4, where $R_{IL12}IL12(0)$ represents the initial (baseline) levels of the R_{IL12}IL12 complex, and the parameter γ allows for a non-linear behaviour. Before viral and mifepristone administration $k_{syn_IFN\gamma}$ is equal to $k_{syn_IFN\gamma}(0)$.

$$k_{syn_IFN\gamma} = k_{syn_IFN\gamma}(0) \times \left(\frac{R_{IL12}IL12}{R_{IL12}IL12(0)} \right)^\gamma \quad (4)$$

The bivariate response model describing simultaneously the time courses of the free observed concentrations of IL12

and IFN γ in serum, taking into account the unobserved bound concentrations of IL12 and IFN γ ($R_{IL12}IL12$, $R_{IFN\gamma}IFN\gamma$, respectively) and unobserved concentrations of total ($R_{T,IL12}$, and $R_{T,IFN\gamma}$) and free (R_{IL12} and $R_{IFN\gamma}$) receptors for IL12, and IFN γ , respectively, are represented by the set of Eqs. 5–10.

$$\frac{dIL12_T}{dt} = k_{syn_IL12} - k_{IL12} \times IL12 - k_{int_RIL12} \times (IL12_T - IL12) \quad (5)$$

$$\frac{dR_{T,IL12}}{dt} = k_{syn_RIL12} - k_{deg_RIL12} \times R_{IL12} - k_{int_RIL12} \times (IL12_T - IL12) \quad (6)$$

$$IL12 = \frac{1}{2} \times \left[(IL12_T - R_{T,IL12} - K_{D_IL12}) + \sqrt{(IL12_T - R_{T,IL12} - K_{D_IL12})^2 + 4 \times IL12_T \times K_{D_IL12}} \right] \quad (7)$$

$$\frac{dIFN\gamma_T}{dt} = k_{syn_IFN\gamma} - k_{IFN\gamma} \times IFN\gamma \quad (8)$$

$$\frac{dR_{T,IFN\gamma}}{dt} = k_{syn_RIFN\gamma} - k_{deg_RIFN\gamma} \times R_{IFN\gamma} \quad (9)$$

$$IFN\gamma = \frac{1}{2} \times \left[(IFN\gamma_T - R_{T,IFN\gamma} - K_{D_IFN\gamma}) + \sqrt{(IFN\gamma_T - R_{T,IFN\gamma} - K_{D_IFN\gamma})^2 + 4 \times IFN\gamma_T \times K_{D_IFN\gamma}} \right] \quad (10)$$

Where $dIL12_T/dt$ and $dR_{T,IL12}/dt$ represent the rate of change of total IL12 ($IL12 + R_{IL12}IL12$) and IL12 receptor ($R_{IL12} + R_{IL12}IL12$) concentrations, respectively. Correspondingly, $dIFN\gamma_T/dt$ and $dR_{T,IFN\gamma}/dt$ represent the rate of change of total IFN γ ($IFN\gamma + R_{IFN\gamma}IFN\gamma$) and IFN γ receptor ($R_{IFN\gamma} + R_{IFN\gamma}IFN\gamma$) concentration, respectively.

The model included irreversible linear elimination for both cytokines described by the first-order rate constants k_{IL12} and $k_{IFN\gamma}$, and occurring in the central (serum) compartment. Receptor turnovers are governed by the zero order and first order rate constants of synthesis (k_{syn_RIL12} , and $k_{syn_RIFN\gamma}$) and degradation (k_{deg_RIL12} , $k_{deg_RIFN\gamma}$), respectively.

An internalization and subsequent degradation process of the $R_{IL12}IL12$ complex accounted by the first order rate constant k_{int_RIL12} was also incorporated into the final model.

Given the endogenous IL12 production, initial conditions (baseline values) of free IL12 and its receptor were represented by $IL12(0)$ and $R_{IL12}(0)$ respectively, parameters to be estimated by the model. Bound IL12 was represented by

$R_{IL12}IL12(0)$, a derived parameter from the quasi-equilibrium assumption (Eq. 11).

$$R_{IL12}IL12(0) = \frac{R_{IL12}(0) \times IL12(0)}{K_{D_IL12}} \quad (11)$$

Baseline values of IFN γ free [$IFN\gamma(0)$] and bound [$R_{IFN\gamma}IFN\gamma(0)$] were codified as derived parameters from the model (Eqs. 12 and 13). At time 0, IFN γ receptors [$R_{IFN\gamma}(0)$] could not be estimated and therefore they were assumed to be 1 for WT mice and 0 for KO mice.

$$IFN\gamma(0) = \frac{k_{syn_IFN\gamma}}{k_{IFN\gamma}} \quad (12)$$

$$R_{IFN\gamma}IFN\gamma(0) = \frac{R_{IFN\gamma}(0) \times IFN\gamma(0)}{K_{D_IFN\gamma}} \quad (13)$$

Consequently, the initial conditions for Eqs. 5–6 and 8–9, which corresponds to the baseline total concentrations, are the sum of the initial free and bound concentrations (*i.e.*, $IL12_T(0) = IL12(0) + R_{IL12}IL12(0)$).

Therefore, $\theta_{DNA/RU}$, RU_{50} , K_{D_IL12} , k_{IL12} , k_{int_RIL12} , k_{deg_RIL12} , $K_{D_IFN\gamma}$, $k_{syn_IFN\gamma}(0)$, $k_{IFN\gamma}$, $k_{deg_RIFN\gamma}$, $IL12(0)$, and $R_{IL12}(0)$ were parameters to be estimated by the model, while $R_{IL12}IL12(0)$, $IFN\gamma(0)$, $R_{IFN\gamma}IFN\gamma(0)$, $k_{syn_IL12}(0)$, k_{syn_RIL12} , $k_{syn_RIFN\gamma}$, were derived parameters (Eqs. 11–16, respectively).

$$k_{syn_IL12}(0) = k_{IL12} \times IL12(0) + k_{int_RIL12} \times (IL12_T(0) - IL12(0)) \quad (14)$$

$$k_{syn_RIL12} = k_{deg_RIL12} \times R_{IL12}(0) + k_{int_RIL12} \times (IL12_T(0) - IL12(0)) \quad (15)$$

$$k_{syn_RIFN\gamma} = k_{deg_RIFN\gamma} \times R_{IFN\gamma}(0) \quad (16)$$

Model Selection

Selection between models was based on the precision of parameter estimates obtained from the analysis of 1,000 bootstrap datasets performed using the software Perl-speaks-NONMEM (19), goodness-of-fit plots, and the minimum value of the objective function value provided by NONMEM (MVOF) and approximately equal to $-2\log$ (likelihood) ($-2LL$). Differences between two hierarchical (nested) models were compared with a χ^2 distribution in which a decrease of 6.63 points in $-2LL$ was considered significant at the 1% level for one extra model parameter. Non-nested models were compared using the Akaike information criteria (AIC) calculated as $MVOF + 2 \times NP$, where NP is the number of parameters in the model (20). The model with the lowest value of AIC, given that the precision of model parameters and data description was adequate, was selected.

Model Evaluation

Despite the naïve pool approach was adopted, simulations using one source of random effect were used to evaluate the descriptive performance of the model since residual error was also a component of the model, although it has to be taken into consideration that the simulated profiles do not represent the overall population. Briefly, visual predictive checks (VPC) were performed by simulating 1,000 new individual profiles for each experiment using the estimated variance of the residual error. Then, 5th, 50th and 95th percentiles of the simulated data were calculated and graphically represented against real data. Agreement between simulations and observations was visually inspected.

External Validation

Similarly, VPC for those experiments not included in the model development were performed and agreement between simulations and observations was inspected to externally validate the selected model.

Applicability of the Model: Simulation of New Dosing Protocols

Different experimental protocols were explored *in silico* in order to find a RU/DNA combination able to maintain sustained IL12 protein levels within the therapeutic range.

RESULTS

Model for Wild-Type Mice

The initial model incorporated the main processes according to the current knowledge regarding the system under study: (1) DNA-based expression of IL12 induced by the administration of RU, (2) IL12-dependent synthesis of IFN γ , and (3) downregulation mechanism on IL12 expression through a regulator (REG) compartment triggered by IFN γ (Supplementary Fig. 1A). However, whereas the corresponding IL12 time profiles for the animals receiving a low viral dose were efficiently captured by the model (data from experiment 1), IL12 after high viral dose were clearly under-predicted (Supplementary Fig. 1B). Given the presence and the role of IL12 membrane receptors on the IL12 dynamics (21,22), a target-mediated disposition, termed first coined by Levy in 1994 to describe those situations where molecules bind with high affinity to their targets influencing the molecule kinetics (23), could be expected and would explain the altered disposition observed after the injection of high viral dose. On these bases, Mager and Jusko proposed in 2001 a generalized pharmacokinetic model of target-mediated drug disposition (TMDD) (17).

Maintaining the previous model structure used to account for the IFN γ kinetics and its negative feedback on the IL12 expression, the full TMDD model was implemented and fitted to the data. Results indicated that not all the parameters from the model were identifiable, a common finding when applying the TMDD model (18,24). In order to resolve this limitation, a rapid binding between the free and bound entities and constant amount of total receptors

($k_{deg_IL12}=k_{int_RIL12}$) (18) were assumed, which provides a precise estimate of K_{D_IL12} and the rest of model parameters, resulting in a better fit compared with the simpler model including Michaelis–Menten elimination. Finally it was observed that the effects of RU over k_{syn_IL12} were better described with a non-linear E_{MAX} type model compared with a linear relationship. A correction factor on the viral dose (DNA parameter) to account for differences between intravenous and intra-peritoneal virus administration was also considered, but was not significantly different from 1 ($p>0.05$). The model selected so far, and represented schematically in Supplementary Fig. 1C, described adequately the kinetics of IL12 and IFN γ . Supplementary Fig. 1D provides an example of model performance showing IL12 as a function of time and dose of RU.

Integrated Model Incorporating Data from Knock-Out Mice

Two plausible scenarios were evaluated to extrapolate the wild-type model previously described to knock-out (KO) mice lacking of IFN γ receptor. First, it was hypothesized that the parameter $k_{IFN\gamma}$ could be reflecting the role of IFN γ receptor on IFN γ elimination. Therefore a simulation exercise was undertaken assuming a value of $k_{IFN\gamma}$ equal to 0 (extreme situation). In the second scenario, the hypothesis that the IFN γ receptor does not affect IFN γ disposition, but does influence the downregulation effect on IL12 expression, was evaluated by deleting the REG compartment from the model. The results obtained from the two simulation exercises are shown in Fig. 3. Blocking IFN γ elimination, the IFN γ profiles at the plateau were well captured, as can be observed in Fig. 3a, but the corresponding levels of IL12 were clearly under-predicted (Fig. 3b). When the inhibitory effects of IFN γ on k_{syn_IL12} were eliminated by cancelling the REG compartment, a smaller discrepancy between IL12 observations and simulations was observed, although IFN γ predictions were not as good as the ones observed when IFN γ elimination was cancelled.

Based on the outcome from the simulations described above, a combination of both scenarios considering that the IFN γ receptors can play an important role, not only on the disposition of IFN γ , but also in the downregulation effect on k_{syn_IL12} , might provide an adequate description of observed IL12 and IFN γ profiles in WT and KO mice.

Therefore, the model developed for wild-type mice was modified, incorporating a target-mediated disposition for IFN γ , and considering the IFN γ receptor complex responsible for the downregulation process triggered over IL12 expression (model represented in Fig. 2). Both IL12 and IFN γ data from the two types of mice were analysed together and a similar process to that described for WT mice was followed: (1) the quasi-equilibrium assumption also provided a precise estimate of $K_{D_IFN\gamma}$, although initial free receptors could not be estimated, and thus the parameter was fix to 1 for WT or 0 for KO mice, (2) an extra REG compartment delaying the inhibitory effect of the IFN γ receptor complex was not required, (3) incorporation of receptor turnover kinetics significantly improved the fit ($p<0.01$), (4) the presence of the internalization processes leading to an irreversible loss of IFN γ was, in this case, not supported by the data and finally, (5) the effects of R_{IL12} on $k_{syn_IFN\gamma}$

Fig. 3. Knock-out mice model extrapolation. TMDD model previously developed for WT mice (Supplementary Fig 1C) is used to simulate the mean profiles of IFN γ (a) and IL12 (b) vs time after 10 consecutive doses of 250 $\mu\text{g/kg}$ of mifepristone 2 weeks after the administration of the low viral dose (experiment 5, DNA=1). Knock-out mice setting is explored by cancelling the rate constant controlling the synthesis and degradation of the regulator compartment (k_{REG} in Supplementary Fig. 1C) or the IFN γ elimination rate constant (k_{IFN} in Supplementary Fig. 1C) and comparing the model predicted levels with the real data (points)

were better described with a non-linear model (Eq. 4) compared with a linear relationship ($p < 0.01$). Incorporation of KO data into the analysis allowed the estimation of an extra linear elimination mechanism of IL12 from the serum compartment (represented by k_{IL12}). Different initial IFN γ concentrations between WT and KO mice were considered by assuming different synthesis or degradation constant rates (see Eq. 12) to account for the different observed concentrations (which were below the quantification limit for WT), however no model improvement was observed ($p > 0.001$). An additional model in which IFN γ exerted its inhibitory effect on the 0 order input rate [$k_{\text{syn_IL12}}(0)$] was tested observing an increase of 26 points in -2LL (worse fit).

The selected model performed well for the different experimental settings, including different RU dosing regimens, viral dose and WT/KO mice (Fig. 4). Despite its complexity, the model provided precise parameter estimates (Table I). Results from the external validation procedure are shown in Fig. 5. Despite of the moderate discrepancies seen in the IFN γ profiles obtained from experiment 8, the model developed was able to successfully describe IL12 and IFN γ levels under the different dosing protocols, providing a further support to the selected model.

Figure 6 shows the behaviour of all main components of the selected model under injection of 1 and 2.5 viral doses and daily IL12 synthesis induction with mifepristone (250 $\mu\text{g/kg}$) over a period of 10 days.

Applicability of the Model: Simulation of New Dosing Protocols

Taking into account the therapeutic goal of the virus system studied in the current analysis, the model developed was used to explore alternative (not tested) dosing protocols aimed at minimizing the downregulation process triggered by IFN γ on the transgene expression.

The modelling approach allowed the estimation of the RU_{50} value (0.629 pmol/mL) for this particular system, observing that when 2,000 $\mu\text{g/kg}$ dose was used, mifepristone concentrations achieved were always kept above the RU_{50} value (data not shown). Therefore, increments on RU dose

above this value, like those used empirically to maintain IL12 expression at the end of the induction cycle (see experiment 3), will show marginal increment of efficacy, as can be graphically observed in Fig. 7a.

Through simulations, we searched for a dosing protocol in which increments in RU would translate into significant increments in the IL12 synthesis rate to compensate for IFN γ receptor complex effects. A protocol where the RU dose was doubled every 2 days (instead of every 3 days), starting with a low dose of 125 $\mu\text{g/kg}$, was finally proposed (Fig. 7b). Outcome from that simulation suggested that this dosing regimen was able to maintain constant levels of IL12. However, IL12 concentrations were below the target therapeutic window for IL12. In order to increase IL12 concentrations without increasing administered RU, increments in the viral load were simulated, observing that a 50% viral dose increment respect to the standard one (DNA=1.5 instead of DNA=1) was enough to reach therapeutic concentrations (Fig. 7b).

DISCUSSION

IL12 is a promising cytokine for treatment of a wide variety of illnesses, such as malignant melanoma or liver cancer. However, clinical studies so far have not been particularly successful, showing low efficacy and high drug toxicity in humans (4,5). Viral vectors have appeared as an interesting alternative to improve drug efficacy. Although this type of system enables us to increase drug concentration in target organs, therefore decreasing negative side effects, promoter silencing induced by cytokine-mediated signals remains the major limitation on its clinical use (6,11). A better understanding of IL12 complex response would help to develop a more effective dosing regimen.

The modelling approach has proven to be helpful to understand the complexity of this type of system better, and to develop more efficient dosing protocols that could translate into future clinical improvements (13,25).

This research aimed to develop a semi-mechanistic pharmacokinetic/pharmacodynamic type model able to simultaneously describe and explore the kinetics of IL12 and its mediator, IFN γ , after the administration of HC-Ad

Fig. 4. Internal model evaluation. Median model predictions (*solid line*) and raw data (*points*) vs time profiles for IL12 (*upper panel*) and IFN γ (*lower panels*) for the low viral dose (DNA=1) of different evaluated protocols. First mifepristone dose is considered to be administered on day 1. *Shadowed areas* correspond to the 90% prediction interval

encoding for mIL12 under a variety of different experimental conditions (Fig. 1).

Non-linear kinetics associated with IL12, and reflected as a non-proportional relationship between the area under the

Table I. Model Parameter Estimates Describing the Time Profiles of IL12 and IFN γ

Parameter		IL12		IFN γ	
Definition	Nomenclature ^a	Estimate	95th confidence interval ^b	Estimate	95th confidence interval ^b
Mifepristone levels triggering 50% of the maximal effect	RU ₅₀ (pmolmL ⁻¹)	0.629	0.22–11.40	–	–
Scaling factor for the $R_{\text{IFN}\gamma}$ inhibitory effect	$R_{\text{IFN}\gamma}\text{IFN}\gamma_{50}$ (pmolmL ⁻¹)	6.18	3.04–14.61	–	–
Maximum synthesis rate given per unit of viral dose	$\theta_{\text{DNA/RU}}$ (au ⁻¹)	5.42	2.58–24.75	–	–
Cytokine synthesis rate constant	$k_{\text{syn}_X(0)}$ (pmolday ⁻¹)	13.87	–	0.023	0.0013–0.051
Shape parameter	γ	–	–	5.87	2.53–6.99
Cytokine elimination rate constant	k_X (day ⁻¹)	2.23	1.045–8.01	2.03	0.27–4.95
Receptor synthesis rate constant	k_{syn_RX} (pmolday ⁻¹)	31.64 ^c	–	1.98 ^c	–
Receptor elimination rate constant	k_{deg_RX} (day ⁻¹)	4.12	2.23–166.57	1.98	0.96–3.36
Dissociation constant	k_{D_X} (pmolmL ⁻¹)	0.018	0.0094–0.22	0.0023	0.00077–0.0066
Complex internalization	k_{int_RX} (day ⁻¹)	4.12 fix	–	NE	–
Baseline free cytokine	$X(0)$ (pmolmL ⁻¹)	0.014	0.0095–0.019	0.011 ^c	–
Baseline receptor complex	$R_X X(0)$ (pmolmL ⁻¹)	3.36 ^c	–	4.78 ^c	–
Baseline free receptor	$R_X(0)$ (pmolmL ⁻¹)	4.32	0.16–13.35	1 fix	–

NE not estimated, AU arbitrary units

^a X IL12 or IFN γ cytokine

^b 95% confidence intervals calculated from the analysis of 1,000 simulated bootstrap datasets

^c Derived parameter

Fig. 5. External model evaluation. Median model predictions (*solid line*) and raw data (*points*) vs time profiles for the high (DNA=2.5) and low (DNA=1) viral dose of different protocols not included during the model building process. First mifepristone dose is considered to be administered on day1. *Shadowed areas* correspond to the 90% prediction interval

plasma concentration *versus* (vs) time curve and the administered dose, has been previously observed. However, due to the high variability in the data, this non-linearity could not be properly characterized (26–28). In our study, data could not be adequately described assuming linear kinetics (Supplementary Fig. 1A). Classic linear pharmacokinetic models consider that the amount of drug interacting with its biological target does not influence the kinetics of the drug, and therefore can be neglected. However, this is not the case of many biological compounds (29,30), where interactions with their receptors play an important role on their disposition, thus exhibiting a target-mediated drug disposition (TMDD) (23). Similarly, IFN γ receptors were found to be relevant, not only in the effects triggered by this molecule, but also in the disposition of the drug (31).

A general model to mathematically characterize drugs showing a TMDD behaviour was published by Mager and Jusko in 2001 (17). These types of models are often over-parameterized and difficult to converge. Instead, a simplification of the above-mentioned model, the quasi-equilibrium approximation (18), was implemented to characterize the dynamic of both cytokines.

Biological IL12 functions are known to be mediated by binding to membrane receptors, primarily expressed on activated NK and T cells (4,32,33). A linear relationship between IL12R β 2 subunit and IFN γ has been previously described (34). However, a better model performance was observed when a

non-linear relationship was tested (Eq. 4). IL12 represents the main stimulus for direct IFN γ synthesis; although, other cytokines such as IL2, also regulated by IL12, are involved in IFN γ production (35). These positive feedbacks could explain the non-linear relation found between IL12-receptor complex and IFN γ (amplification signal). Similar to IL12, downregulation IFN γ -related effects are triggered by the interaction of the cytokine with its receptor (36).

Despite that the mice lacking the IFN γ receptor present an increased susceptibility to pathogen infections and a decrease response preventing tumour development (37), no apparent phenotypic anomalies are observed and the immune system appears to develop normally (38).

The large variability observed in the IL12 and IFN γ response is likely to be caused by differences in liver distribution, hepatocyte's uptake, expression of IL12 and IFN γ , and pharmacokinetics of RU. Whereas data allowed us to discriminate between different possible structural models, estimates of inter-animal variability assigned to a particular parameter in the model would not reflect real variation in that parameter, but the sum of variability from processes that were not controlled in the current setting, being the reason for not using the population approach in the current investigation. Non-available data reflecting pharmacokinetics in plasma and liver of DNA and RU would have helped to interpret inter-animal variability.

Fig. 6. Performance of the main model components. The time profiles of free IL12 and IFN γ cytokines (*left panels*), and their free (R_{IL12} and $R_{IFN\gamma}$, *middle panels*) and bound receptors ($R_{IL12}IL12$ and $R_{IFN\gamma}IFN\gamma$, *right panels*), which are the main components of the model described in Fig. 2, are depicted for the low (DNA1) and high (DNA2.5) viral dose when IL12 expression is induced with a daily RU dose of 250 $\mu\text{g/kg}$ over a period of 10 days

IL12 presented a terminal half-life of approximately 4 h, estimated according to (39) and consistent with previous published results (26,27). The half-life of IFN γ was higher

than expected, probably due to lack of IFN γ -receptor complex internalization in the model. Despite that internalization process has been described previously for IFN γ (40),

Fig. 7. Exploratory analysis of the model. **a** Mean IL12 levels predicted by the selected model over time for the dosing protocol used in experiment 6 (*solid lines*) and for new simulated protocols where the last doses of RU are increased twofolds (*blue*), fourfolds(*green*) or eightfolds (*yellow*) trying to counteract the negative feedback triggered by $R_{IFN\gamma}IFN\gamma$. **b** RU dosing protocol proposed by modelling approach where the doses are doubled every 2 days, starting with a low initial dose of 125 $\mu\text{g/kg}$ and different viral loads are administered in order to achieve sustained IL12 levels within the target therapeutic interval of 1–4.3 pmol/mL (70–300 ng/mL)

data did not support the estimation of two different elimination pathways for IFN γ , which represents a limitation of the current model. A higher number of IFN γ measurements on the decay phase would have allowed us to discriminate between both elimination processes.

Regarding the quasi-equilibrium assumption, the K_{D_IL12} and $K_{D_IFN\gamma}$ estimates of 18 and 2.28 pM, respectively, were in agreement with some of the previous values reported from *in vitro* experiments ranging between 5 and 20 pM (26) for IL12 and around 150 pM (41,42) for IFN γ . We have to take into account the fact that there is a large degree of variability associated with this type of estimate across different studies. For example, Trinchieri *et al.* (32) or Petska *et al.* (36) reported a range of receptor binding characteristics of IL12 and IFN γ between 100 and 600 and 10–1,000 pM, respectively. Differences in cell lines and other experimental conditions might explain at least part of the discrepancy between our results and others. Nevertheless, a careful interpretation of the dissociation rate constant has to be done. It has been described that in certain circumstances, *e.g.*, when the complex internalization process is not negligible compared with the dissociation one, the estimated parameter does not represent only the equilibrium dissociation constant, but rather a function of the association, dissociation, and internalization rate constants (24,43).

Attempts to use the final model (Fig. 2) to describe and estimate model parameters using WT or KO mice alone did not succeed, providing evidence of the importance of having information from different experimental settings in order to characterize a particular system efficiently (29,44).

Despite the fact that in the current study only two entities, IL12 and IFN γ were considered, and recognizing that many other factors contribute to the interaction between the two cytokines (26,36), we do believe that the model developed provides a meaningful interpretation of the nature of the underlying mechanisms. Furthermore, the model was able to predict IL12 and IFN γ profiles of those experiments which had not been considered during the model building exercise (external model validation).

Once the model was developed and validated, new dosing scenarios were explored. Although small increments of RU doses were more efficient to achieve sustained IL12 levels due to the low RU₅₀ value, the induced IL12 concentrations were below the therapeutic value. Modelling simulation exercises showed that a 50% increment in the viral dose administered was enough to obtain IL12 in between the target therapeutic window established in a syngenic colon cancer tumour model (70–300 ng/mL) (10).

CONCLUSION

To summarize the results of the current report, a semi-mechanistic model was developed in the context of gene therapy to describe the kinetics of the interaction between IL12 and IFN γ after the injection of the mifepristone-inducible vector HC-Ad/RUMIL12. The model captures the main processes responsible for the synthesis and degradation of the cytokines, and the downregulation mechanism, providing meaningful parameter estimates and pointing towards the key role that TMDD plays in the system regulation. Examples of model

application, such as the optimization of dosing protocols, have been shown through computer simulations

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