Published in final edited form as:

Immunol Cell Biol. 2011 January ; 89(1): 100–110. doi:10.1038/icb.2010.69.

Inferring relevant control mechanisms for Interleukin-12 signaling in naïve CD4+ T cells

Stacey D. Finley¹, Deepti Gupta², Ning Cheng³, and David J. Klinke II^{3,4}

¹ Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL 60208

² Department of Biomedical Engineering, Northwestern University, Evanston, IL 60208

³ Department of Chemical Engineering, West Virginia University, Morgantown, WV 25606

⁴ Department of Immunology, Microbiology, & Cell Biology, West Virginia University, Morgantown, WV 25606

Abstract

Interleukin-12 (IL-12) is a key cytokine involved in shaping cell-mediated immunity to intracellular pathogens. IL-12 initiates a cellular response via the IL-12 signaling pathway, a member of the JAK/STAT family of signaling networks. The JAK/STAT pathway includes several regulatory elements; however, the dynamics of these mechanisms are not fully understood. Therefore, the objective of this study was to infer the relative importance of regulatory mechanisms that modulate the activation of STAT4 in naïve CD4+ T cells. Dynamic changes in protein expression and activity were measured using flow cytometry and these data were used to calibrate a mathematical model of IL-12 signaling. An empirical Bayesian approach was used to infer the relative strength of different regulatory mechanism in the system. The model predicted that IL-12 receptor expression is regulated via a dynamic, autonomous program that was independent of STAT4 activation. A time scale analysis identified the signaling events that limited the cellular response, including ligand binding and receptor degradation. In summary, a mathematical model of the canonical IL-12 signaling pathway used in conjunction with a Bayesian framework provided high confidence predictions of the system-specific control mechanisms from the available experimental observations.

Keywords

Interleukin-12; Bayesian inference; CD4+ T cells; signal transduction; ordinary differential equations

Introduction

The immune response provides the human body with natural defenses against infectious diseases and wreaks havoc on human health when dysfunctional¹. These natural defenses are coordinated by specialized cells, called CD4+ T helper cells, that release chemical messengers called cytokines^{2,3}. T helper (T_h) cells become polarized into one of three subtypes: T_h1 cells coordinate the response to intracellular pathogens, T_h2 cells defend

Contact Info: david.klinke@mail.wvu.edu, Phone: (304)293-2111 ext 2432, Fax: (304)293-4139, Department of Chemical Engineering, West Virginia University, P.O. Box 6102, Morgantown, WV 26506-6102.

Supplementary information is available at the Immunology and Cell Biology website.

against extracellular pathogens, and T_H17 cells coordinate the autoimmune response⁴. Each of these subsets can be identified by the unique profile of cytokines that they produce. In addition, specific cytokines influence the polarization of naïve T helper cells into the different subtypes. Understanding the role of cytokines in shaping the cellular response is essential for engineering immunotherapies tailored to individuals^{5–7}.

Interleukin-12 (IL-12) is a key cytokine known to promote the differentiation of T cells into T_H1 cells⁸. IL-12 is a heterodimer (IL-12p70) consisting of a 35 kDa subunit (p35) and a 40 kDa subunit (p40) that is expressed in both monomeric (IL12p40) and homodimeric (IL12(p40)₂) forms. IL-12 initiates a cellular response by recognizing and binding to its receptor, IL-12R, which is composed of two subunits: IL-12R β 1 and IL-12R β 2⁹. The β 1 subunit is involved in other cytokine signaling pathways such as IL-23¹⁰, while β 2 is specific to the IL-12 signaling pathway and has been shown to be dynamically regulated during T cell activation⁹. Both IL12p40 and IL12(p40)₂ can bind to IL-12R, acting as antagonists to IL12p70 binding. A mathematical model of this competitive binding reveals the importance of measuring all forms of IL-12 in understanding the bioactivity of IL-12¹¹. Ultimately, regulation of the IL-12 pathway is critical for T_H differentiation¹², and polarization of T_H cells into T_H1 cells requires sustained IL-12 signaling¹³.

The IL-12 signaling network is a member of the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) family of signaling pathways. Signaling through JAK/STAT pathways activates STAT proteins that subsequently translocate to the nucleus, initiating gene expression and protein translation¹⁴. The suppressors of cytokine signaling (SOCS) family of proteins act as negative regulators of signaling in the JAK/STAT pathway. Additional regulators have been found to inhibit signaling through the JAK/STAT pathway, including protein tyrosine phosphatases (PTPs), which are known to dephosphorylate activated JAK, STAT, or cytokine receptors¹⁵, and protein inhibitors of activated STATs (PIAS) that interact with phosphorylated STATs in the nucleus to inhibit their activity¹⁶. TC45, a nuclear PTP, has also been shown to deactivate phosphorylated STAT4 dimers within the nucleus for export back to the cytoplasm¹⁷. Additionally, it has been shown that cellular context plays an important role in influencing the strength of signaling via particular reaction pathways¹⁸. The canonical JAK/STAT pathway incorporates the regulatory mechanisms described above; however, the dynamic role of these different feedback mechanisms in regulating signaling in the IL-12 pathways remains unclear¹⁴. Therefore, the objectives of the current study were to: (1) develop an experimentally validated mathematical model to describe signaling in the IL-12 pathway in naïve CD4⁺ T helper cells, and (2) use the model to infer the relative importance of feedback mechanisms in STAT4 activation within naïve CD4⁺ T cells.

Results

Flow cytometry results

Magnetic bead enrichment from the starting population of Balb/c splenocytes was used to obtain a population of cells that were > 96% positive for CD4⁺ and > 90% positive for both CD4⁺ and CD62L^{high} (i.e., naïve CD4⁺ T cells). As the population of CD4⁺ CD62L^{high} splenocytes may contain a mixture of both central memory and naïve T cells, the activation marker CD44 was used to assess the contribution of the central memory pool. Greater than 95% of CD4⁺CD62L^{high} cells were observed by flow cytometry to express intermediate to low levels of CD44, consistent with a naïve T cell population (i.e., CD4⁺CD62L^{high}CD44^{low}). The results suggest that contribution of the central memory population (i.e., CD4⁺CD62L^{high}CD44^{high}) was minor. In comparison, high levels of CD44 expression were observed in the CD4⁺ CD62L^{low} population, consistent with an effector T cell population (i.e., CD4⁺CD62L^{low}CD44^{high}) (data not shown).

The dynamics of IL-12 signaling leading to STAT4 activation were assessed in naïve CD4⁺ T cells extracted from murine splenocytes. Following a pre-activation period, cells were stimulated and subsequently fixed at various time points ranging from 15 minutes to 24 hours. Flow cytometry was used to measure the extent of cell staining via fluorescence intensity of markers for IL-12R β 1, IL-12R β 2, and phosphorylated STAT4 (pSTAT4). Cells that exhibited non-specific staining were removed by partitioning the population of cells into live and dead cells based upon forward scatter and side scatter characteristic of the sample. On average, 2×10^4 cells were analyzed at each timepoint with $60\% \pm 9\%$ of the population remaining after gating on forward and side scatter characteristics.

The activity of STAT4 (pSTAT4) with respect to IL-12 β 2 is shown in Figure 2. We have defined a data-driven threshold, indicated by the vertical line in all panels, for whether a cell was positive for IL-12R β 2, below which 95% of the unstained cells exhibited a lower level of expression. Additionally, the dotted horizontal line indicates the upper limit of pSTAT4 mean fluorescent intensity (MFI) for 95% of the cell population prior to IL-12 stimulation (see 0 min panel). These data-driven thresholds are shown compared to normalized histograms of IL-12R β 1, IL-12R β 2, and pSTAT4 for unstained, unstimulated, and IL-12 stimulated populations are shown in Figure 3 (panels A, C, and E). The pairwise comparison shown in Figure 2 indicates that the MFI for pSTAT4 was correlated with cells that express IL-12 β 2.

Evolution of IL-12R β 2 and pSTAT4 over time

The protein expression and activity were presented using probability distribution functions (PDFs), enabling a comparison across different samples (Figure S1A–C). This analysis showed that the MFI of IL-12R β 1, IL-12R β 2, and pSTAT4 varied with time. PDFs for IL-12R β 2 at each time point exhibited a unimodal distribution (see Supplemental Figure S1B). In contrast, pSTAT4 exhibited a bimodal distribution (Figure S1C), revealing a heterogeneous population of cells: responsive cells expressing pSTAT4 in response to IL-12 and unresponsive cells that did not activate STAT4. To investigate the cause of the bimodality in pSTAT4 MFI, we stimulated the 2D6 T cell line with recombinant IL-12p70 (20 ng/ml), stained for IL-12R β 1, IL-12R β 2, and pSTAT4, and used flow cytometry to assay for changes in cellular expression and activity (Figure 3). In contrast to the Balb/c primary cells, the PDF for pSTAT4 for the 2D6 cell line exhibited a unimodal distribution, Figure 3F, indicating that the presence of responsive and unresponsive cells may be attributed to heterogeneity in the cell population or stimulation conditions, rather than an inherent characteristic of IL-12 signaling. On the basis of this result, we analyzed the flow cytometry data in order to parse out unresponsive cells and track pSTAT4 in the remaining cells as a function of time. A Gaussian distribution was fit to the unresponsive population and subtracted from the PDF for the total population. The resulting density function was used to characterize the change in pSTAT4 MFI within the responsive population (an example of this analysis is given in Figure S1D).

Using the flow cytometry data, we estimated the median protein expression/activity as a function of time (see Figure 4A–C). The control cells, those receiving no stimulation or anti-IL-4 only, were found to exhibit a similar dynamic change in expression of IL-12R β 1 as cells treated with both IL-12 and anti-IL-4 (Figure 4). Similarly, control cells and cells stimulated with IL-12 exhibited similar expression profiles for IL-12R β 2, suggesting a dynamic program for IL-12 expression not associated with IL-12 stimulation. This initial signaling phase that is independent of IL-12 is consistent with the work of Afkarian and coworkers who show that early activation of IFN- γ , a promoter of IL-12R β 2 expression, by the transcription factor T-bet is independent of stimulation by IL-12¹⁹. Recently, Schulz and coworkers also reported variations in the sensitivity of naïve CD4⁺ T cells to respond to IL-12²⁰. Therefore, we obtained a basal profile of IL-12R expression (Reaction Class 18)

from the control experiments using a superposition of Gaussian distributions (Figure S2). Control cells exhibited negligible activity of STAT4 as expected, and cells stimulated with IL-12 exhibited changes in STAT4 activity that also varied with time (Figure 4). The activity of STAT4 increased with time, up to 2–3 hours after stimulation with IL-12, where maximum pSTAT4 MFI occurred.

As the flow cytometry results provided relative measurements, we determined the basal level of IL-12R to be equal to 130 nM based upon an estimate of the number of receptors per cell (7500 membrane receptors/cell) in PHA-stimulated lymphoblasts, the fraction of receptors present on the cell surface (75%), and a cell radius of $3.5 \mu\text{m}^{11}$. Activity of STAT4 was also normalized for each cell by dividing the observed fluorescence intensity of pSTAT4 by the observed fluorescence intensity of IL-12R β 2 receptor expression. Normalizing STAT4 activity with respect to IL-12R β 2 expression provided an estimate of the activity of the receptor on a cellular basis as a function of time. Changes in activity of the receptor could be due to partitioning of IL-12R within the cell in response to positive feedback mechanisms or due to inhibition via negative feedback mechanisms.

Parameter estimation and model calibration

The mathematical model was used to infer the relative contribution of positive and negative feedback loops in regulating receptor activity. Given that the 33 model parameters were being fit to 44 experimental data points (4 observables, each with 11 time points), the problem is well-posed in theory, and the system is overdetermined. In practice, only a subset of the model parameters can be uniquely defined, and parameter identifiability is useful in determining the identifiable parameters *a priori* (i.e., given unlimited information). The correlation coefficients listed in Table S5 demonstrate that estimates of the forward binding rate (e.g., k_{f6} , k_{f7} , k_{f8} , and k_{f16}) were unique if the corresponding dissociation constants, K_D , were specified. Therefore, we fixed the K_D values according to the values reported by Yamada and coworkers²¹. We equated k_{f6} to k_{f7} , as was done in the model developed by Yamada and coworkers²¹. The Michaelis-Menten constant, K_M , was correlated to the V_m parameter for Reaction Classes 15 and 17. Specifying one of these redundant parameters helps improve the coefficient for estimating the contribution of the pathway, and we set the K_M value based on a previous model of IL-12 signaling²¹. In total, 14 parameters were selected to be fit to experimental data based on their correlation coefficients and values available in the literature.

In contrast to *a priori* parameter identifiability, an empirical Bayesian approach was used to estimate the sensitivity of the model parameters with respect to the available data. An Adaptive Markov Chain Monte Carlo (AMCMC) algorithm²² was used to estimate the expectation values of the model parameters, where simulated annealing provided an initial estimate of the parameter values. Three parallel chains, each containing 1×10^6 steps, were used to estimate the posterior distributions. The simulation of each chain took approximately 720 hours on a single core of a 2.66 GHz Dual-Core Intel Xeon 64-bit processor with 8 GB RAM. The trace and cumulative distributions of the acceptance fraction show that the scaling factor was adjusted in order to maintain the acceptance fraction around 0.2 (Figure S3A–B). The trace of the scaling factor suggests that 1×10^5 steps were required to establish an appropriate proposal distribution (Figure S3C). To minimize the effect of autocorrelation, the chains were thinned by selecting every 500th iteration. A graphical summary of the Gelman-Rubin statistics was used to determine convergence of the Markov chains to the posterior distribution in the model predictions (Figure 5). An initial sequence of 2×10^5 AMCMC steps was required for the three chains to converge. This initial sequence was used as the “burn-in” period.

Traces for each of the parameters were used to estimate the degree of mixing among the three chains (Figure S4). The optimal parameter values were determined using the expectation maximum and are listed in Table S4. Pairwise scatter plots obtained from the three chains following the burn-in period were used to estimate the posterior identifiability of the model parameters (Figure 6). The scatter plots were colored based upon marginal posterior probability density obtained by kernel density estimation. A high value for the correlation coefficient suggests that the parameters were not independently identifiable given the calibration data. There were differences between *a priori* identifiability (Table S5) and posterior distribution in parameter values (Figure 6). For example, k_{f1} and k_{f5} exhibit a higher correlation given the available data, and V_{m17} and k_{18} were not correlated based on available data (Figure 6).

The posterior distribution in the model predictions was obtained by marginalizing the model predictions over all of the parameter values from the Markov chain following the burn-in period. Model simulation revealed that the model was able to capture the long range dynamics of the IL-12 signaling pathway (Figure 7), and convergence of the Markov chains resulted in a small range of predictions.

Discussion

Understanding how biochemical cues are integrated by cells of the immune system to shape immunity remains a major question in the field¹⁴. To address one aspect of this question, we have developed a mathematical model of the IL-12 signaling pathway and calibrated the model to dynamic measurements of IL-12R β 2 expression and STAT4 activation in naïve CD4⁺ T cells derived from Balb/c mice. Given these dynamic calibration data, we also estimated the uncertainty in the model predictions using an empirical Bayesian approach. We incorporated the natural programming of the cellular signaling pathway, as well as feedback loops to describe the control mechanisms at work within the IL-12 signaling network. To our knowledge, this is the first model that integrates kinetic measurements of the dynamic intracellular signaling response of the IL-12 pathway and that establishes a level of confidence in the model predictions. In the following paragraphs, we will describe our predictions related to the relative contributions of postulated regulatory mechanisms that influence sustained IL-12 signaling.

Inference of regulatory mechanisms

The mathematical model developed here was used to infer the relative contributions of different regulatory mechanisms within the IL-12 signaling pathway. We first studied the relative rate of receptor downregulation by activated (ligand-bound receptors) and by inactive receptors. These signaling events were associated with rate parameters k_1 and k_2 and calibrated to the experimental data. The distribution in these parameters indicated very little difference between downregulation of the active and inactive receptor population (Figure 8A), implying that downregulation due to activation of the receptor was not a major factor in regulating of IL-12R expression.

Becskei and Grusby recently reported a positive feedback loop for IL-12-induced IL-12R β 2 expression, whereby activation of STAT4 by IL-12 results in increased IL-12R β 2 expression in naïve CD4⁺ T cells obtained from 129 mice²³. Moreover, Beckseki and Grusby implicitly assume that, in the absence of IL-12 stimulation, the level of IL-12R β 2 expression remains constant. As shown in 4 and in²⁰, the basal expression of IL-12R β 2 is not constant but changes with time in primary naïve CD4⁺ T cells. Assuming that basal expression of IL-12R β 2 is constant and cellular expression of this receptor is measured only under stimulation conditions, the logical interpretation of the dynamic response is that 1) active receptors exhibit a higher degradation rate (as illustrated in Figure 4 by the decline in

IL-12R β 2 in the first 3 hours following stimulation) and 2) the increase in receptors starting at 4 hours is due to a positive feedback loop. In²³, the contributions of this positive feedback loop (i.e., pSTAT4-induced IL-12R β 2 expression) and an autonomous program were quantified by the parameters v_{x4} and b_x , respectively. The reported maximum likelihood values of these parameters imply that the positive feedback pathway is 3500 times greater than the autonomous program pathway. Therefore, we also investigated the relative source of IL-12R β 2, comparing IL-12R β 2 expression via programmed synthesis of IL-12R β 2 mRNA and the positive feedback loop whereby pSTAT4 promotes increased expression of IL-12R β 2. As we have measured cellular response under unstimulated and stimulated conditions, we have reached a different conclusion. The distributions of the reaction rates of the pathways corresponding to an autonomous program for basal synthesis of receptor mRNA and feedback via pSTAT4 revealed that the average reaction rate of pSTAT4 feedback was at least 100 times less than that of the autonomous program of IL-12R β 2 synthesis (Figure 8B). This difference suggests that the positive feedback was not a major factor in regulating IL-12R β 2 expression in our system. This observation is consistent with the reported inability of Balb/c T cells to maintain IL-12 responsiveness despite prior STAT4 activation²⁴. In contrast, T cells derived from the B10.D2 strain increase expression of IL-12R β 2 in response to IL-12 stimulation. This suggests that the contribution of the STAT4-mediated positive feedback loop may be different among inbred mouse strains.

We also evaluated the effect of SOCS binding on the signaling pathway, finding that the fraction of receptor bound by SOCS was quite small (Figure 8C). The average response showed that less than 0.1% of total IL-12R was bound by SOCS and 97.5% of the predictions estimated that less than 0.6% of IL-12R was bound by SOCS, with the maximum occurring at 2 hours following stimulation by IL-12. In addition, the correlation coefficients among the posterior distribution in the parameters, as shown in Figure 8, suggests that an increase in binding of inactive STAT4 to IL-12R (k_f 8) can partially compensate for an increase in SOCS synthesis rate (k_f 23). Under these conditions, SOCS has a limited effect on receptor expression and activation of STAT4. Singh and coworkers²⁵ suggest strong negative feedback by SOCS species following activation of the JAK-STAT signal transduction pathway induced by IL-6 signal; however, in our hands, we observed that this negative feedback loop was not a major contributor to alterations in IL-12R expression. Given the minimal contribution of SOCS we were unable to assess the particular mechanism by which SOCS regulated IL-12 signaling. By obtaining experimental data from a positive control (i.e., stimulating cells with IL-4, which promotes synthesis of SOCS), it may be possible to infer the mechanism of action of SOCS, that is, whether the protein acts to increase IL-12R degradation or decreases the ability of IL-12R to phosphorylate STAT4.

Limitations of the analysis

The results presented here were influenced by the assumptions made *a priori* in developing the model. In particular, we did not explicitly account for the distribution of IL-12-bound receptor among different endosomal trafficking compartments, which has recently been reported to be important for effective IL-5 signaling²⁶. Additionally, we assumed that the rate of ligand-receptor binding and IL-12R mRNA translocation and degradation observed by others was applicable to our system.

Our data suggested cyclical IL-12R expression driven by a cell-autonomous program. Cell-autonomous maintenance of IL-12R β 2 expression has been associated with a genomic region within Chromosome 11 called *Tpml* (T cell phenotype modifier 1)²⁷. The particular genes associated with this locus remain unresolved. Similar oscillations in protein expression resulting from feedback loops have been observed in other biological systems^{28–30}. If we assume that the observed dynamics reflects two sinusoidal functions with periods of 7 and 3.5 hours, respectively, and interpolate system behavior between the 7.5 hour and

24 hour time points, the predicted regulatory mechanisms are similar to the ones shown in Figure 8 (data not shown). A counter-regulatory cytokine, IL-4, also promotes the expression of SOCS3 and inhibits IL-12-induced STAT4 activation in polarized CD4⁺ T_H2 cells³¹. The addition of anti-IL-4 to the stimulation conditions ruled out the contribution of an autocrine negative feedback loop attributed to enhanced IL-4 production by Balb/c T cells³² leading to downregulation of IL12R β 2³³. Regulation of IL-12R β 2 expression in Balb/c T cells by Interferon- γ ³⁴ still remains as a possible explanation. In future studies, it will be important to include experimental measurements of IL-12R synthesis and degradation and SOCS expression in order to address these limitations and further support the model predictions. The model does not exactly reproduce early oscillations in STAT4 activity (Figure 5C), which is closely related to the the input function IL-12R β 2. We chose to represent this IL-12-independent expression profile using 14 evenly spaced Gaussians, and including more Gaussians may result in a better fit of STAT4 activity. Additionally, non-convergence of pSTAT4 during early time points (Figure 5C) may be an artifact of simulation response since this figure reflects variations in STAT4 activity (plotted on the y-axis), and the simulation response is nearly vertical during that time period. Slight variations in parameter values that are consistent with later times would provide large differences in predictions during these early time points.

Concluding thoughts

Mathematical models are tools used to rationalize about the intracellular signaling mechanisms that underpin biological response³⁵. Mathematical models that describe biochemical kinetics are explicit statements about molecular-molecular interactions that are presumed to be important in a system and the corresponding dynamics of these interactions. These interactions give rise to a flow of molecular information in the form of reaction pathways³⁶. The primary goal of the analysis of these reaction pathways is to make predictions: what do we expect to happen in a particular reacting mixture under particular reaction conditions, given our current understanding of molecular interactions? Similarities confirm our explicit statements while differences between the expected behaviors and new data highlight areas of uncertainty in our understanding and provide the engine for scientific progress³⁷. Analogous to experimental studies, the ability of a particular mathematical model to describe a system of interest must include a statement of belief.

Belief derived from a mathematical model is expressed commonly in terms of a single point estimate for the predictions, obtained from the set of parameters that minimizes the variance between model and data³⁸. Given that a model constrains the set of possible states of the system, it is essential to provide an estimate of the uncertainty associated with the model predictions given the available data. A Bayesian view of statistics is a mathematical expression of our beliefs³⁹. Beliefs are established based upon the observation of data and the interpretation of that data within the context of our prior knowledge³⁷. Mathematical models provide a quantitative framework for representing prior knowledge of the detailed biochemical interactions that comprise a signaling network. The unknown parameters of the model can be calibrated against the observed network dynamics. Given the calibration data and the postulated model, the uncertainty in the model predictions can be obtained using an empirical Bayesian approach for model-based inference²².

This work applied an empirical Bayesian approach to infer the relative importance of postulated mechanisms that control IL-12 signaling in naïve CD4⁺ T cells obtained from Balb/c mice. In particular, this work revealed that an IL-12-independent, dynamic program for IL-12R expression was the major contributor to regulating signaling through the IL-12 pathway. The degradation rate for activated receptors was not significantly different from the degradation rate for inactive receptors. The similarity in degradation rates suggested that downregulation of receptor expression was not via a mechanism directly related to the

activity of the receptor. Finally, direct negative regulation of IL-12 signaling via SOCS expression was not a major factor in regulating IL-12 signaling. More generally, this model-based approach can be used to infer control mechanisms in other cell signaling pathways.

Methods

Experimental aspects

Mice—Eight to 12-week old female Balb/c mice were obtained from Hilltop Lab Animals (Scottsdale, PA). Mice were housed in sterilized microisolator cages in the university vivarium, and facility sentinel animals were regularly screened for specific pathogenic agents. All studies were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the West Virginia University Animal Care and Use Committee guidelines.

Antibodies and reagents—Hamster anti-mouse IL-12 receptor $\beta 2$ (HAM10B9), FITC-conjugated mouse anti-hamster IgG1 (G94-56), biotinylated anti-mouse IL-12 receptor $\beta 1$ (114), allophycocyanin (APC)-streptavidin, and phycoerythrin (PE)-conjugated mouse anti-mouse phosphorylated STAT4 (38/p-Stat4 Y⁶⁹³), BD Phosflow Lyse/Fix buffer, BD Phosflow Perm Buffer III, and FcBlock were purchased from BD Biosciences (San Diego, CA). The CD4⁺ CD62L⁺ T cell isolation kit was purchased from Miltenyi Biotec (Auburn, CA). Recombinant mouse IL-12p70, FITC-conjugated rat anti-mouse CD4 (GK1.5), PE-conjugated rat anti-mouse CD44 (IM7), APC-conjugated rat anti-mouse CD62L (MEL-14), affinity purified hamster anti-mouse CD28 (37.51), and rat anti-mouse IL-4 (11B11) were purchased from eBioscience (San Diego, CA). Monoclonal hamster anti-mouse CD3 (145-2C11) was purchased from R&D Systems (Minneapolis, MN). Unless otherwise noted, all primary cell cultures were maintained at 37°C in 5% CO₂ in RPMI 1640 plus supplements (referred to as complete RPMI or cRPMI). The RPMI supplements were 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan UT), 2 mM of l-glutamine (Mediatech Inc., Herndon VA), 50 mM HEPES (Sigma Chemical, St. Louis, MO), 49 μ M β -mercaptoethanol (Sigma Chemical) and 100 μ g/ml of streptomycin and 100 U/ml penicillin (Hyclone). 2D6 cells

Cell isolation and tissue culture—Spleens from Balb/c mice were isolated and a cell suspension was made by mashing the spleens through a nylon screen. Following lysis of red blood cells using tris ammonium chloride, the cells were pooled and washed twice in cRPMI containing 1.5% FBS. Mouse splenocytes were resuspended at 2.5×10^8 /ml. Unpolarized CD4⁺ CD62L⁺ (i.e., naïve) T-cells were subsequently isolated through negative selection by automated magnetic cell sorting, following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Briefly, mouse splenocytes were enriched for CD4⁺ T cells by negative selection through indirect magnetic labeling of non-CD4⁺ T cells using a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against CD8a, CD45R, CD11b, CD25, CD49b, TCR γ/δ , and Ter-119 and magnetic microbeads conjugated to a monoclonal anti-biotin antibody. CD4⁺ CD62L⁺ T cells were subsequently enriched from CD4⁺ splenocytes by positive selection using magnetic microbeads conjugated to a monoclonal rat anti-mouse CD62L antibody. Enrichment for CD4⁺ CD62L⁺ T cells was confirmed by flow cytometry using anti-CD4, anti-CD62L, and anti-CD44 antibodies. 2D6 cells, an IL-12-responsive mouse Th1 cell clone provided by Dr. M. J. Grusby (Harvard Medical School, Boston, MA), were maintained in cRPMI supplemented with 100 mM Sodium Pyruvate (Fisher Scientific, Pittsburgh, PA), 1% MEM non-essential amino acid solution (100X Fisher Scientific) and 6.7 ng/ml IL-12p70.

Pre-activation of naïve CD4⁺ T cells—Signaling via the IL-12 pathways requires the co-expression of both components of the IL-12 receptor: IL-12R β 1 and IL-12R β 2^{9,40}. IL-12R β 1 is expressed constitutively on CD4⁺ T cells. Naïve CD4⁺ T cells were stimulated for 44 hours with plate-bound anti-CD3 and anti-CD28 to upregulate IL-12R β 2⁴¹. Anti-CD3 coated plates were prepared by adding 10 μ g/ml monoclonal anti-mouse CD3 (R&D Systems, Minneapolis, MN) to 96-well culture plates and incubating for 90 minutes at 37°C followed by storage overnight at 4°C. Prior to the addition of the cells, the unbound anti-CD3 was aspirated out of the wells and the wells were washed three times with sterile phosphate buffered saline. Naïve CD4⁺ T cells (2×10^4 cells/well) and 2 μ g/ml of anti-mouse CD28 (ebioscience, San Diego, CA) were added to each of the anti-CD3 coated wells. All primary cell cultures were performed in cRPMI supplemented with 10% FBS, 37°C, and 5% CO₂.

Stimulation of IL-12 signaling—Pre-activated naïve CD4⁺ T cells were divided into three treatment groups. In the first group, cells were stimulated with recombinant IL-12p70 (20 ng/ml) and anti-mouse IL-4 (10 μ g/ml). The addition of anti-IL-4 to the stimulation conditions ruled out the contribution of an autocrine negative feedback loop attributed to enhanced IL-4 production by Balb/c T cells³². The second and third groups functioned as negative controls and were exposed to anti-IL-4 and PBS, respectively. The experiments were performed in duplicate. Prior to IL-12p70 stimulation, 2D6 cells (4×10^4 cells/well) were cultured in 96-well u-bottomed culture plates for 12 hours in the absence of IL-12p70. Cells were assayed by flow cytometry following a 6 hour exposure to IL-12p70 (20 ng/ml) or PBS, as a negative control. The experiments were performed in triplicate. Cells were appropriately stimulated and re-incubated for a series of time points at 37°C at 5% CO₂.

Flow cytometry—Unsorted splenocytes and isolated naïve CD4⁺ T cells were prepared, as described above, and stained with fluorophore-conjugated antibodies specific for the mouse T cell markers CD4, CD62L, and CD44. For extracellular staining, single cell suspensions were washed with ice-cold PBS containing 0.2% sodium azide (Sigma-Aldrich Chemical Co.) (PBSaz) and then incubated with purified rat immunoglobulin and purified mouse immunoglobulin (BD Biosciences) for 30 min on ice to prevent nonspecific binding. Subsequently, the cells were washed, incubated with buffer containing the appropriate antibody reagent for 30 min on ice. The cycle was repeated for any additional antibodies. Finally, the cells were washed twice in PBSaz and fixed in 0.4% paraformaldehyde. For intracellular staining, stimulated cells at the different time points were centrifuged and fixed at the appropriate times by incubating with BD Phosflow Lyse/Fix buffer (BD Biosciences) pre-warmed to 37°C for 10 minutes. Following fixing, cells were washed in PBS and incubated with buffer containing the appropriate antibody reagent for 30 minutes on ice. The cycle was repeated for any additional antibodies. Cells were assayed for total cellular expression of the components of the IL-12 receptor (i.e., anti-mouse IL-12R β 1 and anti-mouse IL-12R β 2, BD Pharmingen, San Jose, CA) and intracellular activation of STAT4 (i.e., phosphorylated STAT4, BD Pharmingen, San Jose, CA) by flow cytometry. Finally, the cells were washed three times in PBSaz and analyzed using a FACS Aria flow cytometer (Becton Dickinson). The fluorescent intensity for each parameter was reported as a pulse area using 18-bit resolution. Single stain controls were used to establish fluorescent compensation parameters. Unstained cells were used as negative flow cytometry controls. Flow cytometry data was analyzed using R/Bioconductor⁴².

Mathematical modeling

A mathematical model of IL-12 signaling was developed to describe the early signaling events following stimulation of IL-12R and to represent the regulatory aspects of the IL-12 pathway. Our model expands upon a model of T helper cell differentiation that was aimed at

simulating the T_h1/T_h2 balance in cytokine signaling networks, including the IL-12 pathway, along with IL-4 and IFN- γ pathways²¹. However, the previous model of IL-12 signaling was based on the dynamics of the IFN- γ pathway. The objectives of our study required the synthesis of a new mathematical model that builds upon elements of this prior work.

Developing a mathematical model for a reaction network involves two aspects. First, one must construct the topology of the reaction network (i.e., the series of biochemical interactions between reacting species). Once the reaction topology is specified, one must select values of the model parameters and initial conditions that are consistent with experimental data used to calibrate the model. Once calibrated, the model can be used to describe the evolution in species' concentration as a function of time and to explore the dynamic implications of different regulatory mechanisms. In the following paragraphs, each of these components of the IL-12 signaling network will be discussed in more detail.

Topology of the IL-12 signaling network—As depicted in Figure 1, a series of reaction classes that represent a canonical JAK-STAT signaling pathway were assembled to create a chemical kinetic model for IL-12 signaling. The collection of molecular species included in the model is listed in Table S1. The kinetics of the reactions were defined using reaction classes based on specific protein-protein interactions (Table 1 and Table S2)⁴³. Although a protein can be part of a larger complex, only a short peptide sequence, a protein motif, provides the point of interaction between proteins⁴⁴. From this perspective, the kinetics are governed by interactions between protein motifs on the various protein complexes. The relationships among all of the biochemical species represented in the model were compiled into a reaction mechanism. The corresponding 28 differential equations were used to describe the evolution of the system with time (Table S3).

Signaling through the IL-12 signaling pathway is initiated by IL-12p70 binding to its receptor, IL-12R. The IL-12 receptor is represented implicitly as a complex of both IL-12R β 1 and IL-12R β 2. IL-12p70 (L), IL-12R (R), and the IL12-IL12R (LR) complex interact with Janus kinase 2 (JAK2)⁴⁵ and tyrosine kinase 2 (TYK2). Binding of JAK2 and TYK2 imparts kinase activity to this activated receptor complex (LRTJ) and activates the STAT4 protein via phosphorylation⁴⁶. Phosphorylated STAT4 then dimerizes and translocates to the nucleus where it promotes the transcription of IL-12R mRNA²³. Nuclear IL-12R mRNA is exported to the cytosol, enabling the synthesis of new IL-12 receptors. In the absence of specific knowledge regarding trafficking of IL-12R⁴⁷, IL-12R is assumed to exhibit basal turnover with enhanced receptor degradation following receptor ligation⁴⁸. In addition, we have accounted for steady-state synthesis of JAK2, TYK2, and IL-12R mRNA, and protein degradation.

To assess the mechanisms for control of IL-12 signaling, we have incorporated three negative regulatory mechanisms: 1) ligand-induced receptor downregulation, 2) inhibition of the activity of activated IL-12R by SOCS, and 3) deactivation of phosphorylated STAT within the nucleus. As discussed above, activating a receptor commonly results in an increase in the rate of receptor degradation (i.e., receptor downregulation). IL-12 promotes the expression of Suppressor of Cytokine Signaling 1 (SOCS1) and 3 (SOCS3)¹³. However, the particular mechanism by which SOCS proteins regulate cytokine signaling remains elusive⁴⁹. In addition, protein inhibitors of activated STATs (PIAS) and protein tyrosine phosphatases (PTP) inhibit the activity of activated STATs within the nucleus⁵⁰. To represent this mechanism, a generic protein phosphatase (XPP) was included in the model that dephosphorylated STAT4 dimers in the nucleus to form STAT4 monomers. Unactivated STAT4 monomers were exported back to the cytosol.

Reactions took place in three locations: extracellular environment, cytosol, and nucleus, and the kinetic constants corresponding to reactions that involved reactants located in different compartments were scaled to account for the different respective volumes⁵¹, with the cytosol serving as the reference volume. Signaling events that occurred at the cell membrane were assumed to be well dispersed within the cytosol. The initial receptor concentration was determined by dividing the total number of receptors present on the cell surface by the average volume of incubation medium per cell, V_m (10^{-5} ml). However, the cytoplasmic water volume, V_{cw} (1.26×10^{-10} ml), is the reference volume, and the receptor concentration was scaled by multiplying by V_m/V_{cw} ⁵¹. Initial concentrations were taken from the literature²¹ or determined using algebraic constraints on conserved species (Table S1).

Model calibration—The resulting biochemical kinetic reaction network specified interactions between proteins (i.e., a set of coupled non-linear differential equations) and was coupled with parameters for each reaction to simulate concentration changes with time. The literature provided an initial source of parameter values for each reaction^{21,52}. The binding rates and kinetic constants for STAT4 phosphorylation and translocation were obtained from the work of Koster and Hauser⁵³. The rates of degradation and translocation of IL-12R mRNA were based on data from Hoffman and coworkers²⁸, assuming degradation and translocation rates are independent of the transcription factor (i.e., NF- κ B versus STAT4). Mass balances provided the rate of steady-state synthesis of nuclear IL-12R mRNA.

The experimental data measuring the expression of IL-12R β 2, the activity of STAT4 (i.e., phosphorylated STAT4), and activity of STAT4 normalized to IL-12R β 2 expression were used to calibrate the mathematical model. Additionally, ligand-receptor binding in human lymphoblastoid cells was used⁵⁴, assuming that ligand-receptor binding is cell-type independent. The model equations were encoded and evaluated in MatLab V7.0 (The MathWorks, Natick, MA). Summed squared error between experimental and simulated measurements was used to determine goodness-of-fit. Estimates for unknown parameter values were determined using these experimental data and were guided by parameter identification analysis. Ultimately, all parameter values, tabulated in Table S4, were determined to be consistent with observed experimental data using a combination of simulated annealing⁵⁵ and an empirical Bayesian approach discussed in more detail in the following sections.

Parameter identification—An important aspect of model development is to identify parameters that can be uniquely determined from the available data. We have used parameter identification analysis to determine whether the parameter calibration problem was initially well-posed⁵⁶. The correlation coefficients between model parameters were calculated, where parameters that were locally identifiable had correlations with all other parameters between -0.965 and 0.965 (i.e., between -1 and 1). Parameters that were not locally identifiable, termed *a priori* unidentifiable, had correlations of > 0.965 or < -0.965 with at least one other parameter (See Supplementary Table S5).

Bayesian confidence interval estimation—A computational Bayesian approach was used to estimate the uncertainty associated with the model parameters given the available experimental data and to determine the interdependence of the parameter values²². We used an Adaptive Markov Chain Monte Carlo (AMCMC) algorithm to generate a sequence of states that represent samples drawn from the posterior distribution of the model predictions, given the uncertainty in the model parameters and the specific calibration data. A starting point in the parameter space was obtained via simulated annealing⁵⁵. Using an unbiased prior distribution, a learning period of 100,000 steps was used to establish the covariance of

the proposal distribution. The proposed steps within parameter space were evaluated using a Metropolis-Hastings algorithm with a targeted acceptance fraction equal to 0.2. The Gelman-Rubin potential scale reduction factor was applied to the model predictions to estimate the convergence of the Markov chain to the posterior distribution of the model predictions^{57,58}. In addition, representative samples from the posterior distribution were obtained by retaining every 500th step of the cumulative chain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

DJK conceived the study; DG and NC performed the experiments; SDF, DG, NC and DJK analyzed the data; and SDF and DJK wrote the paper. The authors thank Kathy Brundage for assistance with the experimental techniques and John Barnett for access to laboratory facilities. The authors declare no competing financial interest. This work was supported by grants from the PhRMA Foundation, the National Cancer Institute (NCI) R15CA123123, and the National Institute of Allergy and Infectious Disease (NIAID) R56AI076221. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCI, the NIAID, or the National Institutes of Health.

References

1. Janeway, C. Immunobiology 5: the immune system in health and disease. 5. Garland Publishing; New York: 2001.
2. Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions in helper T cells. *Advanced Immunology* 1989;46:111–147.
3. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annual Review of Immunology* 1994;12:635–673.
4. Bi Y, Liu G, Yang R. Th17 cell induction and immune regulatory effects. *Journal of Cellular Physiology* 2007;211:273–278. [PubMed: 17311299]
5. Rosenberg SA. A new era of cancer immunotherapy: converting theory to performance. *CA: A Cancer Journal for Clinicians* 1999;49:70–73. [PubMed: 11198888]
6. Martin R, Sturzebecher CS, McFarland HF. Immunotherapy of multiple sclerosis: Where are we? Where should we go? *Nature Immunology* 2001;2:785–788. [PubMed: 11526386]
7. Lam WK, Watkins DN. Lung cancer: future directions. *Respirology* 2007;12:471–477. [PubMed: 17587411]
8. Hsieh CS, et al. Development of Th1 CD4+ T cells through IL12 produced listeria-induced macrophages. *Science* 1993;260:547–549. [PubMed: 8097338]
9. Presky DH, et al. A functional Interleukin-12 receptor complex is composed of two b-type cytokine receptor subunits. *Proceedings of the National Academy of Science* 1996;93:14002–14007.
10. Parham C, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12RB1 and a novel cytokine receptor subunit, IL-23R. *Journal of Immunology* 2002;168:5699–5708.
11. Klinke DJ. The ratio of P40 monomer to dimer is an important determinant of IL-12 bioactivity. *Journal of Theoretical Biology* 2006;240:323–335. [PubMed: 16448670]
12. Sinigaglia F, D'Ambrosio D, Panina-Bordignon P, Rogge L. Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function. *Immunological Reviews* 1999;170:65–72. [PubMed: 10566142]
13. Athie-Morales V, Smits HH, Cantrell DA, Hilken CMU. Sustained IL-12 signaling is required for Th1 development. *Journal of Immunology* 2004;172:61–69.
14. Murray PJ. The JAK-STAT signaling pathway: Input and output integration. *The Journal of Immunology* 2007;178:2623–2629. [PubMed: 17312100]
15. Espert L, Dusanter-Fourt I, Chelbi-Alix MK. Negative regulation of the JAK/STAT: Pathway implication in tumorigenesis. *Bulletin du Cancer* 2005;92:845–857. [PubMed: 16266868]

16. Valentino L, Pierre J. JAK/STAT signal transduction: regulators and implications in hematological malignancies. *Biochemical Pharmacology* 2006;71:713–721. [PubMed: 16426581]
17. ten Hoeve J, et al. Identification of a nuclear Stat4 propeptide tyrosine phosphatase. *Molecular and Cellular Biology* 2002;22:5662–5668. [PubMed: 12138178]
18. Klinke DJ, Ustyugova IV, Brundage KM, Barnett JB. Modulating temporal control of NF-kappaB activation: implications for therapeutic and assay selection. *Biophysical Journal* 2008;94:4249–4259. [PubMed: 18281385]
19. Afkarian M, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nature Immunology* 2002;3:549–557. [PubMed: 12006974]
20. Schulz EG, Mariani L, Radbruch A, Hofer T. Sequential polarization and imprinting of type 1 T helper lymphocytes by Interferon-gamma and Interleukin-12. *Immunity* 2009;30:673–683. [PubMed: 19409816]
21. Yamada S, Tsukada J, Yoshimura A, Kubo M. Computer simulation of the role of SOCS family protein in helper T cell differentiation. *International Immunology* 2006;18:335–345. [PubMed: 16410311]
22. Klinke DJ. An empirical Bayesian approach for model-based inference of cellular signaling networks. *BMC Bioinform* :10.
23. Becskei A, Grusby MJ. Contribution of IL-12R mediated feedback loop to Th1 cell differentiation. *FEBS Letters* 2007;581:5199–5206. [PubMed: 17950290]
24. Guler ML, Jacobson NG, Gubler U, Murphy KM. T cell genetic background determines maintenance of IL-12 signaling: effects on BALB/c and B10.D2 T helper cell type 1 phenotype development. *Journal of Immunology* 1997;159:1767–1774.
25. Singh A, Jayaraman A, Hahn J. Modeling regulatory mechanisms in IL-6 signal transduction in hepatocytes. *Biotechnology and Bioengineering* 2006;95:852–862.
26. Lei JT, Martinez-Moczygemba M. Separate endocytic pathways regulate IL-5 receptor internalization and signaling. *Journal of Leukocyte Biology* 2008;84:1–11. [PubMed: 18388298]
27. Guler ML, et al. Tpm1, a locus controlling IL-12 responsiveness, acts by a cell-autonomous mechanism. *Journal of Immunology* 1999;162:1339–1347.
28. Hoffman A, Levchenko A, Scott ML, Baltimore D. The I κ B-NF- κ B signaling module: Temporal control and selective gene activation. *Science* 2002;298:1241–1245. [PubMed: 12424381]
29. Lahav G, et al. Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nature Genetics* 2004;38:147–150. [PubMed: 14730303]
30. Nelson DE, See V, Nelson G, White MRH. Oscillations in transcription factor dynamics: a new way to control gene expression. *Biochemical Society Transactions* 2004;32:1090–1092. [PubMed: 15506974]
31. Egwuagu CE, et al. Suppressors of Cytokine Signaling Proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *The Journal of Immunology* 2002;168:3181–3187. [PubMed: 11907070]
32. Yagi R, et al. The IL-4 production capability of different strains of naive CD4+ T cells controls the direction of the Th cell response. *International Immunology* 2002;14:1–11. [PubMed: 11751746]
33. Szabo SJ, Jacobson NG, Dighe AS, Gubler U, Murphy KM. Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. *Immunity* 1995;2:665–675. [PubMed: 7796298]
34. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the Interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *Journal of Experimental Medicine* 1997;185:817–824. [PubMed: 9120387]
35. Yaffe MB. Signaling Networks and Mathematics. *Sci Signal* 2008;1:eg7. [PubMed: 18957689]
36. Broadbelt LJ, Pfaendtner J. Lexicography of kinetic modeling of complex reaction networks. *AIChE J* 2005;51:2112–2121.
37. National Research Council (U.S.). Committee on Learning, Research, Practice and Education. *How People Learn: brain, mind, experience, and school*. National Academies Press; Washington, DC: 2000.
38. Banga JR. Optimization in computational systems biology. *Bmc Systems Biology* 2008;2

39. Gelman, A.; Carlin, JB.; Stern, HS.; Rubin, DB. Texts in Statistical Science. Chapman and Hall; Boca Raton, FL: 2004. Bayesian Data Analysis.
40. Presky DH, et al. Analysis of the multiple interactions between IL-12 and the high affinity IL-12 receptor complex. *Journal of Immunology* 1998;160:2174–2179.
41. Park WR, et al. CD28 costimulation is required not only to induce IL-12 receptor but also to render Janus kinases/STAT4 responsive to IL-12 stimulation in TCR-triggered T cells. *European Journal of Immunology* 2001;31:1456–1464. [PubMed: 11465102]
42. Klinke DJ, Brundage KM. Scalable analysis of flow cytometry data using R/Bioconductor. *Cytometry A*. 2009 In press.
43. Faeder JR, Blinov ML, Goldstein B, Hlavacek WS. Rule-based modeling of bio-chemical networks. *Complexity* 2005;10:22–41.
44. Pawson T, Nash P. Assembly of cell regulatory systems through protein interaction domains. *Science* 2003;300:445–452. [PubMed: 12702867]
45. Yamamoto K, et al. Physical interaction between interleukin-12 receptor beta 2 subunit and Jak2 tyrosine kinase: Jak2 associates with cytoplasmic membrane-proximal region of interleukin-12 receptor beta 2 via amino-terminus. *Biochemical and Biophysical Research Communications* 1999;257:400–404. [PubMed: 10198225]
46. Lund RJ, Chen Z, Scheinin J, Lahesmaa R. Early target genes of IL-12 and STAT4 in signaling in Th cells. *The Journal of Immunology* 2004;172:6775–6782. [PubMed: 15153495]
47. Devergne O, Ghiglione C, Noselli S. The endocytic control of JAK/STAT signalling in *Drosophila*. *Journal of Cell Science* 2007;120:3457–3464. [PubMed: 17855388]
48. Lauffenburger, DA.; Linderman, JJ. Receptors: Models for binding, trafficking and signaling. Oxford University Press; New York: 1993.
49. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. *Immunity* 2008;28:477–487. [PubMed: 18400190]
50. Nakahira M, Tanaka T, Robson BE, Mizgerd JP, Grusby MJ. Regulation of signal transducer and activator of transcription signaling by the tyrosine phosphatase PTP-BL. *Immunity* 2007;26:163–176. [PubMed: 17306571]
51. Kholodenko BN, Demin OV, Moehren G, Hoek JB. Quantification of short term signaling by the epidermal growth factor receptor. *Journal of Biological Chemistry* 1999;274:30169–30181. [PubMed: 10514507]
52. Yamada S, Shiono S, Joo A, Yoshimura A. Control mechanism of JAK/STAT signal transduction pathway. *FEBS Letters* 2003;534:190–196. [PubMed: 12527385]
53. Koster M, Hauser H. Dynamic redistribution of STAT1 protein in IFN signaling visualized by GFP fusion proteins. *European Journal of Biochemistry* 1999;260:137–144. [PubMed: 10091593]
54. Chizzonite R, et al. IL-12 receptor I. Characterization of the receptor on phytohemagglutinin-activated human lymphoblasts. *Journal of Immunology* 1992;148:3117–3124.
55. Beers, KJ. Numerical methods for chemical engineering. Applications in Matlab. Cambridge university Press; Cambridge: 2007.
56. Khoo, MCK. Physiological control systems: analysis, simulation, and estimation. IEEE Press; 2000.
57. Gelman A, Rubin D. Inference from iterative simulation using multiple sequences. *Statistical Science* 1992;7:457–474.
58. Brooks S, Gelman A. General methods for monitoring convergence of iterative simulations. *Journal of Computational and Graphical Statistics* 1998;7:434–455.

Figure 1. A simplified schematic diagram of the reaction classes represented in the mathematical model

The model represents IL-12R synthesis and degradation; IL-12 binding to IL-12R; STAT4 dimerization and phosphorylation; JAK2 and TYK2 association with the receptor; activation of the signaling pathways associated with the interaction between STAT4 and the bound receptor complex (LRTJ); dimerization and localization of phosphorylated STAT4; deactivation following the dephosphorylation by STAT4 protein phosphatase; and negative feedback by SOCS and protein phosphatase.

Figure 2. Pairwise density plots for phosphorylated STAT4 versus IL-12R β 2

Each sub-panel corresponds to a different time following IL-12 stimulation. The solid line indicates the expression threshold for a cell to be associated with positive expression of IL-12R β 2. Ninety five percent of the unstained cell fraction was contained below the threshold. The dotted line indicates the upper limit of pSTAT4 MFI for 95% of the CD4+ CD62L+ fraction prior to IL-12 stimulation.

Figure 3. Marginalized probability distribution function (PDF) for IL-12R β 1 (panels A and B), IL-12R β 2 (panels C and D), and pSTAT4 (panels E and F)

Data from the 6-hour time point is shown for Balb/c primary cells (panels A, C, and E) and 2D6 cell line (panels B, D, and F). Three cell populations are shown: unstained cells (black), cells receiving no stimulation (blue), and cells stimulated with IL-12 (red). The dotted line indicates the upper limit of protein expression or activity for 95% of the unstained (panels A–D) or unstimulated (panels E and F) CD4⁺ CD62L⁺ fraction.

Figure 4. Median protein MFI as a function of time

Median values for all experimental groups: cells receiving no stimulation (blue), cells stimulated with anti-IL4 (black), and cells stimulated with anti-IL-4 and IL-12 (red). Changes in fluorescent intensity (see Figure S1) correspond to changes in protein expression or activity: (A) IL-12R β 1 expression, (B) IL-12R β 2 expression, and (C) Phosphorylated STAT4 activity. Two experimental replicates are shown.

Figure 5. Convergence of the model predictions among the three parallel Markov chains
 The y-axis corresponds to the model simulation time. The x-axis corresponds to the cumulative range of AMCMC steps for which the Gelman-Rubin statistic is calculated. (A) Phosphorylated STAT4 normalized to the level of IL-12R β 2 expression on a cellular basis. (B) IL-12 bound to IL-12R (data from Chizzonite⁵⁴). (C) Phosphorylated form of STAT4 reported as a percentage of the maximum STAT4 activity. (D) Total IL-12R β 2 expression. Values less than 1.2 suggest convergence of the chains.

Figure 6. Pairwise comparison of rate parameters
Parameter names are given on the diagonal. Above the diagonal are the pairwise correlation coefficients of the parameters obtained from the thinned Markov chains. Pairwise projections of the marginalized probability density are given below the diagonal. Coloring is based upon the estimated 2-D posterior density distributions. The values in each scatter plot are centered at the mean values determined from three parallel Markov Chains, and each plot axis spans $O(10^{16})$.

Figure 7. The mathematical model for early signaling events reproduces the early dynamics for activation of the STAT4 pathway downstream from the IL-12 receptor

Simulated results from three Markov chains (black, blue, and red lines) are compared against the experimental observations (squares) used to calibrate the mathematical model. Dashed lines indicate 95% confidence interval for the posterior predictions; solid lines indicate the median value. (A) Phosphorylated STAT4 normalized to the level of IL-12R β 2 expression on a cellular basis. (B) IL-12 bound to IL-12R (data from Chizzonite⁵⁴). (C) Phosphorylated form of STAT4 reported as a percentage of the maximum STAT4 activity. (D) Total IL-12R β 2 expression. The initial conditions used in the simulation are listed in Table S1

Figure 8. Comparison of regulation mechanisms predicted from the posterior distribution (A) Distribution in the logarithm of the rate parameters ($\text{Log}(\Theta_i)$) associated with receptor down regulation for inactive (black - parameter k_1) and active (blue - parameter k_2) IL-12 receptors. (B) Distribution of rates of reaction for autonomous program for IL-12R β 2 expression (black - reaction class 18 in Figure 1) and positive feedback where pSTAT4 promotes increased expression of IL-12R β 2 (blue - reaction class 17 in Figure 1) shown as a function of time. Solid line is median value for cumulative Markov chain; dashed lines enclose 95% of the model predictions (i.e., a 95% confidence interval). (C) Range of model predictions for the concentration of IL-12R β 2 bound to SOCS (reaction class 16 in Figure 1) shown as a function of time. Solid line is median value for predictions obtained from the cumulative Markov chain; dashed lines indicate 95% confidence interval in the model predictions, given the postulated mathematical model and experimental data.

Table 1

List of reaction classes.

Reaction class	Description
R1	Degradation of inactive receptor
R2	Degradation of active (ligand-bound) receptor
R3	Basal synthesis of TYK2
R4	Basal synthesis of JAK2
R5	Ligand binding to receptor
R6	TYK2 binding to receptor
R7	JAK2 binding to receptor
R8	Inactive STAT4c binding to activated receptor complex
R9	Phosphorylation of STAT4c by the activated receptor complex
R10	Dimerization of phosphorylated STAT4
R11	Export of STAT4 from cytosol to nucleus
R12	Protein phosphatase binding to active STAT4 in nucleus
R13	Dephosphorylation of STAT4n by protein phosphatase
R14	Export of inactive STAT4 from nucleus to cytosol
R15	Transcription of SOCS mRNA by active STAT4
R16	SOCS binding to activated receptor complex
R17	Transcription of IL-12R mRNA by active STAT4
R18	Autonomous program for basal synthesis of IL-12R mRNA
R19	Export of mRNA from nucleus to ribosomes in cytosol
R20	Synthesis of IL-12R from mRNA
R21	Degradation of mRNA in the cytosol
R22	Transport of intracellular IL-12R to cell membrane
R23	Synthesis of SOCS from mRNA
R24	Protein degradation