Supplementary Materials for

**Systems modeling of molecular mechanisms controlling cytokine-driven CD4+ T cell differentiation and phenotype plasticity**

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**Section 1: Model creation**

The population of CD4+ T cells is functionally and phenotypically heterogeneous consisting of at least four subsets involved in coordinating various aspects of adaptive immunity. Upon antigenic stimulation by antigen-presenting cells, naïve CD4+ T cells (Th0) expand and differentiate into at least three effector cell subsets referred to as Th1, Th2, Th17, and induced regulatory T (iTreg) cells (Figure S1). In addition to the four Th phenotypes studied and modeled in this project other CD4+ T cell phenotypes have been characterized, including transforming growing factor β (TGF-β)-producing CD4+ T cells (Th3) [[1](#_ENREF_1)], IL-10-producing CD4+ T cells (Tr1) [[2](#_ENREF_2)], IL-9-producing CD4+ T cells (Th9) [[3](#_ENREF_3)] and T follicular helper (Tfh) cells located in the follicular regions of lymph nodes and spleen [[4](#_ENREF_4),[5](#_ENREF_5)]. Signaling pathways controlling fates on these phenotypes are closely connected to the four core subsets (Th1, Th2, Th17 and iTreg). Each of these phenotypes is characterized by distinct effector and regulatory functions, which are regulated by a signature pattern of cytokines and multiple transcription factors. The signaling pathways that lead to these four predominant fates are cross-regulated via feedback loops that facilitate a balanced immune response to pathogens or abnormal cells while avoiding chronic inflammation and autoimmunity.

We present for the first time a mathematical and computational model built upon the current paradigms of molecular interactions that occur in CD4+ T cells. This model will help us to elucidate the regulatory mechanisms underlying CD4+ T cell differentiation, identify novel putative CD4+ T cell subsets, and study the dynamics of Th cell differentiation. Previous modeling efforts have also focused on the CD4+ T cell. For instance, Mendoza reported a logical network model for controlling the differentiation process in CD4+ T cells [[6](#_ENREF_6)], however, that model was build upon the Th1 versus Th2 paradigm, without considering Th17 or iTreg subsets. Additional models of immunity are available for predicting the generation of memory cells [[7](#_ENREF_7),[8](#_ENREF_8)] and determining the role of IL-2 in the interplay between effector and regulatory phenotypes [[9](#_ENREF_9)]. There is also a comprehensive review on differentiation of effector CD4+ T cell populations by Zhu and colleagues [[10](#_ENREF_10)]. Recent publications also reported on modeling approaches for specific CD4+ T cell phenotypes, such as the regulation of Th1 by T-bet, IL-12 and interferon-γ (IFN-γ) [[11](#_ENREF_11)] or the regulation of the crosstalk between Th17 and iTreg by quantifying the master regulators [[12](#_ENREF_12)]. Other studies have focused on the interaction between more than two phenotypes using logical models [[13](#_ENREF_13)]. However, our extended ODE-based model is the first to illustrate in a detailed and comprehensive manner the intracellular regulatory networks controlling fate determination for all four phenotypes in a deterministic way (i.e., Th1, Th2, Th17 and iTreg). Specifically, we have extended previous models by adding some new detailed interactions for the Th1/Th2-related pathways, including new pathways controlling plasticity between Th17 and iTreg cells, as well as the crosstalk among these pathways. In addition, in contrast to previous studies and given the initial results of the sensitivity analysis, our structural network model includes the modulation of this process by the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ).

Three distinct signals regulate CD4+ T cell activation and differentiation: a signal from the T cell receptor (TCR) interacting with MHC, a co-stimulatory signal (i.e., CD28 interacting with B7.1 or B7.2 on antigen presenting cells), and a cytokine-driven signal. Other studies have focused on CD4+ T cell proliferation [[14](#_ENREF_14)], TCR signaling [[12](#_ENREF_12)] or co-stimulatory signals [[15](#_ENREF_15)]. In this report, we assemble the knowledge about non-cognate interactions controlling the CD4+ T cell differentiation process (i.e., cytokine milieu, signaling pathways and transcription factors) available in the literature into a comprehensive network model. This is a first step toward a more comprehensive understanding of the dynamics of the CD4+ T cell differentiation process at the systems level. Thus, we are describing activation pathways by phenotype, as well as the inhibitory mechanisms that lead to the induction or suppression of a CD4+ T cell phenotype.

**T helper 1 cells**

A naïve T cell can differentiate into a Th1 phenotype through twomajor signaling pathways which have recently been shown to be interconnected and their expression is coordinated by antigen-induced signaling [[11](#_ENREF_11),[16](#_ENREF_16),[17](#_ENREF_17)]. The first pathway involves antigen recognition through the T cell receptor (TCR) that activates the signaling pathway of IFN-γ, and the transcription factors signal transducer of activation of transcription (STAT)-1 and T-bet. IFN-γ binds to its receptor IFN-γR, on the T cell surface, and activates janus kinase-1 (JAK-1) and STAT-1 [[18-20](#_ENREF_18)] which leads to the expression of T-bet in Th1 [[21](#_ENREF_21),[22](#_ENREF_22)]. Furthermore, T-bet can induce its own transcription [[23](#_ENREF_23)] and is known to induce IFN-γ expression [[24](#_ENREF_24)], thereby creating a positive feedback loop. Independent of IFN-γ a sustained expression of T-bet in human Th2 cells induces Th1 cytokines and represses Th2 cytokines [[24](#_ENREF_24),[25](#_ENREF_25)]. T-bet is also capable of activating suppressor of cytokine signaling (SOCS)-1, which then blocks IL-4R signaling in response to IL-4 stimulation [[26](#_ENREF_26)], therefore inhibiting the Th2 phenotype and favoring Th1 differentiation. In addition to T-bet, another strong activator of SOCS-1 is STAT-1 which also favors Th1 differentiation [[27](#_ENREF_27)]. On the other hand, SOCS-1 can inhibit JAK-1 and block the activation of STAT-1 by IFN-γ *in vivo* [[28](#_ENREF_28),[29](#_ENREF_29)], thereby representing a negative feedback loop that could suppress Th1 differentiation.

The other major pathway for a naïve CD4+ T cell to differentiate into a Th1 phenotype involvesthe IL-12/STAT-4 axis [[24](#_ENREF_24),[30](#_ENREF_30)] by the activation of STAT-4 through the signaling of antigen-presenting cell (APC)-derived IL-12 [[23](#_ENREF_23),[31](#_ENREF_31)], where STAT-4 up-regulates IFN-γ expression [[32](#_ENREF_32)]. Furthermore, STAT-4 is not only capable of inducingTh1 differentiation independently of T-bet, but it is also essentialfor Th1 differentiation in the absence of T-bet [[30](#_ENREF_30)]. Indeed, Furuta et al. (2008) showed that Th1 differentiation was severely impaired in both T-bet–/– CD4+ T cells and STAT4–/– CD4+ T cells, which suggests that STAT-4 activates T-bet directly or indirectly [[30](#_ENREF_30)].

Although IL-18 is not required for the development of Th1 cells, it is essential for the effective induction and activation of Th1 cells by IL-12 [[33](#_ENREF_33)], as it synergizes with IL-12 in the induction of IFN-γ by activating STAT-4 and promoting IFN-γ activation [[34](#_ENREF_34)]. IL-18 signals through the IL-1 receptor associated kinase (IRAK-1) to induce the accumulation of NF-κB[[35](#_ENREF_35)] which then leads to the induction of IFN-γ[[36](#_ENREF_36)]. Indeed, nuclear factor-kappaB (NF-κB) and STAT-4synergize to induce IFN-γ [[37](#_ENREF_37),[38](#_ENREF_38)].

 Next we describe the underlying mechanisms that inhibit naïve T cells from differentiating into a Th1 phenotype. There is evidence that STAT-6 inhibits the IL-12/STAT-4 pathway [[39](#_ENREF_39)] and is required for the down-regulation of IL-18Rα [[40](#_ENREF_40)]. The over-expression of STAT-3 reduces the expression of the trans-acting T cell-specific factor (GATA-3), a transcription factor involved in Th2 differentiation, and T-bet, and hence inhibits the differentiation into Th1and Th2 [[41](#_ENREF_41)]. The transcription factor forkhead box P3 (FOXP3), a marker for Treg cells, inhibits the production of IFN-γ by physically binding to and blocking NF-κB from inducing IFN-γ [[42](#_ENREF_42)]. PPARγ ligands can directlydecrease IFN-γ expression [[43](#_ENREF_43)]. At the same time, however, the inactivation of STAT-3 by PPARγ [[44](#_ENREF_44)] could activate IFN-γ expression as STAT-3 inhibits IFN-γ. In macrophages, PPARγ down-regulates the expression of pro-inflammatory cytokines by antagonizing the activities of transcription factors such as activator protein (AP)-1, STAT and NF-κB [[45](#_ENREF_45)], and in epithelial cells it favors the nucleocytoplasmic shuttling of the activated p65 subunit of NF-κB [[46](#_ENREF_46)]. It remains unknown whether these mechanisms observed in macrophages and epithelial cells play a role in CD4+ T cell differentiation.

**T helper 2 cells**

Naïve T helper cells will differentiate into the effector Th2 phenotype, characterized by the expression of IL-4, IL-5 and IL-13, through two apparently independent pathways, namely, the IL-4/STAT-6 and IL-2/STAT-5 axis [[47](#_ENREF_47)]. GATA-3 is the common link between both pathways[[47](#_ENREF_47)]. Binding of IL-4 to its receptor leads to the phosphorylation of STAT-6 which induces GATA-3 expression[[48](#_ENREF_48)]. GATA-3 is known to activate IL-4[[49](#_ENREF_49)], which creates a positive feedback loop ensuring the stability of Th2 fate.

Enhanced IL-2 signaling by binding to its receptor and inducing STAT-5 is an essential pathway for Th2 differentiation [[50](#_ENREF_50),[51](#_ENREF_51)]. Neutralizationof IL-2 abolishes early IL-4 production without affecting earlyGATA-3 expression [[52](#_ENREF_52)], which suggests alternative mechanisms for activating GATA-3. Experimental results in mice indicate that GATA-3 is capable of inducing its own expression [[49](#_ENREF_49)]. Recently it has been reported that Notch directly regulates GATA-3 expression, and synergistically contributes to Th2 differentiation [[53](#_ENREF_53),[54](#_ENREF_54)]. These findings may explain Th2 differentiation *in vivo* without the stimulation by IL-4.

PPARγ expression in activated T cells is dependent on IL-4 [[55](#_ENREF_55)], indicating a link with the Th2 fate. Direct physical interactions between PPARγ and NFAT can result in inhibition of IL-2 production by CD4+ T cells [[56](#_ENREF_56)]. While IL-4 upregulates PPARγ expression, treatment of CD4+ T cells with PPARγ agonists (i.e., ciglitazone or 15dPGJ2) triggered the physical association between PPARγ and NFATc1, resulting in IL-4 promoter inhibition and decreased IL-4 production [[57](#_ENREF_57)], suggesting the existence of a regulatory mechanism that prevents excessive differentiation towards the Th2 phenotype. Also, 13-hydroxyoctadecadienoic acid, an endogenously generated PPARγ agonist, down-regulated IL-2 production by human peripheral blood T lymphocytes by reducing NFAT and NF-κB binding to the IL-2 promoter [[58](#_ENREF_58)]. Moreover, IL-4 was shown to simultaneously increase the expression of PPARγ and 12-15-lipooxygenase, the enzyme involved in the generation of 13-hydroxyoctadecadienoic acid [[59](#_ENREF_59)]. Thus, it has been proposed that IL-4 indirectly down-regulates IL-2 production by T cells through a PPARγ -dependent mechanism [[58](#_ENREF_58),[59](#_ENREF_59)].

The differentiation into Th2 could possibly be inhibited through a variety of mechanisms that have also been incorporated in our network model. For instance, the over-expressionof SOCS-1 in Th2 cells represses STAT-6 activation and profoundlyinhibits IL-4-induced proliferation [[60](#_ENREF_60)] and SOCS-1 inhibits IL-4R from phosphorylating STAT-6[[61](#_ENREF_61),[62](#_ENREF_62)]. Furthermore, STAT-1 is required for the repression of IL-4-induced gene expression by IFN-γ[[63](#_ENREF_63)]. Also, IFN-γ was shown to inhibit STAT-6 by suppressing its phosphorylation by IL-4R[[64](#_ENREF_64)]. On the other hand, the iTreg cell-derived cytokine transforming growth factor-β (TGF-β) inhibits GATA-3 expression at the transcriptional level, however, it does not interfere with IL-4 signaling [[65](#_ENREF_65)]. FOXP3 interacts with NFAT, such that NFAT becomes unable to induce IL-4 expression [[42](#_ENREF_42)], thereby rearing it unable to activate T cells in response to antigenic stimulation via the TCR [[66](#_ENREF_66)].

**T helper 17 cells**

Th17 cells are characterized by their production of the cytokine IL-17. TGF-β, together with pro-inflammatory cytokines IL-6 or IL-21 and IL-23, orchestrate the differentiation of CD4+ T cells into the Th17 phenotype in a concentration-dependent manner [[67](#_ENREF_67),[68](#_ENREF_68)]. It has been demonstrated that TGF-β synergizes with IL-6 [[69](#_ENREF_69)] or IL-21 [[70](#_ENREF_70),[71](#_ENREF_71)] to promote the expression of IL-17. This is achieved through stimulation of retinoid-related orphan receptor (ROR)γt by IL-6 through the transcription of STAT-3 [[72](#_ENREF_72),[73](#_ENREF_73)], which in turn induces expression of IL-17 [[41](#_ENREF_41),[74](#_ENREF_74)]. While RORγt is essential for the differentiation of naïve CD4+ T cells into Th17 effector cells, IL-23 is required for maintaining and stabilizing the Th17 phenotype[[75](#_ENREF_75)], and it acts through the IL-23R [[76](#_ENREF_76)].

The Th17 differentiation process is very similar in mice and humans [[77](#_ENREF_77),[78](#_ENREF_78)]. As in mice, TGF-β, IL-23 and pro-inflammatory cytokines (IL-1β and IL-6) were all essential for human Th17 differentiation [[79](#_ENREF_79)]. In this regard, TGF-β along with IL-21 and IL-23 stimulate the expression of RORγt, which in turn induces expression of IL-17 [[41](#_ENREF_41),[56](#_ENREF_56)]. Th17 cells also secrete IL-21 [[77](#_ENREF_77)]. IL-6, IL-21 but not TGF-β induced IL-23 receptor up-regulation in stimulated naive CD4+ T cells[[56](#_ENREF_56)].

The differentiation of Th17 cells is antagonized by transcription factors that control the differentiation of other lineages, such as T-bet (Th1), GATA-3 (Th2), and FOXP3 (Treg) [[80](#_ENREF_80)]. T-bet inhibits IL-23 and hence is critical for the stability of the Th17 phenotype[[81](#_ENREF_81),[82](#_ENREF_82)]. GATA-3 acts as an inhibitor of Th17 [[83](#_ENREF_83)], this could be mediated by the inhibition of STAT-4, a promoter of IL-17 expression. FOXP3 inhibits the RORγt-driven transcription of IL-17 by directly suppressing RORγt [[68](#_ENREF_68),[84](#_ENREF_84)]. Furthermore, the IL-2/STAT-5 axis constrains Th17 [[78](#_ENREF_78)] in part through a FOXP3-dependent mechanism, since STAT-5 activates FOXP3 [[85](#_ENREF_85)] as well as through the inhibition of the STAT-3/IL-21 pathway [[86](#_ENREF_86)]. Double positive FOXP3 RORγt T-helper cells have been identified as an intermediary that displays suppressive function [[87](#_ENREF_87)]. Of note, the equilibrium of this double positive balance coexist and it is tightly controlled, suggesting that a perturbed equilibrium coming from a change in cytokine milieu might lead to a skewed phenotype [[88](#_ENREF_88)]. In line with this fact, IL-2 signaling via STAT-5 constrains Th17 generation [[89](#_ENREF_89)] and IL-2 has been found to regulate the development of Th17 via FOXP3+ regulatory T cells[[90](#_ENREF_90)].

Another known inhibitor of Th17 differentiation is PPARγ as its activation can inhibit STAT-3 and hence contribute to the down-regulation of IL-17 through the IL-6/STAT-3/RORγt/IL-17 axis [[91](#_ENREF_91),[92](#_ENREF_92)]. Although TGF-β alone is not capable of inducing IL-17 and hence producing Th17, it is necessary for differentiation into Th17 and its absence induced a shift from a Th17 profile to a Th1-like profile [[77](#_ENREF_77),[79](#_ENREF_79)]. Moreover, PPARγ is a key negative regulator of human and mouse Th17 differentiation since it reduced RORγt transcription on a single-cell level [[93](#_ENREF_93)].

**Regulatory CD4+ T cells**

Induced or adaptive Treg (iTreg) cells can be generated from naïve CD4+ T cells by the stimulation of TCR and in the presence of TGF-β1 [[94](#_ENREF_94),[95](#_ENREF_95)] and the absence of IL-6 [[69](#_ENREF_69)]. TGF-β induces the expression of FOXP3, which is the master regulator for Treg [[95-97](#_ENREF_95)], and the IL-2/STAT-5 pathway is essential for the up-regulation of FOXP3 [[98](#_ENREF_98)]. The participation of TGF-β in the differentiation of Th17 cells places the Th17 lineage in close relationship with CD4+CD25+FOXP3+ iTregs, as TGF-β also induces differentiation of naive T cells into FOXP3+ iTregs in the peripheral immune compartment [[77](#_ENREF_77)]. The key difference that drives a TGF-β-stimulated CD4+ T cell towards Th17 or iTreg is the presence or absence of IL-6, respectively. Interestingly, iTreg cells can differentiate into pathogenic Th17 in the presence of IL-6 and/or IL-23 [[99](#_ENREF_99)], indicating plasticity in lineage commitment.

STAT-1 is also critical for the induction of iTreg cells. STAT1-deficient mice developed a functional impairment of iTreg cells [[100](#_ENREF_100),[101](#_ENREF_101)]. Recently, it was shown that FOXP3expression is boosted by IFN-γthrough theactivation of STAT-1 which then directly binds to the FOXP3 promoter [[102](#_ENREF_102)].

PPARγ ligands enhance the differentiation of CD4+ T cells into iTreg cells [[103](#_ENREF_103),[104](#_ENREF_104)], although the underlying mechanisms are incompletely understood. Additionally, PPARγ ligands inhibit the production of pro-inflammatory cytokines, including IL-6 [[45](#_ENREF_45)]. In turn, IL-6 inhibits the expression of FOXP3 and hence favors Th17 over the iTreg phenotype [[96](#_ENREF_96),[105](#_ENREF_105)]. Thus, in the presence of PPARγ activation there is less IL-6 and a suppressed IL-6 mediated inhibition of FOXP3 that will favor the iTreg phenotype and facilitate anti-inflammatory responses and prevention of autoimmune disease.

Differentiation of CD4+ T cells into iTreg is inhibited through multiple mechanisms, including negative regulation of FOXP3 expression via GATA-3 [[106](#_ENREF_106)], IL-4-mediated inhibition of FOXP3 through STAT-6 [[107](#_ENREF_107)], and inhibition of TGF-β-induced FOXP3 by IL-6 and IL-21 [[70](#_ENREF_70)]. The latter mechanism of inhibition of iTreg differentiation appears to be mediated via STAT-3 activation, since IL-6 fails to inhibit FOXP3 in STAT-3-deficient mice [[108](#_ENREF_108)].

Interestingly, IFN-γ-deficient-mice had more FOXP3-positive cells than wild-type mice in all secondary lymphoid organs except the thymus [[109](#_ENREF_109)]. However, T-bet- or IL-4Rα-deficient mice did not show a similar increase. *In vitro* differentiation studies showed that conversion of naïve CD4+ T cells into FOXP3-positive iTreg cells by TGF-β was significantly inhibited by IFN-γ in a STAT-1-dependent manner. In an earlier study [[110](#_ENREF_110)], autocrineIFN-γ production regulated TGF-β-driven FOXP3 expression in iTreg and suppressed the conversion ofnaïve CD4+ T cells into FOXP3+ iTreg cells. However, IFN-γ is critically required for the conversion of naïve T cells to iTregs in a mouse model of multiple sclerosis [[101](#_ENREF_101)]. Furthermore, in human iTreg differentiation, a mechanismby which the STAT-1-activating cytokines IL-27 and IFN-γ amplifyTGF-β-induced FOXP3 expression is revealed [[102](#_ENREF_102)]. Finally, recent reports show that the transcription factors for Th1, Th2, and Th17 cells, T-bet, GATA-3, and RORγt, respectively, can also be co-expressed in some Treg cells [[111-113](#_ENREF_111)], thereby indicating the existence of intermediate phenotypes. However, the molecular network leading to these intermediate phenotypes and their function remain largely unknown. The better understanding of the dynamics of iTreg differentiation is important for driving the informed development of possible Treg cell-based therapeutics against immune-mediated diseases.

**Importance of PPARγ in CD4+ T cell differentiation**

Inflammation is at the core of most human diseases, including chronic, infectious and immune-mediated. Activation of PPARγ, a widely expressed transcription factor, represents a conserved anti-inflammatory mechanism involved in the prevention of cancer [[114](#_ENREF_114),[115](#_ENREF_115)], diabetes [[116-118](#_ENREF_116)], atherosclerosis [[119](#_ENREF_119)], obesity [[120](#_ENREF_120)], infectious [[121-125](#_ENREF_121)] and immune-mediated diseases [[126-129](#_ENREF_126)]. Thus, modeling the mechanisms by which PPARγ regulates CD4+ T cell differentiation and function will facilitate a rational development of anti-inflammatory drugs and immunotherapeutics.

At the cellular level, iTreg express 10-fold greater amounts of PPARγ than Th1 cells [[130](#_ENREF_130)] and PPARγ is required for naturally occurring Treg-mediated protection from colitis [[129](#_ENREF_129)]. Moreover, PPARγ has been identified as a key down-regulator of differentiation of CD4+ T cells into Th17 [[131](#_ENREF_131)] a phenotype associated with inflammation. In macrophages, PPARγ favors a switch from a pro-inflammatory “classically activated” M1 to an M2 “alternatively activated” anti-inflammatory phenotype [[132](#_ENREF_132)]. Since PPARγ is ubiquitously expressed in the gut, tracing clinical improvements from therapeutic interventions with thiazolidinediones (TZD) and other PPARγ ligands back to concrete PPARγ-initiated immunological mechanisms has proven extremely challenging. PPARγ activity delineates the susceptibility to intestinal inflammation ranging from highly pro-inflammatory (low expression or activation) to anti-inflammatory (high expression or activation) states. We have developed a multiscale model of the intestine to understand how PPARγ modulates the immune response dynamics, gut pathology and anti-inflammatory responses [[133](#_ENREF_133)]. The initial level of granularity was cellular (immune and epithelial cells), with multiple tissues and compartments such as lumen, colonic lamina propria (LP) and mesenteric lymph nodes (MLN) [[133](#_ENREF_133)].

Here we present a higher resolution structural model network with molecular granularity that illustrates the principal pathways controlling the CD4+ T cell differentiation process towards Th1, Th2, Th17 and iTreg. An additional and novel feature of our model is that we describe the role of PPARγ as a central modulator of CD4+ T cell differentiation and function.

**Section 2: Modeling process**

Generating a mathematical model usually is comprised of three steps: first, a translation from the literature into a structural network is needed. The architecture of the model has to be assembled based on literature fates. Secondly, data extracted from the literature and data generated by our laboratory is inserted in the model to adjust the dynamics of the model and ensure the correct trends and behaviors of different molecules in the model. This process is known as ‘parameter estimation’. Once the parameters are set, a quality control check is needed to guarantee that signaling pathways are being activated promptly at the correct time with the right signal.

At this point, the model is ready to start running *in silico* experimentation and generating predictions with the right initializations. Ultimately, computational results will be generated, *in vitro* and *in vivo* validation studies are performed and the data generated in those studies is used to re-calibrate the model, using ‘parameter estimation’ again, thus closing and completing the modeling process. This iterative process is outlined in Figure S2.

**Parameter estimation for dynamics adjustment**

Once all the relationships between molecules were set, they were incorporated in the CellDesigner diagram representing a single CD4+ T cell (Figure 1). This diagram represents the cellular response of one CD4+ T cell activating and inhibiting reaction that take place in three different places: the extracellular environment, the cytoplasm and the nucleus space. Since CellDesigner [[134](#_ENREF_134)], a software package that enables users to describe molecular interactions using a well-defined and consistent graphical notation, and our MIEP-developed modeling software, the COmplex PAthway SImulator (COPASI) [[135](#_ENREF_135)] are Systems Biology Markup Language (SBML)-compliant an import was made into COPASI and the rate laws were adjusted to create the ordinary differential equations (Figure S3). To model CD4+ T cell differentiation, the hill function and mass action equations were used. While the Hill Coefficient allowed us to quantify the effect of a ligand binding a macromolecule through cooperative binding, mass action laws can represent dynamic equilibriums for elementary reactions, considering products as a proportion of the participating molecules in the reaction.

The parameter estimation computational approach was used to determine the unknown constants driving the dynamics of the model. Briefly, we used the Particle Swarm Optimization (PSO) [[136](#_ENREF_136)] algorithm to obtain computational values for our model parameters in order to fit our experimental data (Table S1) to the model. PSO is a global search algorithm and thus depends only minimally on the initial guess of each parameter and therefore avoids the subjective estimation caused by initial guesses in local methods as Levenberg-Marquardt. PSO has been used in other publications for the same purpose [[137](#_ENREF_137)].

Given the complexity of the model, the parameter estimation task was split into different sub-estimations that would run faster. Seven different parameter estimations were run successfully, including a parameter estimation for each phenotype (Th1, Th2, Th17 and iTreg), plus an extra one for PPARγ calibration, one for the Th1/Th2 crosstalk and a last one called ‘global parameter estimation’ that would include all these last six mentioned. Next, the ‘non-zero-gradient’ approach was performed. This step consists of assessing all the values with the gradients and check, value per value and parameter per parameter, which of those have the lowest or highest gradient. This approach can inform of which values have to be used for each phenotype and reaction. For instance, if we want to assess the parameter named re10.K1 and this reaction is involved in the Th1 phenotype we want to use the value that has the highest/lowest gradient in our results. In this case, it would coincide with the Th1 parameter estimation. An example is shown in Table S2. As an example, the parameter K1 in reaction number 10 has the lowest gradient in the results of the Th1/Th2 cross-talk parameter estimation. So when uploading these numbers to the model, re10.K1 will have a value of 64.1808, which is the one obtained from the task.

The results on the parameter estimation using Particle Swarm shows a good fitting between the experimental data and the values computationally estimated by COPASI with reduced weighted error (Table S3 and Figure S4). These values are then implemented in the reactions and rate laws to adjust the dynamics of the model, based on the model assumptions considered for the CD4+ T cell model (Table S4).

Once this step is completed, quality control is performed. Using the proper initialization given by literature and represented in Table S5, the system is induced to the four phenotypes and checked to reproduce the correct up- and downregulation of specific molecules.

These four phenotype checks are the result of our CD4+ T cell modeling efforts after calibration and they provide evidence that our computational and mathematical model is capable of reproducing the behaviors of the four CD4+ T cell phenotypes in terms of cytokines, inductors and transcription. In addition, we demonstrate that the calibration process has been run successfully and the dynamics of the CD4+ T cell differentiation network model are adjusted to mimic immunological behaviors characteristic of each phenotype (Figure S5).

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