Title

Quantitative modelling explains distinct STAT1 and STAT3 activation dynamics in response to both IFNγ and IL-10 stimuli and predicts emergence of reciprocal signalling at the level of single cells.

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Abstract

Cells use IFNγ-STAT1 and IL-10-STAT3 pathways primarily to elicit pro and anti-inflammatory responses, respectively. However, activation of STAT1 by IL-10 and STAT3 by IFNγ is also observed. The regulatory mechanisms controlling the amplitude and dynamics of both the STATs in response to these functionally opposing stimuli remains less understood. Here, our experiments at cell population level show distinct early signalling dynamics of both STAT1 and STAT3(S/1/3) in responses to IFNγ and IL-10 stimulation. We built a mathematical model comprising both the pathways that quantitatively captured the dose-dependent dynamics of S/1/3 in response both IFNγ and IL-10 stimulations. As both the functionally opposing cues activate S/1/3 we next predicted a co-stimulation scenario (IL-10 and IFNγ applied simultaneously) which suggests signalling dynamics of STAT3 would remain robustly IL-10 driven which it ensures through strong induction of SOCS1, a negative regulator of IFNγ pathway; the prediction was subsequently validated. Next, to understand the effect of protein expression heterogeneity in the robustness of STAT3 dynamics in co-stimulation scenario we performed single cell simulations. The simulations show the emergence of two reciprocally responding subpopulations in response to an identical signal; in one subpopulation co-stimulation enhances STAT3 and inhibit STAT1 phosphorylation, and vice versa in the other subpopulation. Analysing the distribution of protein concentrations in single cells we identified key proteins whose relative concentration regulates the STATs responses in the reciprocal subpopulations. Finally, through targeted perturbation of individual cellular states, we could tune STAT1 and STAT3 responses in a desired way. Taken together, our data-driven modelling at the population level and single cell simulations uncover plausible mechanisms controlling STAT1 and STAT3 amplitude and dynamics in response to functionally opposing cues. Therapeutic potential of this study may be explored further.

Introduction

Information encoded in the dynamics of signalling pathways triggers a plethora of biological processes like cell growth, proliferation, apoptosis or developmental lineage commitments [1-12]. A sustained (NGF stimulation) or transient (EGF stimulation) MAPK signalling dynamics, for instance, is linked to cell differentiation and proliferation [8]. Sustained or transient dynamics of the SMAD2 transcription factors during TGFb signalling is shown to trigger growth inhibition [13, 14] or progression of EMT[15]. Dose dependent dynamics of signalling pathways is also observed to have distinct cellular responses: in CD40

receptor signalling in macrophages a dose-dependent reciprocal dynamics of ERK and p38MAPK is observed [11, 12] where ERK(p38MAPK) activated anti(pro)-inflammatory cellular responses. Similarly, in calcium signalling, NF-kB and JNK are selectively activated by a large transient rise in the intracellular calcium ion concentration(Ca2+) whereas NFAT is activated by a low, sustained Ca2+ plateau [3]. Further, signalling pathways like IL-10 uses sustained STAT3 signalling to elicit anti-inflammatory(AIF) responses whereas IL-6-receptor uses transient STAT3 signaling to trigger expression of pro-inflammatory(PIF) genes [16]. PIF signalling through the canonical interferon gamma(IFNγ)-STAT1 pathway is also transient in nature [17].

The regulatory mechanism controlling the dynamics of signal response in the IFNγ-STAT1 and IL-10-STAT3 signalling pathways are studied separately through experiments [17, 18] and mathematical modelling [19], for instance, the roles of transcriptionally induced negative regulators like SOCS1 and phosphatases like SHP-2 are identified as important regulators of STAT1 dynamics in IFNγ signalling [18-22]. On the other hand, IL-10-STAT3 signalling is usually of long duration in the absence of explicit negative feedback loops where signal termination is observed to occur via negative regulation of the IL-10 receptor 1(IL-10R1) through ubiquitination, endocytosis, and degradation [23]. Although STAT1 and STAT3(S/1⁄3) are primary/canonical responders of the IFNγ and IL-10 pathways respectively, the STATs are also observed to be cross-activated: STAT1 phosphorylation by IL-10 [24] and STAT3 phosphorylation by IFNγ[25] are observed, although, regulatory mechanisms that control the amplitude and dynamics of S/1⁄3 activation in response to the two functionally opposing cues are not quantitatively explored. Systematic study of such dose-dependent dynamics in a signalling pathway facilitates better understanding of the amplitude and duration of signalling at different activation scenarios [14, 26, 27], and further, such data can be used to calibrate mathematical models [14, 26] for gaining better insight into the systems regulatory processes. Such models calibrated and validated at the cell population level can also be extended to single cell level and effect of protein expression heterogeneity on a pathway's signal response can be systematically investigated [14, 28, 29].

Here, firstly our experiments show the quantitative distinction of amplitude and dynamics of S/1⁄3 in response to the different doses of IFNγ or IL-10 stimulus. To understand the regulatory mechanisms controlling S/1⁄3 activation at different doses we built a quantitative model of IFNγ or IL-10 pathways. The calibrated models suggested 1. Signal inhibition at the receptor level (in both IFNγ and IL-10 pathways) 2. IFNγ dose-dependent activation of a STAT1⁄3 phosphatase (IFNγ pathway) as two key mechanisms controlling the observed dynamics at different doses. Notably, induction of SOCS1 (the feedback inhibitor of IFNγ pathway) was much stronger in response to IL-10 stimulation as compared to IFNγ stimulation. As both IFNγ or IL-10 pathway activated S/1⁄3 we next simulated a co-stimulation scenario (IL-10 and IFNγ applied simultaneously) to understand the how both the STATs dynamics responses would change in response to the co-stimulation scenario. The simulations predict STAT3 amplitude would primarily remain IL-10 driven during co-stimulation which the former ensures through a stronger activation of SOCS1; subsequent experiments validated the prediction. We next extended the population level model to study how cell-to-

cell variability in protein expression would impact the S/1/3 signalling dynamics at the level of single cells, particularly in the co-stimulation scenario. The single cell simulations suggest, in addition to cells exhibiting responses like the population level experiments (where STAT3 dynamics is robustly comparable between IL-10-only and co-stimulation) two subpopulation with reciprocal STAT1 and STAT3 activation profiles can emerge due to cell-to-cell variability. Analysing the distribution of protein concentrations from these reciprocal subpopulations we identified key contributors that determine the S/1/3 responses in the reciprocal cells.

Results

STAT1 and STAT3 phosphorylation in response to different doses of IL-10 and IFNγ

We took the peritoneal macrophages obtained from BALBc mice and stimulated them with increasing doses(0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 40.0 ng/ml) of either IL-10 or IFNγ ligands(details in methods). We observed: STAT1 phosphorylation is proportional to the increasing dose of IFNγ stimulation (Figure 1A) but IL-10 induced STAT1 phosphorylation showed as bell-shaped response with strongest activation at 2.5 and 5ng/ml of IL-10 (Figure 1B). STAT3 phosphorylation showed a gradual increase with escalating doses of IL-10(Figure 1B). STAT1 phosphorylation in response to IFNγ treatment is maximal and comparable between the doses 2.5-10ng/ml(Figure1A). For both IFNγ and IL-10 stimulation of 1ng/ml weak induction of the cross-activated STATs is observed; at 5ng/ml of both canonical and cross-activated S/1/3 have high phosphorylation and at 20ng/ml cross-activated STATs amplitude are inhibited in both the pathway.

Next, to understand the regulatory processes controlling the dose-dependent responses of the canonical and cross-activated STATs we selected three doses of IFNγ and IL-10: low(L), medium(M) and high(H), which are respectively 1.0 ng/ml, 5.0 ng/ml and 20.0 ng/ml for both ligand types. The representative immunoblots are shown in figure S1. The selected doses were used for the kinetic studies which we subsequently used to calibrate the S/1/3 dynamics in a mathematical model. The peritoneal macrophages were stimulated with L, M and H dose of IFNγ and IL-10 stimuli and S/1/3 dynamics were captured at different time points. For IFNγ treatment STAT1 phosphorylation increases as the signal dose increases, however, M and H don't exhibit significant differences in phosphorylation, indicating signal saturation close to M dose upwards (Figure 1C, 1st panel). STAT3 phosphorylation upon IFNγ stimulation however exhibits a distinct profile: at M dose STAT3 is rapidly phosphorylated to a high peak amplitude and gets rapidly inhibited as well, but at L and H doses STAT3 phosphorylation is relatively negligible (Figure1C, 2nd panel). Such bell-shaped activation dynamics of signalling intermediates is also observed in earlier studies[30]. In response to IL-10, the STAT1 phosphorylation peaks at M dose and a slightly inhibited response can be observed at the H dose(Figure 1D, 1st panel). Unlike STAT1 in H dose of IFNγ stimulation, STAT3 phosphorylation amplitude decreases at the H dose of IL-10 stimulus(compare Fig. 1C, 1st panel Fig. 1D, 2nd panel). Such dose-dependent inhibition usually indicates presence of negative regulators induced/activated as a function of applied signal [11, 14,

31, 32]. SOCS1 being a commonly induced negative regulator in IFNγ signalling [19, 20, 22], we experimentally investigated SOCS1 induction upon M dose of IFNγ (Fig2E, 1st panel, dashed line). SOCS1 induction was also checked for IL-10 stimulation (Fig2E, 1st panel, solid line). The experiments show SOCS1 induction remains negligible upon IFNγ stimulation, but a much stronger induction is observed upon IL-10 treatment(Fig 2E, 1st panel, solid line). We also captured the dynamics of SOCS3 induction which is a target gene downstream to both the stimulation types(Fig 2E, 2nd panel). Notably, SOCS3 in principle can inhibit IFNγ signalling, but the relative inhibitory strength of SOCS3 is observed to be negligible compared to SOCS1 [33], hence w.r.t IFNγ stimulation we studied SOCS3 only as a target gene.

Quantitative modelling captures dynamics of STAT3 and STAT1 activation at different doses of IL-10 and IFNγ ligand

We used the observed dynamics of S/1/3 to calibrate a mathematical model comprising both IFNγ or IL-10 pathways. Such data-trained models usually help understand the regulatory interactions between pathway components and offer predictions that can be validated experimentally [14, 34, 35].

Our model comprises three modules

I. A Simplified receptor activation module for both IFNγ and IL-10 activation.
II. A detailed STAT1 and STAT3 phosphorylation module in response to both the IFNγ and the IL-10 stimulation.
III. A simplified transcriptional induction module where both SOCS1 and SOCS3 are induced as a function of activated STATs.

IFNγ pathway model

Figure 2A schematically shows the minimal models of IFNγ pathway. The model has a simplified step of receptor activation where details of the interaction between IFNγ Receptor(IFN-R) and the Janus kinases JAK1 and JAK2 [19] are simplified to one step activation process [21]. As the ligand binds to the receptor the active receptor complex (IFN-LR) phosphorylates the transcription factors STAT1 and STAT3. Both the STATs undergo dimerization and forms transcriptionally active complexes [19] which in turn induces target genes such as SOCS1 and SOCS3. The time delay in transcriptional induction is captured with Hill functions [14]. Dephosphorylation of STAT1 and STAT3 are assumed to be carried out by phosphatases such as SHP2[18, 36, 37] which we generally named in our model as “Phos”. We tested this simplified IFNγ model by fitting it against the dose-dependent kinetics of STAT1, STAT3 as well as the SOCS1 and SOCS3 data at the M dose simultaneously. The model quantitatively captures the distinct dynamics of STAT1 phosphorylation at: L(Fig2B, 1st row, 1st column), M(Fig2B, 1st row, 2nd column) and H (Fig2B, 1st row, 3rd column) doses. Similarly, phosphorylation dynamics of the cross-activated STAT3 is fitted for L(Fig2B, 2nd row, 1st column),

M(Fig2B, 2nd row, 2nd column) and H(Fig2B, 2nd row, 3rd column) doses. SOCS1 and SOCS3 induction kinetics at M dose was also fitted together with the S/1/3 data (Fig2B, 3rd row). Details of model building is shown in the methods section and in the supplementary text TS1.

IL-10 pathway model

Figure 2C shows the schematics of IL-10 receptor-mediated phosphorylation of STATs and the transcriptional induction of SOCS1/3. Similar to the IFNγ pathway, the explicit steps of IL-10 receptor1(IL-10R1) and receptor 2(IL-10R2) binding to JAK1, Tyk2 kinases leading to the formation of active signalling complex[38] is simplified to one step activation process. STAT1/3 phosphorylation steps are explicitly modelled and dephosphorylation of STAT1/3 is assumed to be carried out by the phosphatase Phos(such as SHP2 [36,37]). Negative regulators of IL-10 signalling that works by degrading the activated IL-10 receptor is considered in the model calibration process and we name it as IL-10Ri; such negative regulators are observed to act by sequestering the IL-10 receptor 1(IL-10R1) which leads to subsequent ubiquitination and degradation [20]. Figure 2D shows the STAT1/3 dynamics in the model upon IL-10 stimulation with L, M, and H doses. As both the pathways are built as one model with common signalling intermediates we calibrated both IL-10 and IFNγ models simultaneously to their respective datasets. During the model calibration the common signalling intermediates and biochemical parameters were constrained to have have one value which is stimulus independent.

Mechanism controlling dose dependent kinetics of the STATs

The observed bell-shaped dose response of STAT3 in both IFNγ and IL-10 stimulation, as well as the graduating pattern in activation of STAT1 for both M and H dose of IFNγ stimulation, is captured simultoneously by our simplified model. For the optimal calibration of the model to all the training datasets we require a negative regulation of signal at the receptor module (inhibition of IFN-LR and IL-10-LR) through their respective inhibitors: stoichiometric inhibition of IFN-LR by SOCS1 and IL-10-LR by IL-10Ri, respectively, as one primary mechanism, that controls S/1/3 dynamics in both the pathways. Additionally, in the IFNγ pathway a dose-dependent activation of the Phos was required to reproduce the sharp rise and rapid fall of STAT3 at M dose(Figure 2B, 2nd row, 2nd column). Such dose dependent activation of phosphatase is studied elsewhere, where S/1/3 phosphatase SHP-2 is activated in a JAK1 dependent manner [39] or the phosphatase SHP-1 is activated in a JAK2 dependent manner [40]. Here we assumed Phos is activated as a function of active IFN-LR which in our simplified receptor module represents a signalling complex comprising activated JAK1, JAK2, the ligand and both the IFNγ receptor1 and 2. Figure S2 shows phosphorylation amplitude (normalized to maximum) of STAT3p and the respective amount of active Phos as a function of IFNγ signal dose. The signal-dependent activation of Phos coupled to inhibition of IFN-LR by

the basally present SOCS1 are the two key regulatory mechanisms controlling the observed S/1/3 responses in the IFNγ pathway. SOCS1 is a commonly reported feedback regulator in the IFNγ pathway but our experiments show negligible SOCS1 induction upon stimulation (Fiure1E, top panel, dotted line). The model fitting however suggests that sequesteration of IFN-LR by basally present SOCS1 controls the later's availability to the downstream S/1/3. Further, the model suggests that sequestration of basal IFN-LR by SOCS1 can regualtes the extent of dephosphorylation of S/1/3 by the Phos, which can be shown in the model by blocking the sequestration (Figure S3).

Prediction and validation of a co-stimulation scenario: IL-10 pathway inhibits IFNγ pathway to ensure robust STAT3 dynamics.

In our experimental system SOCS1 is strongly induced upon IL-10 but not upon IFNγ stimulation, so intuitively, if both these functionally opposing pathways are simultaneously activated(co-stimulation) an IL-10 driven negative regulation of the IFNγ pathway can be expected. To quantitatively understand the consequence of IL-10 induced SOCS1 on the IFNγ pathway we simulated both the pathways simultoneously. We chose the M doses of both the ligand types as both the canonical and cross-activated STATs are strongly activated in this dose. Figure 3A depicts the co-stimulation scenario and the predictions for dynamics of STAT3(Fig 3B), STAT1(Fig 3C) and SOCS1(Fig 3C) are shown. To achieve robust predictions we used 40 independently fitted models with comparable goodness of fit(see in methods for details). The models predict: induction of SOCS1 by IL-10 would lead to a stronger inhibition of IFNγ signalling as excess the SOCS1 strongly sequesters IFN-LR, hence blocking its access to S/1/3. So cells simultoneously subjected to anti-inflammatory (IL-10) and pro-inflammatory(IFNγ) signal are predicted to robustly maintain the amplitude and dynamics of STAT3. Subsequent experiments quantitatively validate that STAT3 dynamics remain strongly IL-10 driven as predicted by the model (Figure 3B, black filled circles). Notably, the experimentally observed STAT1 amplitude is higher than the predicted amplitude (Figure S4A) which is not explained by the present model. However, we also observed, despite the differences in amplitude the shape of the trajectories of STAT1 are strongly comparable between the prediction an validation datasets, as we can reproduce the experimentally observed STAT1 trajectory by simply multiplying the corresponding model trajectory by a scaling factor(Figure 3C). Figure 3D shows experimental validation of the SOCS1 expression dynamics in co-stimulation which is closely comparable to IL-10 only stimulation scenario (compare figure 3D with figure 2D, 3rd row, 1st column); this further validates our hypothesis that IL-10 pathway inhibits IFNγ pathway during co-stimulation using negative regulators like SOCS1. The SOCS1 expression dynamics is also predicted using the 40 independent models with similar goodness of fit obtained using local multistate optimization [41].

Next, we extended our population level model to single cell level and investigated the effect of cell-to-cell variability[38, 39] on the signalling dynamics of STAT1 and STAT3. It is observed that isogenic cells have heterogeneity in their protein expression that subsequently results in heterogeneity in signalling dynamics [14, 42-45]. Such heterogeneity in signal dynamics has phenotypic consequences; for instance, individual cells with a certain signalling state can become cancerous and in rare cases can even become drug resistant [46, 47]. Hence understanding signal processing at the level of individual cells remain as an important topic of investigation both conceptually [48] and experimentally[14, 44]. Based on our findings at the cell population level we next investigated if the robustness of STAT3 amplitude and dynamics in co-stimulation is also robustly maintained at the level of single cells.

Single cell simulations show emergence of reciprocally signalling subpopulations during co-stimulation

To this end, we have extended the population-level model to understand the effect of protein expression heterogeneity on S/1/3 dynamics in co-stimulation(details in methods). The single cell simulations show, in addition to cells exhibiting responses observed in the population level(C0 cell; here STAT3 responses are comarable between IL-10 only stimulation and co-stimulation), two distinct subpopulation of cells with functionally opposing responses can also emerge due to cell-to-cell variability. The subpopulation with reciprocal cell type 1(Rc-t1) has high STAT3 and low STAT1 activation(Fig 4A, 1st column, 1st and 2nd row, red line) compared to their respective population level (Fig 4A, 1st column, 1st and 2nd row, blue line, normalized to maximum amplitude). The other subpopulation (Rc-t2) has low STAT3 and high STAT1 activation in co-stimulation (Fig 4A, 1st column, 2nd row, green line). Figure 4 shows the normalized STAT3 dynamics from C0, Rc-t1 and Rc-t2 subpopulation (1000 cells in each subpopulation) with their respective median and standard deviations.

To mechanistically understand how the reciprocal subpopulation emerges we analysed the protein concentration distribution in the three subpopulations. Such comparison uncovers the sensitivity of single-cell variables(protein concentrations) unique to a given subpopulation[14]. Figure 4B compares the distribution of the protein concentration for C0, Rc-t1 and Rc-t2 cell types. The analysis show Rc-t1 subpopulation has a high level of IL-10R and low level of IL-10Ri compared to their respective values in C0(Figure 4B, 1st row, 1st and 2nd column), and further, the ratio of positive and negative regulator [IL-10R]/[IL-10Ri] is distinct in the cells of Rc-t1 subpopulation (Figure 4C, 1st row). Rest of the single cell variables are not strongly separable between C0 and R1-ct subpopulation (Figure 4B). On the other hand, Rc-t2 subpopulation has a relatively lower basal $SOCS1_B$ (Figure 4C, 2nd row). while rest of the variables in Rc-t2 don't exhibit sharp differences from either C0 or Rc-t1.

In Rc-t1 cells stronger expression of IL-10R and a weak expression of IL-10Ri results in a stronger induction of SOCS1 through the IL-10 pathway; the abundant SOCS1, in turn, ensures stronger inhibition of IFN-LR complex. Inhibition of IFN-LR also results in lesser amount of activated S/1/3 phosphatase Phos, and

consequently, a stronger and persistent STAT3 activation results. In Rc-t2 cells, weaker $SOCS1_B$ activation ensures lesser inhibition of IFN-LR through sequestration and a resultant increase in the level of active Phos in Rc-t2. Effect of higher level of Phos in Rc-t2 is more pronounced in STAT3 dephosphorylation (Figure 4A, compare green lines in 1st and 2nd row) which is also influenced by the respective dephosphorylation rates of STAT1 and STAT3.

Subpopulation responses can be altered by tuning only one/two critical single-cell variable

Experimental studies show protein concentration mixing [43] due to processes like cell division and the inherent noise associated to expression/degradation of proteins in individual cells [49-51] leads to stochastic changes in the cellular states of individual cells. To understand the significance of single-cell variables with distinct distribution in the three subpopulations, we next performed a set of perturbation studies where we perturbed the signalling states of cells in one subpopulation by systematically replacing the single cell variables from a subpopulation with different signalling state. As the cells in different subpopulations emerge as a function of cell-to-cell variability, this analysis is designed to systematically understand the effect of expression noise on the most sensitive proteins of the pathway. For instance, we took one C0 cell and replaced a single cell variable (like STAT1) with its counterpart from an Rc-t1 cell(C0 -> Rc-t1). To obtain good statistics a variable in one cell of C0 subpopulation is replaced with its counterpart from 1000 other cells from Rc-t1 and this was repeated for each cell of C0 subpopulation; we show 100 representative cells in the heatmaps (Figure 5). We studied the effect of such perturbations in all pairwise combinations of the subpopulations (C0 -> Rc-t1 , Rc-t2 ->C0 , C0 -> Rc-t2 , Rc-t2 -> Rc-t1 , Rc-t1 -> Rc-t2). In Figure 5A each row shows the median fraction of Rc-t1 cells transformed to a C0 cell types when a given variable in the Rc-t1 cell is replaced by its counterpart from 1000 C0 cells. The analysis show, emergence of the two reciprocal subpopulations Rc-t1 an Rc-t2 can be attributed to the differences in the values of a small number of single-cell variables. The protein concentration distributions already show a low basal SOCS1 concentration ($SOCS1_B$) in the Rc-t2 subpopulation (Figure 4A, 2nd row, 1st column), hence, in Rc-t2 ->C0 relatively more frequent occurrences of C0 cells are observed when the SOCS1 concentration in Rc-t2 is replaced by its counterpart from C0 cells(Figure 4C, 2nd row). The analysis suggests that $SOCS1_B$ is a key determinant for the emergence of Rc-t2 subpopulation. The perturbation analysis also show significance of IFNR for the Rc-t2 -> Rc-t1 transformation (Figure 5A, 2nd row, 2nd column), which is not evident only from the protein concentration distributions (Figure4B). Next, as the ratio ILR0R/IL-10Ri is distinct in Rc-t1 we studied the effect of replacing the ratio from the other two subpopulations; figure 5B(1st row) shows the dramatic increase in the median frequency of Rc-t1 -> C0 transition when both ILR0R and IL-10Ri were simultoneously replaced from C0 to Rc-t1. Similarly, when both IFNR and its inhibitor $SOCS1_B$ is replaced between the pair of reciprocal subpopulation, the frequency of Rc-t2 -> Rc-t1 or Rc-t1 -> Rc-t2 transitions dramatically increased (compare figure 5B, 2nd row and figure 5A, 2nd row 2nd column; figure 5B, 3rd row and

figure 5A, 3rd row 2nd column). Hence our analysis shows, a combinatorial change of protein concentrations of key single-cell variables driven by the cell-to-cell variability can result in either high STAT1(low STAT3) or high STAT3(low STAT1) signalling at the level of individual cells, with a potential to generate opposing cellular responses.

Discussion

IL-10-STAT3 and IFNγ-STAT1 signalling axis primarily elicit the anti-inflammatory(52-54) and pro-inflammatory(55, 56) cellular responses, respectively. The signalling by STAT1 and STAT3 are observed to be functionally opposing in a spectrum of biological processes; activation of macrophages is enhanced by STAT1 and inhibited by STAT3, cell proliferation is inhibited by STAT1 and promoted by STAT3, and in Th differentiation, STAT1 promotes Th1 responses and STAT3 inhibits Th17 response [57, 58]. Transcriptionally active STAT1 induces death receptor expression to promote apoptosis and it negatively regulates the expression of several oncogenes [59, 60]; in contrast, constitutive activity of STAT3 is essential for the survival of many primary tumour cells[59]. Also, transcriptional targets of STAT3 are many anti-apoptotic genes that promotes tumour cell proliferation [59, 61]. However, studies also show that STAT1 is activated by IL-10 receptor and STAT3 can be activated during IFNγ signalling [24, 25, 62-64]. The dynamics of the STATs signalling also critically determines the cell fate decisions: IFNγ-STAT1 signalling or IL6-STAT3 signalling is transient and pro-inflammatory but anti-inflammatory IL-10-STAT3 signaling is relatively sustained [16, 23]. In this study, firstly we asked

1. How the dynamics of canonical and non-canonical STATs are regualted in the IFNγ and the IL-10 pathway ?
2. How the opposing signaling information applied simultaneously would get integrated at the level of STAT activation ?

To address these questions we first conducted experiments at the cell population level, and through immunobloting, captured the kinetics of STAT1 and STAT3 activation at different doses of IFNγ and IL-10 signal. Our kinetic studies show, in addition to activating their canonical signaling partners the IFNγ and IL-10 receptors also cross-activates the non-canonical STATs. Such cross-activated STATs, especially STAT3 activated in IFNγ exhibited very high amplitude at a M dose of IFNγ but remained inhibited in both L and H doses. This indicated, in signal strengths around the M a stronger STAT3 activation is possible through the IFNγ pathway. STAT3 activation in the canonical in IL-10-STAT3 pathway also exhibited a bell-shaped dose-response with maximum and lowest amplitude observed in M and H doses respectively. STAT1 activation exhibited a consistent reponse to increasing dose of IFNγ where amplitude and dynamics are not significantly different between M and H doses, indicating signal saturation. The cross-activated STAT1 in response to IL-10 is weakly activated which also peaks at the M dose.

To understand the regulatory mechanisms controlling the dose-dependent activation/inhibition of the STATs we constructed a simplified mathematical model of both the IFNγ and IL-10 pathways and calibrated the model to experimental data. The model quantitatively captured the dynamics of S/1/3 in both the signaling pathways and mechanistically explains the control of S/1/3 signaling (A) In the IFNγ pathway negative regulation of the active signaling complex by a negative regualtor such as SOCS1 coupled to signal-dependent activation of a STAT1/STAT3 phosphatase determines the dose-dependent S/1/3 reponses(B)In IL-10 pathway negative regulation of active IL-10 receptor by a receptor inhibitor (IL10Ri) regulate the S/1/3 responses. Our experiments also show strong SOCS1 induction upon IL-10 stimulation, but not IFNγ stimulation. To quantitatively understand the effect of IL-10 mediated SOCS1 induction on the S/1/3 signaling by IFNγ we next predicted a co-stimulation scenario where both IL-10 and IFNγ were applied simultaneously. The model predicted that SOCS1 induced by IL-10 would inhibit signaling by IFNγ pathway and STAT3 amplitude and dynamics would primarily remain IL-10 driven, this was validated exprimetnally. The robust maintenance of primary anti-inflammatory axis (IL-10-STAT3) in the co-stimulation at cell population level led us to investigate if similar robustness in STAT3 responses are also predomiant at the level of the single cells. The signal-response at the level of single cells usually determine an individual cell's fate in response to the external signal results in distinct gene expression profiles [50, 65] or phenotypic outcomes [14]in individual cells.

To computationally explore the consequences of protein expression heterogeneity in S/1/3 signaling in co-stimulation we next extended introduced protein expression heterogenity in the population-level model [14, 28] and simulated thousands of single cells and studied the the STAT1 and STAT3 dynamics in individual cells. Our simulations and analysis show, in addition to single cells with responses similar to population level, where STAT3 response in a given cell is comarable between IL-10 only stimulation and co-stimulation, two distinct subpopulation of cells with reciprocal STAT1 and STAT3 responses also emerged at the level of single cells. The reciprocal activation of STAT1 and STAT3 resulting as a function of protein expression noise may also trigger distinct cell fates. To understand further the cellular states in the reciprocally signaling cells we analyzed the protein concentration distributions in cells from each of the subpopulations.

Our analysis show the two reciprocal subpopulations Rc-t1 and Rc-t2 have distinct distribution of some key variables: in Rc-t1 cells IL-10R expression is high and its inhibitor IL-10Ri is expressed in low concentration whereas in Rc-t2 cells the basal SOCS1($SOCS1_B$) is lowest among the three subpopulations. The significance of distinct distribution of IL-10R and IL-10Ri was more pronounced in Rc-t1 when we compared a ratio [IL-10R]/[IL-10Ri]. The analysis suggests that distinct subpopulation with opposing signal responses can simply emerge when one(in Rc-t2) or two(in RC-t1) key single cell variables have their concentrations in the desired range. The frequency of occurrence of each subpopulation is different but all three subpopulations can be robustly obtained in multiple independent simulations(when several thousand cells were simulated in each independent run).

Our model next explored if the reciprocal responses can be altered by altering the cellular states via targeted perturbation. It is argued that understanding the control of signal response in individual cells has the potential to help design better interventions against complex diseases like cancer[66] and understand the mechanims of drug resistance in individual cells [67, 68]. Here we asked if certain minimal perturbations in single cell variabiles can alter the reciprocal cellular states in a desired way. To do so we performed a perturbation analysis where we systematically swap the values of single-cell variables(one variable at a time) between the cells of a pair of subpopulation and compared the responses of a cell before and after such perturbation. We found C0 to Rc-t2 transformation or vice-versa is critically dependent on $SOCS1_B$; for instance, replacing the value of $SOCS1_B$ from cells in C0 subpopulation into cells in Rc-t2 subpopulation resulted in a significant number Rc-t2 to C0 transformation. The transformation from Rc-t1 however required simultaneous replacements of IL-10R and IL-10Ri. Systematic perturbation of cellular states thus showed targeted inhibition/overexpression of few single-cell variables can potentially be used to enhance/inhibit STATs signal responses in a cell population. Notably, each of the three subpopulations has distinct single cell variable that critically determines the signaling state of the subpopulation; for instance, if an intervention requires more Rc-t2 cell types it can be achieved by inhibiting the $SOCS1_B$ expression, whereas, occurances of Rc-t1 cells can be enhanced by adjusting the ratio [IL-10R]/[IL-10Ri] through overexpression/inhibition. Our simulations suggest ccurrence of these reciprocal sub-population (Rc-t1 and Rc-t2) are relatively less frequent and the dominant cell types are the average responders (C0), which is perhaps why we didin't observe such responses in our immunoblot analysis. In the cases where non-genetic cellular heterogeneity is the plausible cause underlying occurrences of rare cell types with deleterious physiological consequences [46, 69, 70], results from our study may be explored further to design possible therapeutic interventions.

Materials and methods

Experimental protocol: Balb/c derived macrophages were treated with increasing doses of recombinant IL-10 and IFNγ protein. The cells were then lysed and processed for immunoblotting. Dose response studies were used for selecting the high (20ng/ml), medium (5ng/ml) and low (1ng/ml) doses of both cytokines and kinetic studies were performed at these three selected doses.

Western blotting: After treatment with the indicated reagents, cells were washed twice with chilled PBS and lysed in cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 10%glycerol, 1mMEDTA, 1mMEGTA, 1%NonidetP-40, protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitor mixture (Pierce)]. Lysates were centrifuged (10,500 rpm, 10mins) and supernatants were collected. Protein was quantified by using the Bradford reagent (Pierce) and an equal amount of protein was run on

SDS–PAGE. Resolved proteins were blotted to PVDF (Millipore) and then blocked with 5% nonfat dried milk in TBST [25 mM Tris (pH 7.6), 137 mM NaCl, and 0.2% Tween 20]. Membranes were incubated with primary antibody at 4˚C overnight, washed with TBST, and incubated with HRP-conjugated secondary Ab. Immunoreactive bands were visualized with the luminol reagent (Santa Cruz Biotechnology). The STAT1 antibody we used detected both splice variants of -STAT1 (Tyr701), p91 STAT1α and p84 STAT1β, here we detected STAT1α. The STAT3 antibody we used is bound to tyrosine phosphorylated STAT3 molecules of both isoforms STAT3α(86kDa) and STAT3β (79kDa).

Reagents: Antibodies specific for p-STATI (Tyr-701), STAT1, p-STAT3 (Tyr-705) and STAT3 were purchased from Cell Signaling Technology (Danvers, MA) and those for SOCS1, SOCS3 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Soluble mouse recombinant IL-10 and IFNγ were procured from BD Biosciences (San Diego, CA). RPMI 1640 medium, penicillin-streptomycin and fetal calf serum were purchased from Gibco®-ThermoFisher Scientific ((Life Technologies BRL, Grand Island, NY). All other chemicals were of analytical grade.

Animals and cell culture: BALB/c mice originally obtained from Jackson Laboratories (Bar Harbor, ME) were bred in the National Centre for Cell Science's experimental animal facility. All animal usage protocols were approved by the Institutional Animal Care and Use Committee. 3% thioglycolate-elicited peritoneal macrophages were isolated from Balb/c mice and cultured in RPMI containing 10% FCS. Adherent cells were washed and maintained in a water jacketed CO2 incubator at 37°C for 48hrs to allow them to reach resting stage. Serum-free medium was added to the cells for 4hrs before stimulation.

Mathematical model building:

The pathway schemes in Figure 2A and 2C and 3A were be converted to a set of ordinary differential equations (Supporting Material) which captures the dynamics of signalling in both FNγ and IL-10 pathways. We have both the pathways built within one model where we preferentially switch on either the IFNγ or IL-10 pathways for individual pathway stimulation scenarios, or activate both the pathways simultoneously in a costimulation scenario. Below we explain the model rections specific to each of pathway as well as reactions common to both the pathways.

I. Receptor activation and engagement

A. IFNγ pathway

The ligand (IFNγ) bindss to the receptor (IFNR) to form an active signaling complex IFN-LR. We lumped several steps of receptor activation[19, 20] into a one step receptor activation process [21] assuming reversible kinetics .

IFNR + IFN <=> IFN-LR --------- (1)

The signling complex(IFN-LR) next carries out phosphorylation of the STAT1 and STAT3(shown in details in section 'STAT1 and STAT3 signaling', below); activates the STATs phosphatase Phos and also interact with the negative regualtor SOCS1.

STAT1 => STAT1p ; IFN-LR ---------(2)

STAT3 => STAT3p ; IFN-LR ----------(3)

Phosi => Phos ; IFN-LR ----------(4)

IFN-LR + SOCS1 <=> [IFN-LR. SOCS1] ------ (5)

While in the reactions (1) – (4), IFN-LR acts as a modifier/enzyme, reaction (5) shows sequeatraion of IFN-LR by its negative regulator SOCS1 [19] resulting in an functionally inactive complex [IFN-LR.SOCS1] that blockes the access of downstream substrats STAT1 and STAT3 to their activator IFN-LR.

B. IL10 pathway

Similar to the IFNγ pathway the IL10 receptor also binds to ligand and become functionally active. In our simplified model of the pathway the explicit receptor activation deactivation steps are simplified to a one step activation and deactivation process .

IL10R + IL10 <=> IL10-LR --------- (6)

The acive signaling complex IL10-LR phosphorylates and activates STAT3 and STAT1(explained in details in the section 'STAT1 and STAT3 signaling', below). A receptor level inhibitor that is observed to act by targeting the IL10R1 [23] is considered in our model as the negative regualtor of IL10-LR.

STAT1 => STAT1p ; IL10-LR ---------(7)

STAT3 => STAT3p ; IL10-LR ----------(8)

IL10-LR + IL10Ri <=> [IL10-LR . IL10Ri] ------ (9)

[IL10-LR . IL10Ri] -> IL10-LR --------- (10)

[IL10-LR . IL10Ri] -> IL10Ri --------- (11)

=> IL10Ri --------- (12)

IL10Ri => ..... (13)

As observed experimetnally[23], IL10Ri production and degradation is implememted in our model as stimulation independent proesses; reaction (12) and (13) depicts the basal production and degradation of IL10Ri. The differential equations below captures the dynamics of IL10 receptor activation and its inhibition by IL10Ri.

II. STAT1 and STAT3 signaling

In addition to equations [2], [3], [7] and [8] representing the activation of IFNγ and IL-10 specific activation STAT1 and STAT3, in the third condition, co-stimulation, STAT1 and STAT3 are activated by both the stimulus types simultoneously.

STAT1 => STAT1_act ; IFN-LR , IL10-LR ----- (14)

STAT3 => STAT3_act ; IFN-LR , IL10-LR ----- (15)

Studies show competition of STAT1 and STAT3 for the active IFNγ receptor [58] which is impliment in our model. In the same lines, we implimented competition of STAT1 and STAT3 for the access to IL10 (see differential equations for x8 and x10). STAT1_act and STAT3_act are the activated/phosphorylate froms of STATs which subsequently undergo dimeriazation and in turn become transcriptionally active[ref]

STAT1_act + STAT1_act -> STAT1_act_Dm

STAT3_act + STAT3_act -> STAT3_act_Dm

III. Transcriptional induction of SOCS1 and SOCS3

Transcriptional induction of SOCS1 and SOCS3 were experimetnally tested upon both IFNγ and IL10 signaling. Both the SOCS are induced relatively strongly upon IL10 signaling compared to IFNγ signaling (Figure 2B and 2D), especially SOCS1 induced upon 1L10 signaling is ~3 fold higher compared to IFNγ signaling. Transcriptional induction process of SOCS1 and SOCS3 in both IFNγ and IL10 pathways is depicted below.

In absence of external signal basal production and degradation of the SOCS is given as

=> SOCS1 ----- (16)

=> SOCS3 ----- (17)

SOCS1 => ----- (18)

SOCS3 => ----- (19)

Where reaction (16)&(17) represents production and reaction (18) & (19) represents degradation of the SOCS shown.

Upon IFNγ and IL10 signaling both the SOCS are transcriptionally induced as a function of dimeric STAT1 (in IFNγ pathway) or dimeric STAT3(in IL10 pathway). Experimental studies show during IFNγ signaling in STAT1 null mice SOCS3 but not SOCS1 is induced and in STAT3 null mice SOCS3 is blocked [58]. Thus in the IFNγ pathway we model SOCS1 and SOCS3 induction as functions STAT1_act_Dm and STAT3_act_Dm respectively. Similalry assumpotions were made for SOCS1 and SOCS3 induction in the IL10 pathway. Additionally, studies show efficient promoter binding and gene expression by STAT1 and STAT3 also depends on othre complex factors like availability of other cofactors, for instance, during IFNγ stimulation, occupation of contiguous DNA-binding sites for Stat1 and the transcriptional activator Sp1 are both required for full activation of certain genes [74]. Similarly gene expression in IL10 pathway are dependent on sp1 and sp3 cofactors [75]. Also cofactors like CoaSt6 selectively enhances the expression of genes certein pathways but not others [76] and complexity of sch regulations increase as competition of bind to different cofactors emerge between STAT1 and STAT3 [77] where expression/activation of the cofactors can be further controlled by specific input stimuli [74]. Thus it seems plausible that relative abundance of cofacators of STAT1(STAT3) for SOCS1(SOCS3) induction in IFNγ or IL10 stimualtion could be differeent. To accomodate the possibility of such differences in STAT1(STAT3) mediated induction of SOCS1(SOCS3) between IFNγ and IL10 pathways we allowed respective differerences in induction rates and Km values (that were determined during the model fitting process). Differential equations below captures basal production/degradation as well as transcriptional induction dynamics of SOCS1 and SOCS3.

Model equations

The differential equations below captures the information propagation in both IFNγ and IL10 pathways. The xs' are model species and the ps are model paramters, names of the species and paramter as well as their bestfict values are detailed in supplementary table TS1.

= x5 * p2 - x1 * x2 * p1 * p44 + p46 * p47 * p44.

= x5 * p2 - x1 * x2 * p1.

= -(x5^p43* x3 * p3)/(x5^p43+ p4^p43).

= (x5^p43 * x3 * p3)/(x5^p43 + p4^p43).

= x1 * x2 * p1 - x5 * p2 + p44 * x6 * p6 + p44 * x6 * p17 - x5 * p44 * x11 * p5.

= x5 * p44 * x11 * p5 - p44 * x6 * p17 - p44 * x6 * p7 - p44 * x6 * p6.

= x4 * x8 * p9 + x4 * x13 * p9 - (x5 * p44 * x7 * p8)/(p10 * (x7/p10 + x9/p11 + 1)) - (x17 * IL10_on * x7 * p26)/(p28 * (x7/p28 + x9/p29 + 1)).

= x4 * x13 * p9 - x4 * x8 * p9 - 2 * x8^2 * p12 + (x5 * p44 * x7 * p8)/(p10 * (x7/p10 + x9/p11 + 1)) + (x17 * IL10_on * x7 * p26)/(p28 * (x7/p28 + x9/p29 + 1)).

= x4 * x10 * p15 + x4 * x14 * p15 - (x5 * p44 * x9 * p14)/(p11 * (x7/p10 + x9/p11 + 1)) - (x17 * IL10_on * x9 * p27)/(p29 * (x7/p28 + x9/p29 +1)).

= x4 * x14 * p15 - x4 * x10 * p15 - 2 * x10^2 * p13 + (x5 * p44 * x9 * p14)/(p11 * (x7/p10 + x9/p11 + 1)) + (x17 * IL10_on * x9 * p27)/(p29 * (x7/p28 + x9/p29 + 1)).

= p16 - x11 * p17 + p44 * x6 * p6 + p44 * x6 * p7 - x5 * p44 * x11 * p5 + (p44 * p20 * p16 *(x13/p30)^p41)/((x13/p30)^p41 + 1) + (IL10_on * p34 * p16 * (x14/p32)^p39)/((x14/p32)^p39 + 1).

= p18 - x12 * p19 + (p44 * p21 * p18 * (x13/p31)^p42)/((x13/p31)^p42 + 1) + (IL10_on * p35 * p18 * (x14/p33)^p40)/((x14/p33)^p40 + 1).

= x8^2 * p12 - x4 * x13 * p9.

= x10^2 * p13 - x4 * x14 * p15.

= x17 * IL10_on * p23 + p48 * p45 * p49 - x15 * x16 * p45 * p22.

= x17 * p45 * p23 - x15 * x16 * p45 * p22.

= p45 * x18 * p25 - x17 * p45 * p23 + p45 * x18 * p37 - x17 * x19 * p45 * p24 +x15 * x16 * p45 * p22.

= x17 * x19 * p45 * p24 - p45 * x18 * p25 - p45 * x18 * p37 - p45 * x18 * p38.

= p45 * p36 + p45 * x18 * p38 - x19 * p45 * p37 + p45 * x18 * p25 - x17 * x19 * p45 * p24

.

The model comprising both the patways is calibrated to the experimetnal data (Figure 2), and further, the calibrated model is used for making predictions that we validated experimetnally(Figure 3). Details of model calibration and validation steps can be found in supplmentary file S1.

Single cell simulations

To convert the calibrated population average model to single cell level we adopted an ensemble modelling approach in which a population of single cells are generated by sampling the protein concentration assuming protein expression noise at the level of single cells follow a log-normal distribution[14, 43]. Several studies show the distribution of protein concentration in individual cells were drawn from lognormal distirubutions[28, 29, 43, 46], where, for a given protein its population average value from the best-fit model is used as the median of the generated distribution.

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Figure legends :

Figure 1: Dose-response and kinetic studies of STAT1 and STAT3 phosphorylation upon IL-10 or IFNγ stimulation. 48 hrs rested Balb/c derived peritoneal macrophages, cultured in RPMI 1640 with 10% fetal bovine serum were subjected to serum starvation for 3hrs. The cells were stimulated with increasing concentrations (0.5ng/ml, 1.0ng/ml, 2.5ng/ml, 5.0ng/ml, 10.0ng/ml, 20.0ng/ml, 40.0ng/ml) of (A) recombinant IL-10 protein or (B) recombinant IFNγ protein for 15mins, then washed with ice-cold phosphate-buffered saline and lysed with RIPA lysis buffer containing protease and phosphatase inhibitors. The cell lysates were further processed for immunoblotting and probed for and total STAT1 and STAT3

proteins. (C) Kinetics of STAT1 and STAT3 phosphorylation by high (20ng/ml), medium (5ng/ml) and low (1ng/ml) doses of IFNγ stimulation.(D) Kinetics of STAT1 and STAT3 phosphorylation by high (20ng/ml), medium (5ng/ml) and low (1ng/ml) doses of IL-10 stimulation. (E) Kinetics of SOCS1 and SOCS3 expression on stimulation with medium dose of IL-10 or IFNγ. Data in (C-E) represents mean ± s.d. of three sets.

Figure 2: Quantitative modeling of STAT1 and STAT3 dynamics at different doses of IL-10 and IFNγ. (A) Schematic representation of the IFNγ pathway. The blunt heads solid lines represent catalysis; arrowheads with solid lines represent binding, unbinding, phosphorylation and dephosphorylation; blunt-headed dashed lines represent transcriptional induction. Upon ligand(IFN) binding the receptor(IFNR) forms an active signaling complex(IFN-LR) which triggers the activation of STAT1(STAT1→ STATp) and STAT3(STAT3 → STAT3p) through phosphorylation. STAT1p and STAT3p undergo dimerization to become STAT1p_Dm and STAT3p_Dm, respectively. Transcription induction of target genes such as SOCS1 and SOCS3 is shown. SOCS1 is a negative feedback regulator of IFNγ signaling which forms a functionally inactive complex [SOCS1.IFN-LR] and inhibits the signaling. A STAT1⁄3 phosphatase is also activated (Phosi → Phos) by IFN-LR. (B) IFNγ pathway in the model was calibrated to STAT1⁄3 activation dynamics at three different doses of applied signal 1ng/ml(L), 5ng/ml(M) and 20ng/ml(H). The pathway was also calibrated to the dynamics of SOCS1 and SOCS3 induction at M. (C) schematics of IL-10 signaling pathways in the model is shown. Notation of the arrows is kept same as described in the IFNγ pathway. Both STATs are activated by IL-10 stimulation. Upon ligand (IL-10) binding the receptor (IL-10R) forms an active complex (IL-10-LR) which activates both STATs. At the transcriptional level induction of SOCS1 and SOCS3 takes place. IL-10Ri represents an inhibitor of IL-10 signaling which acts by sequestrating and degrading the IL-10 receptor. (D) IL-10 pathway in the model is calibrated to STAT1⁄3 phosphorylation dynamics at L, M and H doses of IL-10 stimuli and SOCS1, SOCS3 expression dynamics at M dose .

Figure 3. Prediction of STAT1 and STAT3 dynamics in co-stimulation (IL-10 + IFNγ).

(A) Schematic representation of the co-stimulation scenario. Here both IL-10 and IFNγ signal were applied simultaneously. The cyan and orange colored dashed lines with blunt heads represent IFNγ and IL-10 pathway specific STAT1⁄3 activation, respectively. The stimulus type independent process like STAT1⁄3 dimerization or their dephosphorylation step is shown with black colored arrows. (B) STAT3 dynamics upon co-stimulation. The shaded area shows predictions from 50 independent fits with the similar goodness of fit and the black dots represent experimental data. (C) STAT1 dynamics upon co-stimulation, shaded area shows predictions (with amplitude correction) from the 40 independent fits and black dots shows the data (D) SOCS1 induction upon co-stimulation. The shaded areas show prediction range and the black dots show experimental data.

Figure 4: Cell-to-cell variability in protein expression results in reciprocally signaling subpopulations (A) Normalized dynamics of STAT3 and STAT1 is shown from three distinct subpopulations C0, Rc-t1 and Rc-t2.

STAT3 activation in a cell in C0 is closely comparable between it's IL-10-only and co-stimulation responses. Blue line shows median of the ratio $STAT3_{co\text{-}stimulation}/STAT3_{IL\text{-}10}$ (upper panel) and $STAT3_{co\text{-}stimulation}$ /$STAT3_{IL\text{-}10}$ (lower panel) from 1000 C0 cells. Red line shows these ratios from Rc-t1 cells and green line shows the ratios from Rc-t2 cells, with 1000 cells in each subpopulation. (B) Distribution of single-cell variables(protein concentrations) in C0, Rc-t1, and Rc-t2 subpopulation with 1000 cells in each subpopulation.(C) Distribution of the most distinct variables in the Rc-t1 or Rc-t2 subpopulation is shown comparably.

Figure 5: Systematic replacement of single-cell variables identifies most sensitive perturbation targets specific to different subpopulations.

(A) Protein concentrations subjected to cell-to-cell variability were systematically replaced between the 6 pairs of subpopulations. In each heat map the x-axis direction shows the 100 representative cells from a subpopulation subjected to such parameter replacement. The notation Rc-t1 → CO, for instance, would mean single-cell variables in Rc-t1 cells are replaced with their counterparts from C0 cells, following which, we calculate the fraction of CO cell emerging from the Rc-t1 subpopulation. Each column in a row, for example in the 1st row 1st column of the heat map titled "Rc-t1 → CO", shows the number of C0 cells emerging per Rc-t1 cell when IL-10Ri is replaced from 500 other C0 cells; the result is normalized to show the fractions where fraction = 1 means replacement of IL-10Ri from 500 C0 cells to Rc-t1 would result in 500 C0 cells. For all the heat maps the same procedure is followed, the title of the heat map shows the pair of subpopulation studied and the y-direction shows the protein concentrations that were replaced between the pairs. For each protein concentration, 100 representative cells are sorted according to their observed frequency of transformation to the respective new type of cells. (B) The significance of change in activator to inhibitor ratio in either IL-10 or IFNγ pathway. Replacing both IL-10R and IL-10Ri from C0 to Rc-t1 resulted in dramatic increase in the Rc-t1 to C0 transformations, as compared to, only IL-10R or IL-10Ri replacement(compare figure 5A, heat map titled Rc-t1 → CO).

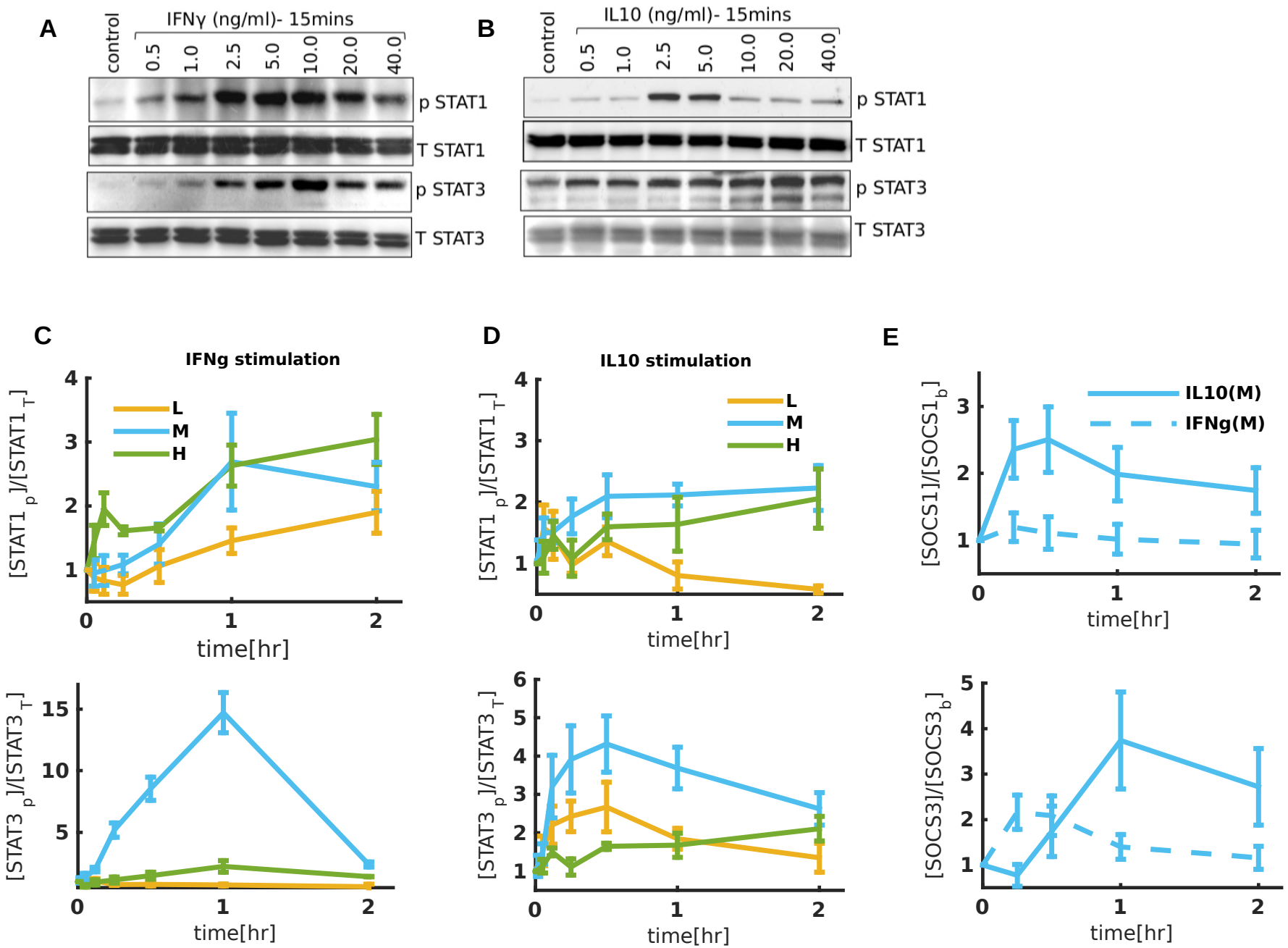
Supplementary figure.

Figure S1. Kinetic studies of STAT1 and STAT3 phosphorylation at low(L), medium(M) and high(H) dose of IL10 and IFNγ stimulation. Kinetics study of STAT1(A) and STAT3(B) phosphorylation at high (20ng/ml), medium (5ng/ml) and low (1ng/ml) doses of recombinant IL10 and IFNγ proteins. Balb/c derived macrophages were stimulated for 3', 7', 15', 30', 60' and 120' with the mentioned doses of IL10 or IFNγ, lysed and processed for immunoblot analysis of STAT1 and STAT3 phosphorylation.(C) Kinetics of SOCS1 AND SOCS3 expression on stimulation with MD of IL10 or IFNγ for 15', 30', 60' and 120' is shown.

Figure S2. IFNγ dose dependent activation of STAT3 and it's phosphatase. Simulation result show the IFNγ dose depedent activation of phosphatase Phos and the respective maximum amplitude of STAT3. A bell shaped dose-response in STAT3 activation and and switch like Phos activation is observed.

Figure S3. Basal SOCS1(SOCS1$_B$) positively regualtes STAT1 and STAT3 activation amplitudes. Both STAT1 and STAT3 activation amplitudes are reduced when SOCS1 binding to the active IFNγ signaling complex IFN-LR is inhibited. In absence of SOCS1$_B$ rapid rise and fall of STAT1 and STAT3 trajectories can be observed (dashed line in both A and B), demonstrating the positive regualtion of STAT1 and STAT3 activation by SOCS1 in the IFNγ pathway.

Figure S4. STAT1 and STAT3 activation in response to co-stimulation (IL-10 + IFNγ)treatment. (A) Prediction from 40 independently fitted models with similar goodness of fit. The shaded area shows range of predictions and the blue filled circles show data. The model and data trajectories are closely comparable by multiplying the model trajectory with a scaling factor which we show in Figure 3C. (B) Representative immunoblots for co-stimulation is shown. The experimental procedure is same as described in figure S1A or S1B. (C) Kinetics of SOCS1 and SOCS3 expression on stimulation with medium dose of IL-10 or IFNγ. One representative dataset is shown.


A
IFNγ (ng/ml)- 15mins
control
0.5
1.0
2.5
5.0
10.0
20.0
40.0
p STAT1
T STAT1
p STAT3
T STAT3
B
IL10 (ng/ml)- 15mins
control
0.5
1.0
2.5
5.0
10.0
20.0
40.0
p STAT1
T STAT1
p STAT3
T STAT3
C
IFNg stimulation
L
M
H
[STAT1 p]/[STAT1 T]
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time[hr]
D
IL10 stimulation
L
M
H
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time[hr]
[STAT3 p]/[STAT3 T]
time[hr]
E
IL10(M)
IFNg(M)
[SOCS1]/[SOCS1 b]
time[hr]
[SOCS3]/[SOCS3 b]
time[hr]

Figure 1

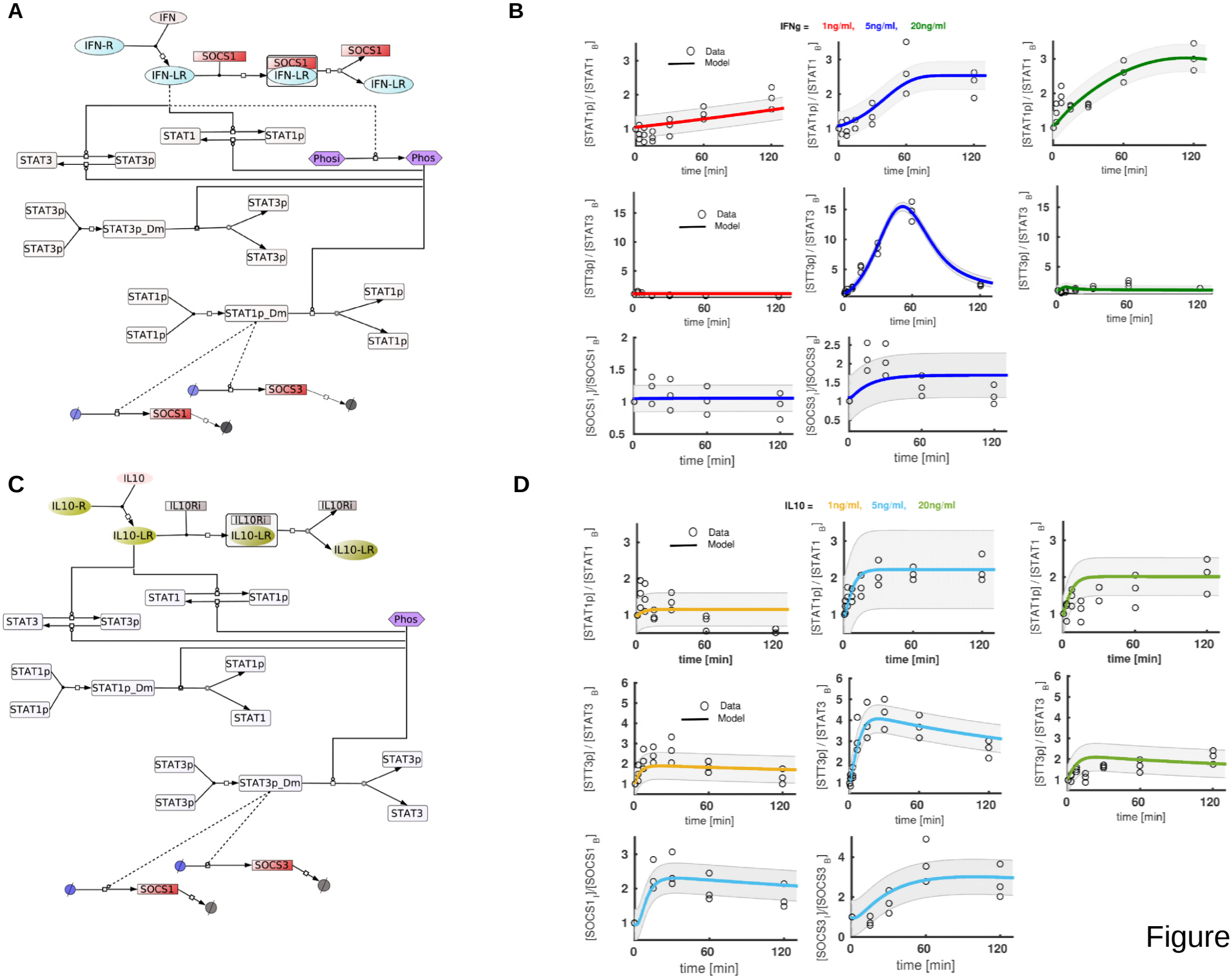
A
IFN
IFN-R
IFN-LR
SOCS1
SOCS1
IFN-LR
SOCS1
IFN-LR
STAT1
STAT1p
STAT3
STAT3p
Phosi
Phos
STAT3p
STAT3p_Dm
STAT3p
STAT3p
STAT1p
STAT1p_Dm
STAT1p
STAT1p
SOCS3
SOCS1
B
IFNg = 1ng/ml, 5ng/ml, 20ng/ml
Data
Model
[STAT1p] / [STAT1 B]
[STT3p] / [STAT3 B]
[SOCS1 I]/[SOCS1 B]
[SOCS3 I]/[SOCS3 B]
time [min]
C
IL10
IL10-R
IL10-LR
IL10Ri
IL10Ri
IL10-LR
IL10Ri
IL10-LR
STAT1
STAT1p
STAT3
STAT3p
Phos
STAT1p
STAT1p_Dm
STAT1p
STAT1
STAT3p
STAT3p_Dm
STAT3p
STAT3
SOCS3
SOCS1
D
IL10 = 1ng/ml, 5ng/ml, 20ng/ml
Data
Model
[STAT1p] / [STAT1 B]
[STT3p] / [STAT3 B]
[SOCS1 I]/[SOCS1 B]
[SOCS3 I]/[SOCS3 B]
time [min]

Figure 2

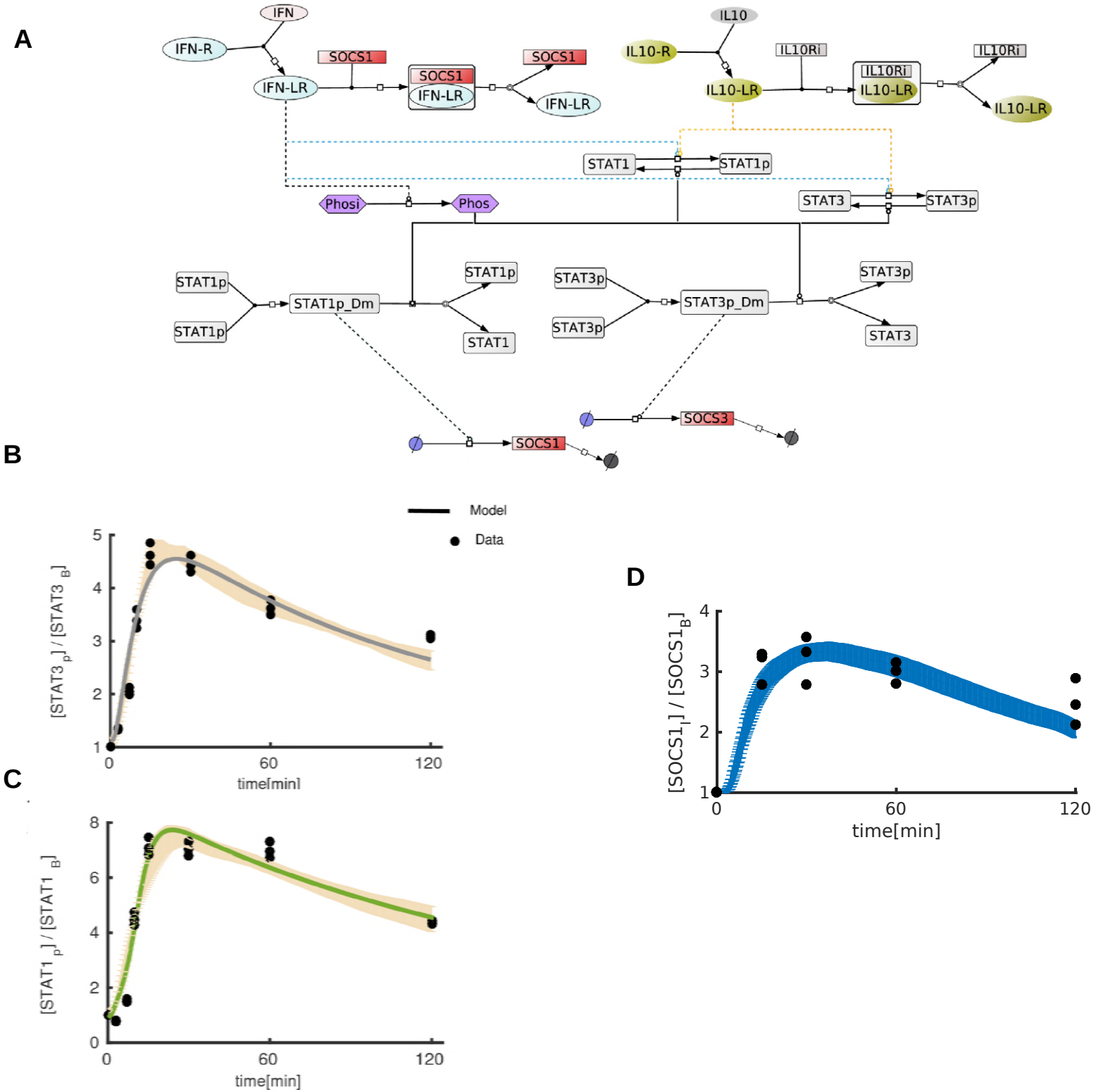
A
IFN
IFN-R
IFN-LR
SOCS1
SOCS1
IFN-LR
SOCS1
IFN-LR
IL10
IL10-R
IL10-LR
IL10Ri
IL10Ri
IL10-LR
IL10Ri
IL10-LR
STAT1
STAT1p
Phosi
Phos
STAT3
STAT3p
STAT1p
STAT1p
STAT1p_Dm
STAT1p
STAT1
STAT3p
STAT3p
STAT3p_Dm
STAT3p
STAT3
SOCS3
SOCS1
B
Model
Data
[STAT3p] / [STAT3B]
time[min]
C
[STAT1p] / [STAT1B]
time[min]
D
[SOCS1I] / [SOCS1B]
time[min]

Figure 3

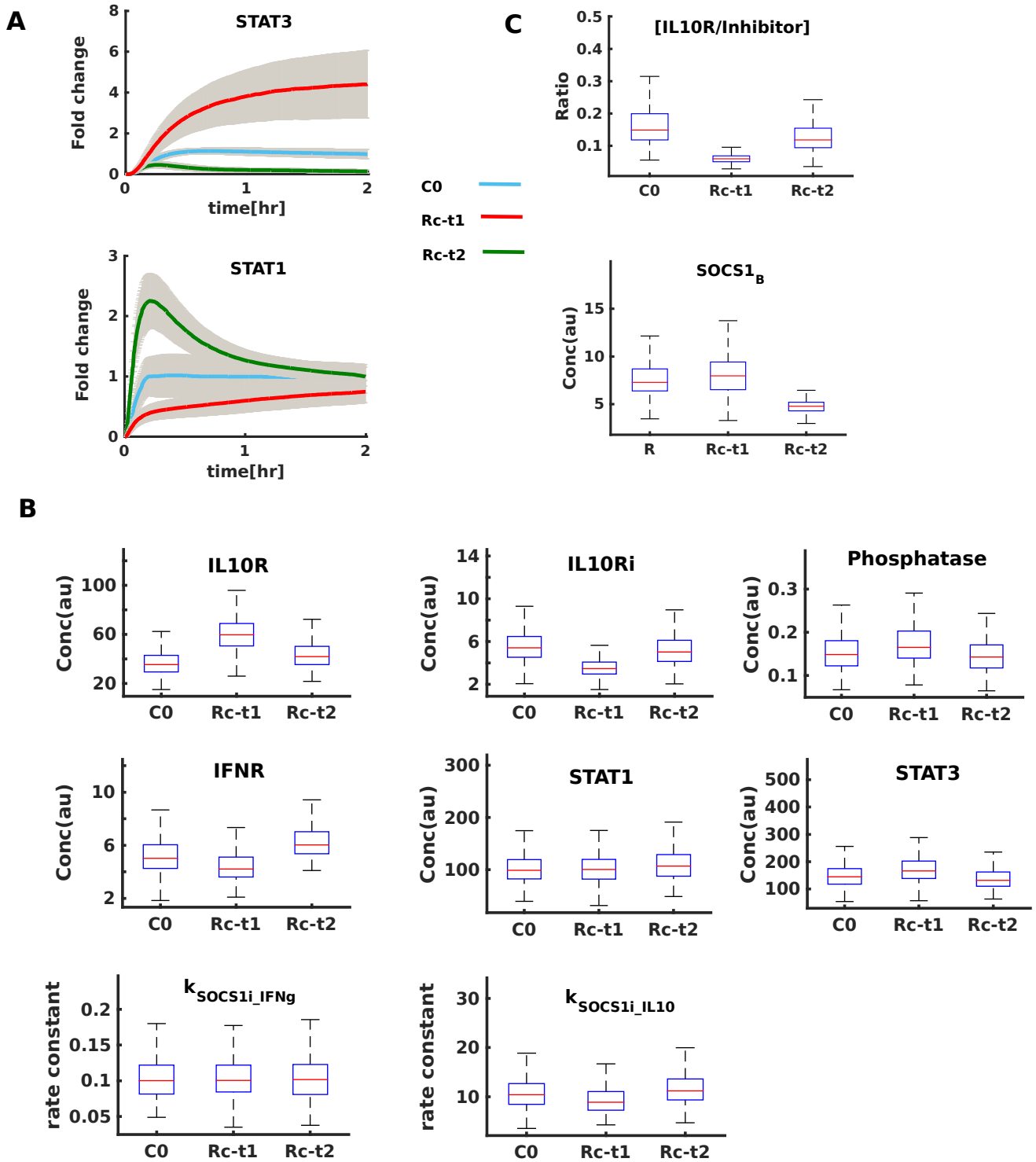
A
STAT3
Fold change
time[hr]
STAT1
Fold change
time[hr]
C0
Rc-t1
Rc-t2
C
[IL10R/Inhibitor]
Ratio
C0
Rc-t1
Rc-t2
SOCS1B
Conc(au)
R
Rc-t1
Rc-t2
B
IL10R
IL10Ri
Phosphatase
IFNR
STAT1
STAT3
Conc(au)
kSOCS1i_IFNg
kSOCS1i_IL10
rate constant
C0
Rc-t1
Rc-t2

Figure 4

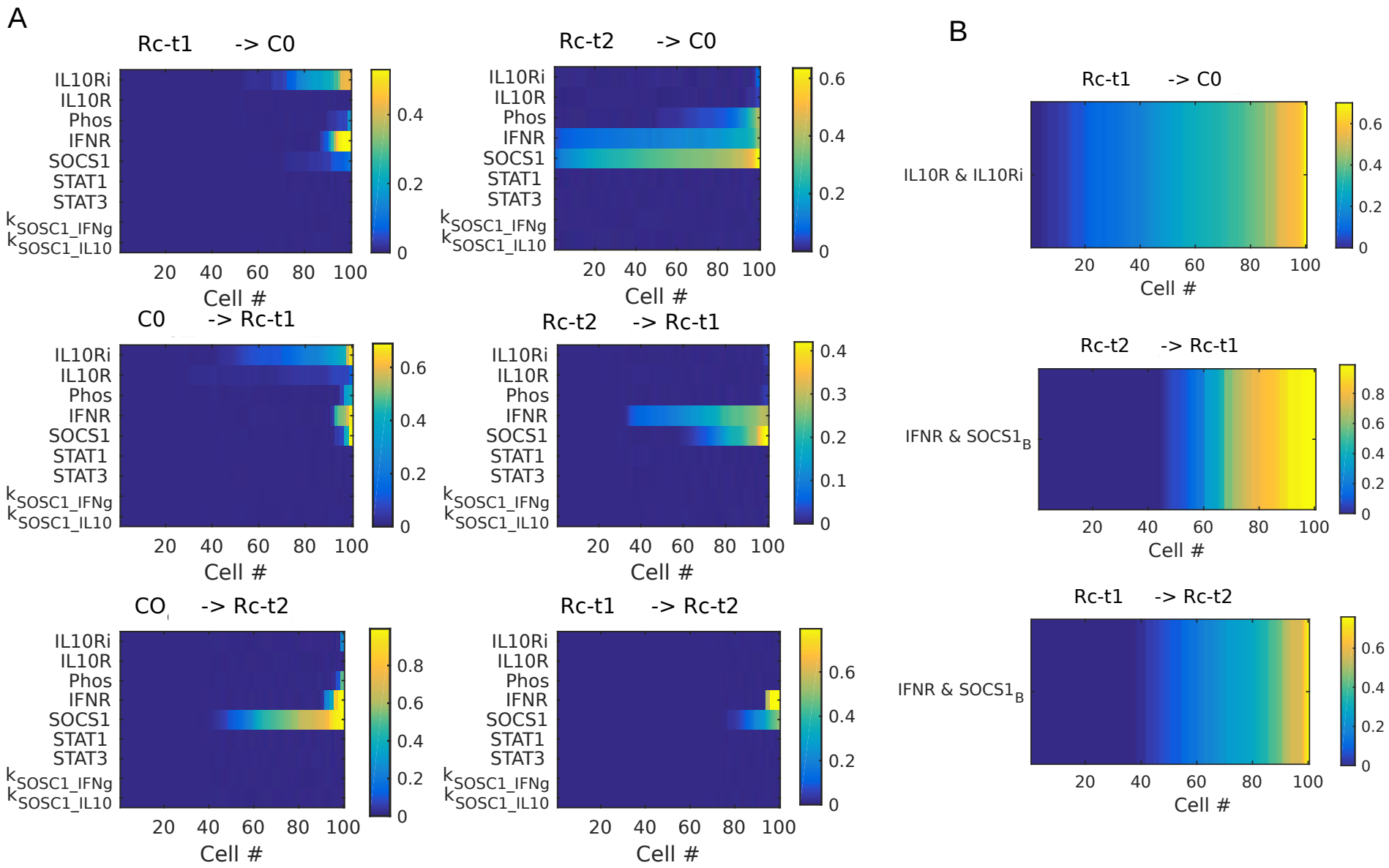
A
Rc-t1 -> C0
Rc-t2 -> C0
C0 -> Rc-t1
Rc-t2 -> Rc-t1
CO -> Rc-t2
Rc-t1 -> Rc-t2
IL10Ri
IL10R
Phos
IFNR
SOCS1
STAT1
STAT3
kSOSC1_IFNg
kSOSC1_IL10
Cell #
B
Rc-t1 -> C0
IL10R & IL10Ri
Rc-t2 -> Rc-t1
IFNR & SOCS1B
Rc-t1 -> Rc-t2
IFNR & SOCS1B

Figure 5