

How macrophage-encoded bistable circuit generates tissue memory in concert with IECs

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In the colon tissue, we have intestinal epithelial cells (IECs) lining the colon, with macrophages interspersed below them. We found that inducing mild inflammation using a weaker dose of dextran sodium sulfate (DSS) in drinking water is protective against a higher DSS challenge dose, akin to vaccination. We found that the mild inflammation greatly induces IL18 in colon tissue, most of which is derived from epithelium. We also found that macrophages are the ones that mediate the protection. Removing those "primed" macrophages thru clodronate liposomes failed to protect mice against the challenge DSS dose. So, in summary, the mild inflammation leads to an epithelial response (i.e. secretion of IL18) that influences the macrophages in the tissue and changes their metabolic state to be protective.

So, the question is what are the design principles in this colon tissue that allows for protection to happen? To be more specific, how is it that macrophages are able to generate and retain that memory, especially since IECs have a higher rate of renewal?

A macrophage-IEC circuit

We found that epithelium releases IL18, which activates the cGAS-cGAMP-STING pathway (IFN-beta is a downstream reporter of that pathway) in macrophages. Macrophages release cGAMP which then can activate IL18 activity in epithelium. This is in essence a positive feedback loop. Because of this positive feedback loop and the potential for a "tissue memory" to form, this can be a manifestation of a bistable circuit. Since tissues are communities of cells, this cross-talk of IL18/cGAMP among IECs/macrophages can just be one of many manifestations of a generalizable principle that tissues can utilize bistable circuits to encode memory.

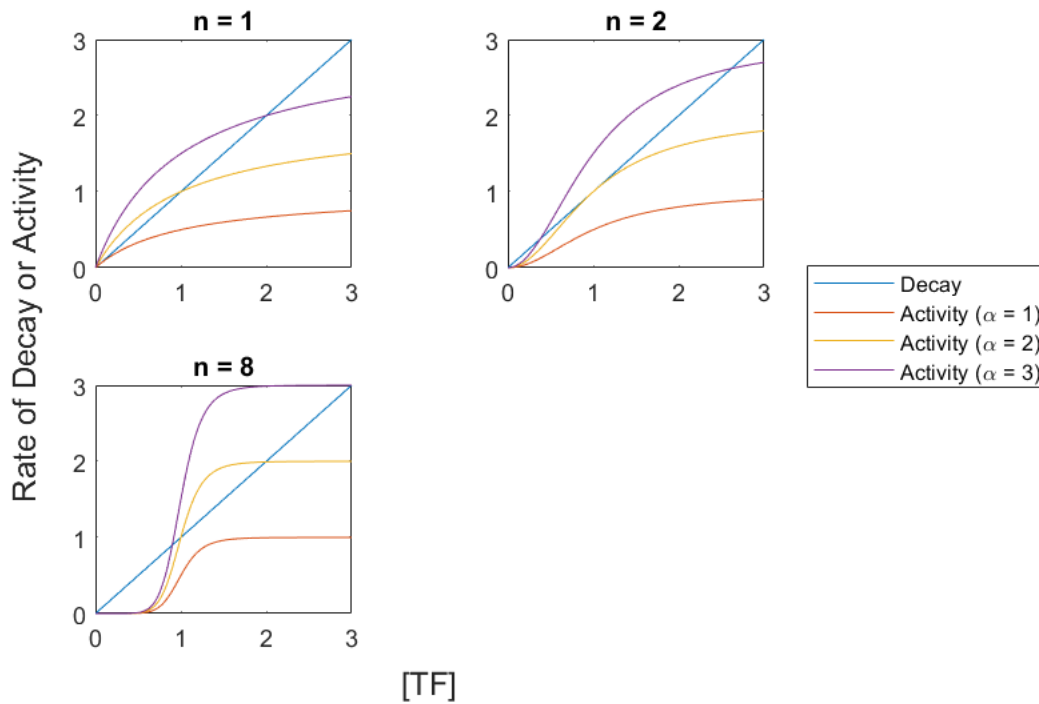
What is a bistable circuit?

Bistability is a phenomenon when a system can exist in 2 stable states (e.g. ON or OFF). A hallmark aspect of bistability is that it relies on initial conditions or history of the system to inform which of the 2 stable states the system will occupy. A key feature of bistability is that it relies on positive feedback loops in order to generate an "ultrasensitive" response. We are studying how exposure to IL18 activates an intracellular process or transcription factor (TF) in macrophages that changes their cell state (i.e. ON or OFF). See below where

an intracellular TF x is degraded linearly and produced at $\alpha \frac{x^n}{1 + x^n}$, where α is the maximal response and n is the coefficient that controls the "ultrasensitivity" of the positive feedback loop. Greater the n , the stronger the positive the feedback loop such that it reaches the maximum value much quicker (i.e. the time to reach 1/2 max response is much quicker).

Below, we will examine how activity rate changes for different ultrasensitivity strengths ($n = 1,2,8$) and different response strengths ($\alpha = 1,2,3$).

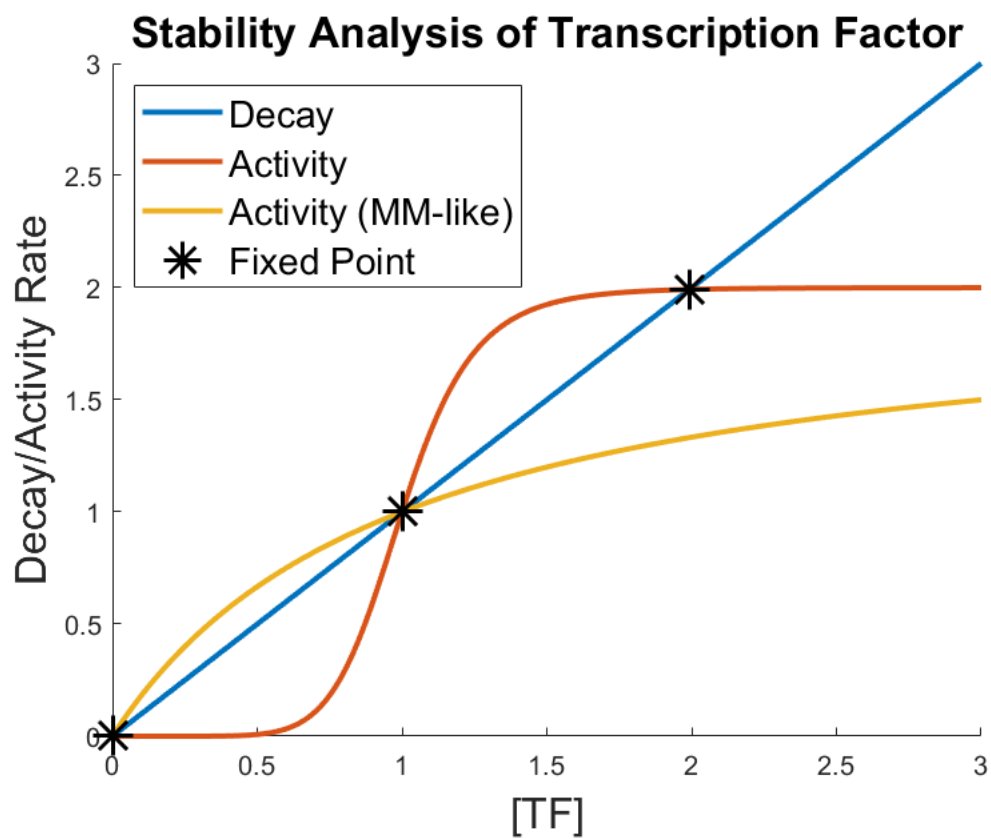
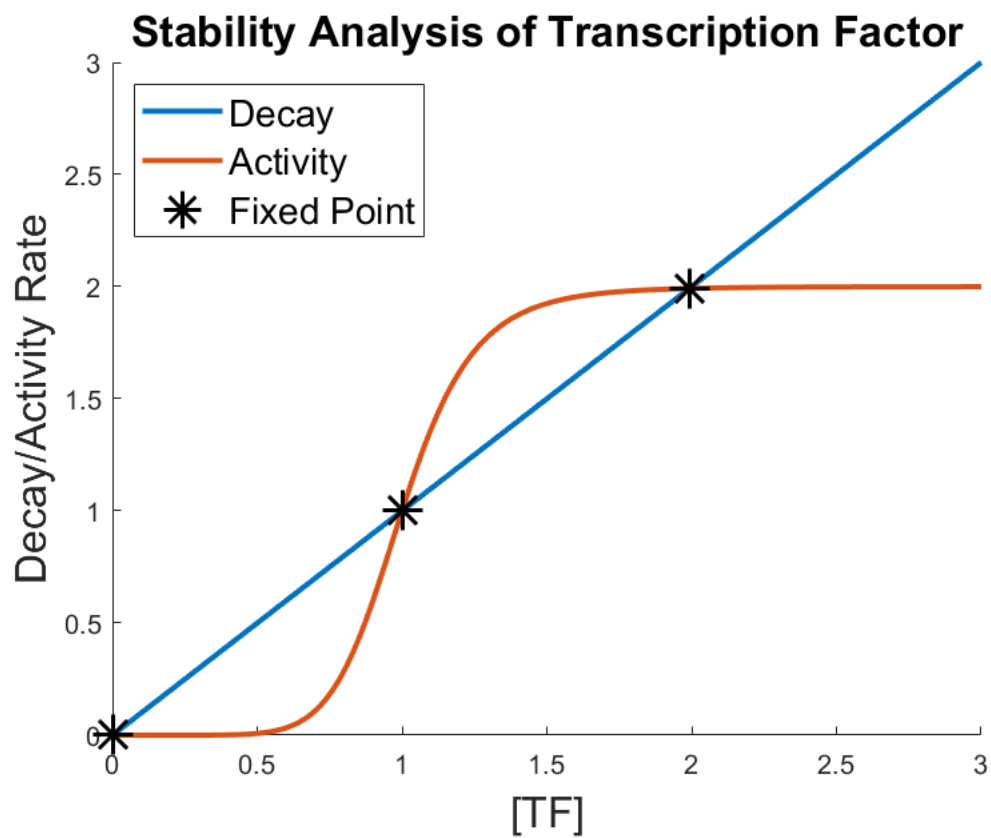
Activity vs Decay of an intracellular TF



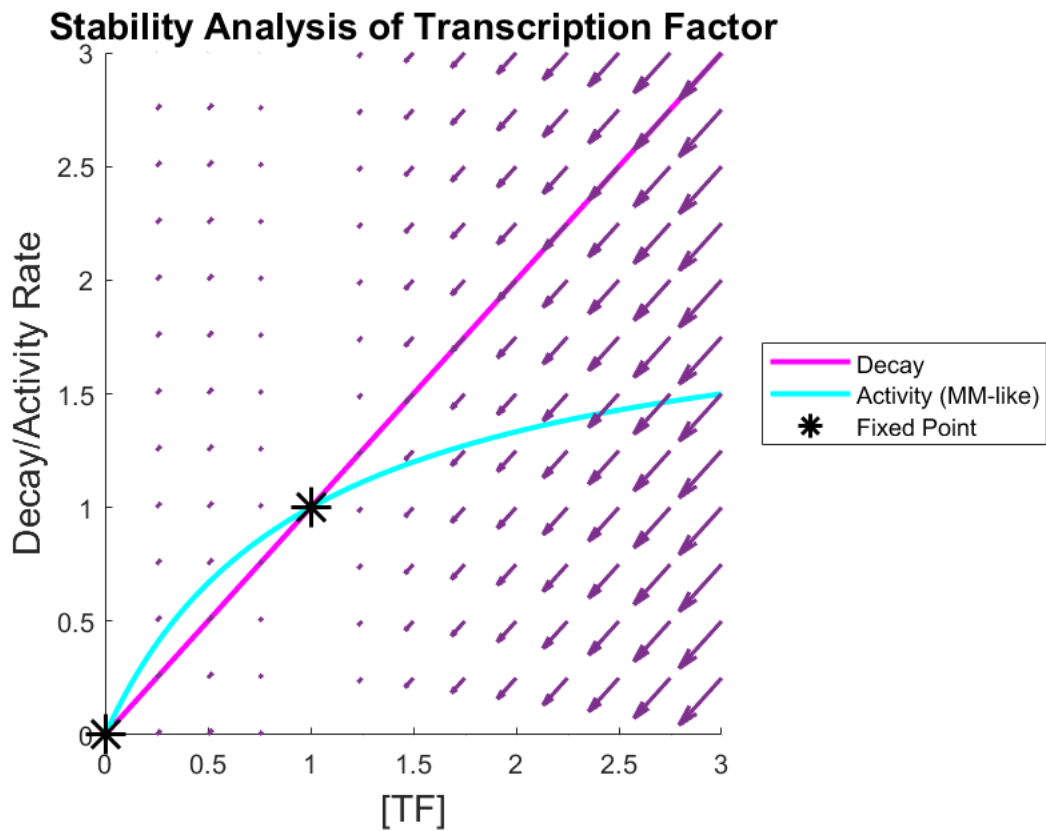
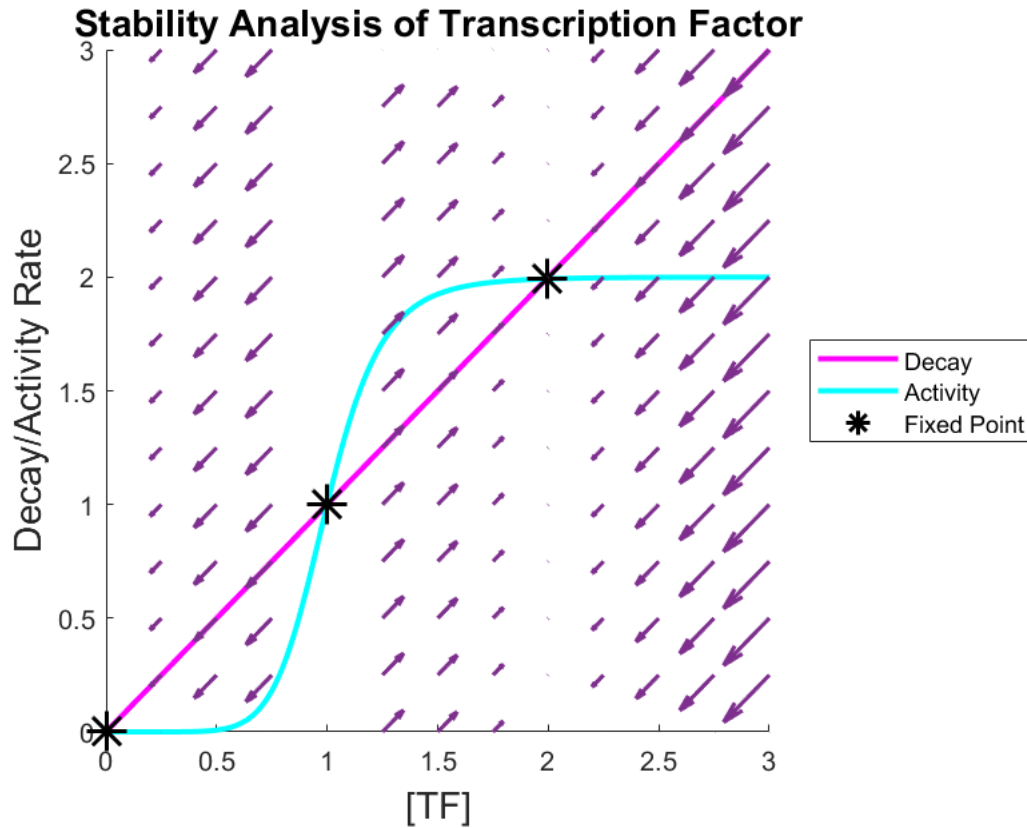
As you can see from the graphs, at low ultrasensitivity ($n=1$), the activity curves only intersect the decay curves twice (at 0 and another value). However, by increasing the ultrasensitivity (i.e. increasing n), the activity curve of the TF becomes more sigmoidal that is able to intersect with the decay curve 3 times (in the case of $n=8$ and $\alpha=3$). This is super important! Wherever the activity curve intersects the decay is called a "fixed point", where the TF's activity and decay rates are equivalent and results in a steady-state. But how do these fixed points tell us how macrophages can reach their stable ON or OFF states?

How to characterize stability of the system using fixed points?

Let's take a look at the graph of the activity curve with $n=8$ and $\alpha=2$ and find where they intersect with the decay curve. We can also set $n=1$ to mimic the hyperbolic Michaelis-Menten-like kinetics (MM-like).



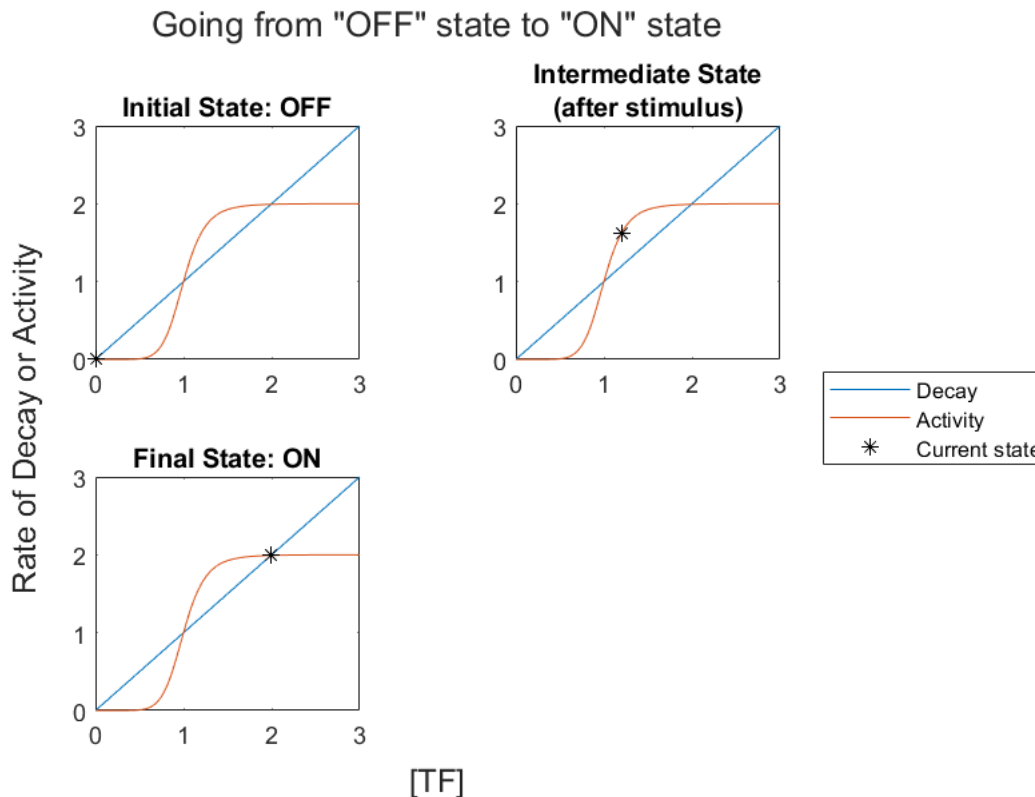
We can see there are 3 intersections at $[TF]$ (or x) = 0, 0.9, and 2. What is the behavior of our TF activity/decay system near these fixed points? We can use quiver plots to showcase the vector fields.



We can clearly see from our quiver plot that around the middle intersection ($[TF]=1$), the arrows are pointing to the left for the TF to go to 0 or to the right for the TF activity to go to 2. This means our middle intersection is an "unstable" fixed point whereas the left and right fixed points at $[TF] = 0$ (OFF), 2 (ON), respectively, are "stable" fixed points. So, if we are slightly to the right of the unstable fixed point, our TF will go to the ON position. If we are slightly to the left of the unstable fixed point, our TF will go to the OFF position. Similarly, if we go to the right of our stable "ON" fixed point ($[TF]>2$), our decay rate exceeds our activity rate, so we will return to that stable "ON" fixed point. All in summary, this means having an **ultrasensitive and strong enough positive feedback loop** can lead us to iterate between the stable ON and stable OFF states. But how do we transition from 1 steady-state fixed point to another?

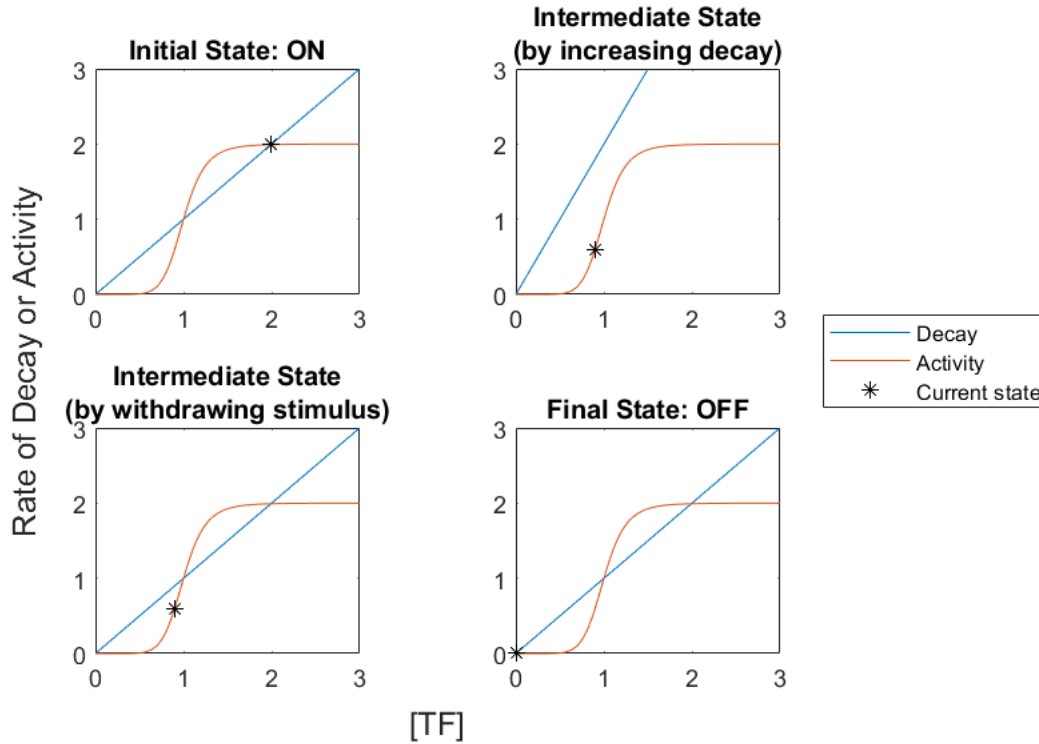
Switching between ON, OFF states

One of the most important things we need to understand is how we can switch between the OFF (0) steady-state to the ON (1) steady-state and vice versa. To switch from OFF to ON, we need to be able to pass a certain threshold of TF concentration in order to reach the ON state. This is typically seen in a dose escalation study where there is a sharp transition from an OFF state to an ON state.



However, going from ON to OFF is more interesting. Basically, it requires us to make our decay rate exceed our activity rate (even transiently!). This can be accomplished by cell division, dilution, withdrawing of our stimulus that induces TF activity, etc. We can see the transition below for 2 cases, where we either increase decay rate or by withdrawing the stimulus:

Going from "ON" state to "OFF" state



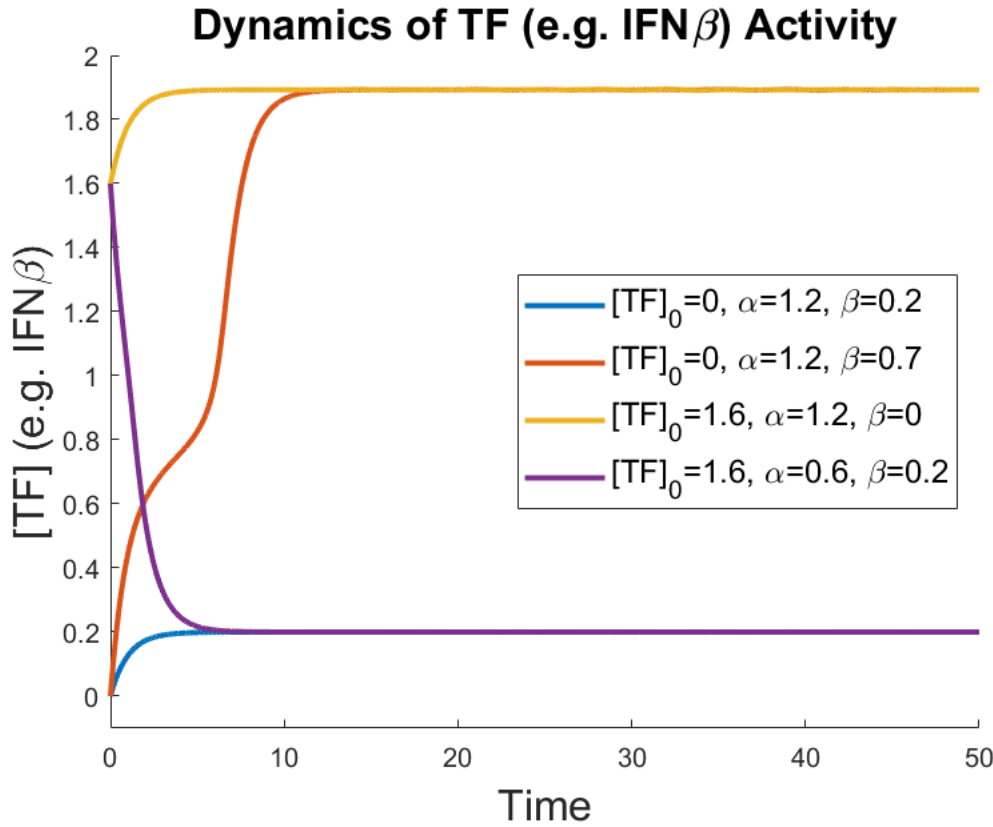
Translating to an experimental system where we have to stimulate macrophages

Okay, so now we have an intuition for bistable systems and how we can switch between ON and OFF states. Let's translate this to our macrophage system. We have IECs that secrete IL18, which then impacts macrophages by signaling thru NCC, activating STING, and leading to IFN β expression. In addition, the STING activation leads to release of cGAMP from the macrophages, which in turn activates the IECs to secrete more IL18. So, since the macrophages are the ones that retain the protective memory due to IL18 stimulation, we shall focus on the macrophages and how they participate in a bistable circuit.

Let's focus on the macrophages' ability to have a memory that may be enabled by a bistable circuit. Let's say that macrophages produce a TF or program that lets it retain the protective memory. So, we need to change our model a bit to account for the stimulus of IL18 that eventually activates that TF (which we can experimentally read out using IFN β). We will say that stimulus causes TF activity at rate β , so our transcription factor has

the rate $\frac{dx}{dt} = \beta + \alpha \frac{x^n}{1 + x^n} - x$ with α being the positive feedback strength and n controlling our ultrasensitivity.

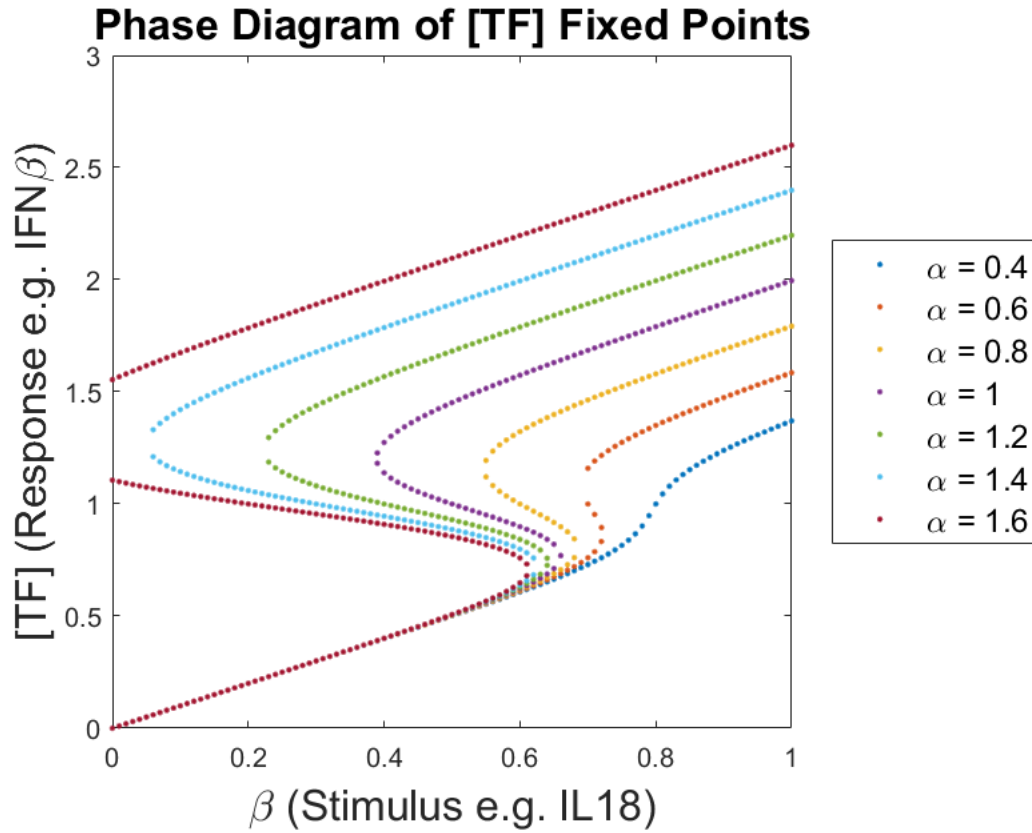
Let's take a look at how those trajectories look like:



As we can see from the above graph, there are 2 steady-state values: $[TF]=0.2$ (i.e. OFF) and $[TF]=1.9$ (i.e. ON). When we do not have any $[TF]$ to start with ($[TF]_0=0$), β induces the TF when it is sufficiently high (red curve, where $\beta=0.7$). However, if we do have some starting TF ($[TF]_0=1.6$), then our system is either 1) able to maintain TF expression in the absence of stimulus β (yellow curve where $\beta=0$) or 2) decline to 0 if the feedback strength α is lowered (purple curve where $\alpha=0.6$). The former case is akin to saying that our macrophages can stay ON even if there is no more IL18 stimulation.

How does stimulus and positive feedback affect our ON/OFF steady-states?

Assuming that $n=8$, how do the stimulation and positive feedback strength (i.e. values of β and α) influence our fixed points? We can accomplish by generating a phase diagram that shows the fixed points as a function of stimulus (β) and positive feedback strength (α).

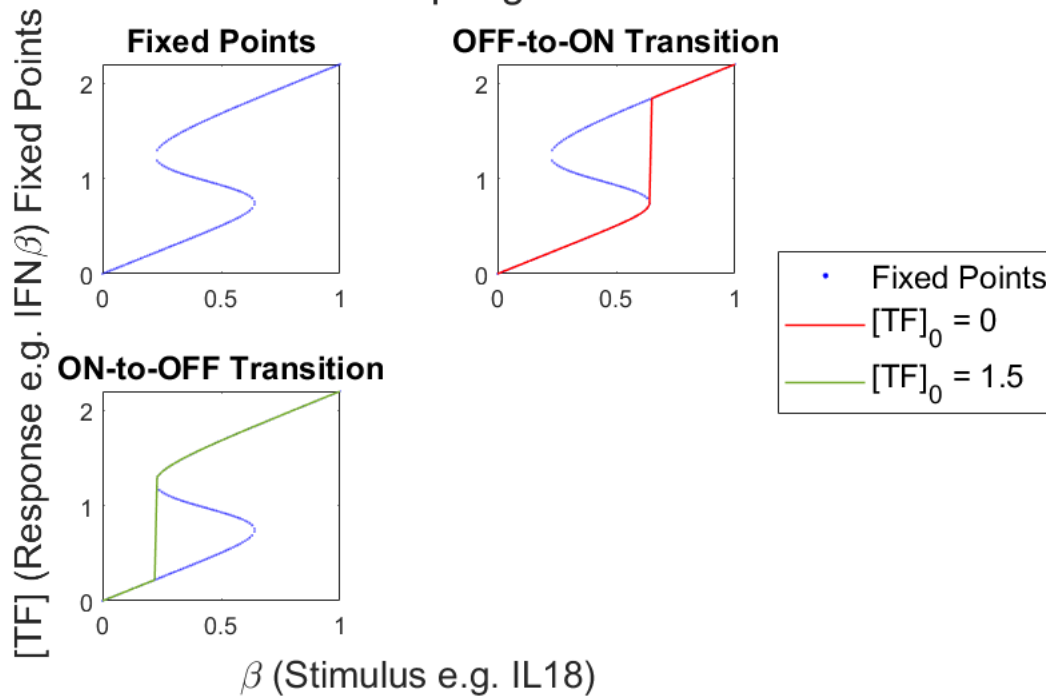


We can see that at a given ultrasensitivity ($n=8$), increasing positive feedback strength α allows us to have 3 fixed points at a given stimulus β . One of the fixed points will be the unstable point discussed earlier while the other 2 are the stable steady-states at the OFF and ON positions.

Positive feedback in a bistable system leads to hysteresis, a proxy for memory

Perhaps most importantly, we can see from the above graph that increasing the positive feedback strength α leads to a greater range of stimulus β that occupies an unstable fixed point, which can then subsequently transition to either the OFF or ON steady-states. That means there is more of a mismatch in the thresholds to go from OFF to ON and vice versa, i.e. the system exhibits hysteresis. This hysteresis can be a form of memory as it relies on the initial state of the macrophages and makes it more difficult to transition from one steady-state to another.

Hysteresis Encodes a Memory Response in Macrophages

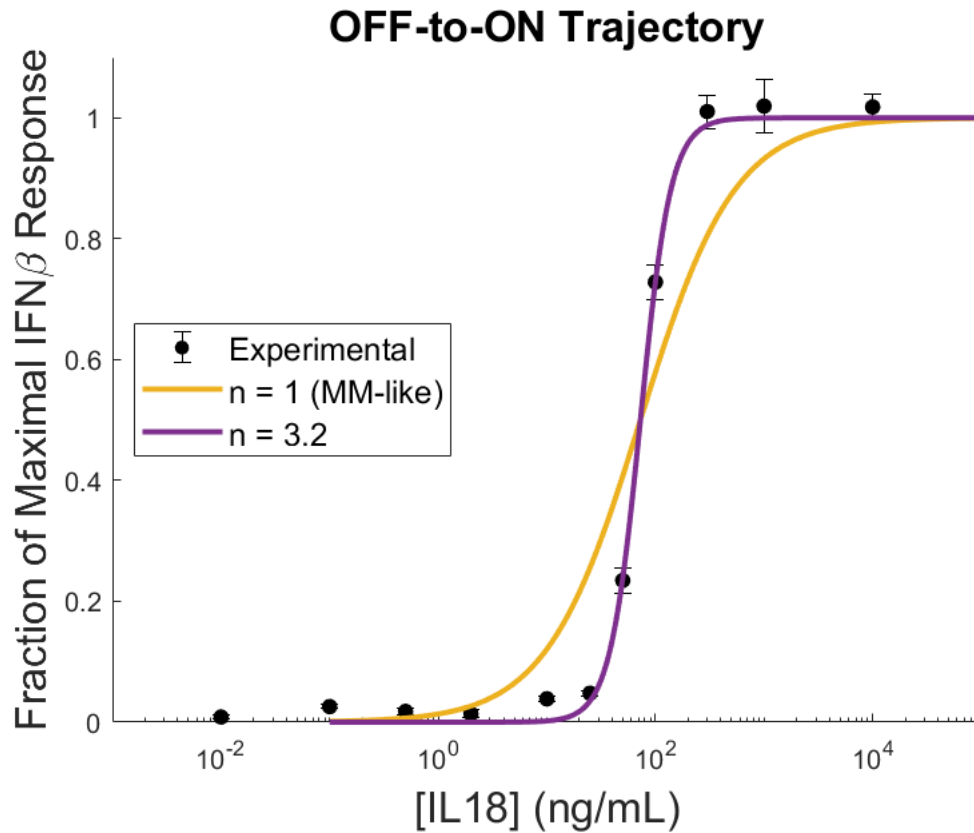


As you can see from the above graph, there is a mismatch in the thresholds of stimulus β to turn ON and to turn OFF the macrophages. If the macrophages are initially OFF ($[TF]_0=0$), then they need more IL-18 (~ 0.6) to turn them ON. Conversely, if the macrophages are already ON ($[TF]_0=1.5$), then they need much less IL-18 (~ 0.3) to turn them OFF. So, if we note a difference in these transition thresholds experimentally using IL18 as the stimulus and $IFN\beta$ as the response signal, there is most likely evidence of the macrophages exhibiting bistable behavior.

Examining experimental data

Characterize the OFF-to-ON transition and validate the ultrasensitivity assumption

We can read in actual data from the flow cytometry experiment and fit our activity curve to it.

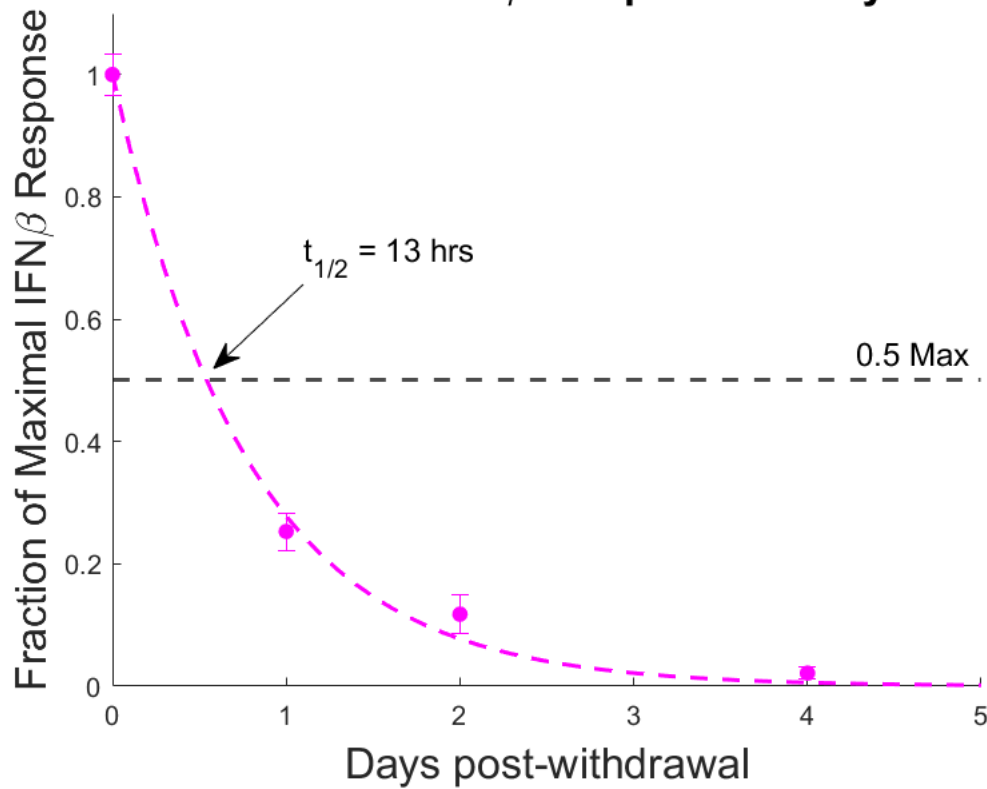


We want to show that our experimental data does not fit Michaelis-Menten type kinetics ("MM-like") that can be approximated with a Hill coefficient $\frac{x^n}{K^n + x^n}$, but with $n=1$ (MM-like, yellow curve). Using curve fitter, we found $K = 138.1$, and $n = 3.2$. This demonstrates that the BMDM's IFN β response to IL18 stimulation is ultrasensitive/cooperative as it does not fit the hyperbolic behavior expected from Michaelis-Menten like responses.

Characterize the decay of IFN β signaling after withdrawing IL18 stimulus and validate it fits an exponential decay

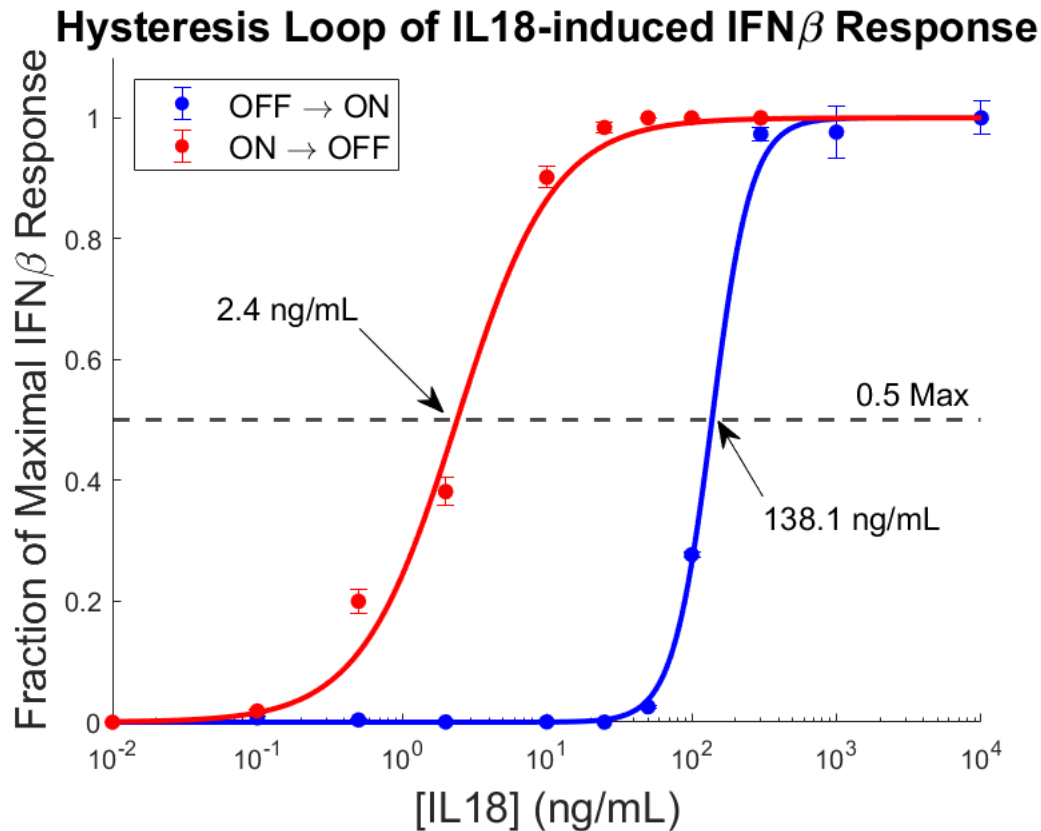
We need to validate that the IL18 stimulation does not induce a durable IFN β response in BMDMs (i.e. it induces reversible IFN β response) for the time scale we are considering. As a result, BMDMs were stimulated with IL18 for 2 days, withdrawn, and then IFN β signaling was measured over a time course of 4 days after the IL18 withdrawal.

Kinetics of IFN β Response Decay



Characterize the ON-to-OFF transition and the memory-via-hysteresis

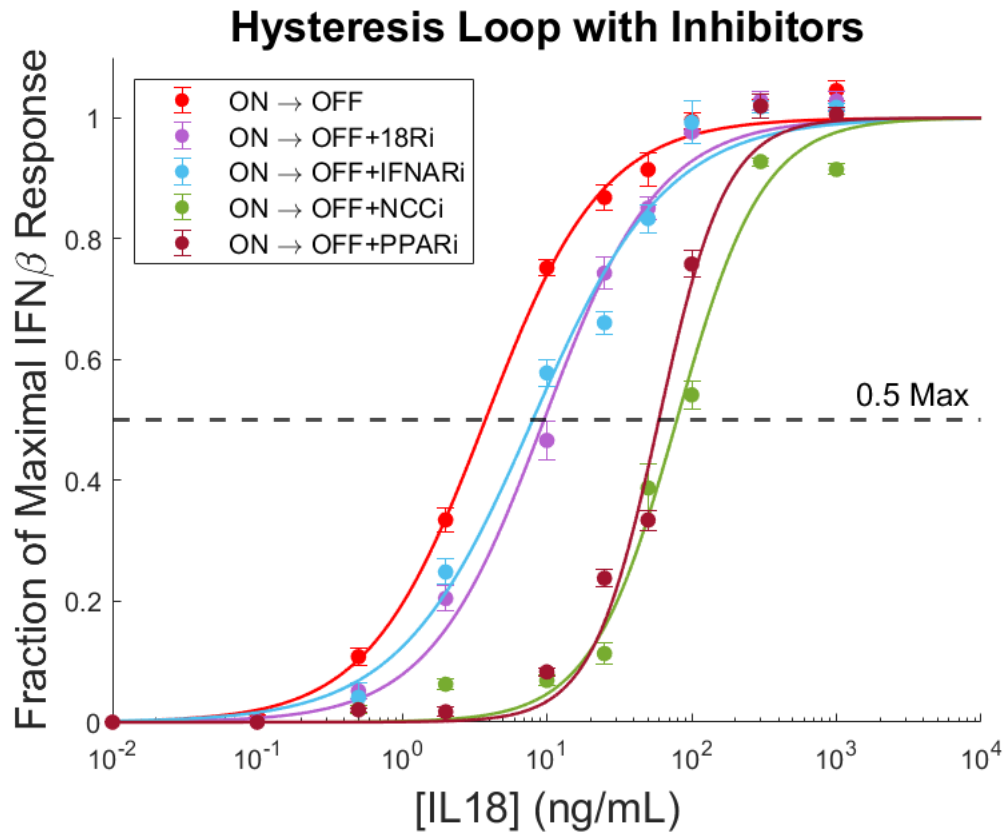
We want to see the mismatch in IL18 requirements (i.e. hysteresis) between turning ON the IFN β response in naive ("OFF") BMDMs and turning OFF the IFN β response in activated ("ON") BMDMs. The results are below and curves were fit to the Hill equation.



Whereas the OFF-to-ON trajectory had $K=138.1$ ng/mL and $n=3.2$, the ON-to-OFF trajectory had $K=2.4$ ng/mL and $n=1.3$. As K represents the IL18 concentration needed to reach half of the maximal response value, we can see that the hysteresis loop spans a 58-fold difference between these K values.

Characterize what intracellular signaling is required for the memory-via-hysteresis

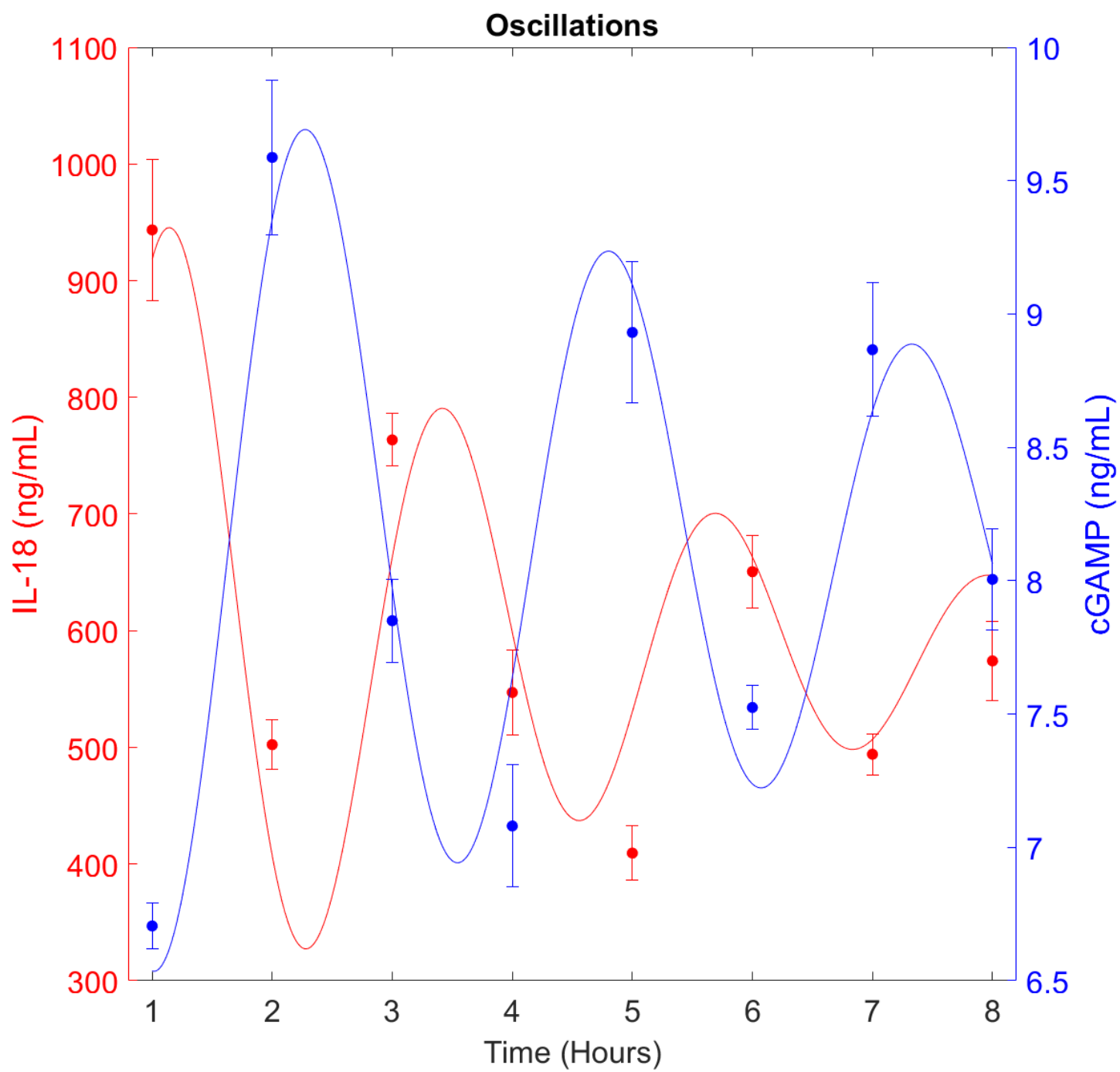
This hysteresis in the IL18-IFN β axis in BMDMs must be relying on some intracellular processes, so we wanted to identify which ones. We hypothesized that NCC signaling and fatty acid oxidation may play a role in maintaining this hysteresis. So, we pretreated BMDMs with IL18 to turn them ON, and then we gave BMDMs an inhibitor against NCC (NCCi) or an inhibitor against PPAR α (PPARi), which is a transcription factor that drives fatty acid oxidation. Then, the IFN β response was measured after the treatment with inhibitors. We would expect that the inhibitors would cause IFN β treatment to be between the ON-to-OFF trajectory (because that would mean that hysteresis is fully maintained, so it is the upper bound), or the OFF-to-ON trajectory (because the inhibitors would totally abolish the hysteresis effect, so it would be the lower bound).

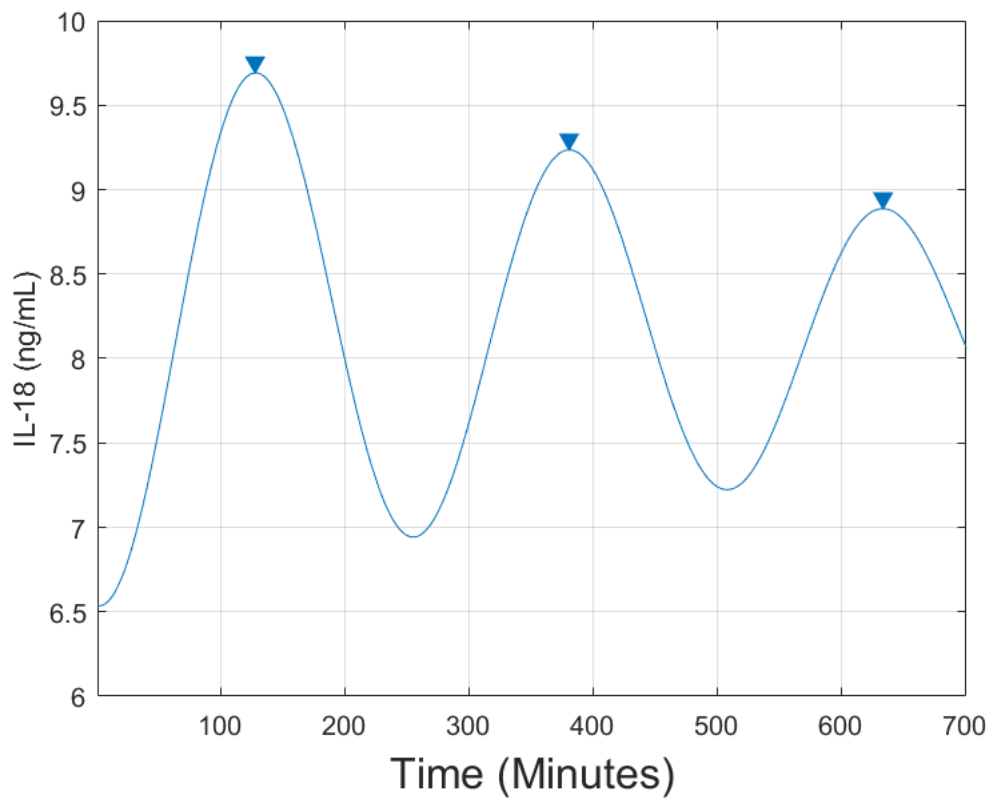
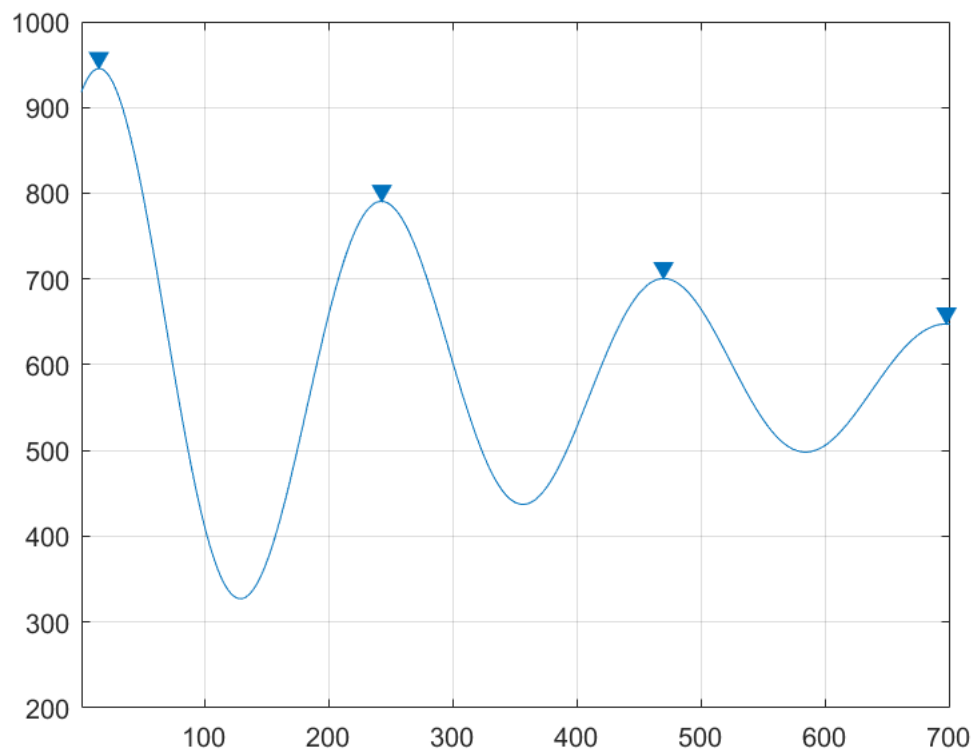


As we can see from the above graph, BMDMs that received treatment of NCCi or PPARi had a greatly diminished IFN β response with a 0.5 Max value at approximately 35 ng/mL, indicating that NCC signaling and fatty acid oxidation are processes required for BMDMs to maintain their IFN β response after IL18 stimulation.

The IL-18-cGAMP circuit is oscillatory in nature

How can macrophages maintain the activity of the bistable program? We hypothesized that it may be due to oscillations of IL-18 in the environment. Indeed, that is what we have found.

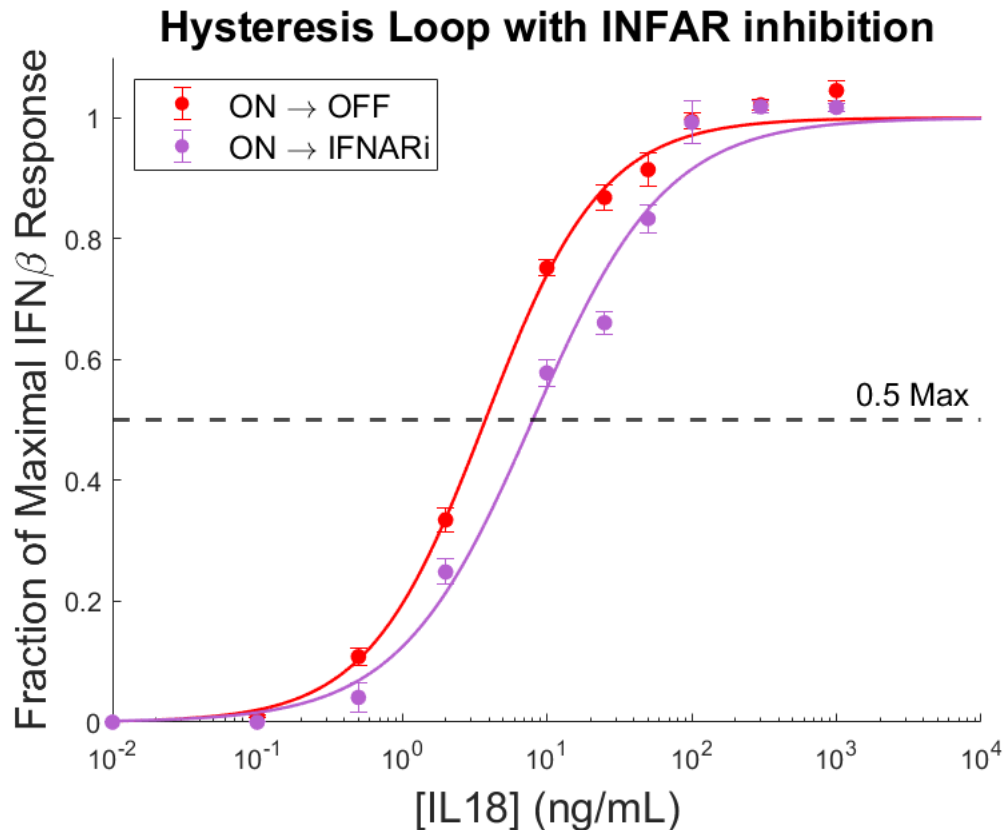




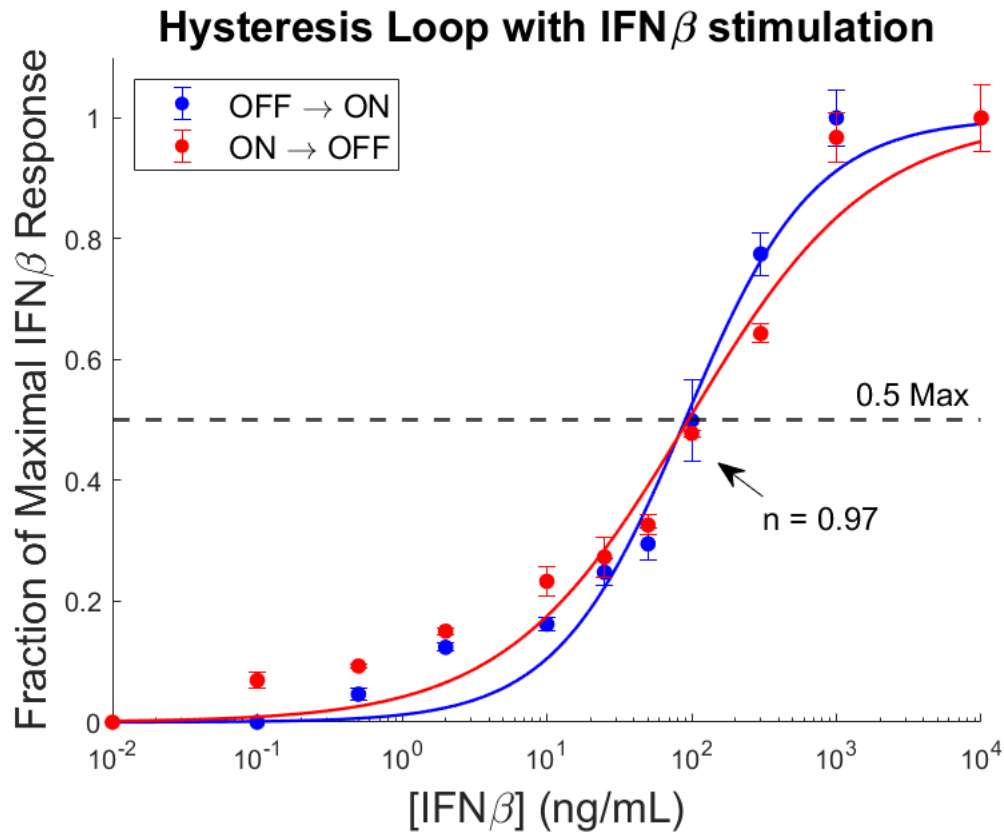
The rsquare values of the IL18 and cGAMP curves are 0.78 and 0.91, respectively. We also find that their periods of the IL18 and cGAMP curve fits are 2.28 and 2.53 hours, respectively. The lag-time between the curves is ~1.4 hours.

Probing role of IFNb and IFNAr signaling on hysteresis

We have now established that IL18 stimulation launches an intracellular bistable circuit in macrophages through mitochondrial fatty acid oxidation and cGAMP signaling. However, a confounding circuit is IFNa and IFNb signaling as both can potentially induce FAO. Let's first show whether the hysteresis from the IL18-induced circuit relies on IFNa signaling by giving an antagonist (IFNARi) against the IFNa receptor IFNAR.



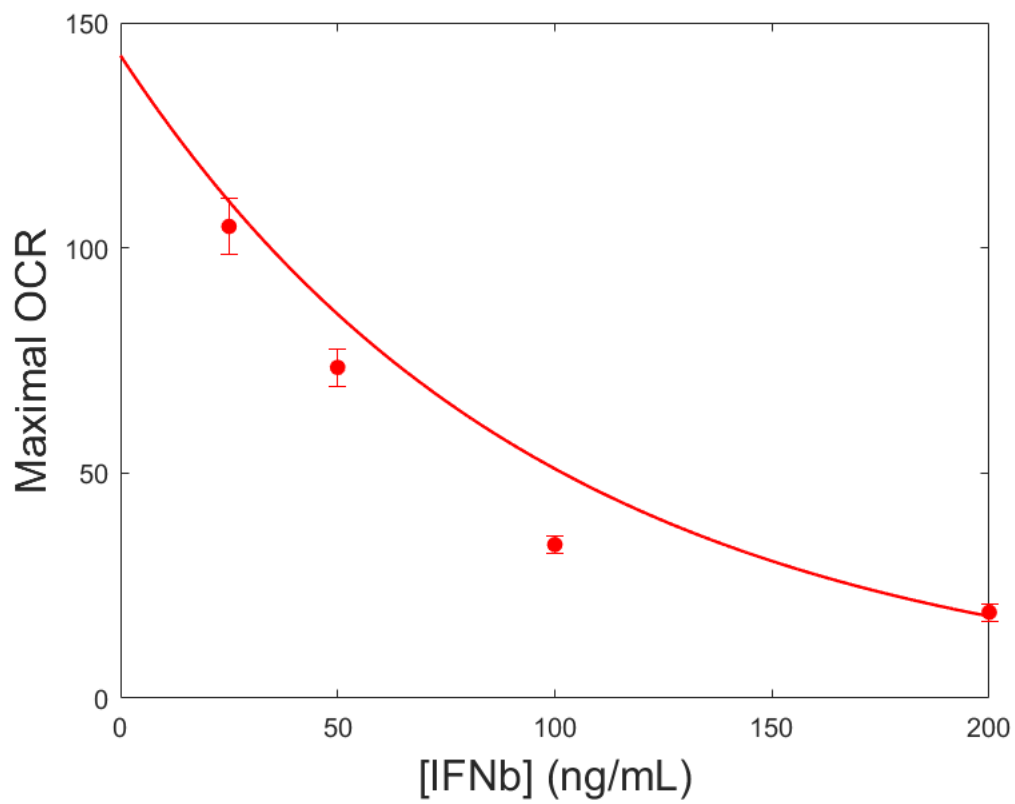
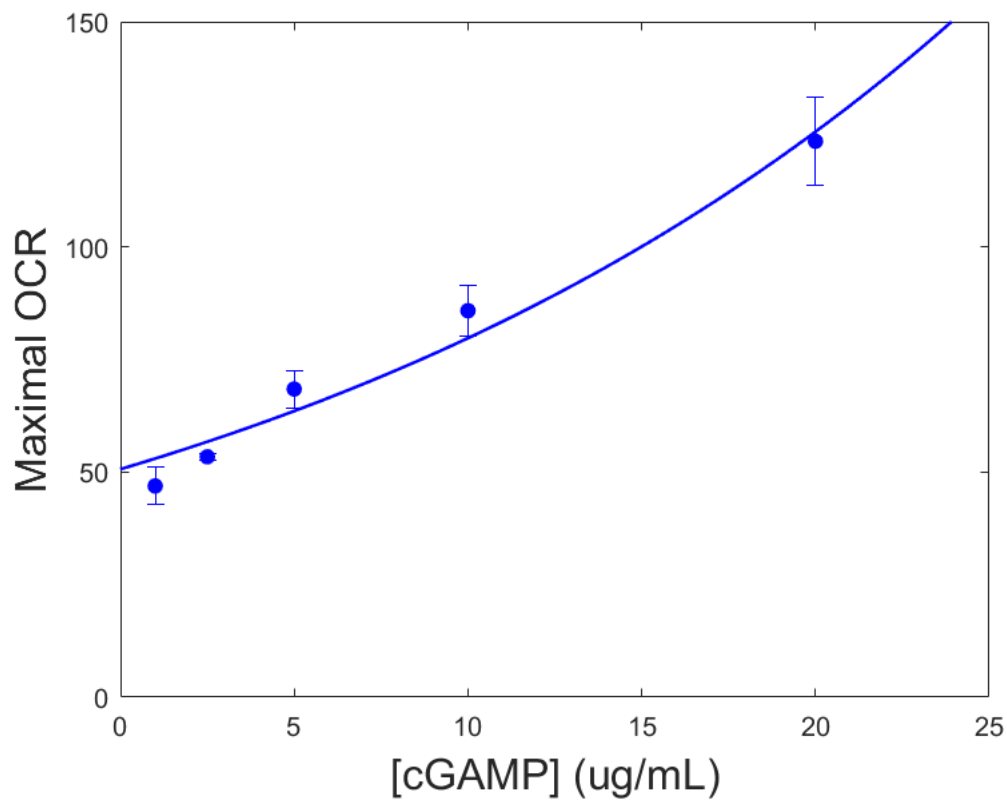
So we can see here that the IFNAR signaling is rather dispensable for the hysteresis exhibited by IL18's circuit. Now, can IFNb make a similar circuit?



So, we can see here that although IFN β stimulation does lead to elevated FAO (as seen with the increase in IFN β reporter signal), there is no hysteresis when IFN β is withdrawn. This is markedly different behavior in contrast to the hysteresis seen with IL18 stimulation, which implies that the IL18-induced circuit operates through a different feedback mechanism than the one used by IFN β stimulation.

Effect of cGAMP, IFN β on maximal OCR

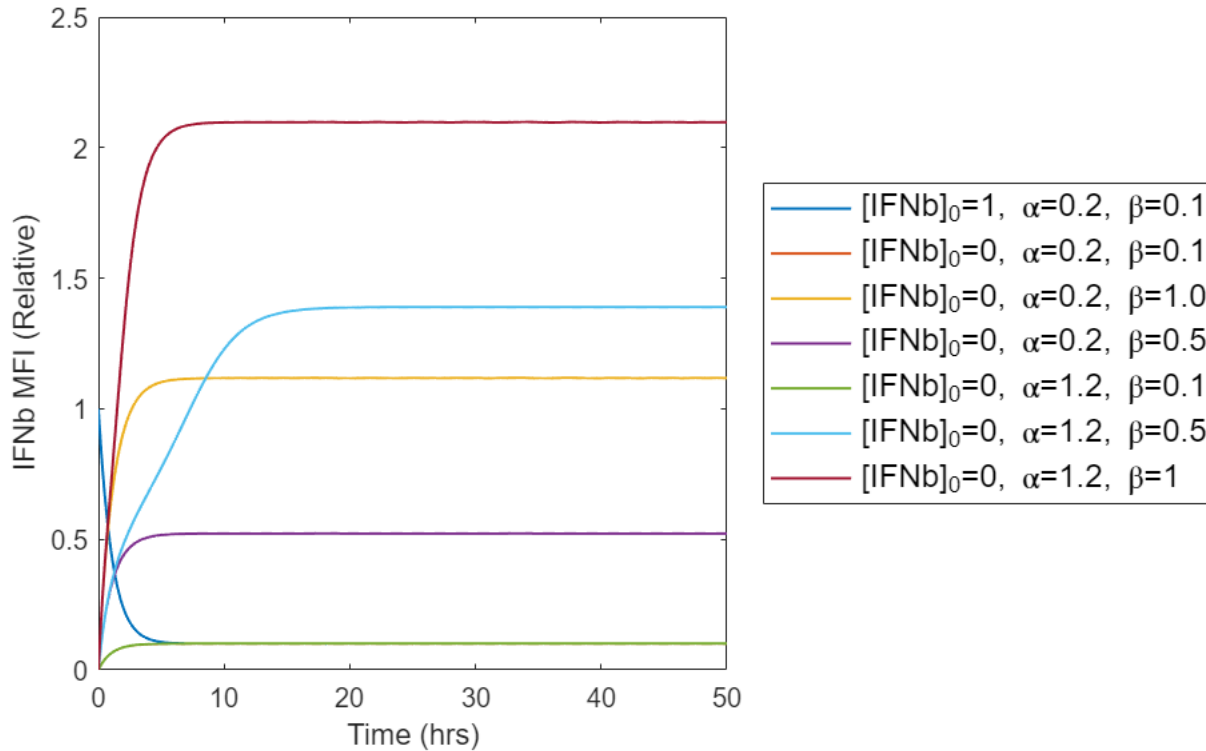
We found that cGAMP increases maximal OCR while IFN β decreases it on BMDMs.



As you can see, there is an exponential increase in OCR with cGAMP treatment and exponential decrease in OCR with IFNb treatment.

Fitting ODE model to experimental time-course

We want to estimate the parameters of the differential equation $\frac{dx}{dt} = \beta + \alpha \frac{x^n}{1 + x^n} - x$. Let's first visualize how it would look like



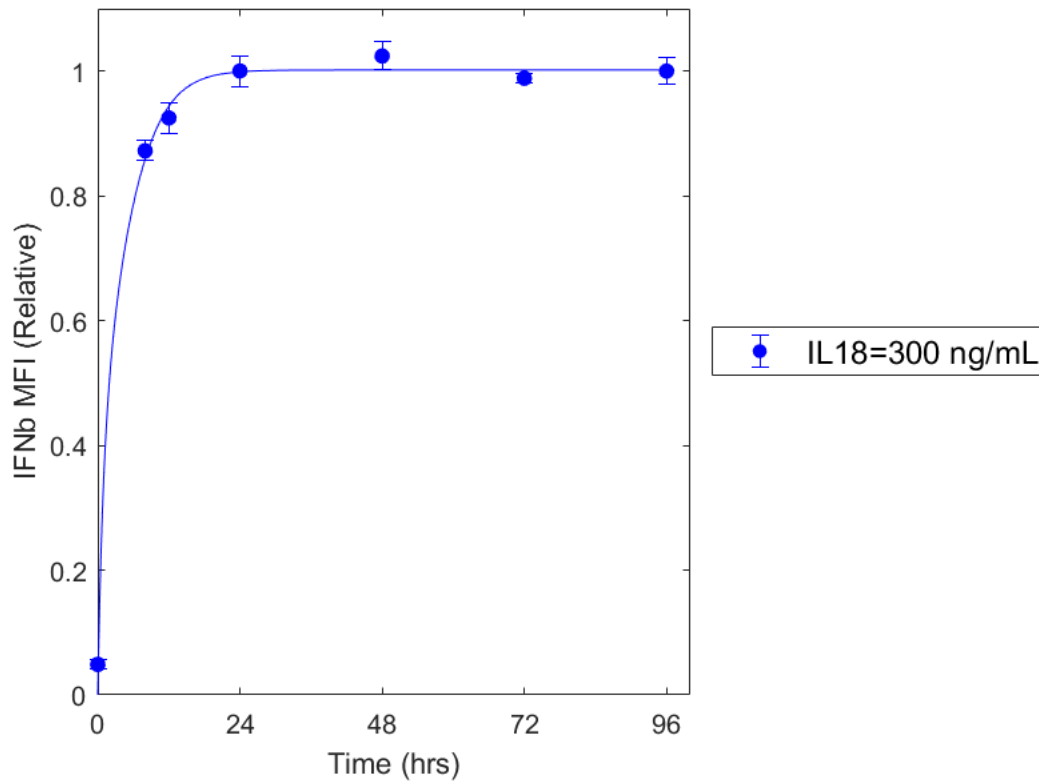
Now let's see if we have experimental data whether we can fit to the above equation

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Local minimum possible.

lsqcurvefit stopped because the final change in the sum of squares relative to its initial value is less than the value of the function tolerance.

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As we can see, the experimental data can be nicely fit to the differential equation $\frac{dx}{dt} = \beta + \alpha \frac{x^n}{1 + x^n} - x$ where $n = 3.2$ (as we previously determined when fitting the experimental data of sigmoidal activation to the Hill function). Namely, the β, α value pair is $[0.534, 0.932]$ for 300 ng/mL doses. Most importantly, the BMDMs reach maximal activation within 24 hours of IL-18 stimulation, thus validating that a steady-state is reached by 24 hours.