



Cyclic Attractors Are Critical for Macrophage Differentiation, Heterogeneity, and Plasticity

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Adaptability, heterogeneity, and plasticity are the hallmarks of macrophages. How these complex properties emerge from the molecular interactions is an open question. Thus, in this study we propose an actualized regulatory network of cytokines, signaling pathways, and transcription factors to survey the differentiation, heterogeneity, and plasticity of macrophages. The network recovers attractors, which in regulatory networks correspond to cell types, that correspond to M0, M1, M2a, M2b, M2c, M2d, M2-like, and IL-6 producing cells, including multiple cyclic attractors that are stable to perturbations. These cyclic attractors reproduce experimental observations and show that oscillations result from the structure of the network. We also study the effect of the environment in the differentiation and plasticity of macrophages, showing that the observed heterogeneity in macrophage populations is a result of the regulatory network and its interaction with the micro-environment. The macrophage regulatory network gives a mechanistic explanation to the heterogeneity and plasticity of macrophages seen *in vivo* and *in vitro*, and offers insights into the mechanism that allows the immune system to react to a complex dynamic environment.

Keywords: boolean network, macrophage, oscillation, cycles, differentiation, heterogeneity, plasticity, regulatory network

INTRODUCTION

The balance between inflammatory and anti-inflammatory immune responses is crucial to maintain homeostasis in the face of the diverse immune challenges an organism meets. Macrophages are cells essential to immunity. They recognize pathogens and pathogen-derived molecules, collaborate with other cells of the innate and adaptive immune system, and are critical players both in chronic inflammation and in tissue regeneration (Sica and Mantovani, 2012; Varol et al., 2015; Vannella and Wynn, 2017; Li et al., 2019; Locati et al., 2020). Macrophages are characterized by their diversity and plasticity. Depending on the signals received, non-polarized M0 macrophages can be polarized into two main types: classically activated macrophages or M1, characterized by a pro-inflammatory profile, and alternatively activated macrophages or M2, which promote proliferation and repair (Mendoza-Coronel and Ortega, 2017; Funes et al., 2018).

M0 macrophages are usually monocytes differentiated into M0 macrophages in the presence of GM-CSF that have not been exposed to any pro or anti-inflammatory stimulus or environment that promotes their activation, cytokine production, and functional polarization (Kumar, 2019). M1 polarization is generally triggered by the stimulation of TLRs, or by cytokines such as IFN γ and GM-CSF, which lead to high production of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-12, and IL-23, in addition to a low expression of IL-10 (Lehtonen et al., 2002; Park et al., 2009; Weber et al., 2010; At et al., 2011; Lawrence and Natoli, 2011; Liu et al., 2014; Bally et al., 2015; Funes et al., 2018; Hamilton, 2019; Wang et al., 2019; Petrina et al., 2021). M2 polarization has been subdivided into M2a, M2b, M2c, and M2d macrophages, due to diverse transcriptional programs and stimuli involved (Huang et al., 2018). The M2a macrophages are derived from M0 cells stimulated by IL-4 and IL-13, they release high levels of IL-10 and TGF- β using transcriptional factors (TFs) such as STAT6 and IRF4, and are involved in proliferation and tissue repair functions (Bouhlef et al., 2007; Chawla, 2010; Gordon and Martinez, 2010; Ma et al., 2015; Arora et al., 2018; Wang et al., 2019). A combination of TRL ligands generates M2b macrophages and immune complexes (IC) as well as IL-1R ligands, yielding both pro-inflammatory cytokines, such as IL-1 β , IL-6, TNF- α , and IL-12, and anti-inflammatory cytokines such as IL-10. The signaling pathways stimulated involve MAPKs, PI3K/Akt, and, ultimately, NF- κ B. M2b macrophages have a role in the regulation of inflammatory responses (Lucas et al., 2005; Park et al., 2009; Zhang et al., 2009; Luo et al., 2010; Weber et al., 2010; Foey, 2014; Liu et al., 2014; Bally et al., 2015; Wang et al., 2019). M2c macrophages arise upon IL-10 stimulation. They express high levels of IL-10, which induces the phosphorylation of STAT3, thus negatively regulating the production of pro-inflammatory cytokines (Hutchins et al., 2013; Ma et al., 2015; Wang et al., 2019). M2d macrophages are induced by the costimulation of the adenosine A2 receptor and TLR, expressing levels of IL-10 as well as IL-12, and characterized by presenting properties of tumor-associated macrophages that carry out angiogenesis and tumor progression (Leibovich et al., 2002; Grinberg et al., 2009; Park et al., 2009; Colin et al., 2014; Arora et al., 2018; Wang et al., 2019; Anders et al., 2021). However, these are not the only possible expression patterns, as variations have been found *in vitro* and *in vivo*.

Given the plasticity, heterogeneity, and adaptability of macrophages and their role in the immune system, it is important to understand their phenotypic landscape, the conditions in which they originate, and the possible transitions between subsets. Macrophage differentiation can be seen as a continuum between M1 and M2 phenotypes, where these cells can express different profiles and concentrations of cytokines, receptors, and transcription factors (Sica and Mantovani, 2012; Palma et al., 2018). At the same time, not all combinations of key molecules like IL-12, IL-10, IL-6, or VEGF are possible. For example, the IFN γ -induced and IL-4-induced programs inhibit each other in the cell, leading to heterogeneous populations in environments with

mixed signals (Munoz-Rojas et al., 2021). Furthermore, it is known that the signaling pathways have inhibitory mechanisms that lead to self-regulation, causing oscillations in the expression of cytokines like IL-6, which are expected as part of the physiological behavior of macrophages, but that can also act against the host in pathological scenarios (Wang et al., 2013).

Regulatory networks are a valuable tool to bridge the molecular regulation of a cell with its phenotype and have been used to study the differentiation of hematopoietic cells (Saez-Rodriguez et al., 2007; Naldi et al., 2010; Martinez-Sanchez et al., 2015; Liquitaya-Montiel and Mendoza, 2018; Ramirez and Mendoza, 2018; Palma et al., 2018; Ramirez et al., 2019; Avila-Ponce de León et al., 2021) and the plasticity of macrophages (Palma et al., 2018; Ramirez et al., 2019; Avila-Ponce de León et al., 2021). Boolean networks integrate qualitative data about the interactions between cytokines, signaling pathways, and transcription factors to predict differentiation and plasticity. They allow testing different hypotheses and determine how the regulatory structure impacts complex cellular behaviors, all of this, with only a few parameters (Kauffman, 1969; Albert and Thakar, 2014).

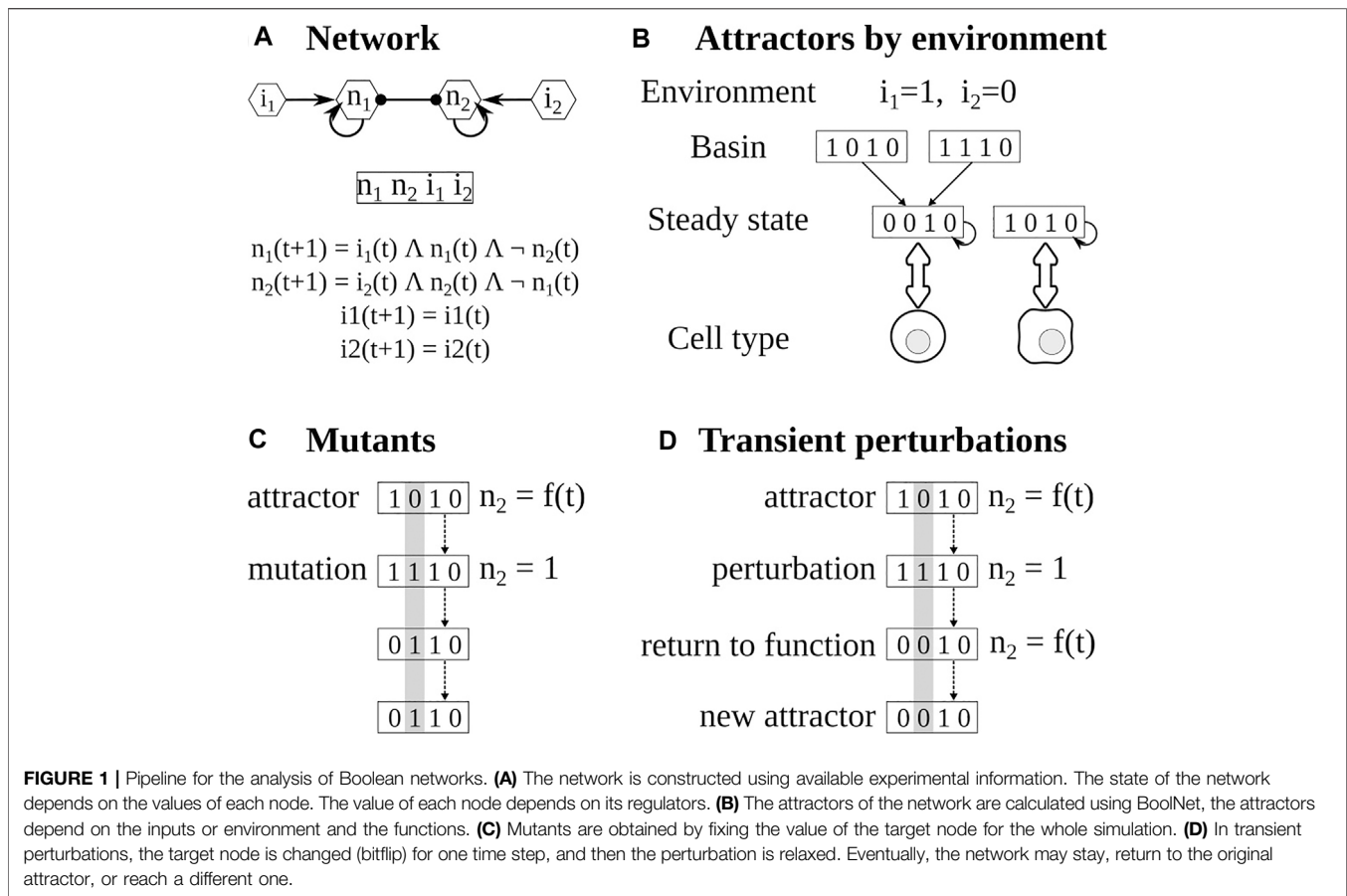
This paper presents a Boolean model of the regulatory network that underlies macrophage differentiation, extending previous approaches (Palma et al., 2018; Ramirez et al., 2019). The model recovers the M0, M1, M2a, M2b, M2c, M2d, and other M2-like cell types, including several cyclic attractors that reproduce known experimental data. Then, we use the model to study how the classic polarizing environments and mixed combinations of extrinsic signals affect the stability of these cells. We show that the plasticity, heterogeneity, adaptability, and variable levels of expression of key cytokines in macrophages result from the structure of the regulatory network.

MATERIALS AND METHODS

All the datasets, scripts, tables, and images used in this study can be found in the repository https://github.com/mar-esther23/Macrophage_Differentiation.

A Boolean network consists of nodes representing molecular components (i.e., cytokines, signaling pathways, transcription factors) and edges representing the interactions between them. The value of the nodes is a discrete variable: one if the node is functional and 0 if it is not functional. The value of a node i at the time $t+1$ depends on the value of its regulators at time t , according to a logical function that recapitulates available biological information. The state of the network at $x(t)$ depends on the values of all its nodes and will evolve through time as the regulatory functions are evaluated. Eventually, the system will arrive at an attractor, which corresponds to a cell type. These attractors can be steady states when $x_t = x_{t+1}$ or cycles when $x_t = x_{t+\tau}$ (Kauffman, 1969; Albert and Thakar, 2014).

We constructed the macrophage regulatory network according to previous models (Palma et al., 2018) and available information (Leibovich et al., 2002; Yoshimura et al., 2007; Chang et al., 2013; Wang et al., 2013; Liu et al.,



2014; Wilson, 2014) among others which can be seen in (Supplementary Table S1, Figure 1A). The dynamical analysis of the network was done using the packages BoolNet (Mussel et al., 2010) and BoolNetPerturb (Martinez-Sanchez et al., 2018). Synchronous updating was used in all simulations.

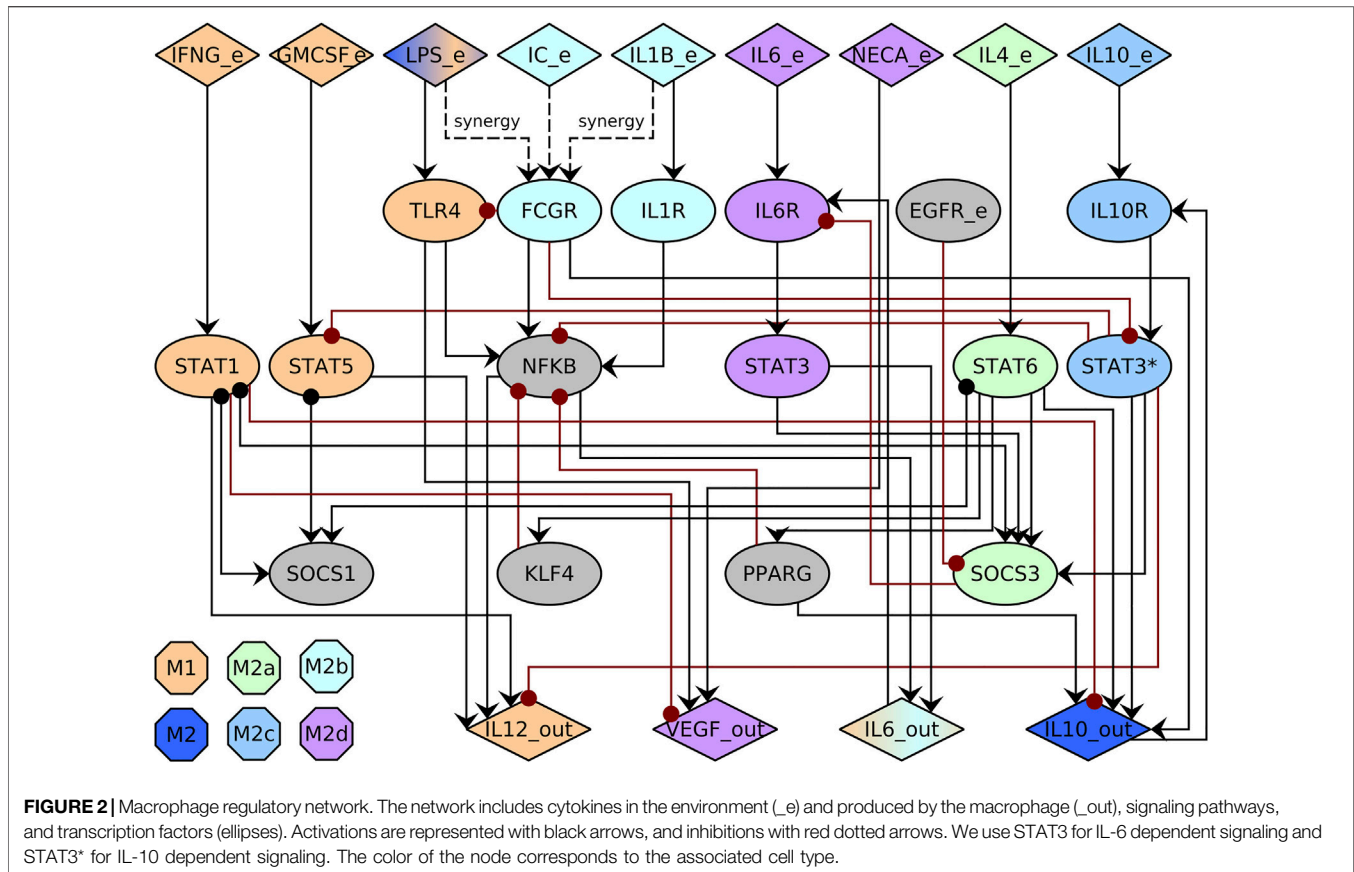
We determined attractors of the network and classified them depending on the expression of both the characteristic transcription factor and cytokine. In every case, we focused on the presence and absence of the nodes that correspond to common cell type markers, and ignored the value of the other nodes (Kauffman, 1969; Albert and Thakar, 2014; Martinez-Sanchez et al., 2015). The basin of attraction of a network is the set of states that lead to an attractor (steady state or cycle) in the simulation.

To determine the effect of the micro-environment, we first determined the cytokines present in each polarizing environment (Kauffman, 1969; Albert and Thakar, 2014; Martinez-Sanchez et al., 2015), then, we fixed the corresponding input nodes according to the cytokines present or absent in that environment and then determined the resulting attractors (Figure 1B). This is modeled by changing the input function $i_{t+1} = i_t$ to $i_{t+1} = 0$ or $i_{t+1} = 1$ depending on the presence or absence of the cytokines in the environment.

To further verify the model, we simulated the knock-out and overexpression of target nodes by setting their values to 0 or 1

and comparing the resulting attractors with known mutants (Kauffman, 1969; Albert and Thakar, 2014; Martinez-Sanchez et al., 2015) (Figure 1C). Furthermore, we checked that the attractors found with synchronous updating could be found using asynchronous updating.

The expression pattern of a cell can change in response to changes in both internal and environmental factors. We focused on the effect of small transient perturbations. For example, due to stochastic effects, a transcription factor may not be activated because the polymerase fails to bind to its DNA sequence for a time, even if the rest of the regulators are present. This can be modeled as the corresponding node having a value of zero for a time step, and then the perturbation will be relaxed and the node will acquire a new value depending on its regulators (Figure 1D). On the other hand, a cell may be subjected to a small peak of a cytokine in its environment. This can be modeled as the extrinsic cytokine node having a value of one for a time step and then returning to its original value. The attractor of the system, which corresponds to the cell type, may change or not depending on the regulatory network, the original state of the network, and the perturbed node. To study the stability and plasticity of the system for each microenvironment we took its attractors and modified for one time step the value of the node (bitflip), then the perturbation was relaxed, and the resulting attractor was determined (Martinez-Sanchez et al., 2018, 2015).



RESULTS

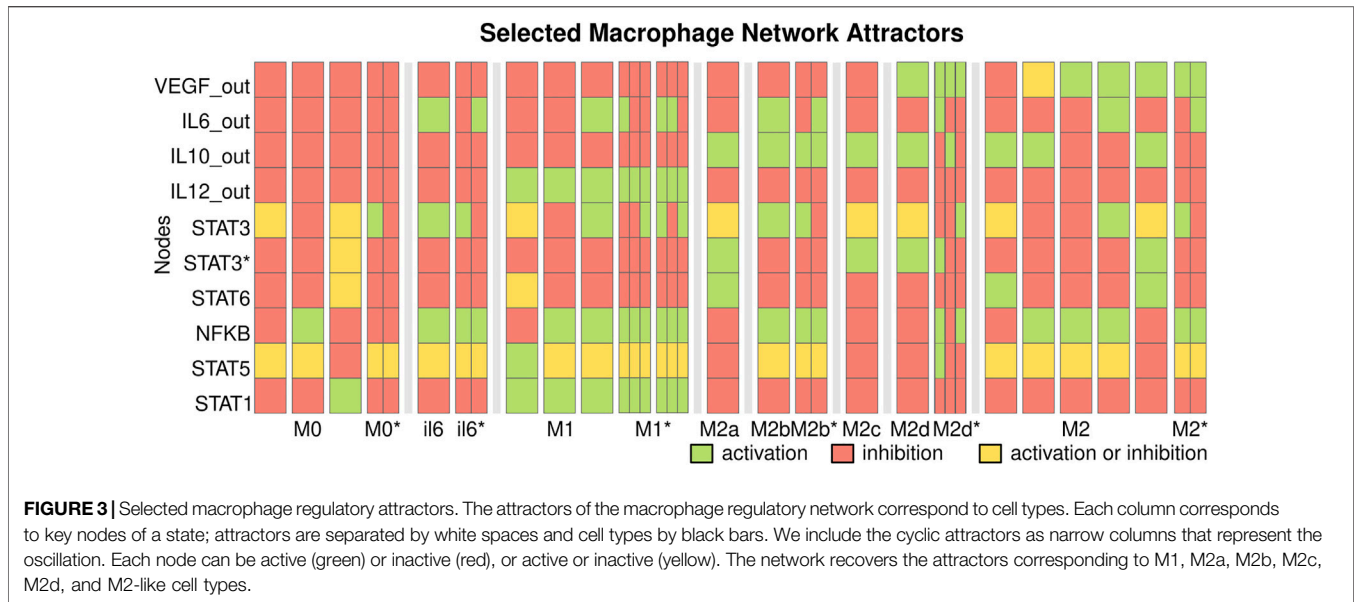
Macrophage Differentiation Patterns Emerge From Feedback Between Transcription Factors, Cytokines, and Signaling Pathways

We expanded the previously published macrophage regulatory networks (Palma et al., 2018; Ramirez et al., 2019). In this network, we included multiple molecules like transcription factors, STAT proteins, cytokine receptors, SOCS proteins, and cytokines, among others. We only included direct interactions that have been experimentally validated (**Supplementary Table S1**, **Supplementary Table S2**) to include IL6 (Chang et al., 2013; Wang et al., 2013; Liu et al., 2014; Wilson, 2014), NECA (Leibovich et al., 2002), EGFR (Wang et al., 2013), and SOCS3 (Yoshimura et al., 2007; Wang et al., 2013; Wilson, 2014). Then, we simplified the network using GINSIM (Gonzalez et al., 2006). The resulting network has 29 nodes and 52 interactions (**Figure 2**, **Supplementary Table S3**). We assumed that different pathways mediate IL-6 and IL-10 signaling by STAT3 and marked them as STAT3 for IL-6 dependent signaling and STAT3* for IL-10 dependent signaling. The state of a node represents whether the biological component is active (1) or inactive (0). A node is active if it can alter the regulation of other nodes.

Then, we determine the macrophage cell types by calculating the attractors, steady states, and cycles of the network (Kauffman, 1969) and label them. An attractor corresponds to a cell type if the characteristic signaling pathways, transcription factors, and produced cytokines are present (**Supplementary Table S4**). The network recovers 44 steady-state attractors and 358 cyclic attractors of size 2, 3, and 6 which correspond to 'M1', 'M2b', 'M2a', 'M2c', 'M2d', 'M2' (M2-like), 'M0', and 'il6' cell types (**Figure 3**, **Supplementary Figure S2**, **Supplementary Table S5**).

M1 macrophages produce IL-12 and may produce IL-6 and activate the pathways for STAT1, STAT5, or NFKB. M0 and cyclic M0* macrophages do not produce any cytokines and correspond to naive macrophages. They are usually found in simulated environments with neither extrinsic cytokines nor contradictory extrinsic signals that inhibit each other through SOCS proteins. The attractors labeled 'il6' produce IL-6 but no other extrinsic cytokines. The steady states il6 macrophages are only found when there is EGFR_e in the microenvironment and may correspond to inflammatory pathogenic states as those seen in cancer (Wang et al., 2013) and severe COVID-19 (Matsuyama et al., 2020; Merad and Martin, 2020). In contrast, the cyclic il6* attractors may correspond to pathogenic states or be non-fully differentiated macrophages.

M2 macrophages produce IL-10 or VEGF, and they can be classified into different subtypes depending on the cytokines produced and active signaling pathways. The M2a subtype



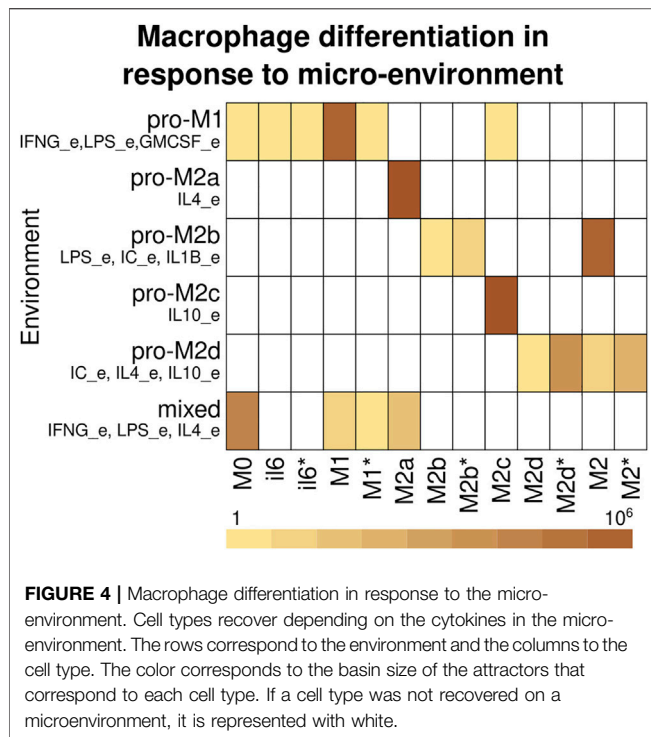
produces IL-10 and activates the STAT6 pathway, M2b produces IL-10 and IL-6, M2c produces IL-10 and activates the IL-10 dependent STAT3* pathway, M2d macrophages produce VEGF, IL-10 and no IL-12. The model recovers steady states corresponding to these cell types, including cyclic attractors for M2b with an IL-6 dependent STAT3 oscillation. We also recover M2-like subsets that produce IL-10 and VEGF or IL-6.

Most cyclic attractors present oscillations in the IL6/STAT3/SOCS3 circuit, which may affect the downstream production of IL6_out. The self-inhibition of the IL6 pathway causes these oscillations: STAT3 induces SOCS3 expression, which inhibits IL6R and STAT3 phosphorylation, causing a repressed circuit and oscillations. The IL10/STAT3* pathway also presents oscillations that may affect SOCS1, NFKB, and STAT5. These oscillations can be caused by inhibition by other pathways, for example, IL6. The self-inhibition caused by SOCS3 and the other cycles may have a role in limiting the production of IL-6 by macrophages and the associated hyperinflammation. Given the hypothesis that macrophage differentiation is a continuum (Sica and Mantovani, 2012; Palma et al., 2018) these M2-like and cyclic states may be a mechanism to regulate the production of cytokines by macrophages.

The number of attractors associated with a cell type does not necessarily correspond to the number of states that reach the attractors of that cell type (basin of attraction). The biggest basin is M0, followed by M2, M2a, and M1, while il6, M0*, and il6* had the smaller basins. In general, the cell types labeled as cyclic (*) had smaller basins.

To validate the model we verified whether the attractors were robust to asynchronous updating. All the steady states were robust to the change in update schema. While most of the attractors of sizes 2 and 3 were unstable we found asynchronous attractors of size 6 or more that correspond to M0*, il6*, M2b*, and M2*, but lost the M1* and M2d*.

To further validate the model, we compared the knock-out and over-expression simulations with experimental data (Supplementary Figure S3, Supplementary Table S6). In general, the predictions made by the model correspond to the observed biological data (Supplementary Table S7). For instance, in STAT1-null macrophages stimulated with IFN- γ and Pam3CSK4, a dose-dependent decrease in IL-12 has been experimentally observed compared to wild-type macrophages (Kim et al., 2015). This phenomenon is recovered by a network simulation of a STAT1 knock-out, where we see that attractors completely lose IL-12 production, causing the disappearance of M1. In the same way, it has been experimentally obtained that inhibition of PPAR γ -dependent gene expression significantly decreases the production of IL-10 mediated by LPS, which is recovered in the simulations in which a knock-out of PPAR γ was set, obtaining a decrease in IL-10-producing attractors (Majai et al., 2007). Furthermore, regarding overexpression, there is the experimental case where *in vitro* STAT6 has been overexpressed, causing a promotion of M2 macrophages (Gong et al., 2017). An overexpression simulation in STAT6 recovers this last, which causes a higher proportion of M2 attractors and a decrease in M1. However, these simulations do not recover the expected behavior in the case of the NF κ B mutant. The NF- κ B pathway is a highly complex protein, but our network simplifies it to a single node, so this discrepancy is probably the result of the modeling decisions. In this mutations experiments, the most stable states were the M0*, il6*, and M2d macrophages. On the other hand, the more sensitive cell types are M0 and M1. The nodes that tended to cause more changes in differentiation if mutated are IL12_out and IL10_out, which affect the cytokine profile, followed by STAT1, STAT6, and IL-10 mediated STAT3 activation (STAT3*). Furthermore, this analysis predicts the effect of knock-out and over-expression mutants that have not been tried experimentally.



Role of the Micro-Environment in Macrophage Differentiation and Stability

Macrophage differentiation does not occur in a vacuum but in response to the micro-environmental signals (**Supplementary Table S8**). To determine the role of the microenvironment, we determined the attractors associated with a cell type and their combined basin size in different microenvironments (**Figure 4**, **Supplementary Table S9**). The pro-M1 microenvironment contains IFNG_e, GMCSF_e, and LPS_e, and the model mainly recovers the presence of M1 attractors with a small number of M1*, M0, il6, il6* attractors. The pro-M2a environment contains IL4_e, and the model recovers only M2a attractors. The pro-M2b environment contains LPS_e, IC_e, and IL1B_e, and the model recovers M2, M2b, and M2b*. The pro-M2c environment contains IL10_e, and the model recovers only M2c attractors. The pro-M2d environment contains IC_e, IL4_e, and IL10_e, and the model recovers M2d, M2d*, M2, and M2* attractors. The mixed environment contains LPS_e, IFNG_e, and IL4_e, which are associated with M1 and M2a polarization, and recovers M0, M1, M1*, and M2a attractors, which are in accordance with the heterogeneity observed in macrophage populations subjected to *in vitro* co-stimulation with these same cues (Munoz-Rojas et al., 2021).

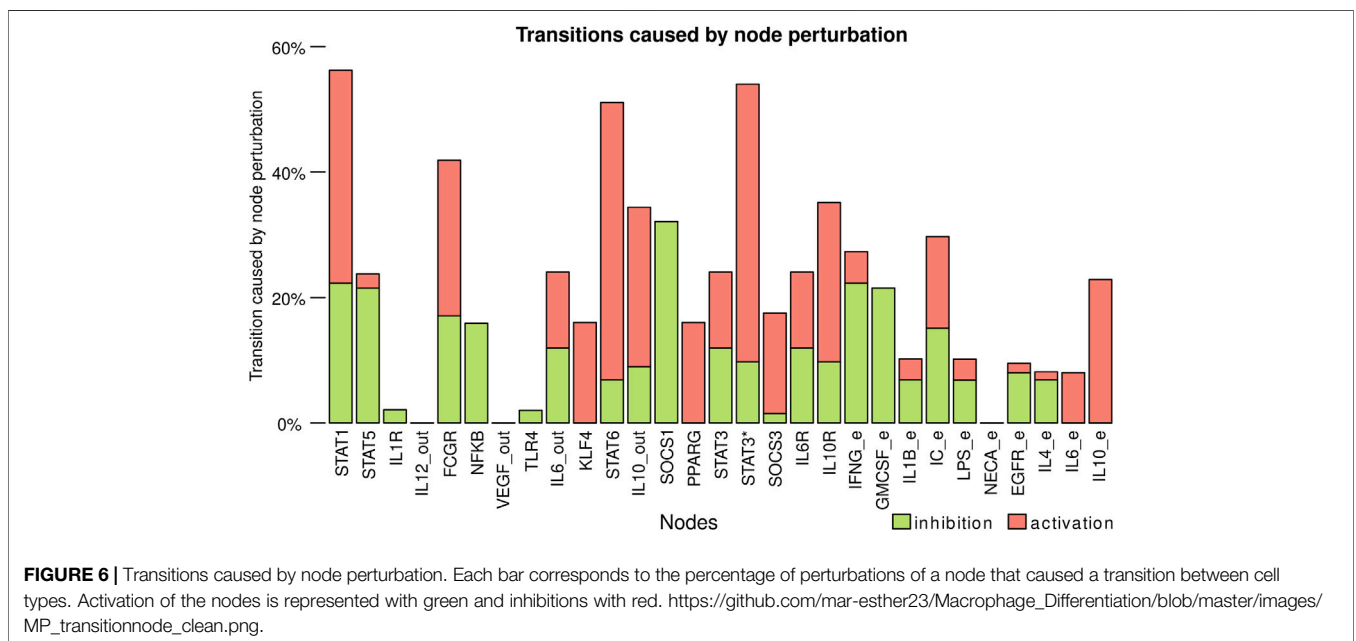
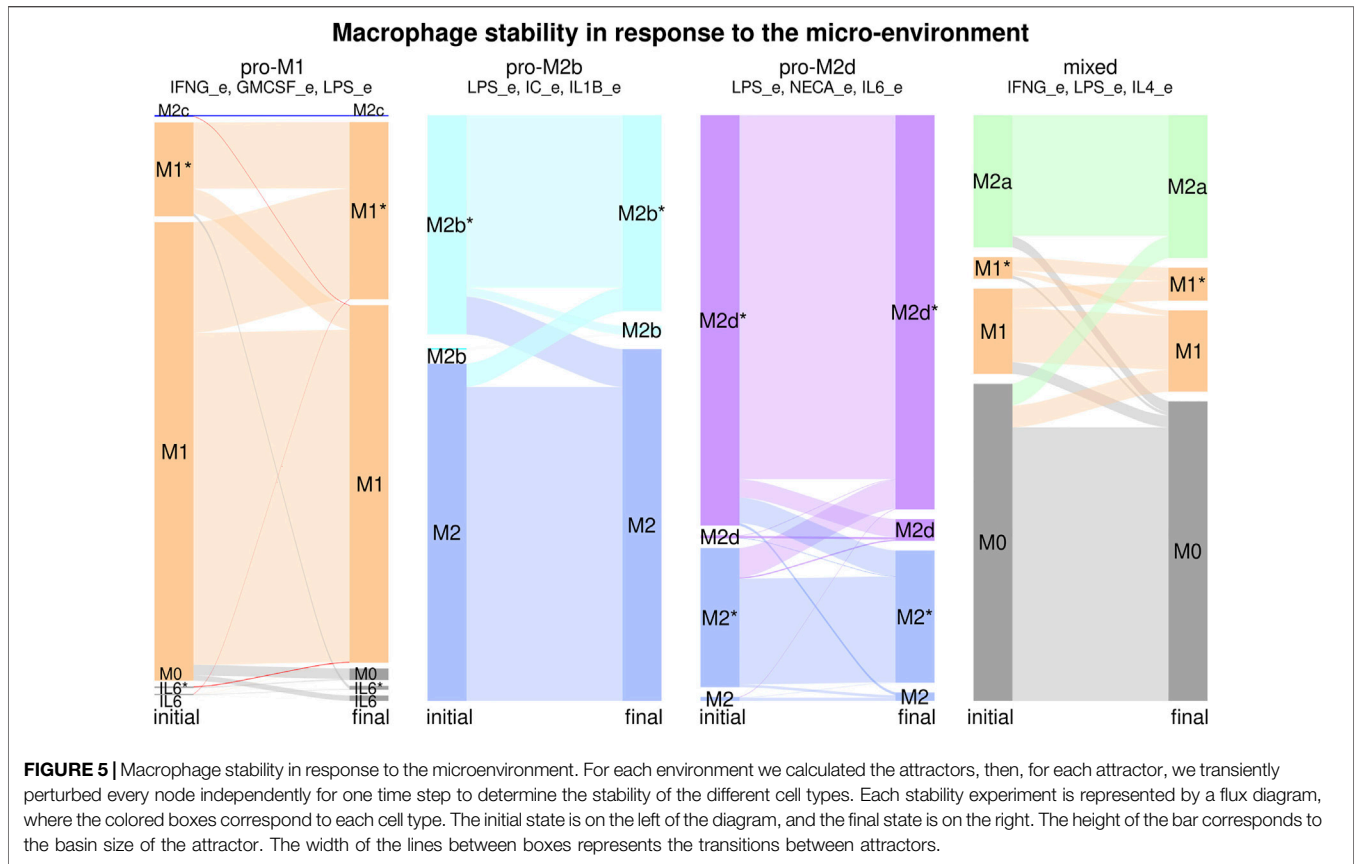
Macrophage differentiation is not a wholly deterministic process, but it can be affected by transient changes in the environment, stochastic noise during transcription, translation and signaling events, and other types of noise. Furthermore, the environment and the internal state of the cell can have small changes in response to the progress of a pathological state. To study this for each of the six environments, we took the recovered

attractors and perturbed each node one by a time step, and determined if that changed the resulting attractor and cell type (**Figure 5**, **Supplementary Table S10**). In the pro-M1 environment, most of the transitions are between M1 and M1*, with a bias towards the cyclic M1* attractors. Most M1* attractors have oscillations in the IL6/STAT3/SOCS3 pathway, and some of them have oscillations in IL6_out. There is a small number of transitions towards M0 and il6/il6* that increase in percentage, which may have a role *in vivo* by limiting the production of IL-12 as the infection progresses toward resolution. In the pro-M2b environment, there is a small number of transitions between M2 and M2b/M2b*, with a slight bias towards M2. In the pro-M2d environment, there are also transitions between M2/M2* and M2d/M2d*. In the pro-M2a and pro-M2c environments, there are only M2a and M2c attractors, so these are stable. In the mixed environment, there are transitions from M0 to and from all differentiated environments but not between M1 and M2a attractors, which may indicate that the plasticity between these cell types requires longer signals, especially in mixed environments, and that a temporal cease of cytokine production precedes a transition between M1 and M2 cell types.

In general, we can say that each microenvironment favors the differentiation and stability of the associated cell type, even in some cases where there is a small number of attractors associated with an additional cell type. The exceptions are the pro-M2b and pro-M2d environments, where there is a strong presence of M2-like attractors; however, this can be seen as part of the phenotypic plasticity of macrophages. If we consider all possible combinations of cytokines, most of the cell types were highly stable, with only a small proportion of transitions between subsets. The cyclic attractors associated with a cell type were highly stable, as the oscillations seem to be the result of the network topology and not a dynamical artifact.

To determine the key nodes for the dynamic stability of the model, we determined which nodes caused more changes between cell types when transiently perturbed (**Figure 6**, **Supplementary Table S10**). The nodes that caused more transitions between cell types were STAT1, IL-10 mediated STAT3*, and STAT6, which are associated with the signaling pathways of key cytokines in macrophage differentiation. IC_e and FCGR also had an essential role in the stability of the model, as they regulate both NFkB, STAT3*, and IL10_out. STAT1, STAT3*, STAT6, and FCGR have a higher number of out-going edges and directly or indirectly modulate the activation and inhibition of different circuits of the network. The activation of SOCS1 also has a relevant role, as its activation inhibits the STAT1, STAT5, and STAT6 nodes. The nodes that cause fewer transitions between cell types are IL12_out, VEGF_out, NECA_e, IL1R, and TLR4.

In general, of the single state transient perturbations 21% resulted in a change of cell type and 2.96% were transitions between M1 and M2 states. We also simulated all possible double node perturbations 34.44% resulted in a change of cell type and 5.02% were transitions between M1 and M2 states. We also realized a Derrida curve (Derrida and Pomeau, 1986) to determine how sensible the system was to perturbations in the



value of the states. For perturbations of Hamming distance 1 on average the change was of 4.18 nodes and increased towards 8.47 nodes on average as the number of perturbed nodes increased

(Supplementary Figure S3). It is worth taking into account that the high number of input nodes, that represent the micro environment, heavily influence these results. For example, on a

micro-environment with only M2 attractors it is impossible for there to be a transition towards M1, as the cell type is not stable. Furthermore, not all possible micro-environments can be found *in vivo*, which implies that while a transition may be possible in the system it might not be observed *in vivo*.

DISCUSSION

In this paper, we propose an actualized regulatory network of cytokines, signaling pathways, and transcription factors to study the differentiation, heterogeneity, and plasticity of macrophages. This network allows us to give a mechanistic explanation of the dynamic behavior of these cells in response to different micro-environments. Furthermore, the network recovers multiple cyclic attractors that are both stable to perturbations and in accordance with previous experimental observations (Wang et al., 2013), showing that these oscillations are the result of the structure of the network and suggesting that they may have a biological function. In fact, the biological relevance of oscillatory behavior seems to lie in exquisitely regulated phenomena like the activation and nuclear translocation of NF κ B. Cheng et al. (2021) recently demonstrated that macrophages respond to different proinflammatory micro-environments with oscillatory or non-oscillatory activity of NF κ B. ChIP-seq data and computational modeling analysis reveal that NF κ B presents oscillatory behavior in the majority of stimulated cells, however, only non-oscillatory behavior leads to sufficiently prolonged chromatin accessibility, favoring gene expression. This phenomenon is regulated by the NF κ B inhibitor I κ B α , which may allow sensing of the micro-environment while refraining the cell from secreting inflammatory mediators until the concentration of a certain cue reaches a specific threshold. All of it without changing the cell phenotype. Therefore, we can also speculate that inflammatory disorders may arise or be sustained by macrophages whose oscillation is skewed towards non-oscillatory behavior. The macrophage regulatory network recovers steady state and cyclic attractors that correspond to M0, M1, M2b, M2d, M2-like, and IL-6 producing macrophages. Cyclic attractors represent 89% of the total attractors, but their combined basins of attraction are only 11% of the total state space. However, it is worth noting that most of these attractors are stable, and when perturbed, most perturbations lead to a cycle of the same cell type. Oscillatory activation of STAT3 with its downstream effect in IL-6 production in macrophages has been previously reported (Wang et al., 2013). The oscillations in the macrophage regulatory network are the result of the IL-6R/STAT3/SOCS3 pathway, and the crosstalk with other signaling pathways, like those mediating the activation of STAT3* via STAT6, STAT5, or IL-10, which are circuits commonly observed in immune cell regulatory networks.

In vivo and *in vitro* macrophages express marker molecules and cytokines in an expression range, which can be observed in a flow cytometric analysis as the spread of the population on a dot plot or the width of a histogram (Munoz-Rojas et al., 2021). The expression range can vary depending on the cell type, the molecule being measured, and the pathological state. For

example, the levels of IL-6 produced by macrophages in cancer and COVID-19 are associated with the severity of the disease (Wang et al., 2013; Matsuyama et al., 2020; Merad and Martin, 2020). How do macrophages, and other immune cells, generate and regulate the expression range is an open question. Oscillations, and their associated cyclic attractors, could be a mechanism to create variability in the expression range of a molecule. For example, when averaged over time, the oscillations in STAT3 activation, and their downstream targets in M1 macrophages could create diverse expression levels that depend on the structure of the network. These oscillatory circuits could be further finely tuned by other mechanisms like the induction of signaling pathways, transcriptional regulation or stochastic effects like variations in local cytokine concentration, noise in signaling pathways, transcription factors binding, etc. Cyclic attractors are usually ignored when studying Boolean dynamics in hematopoiesis (Alvarez-Buylla Rocas et al., 2018), but the relevance of the oscillatory dynamics *in vivo* and in this network (Wang et al., 2013; Munoz-Rojas et al., 2021) indicates that more methods should be developed to study cyclic attractors and determine when and how they have a functional role.

The macrophage regulatory network also allows us to study the effect of the environment on the heterogeneity and plasticity of macrophages. All the polarizing environments favor the attractor associated with it, for example, in a pro-M2a environment, we found M2a attractors. At the same time, in most of the environments we studied (pro-M1, pro-M2b, pro-M2d, and mixed), there was more than one possible cell type, which implies that the heterogeneity in macrophages populations is a result of the regulatory network. This was especially notable in the mixed environment (LPS + IFN γ + IL4), where we recover M0, M1, and M2a macrophages (Munoz-Rojas et al., 2021). The specific differentiation pathway a cell follows is also a result of the regulatory network, the internal state of the cell, and stochastic events. Studying the basins of attraction of the different cell types and their sensibility to stochastic events may allow us to understand the heterogeneity of macrophage populations better. For example, *in vitro* stimulation with a combination of LPS, IFN- γ and IL-4 produces heterogeneous populations with M1 and M2 sub-populations (Munoz-Rojas et al., 2021). In this study Muñoz-Rojas et al. use a combination of molecular and cell biology techniques, including single-cell RNA sequencing (scRNA-seq), to ascertain the global transcriptional programs that lead to the observed heterogeneity. Similar results have been observed *in vivo*, where scRNA-seq of macrophage populations has also shown the coexistence of two clearly defined subpopulations in adipose tissue that do not follow the classic M1/M2 paradigm and whose proportions vary depending on the micro-environment (Grosjean et al., 2021). The differentiation of each individual cell depends on the initial state of the cell (transcription factors expressed and active signaling pathways) and stochastic events (local cytokine concentration, noise in signaling pathways, transcription factors binding, etc.), which generates an initial variability. Such variability determines which pathways of the regulatory network activate and which to inhibit, to polarize the individual cells into clearly defined

subpopulations, thus maintaining a heterogeneous population. These results coincide with our findings that in mixed environments M0, M1, and M2 macrophages coexist, implying that the design and performance of our network are appropriate to recover the outcomes of complex scenarios reported *in vivo* and *in vitro* after extensive analyses.

The model also allowed us to study macrophage plasticity. The environment determines plasticity because it limits the accessible cell types and modulates the effect of perturbations. In general, most perturbations did not cause changes in the labeled cell type. However, transitions between a steady state and a cyclic attractor of the same cell type were common, which means that cells that may be classified as the same cell type given their membrane markers may have different internal states, creating a hidden source of heterogeneity to respond to changes in the micro-environment. In polarizing environments (pro-M1 and pro-M2), most of the transitions were towards the favored cell type. There was a high level of transitions among the different subtypes of M2 attractors but limited transitions towards M1. This seems to indicate that M2 attractors are more closely related to one another than to M1 attractors, fine tuning their regulatory activity and creating a continuum of M2-like states. The multiple inhibitions between M1 and M2 transcriptional programs help the system maintain a stable inflammatory or regulatory program, making these the two poles of macrophage differentiation. This is especially relevant when taking into account the key role of macrophage differentiation and plasticity in COVID-19 and cancer (Wang et al., 2013; Li et al., 2019; Matsuyama et al., 2020; Merad and Martin, 2020). Perturbations that favor M2 macrophages can favor transitions towards more aggressive cancers, even in situations where perturbing the cancer cells may not be enough to change the steady state behavior of the system (Li et al., 2019). This seems to imply that there are a series of feedback loops between the tissues, the environment, and immune cell populations that are crucial to understanding complex diseases. Understanding these feedback loops will require us to conceive disease as a system where the cytokine and cellular environment play a key role.

The mixed environment (LPS + IFN- γ + IL4) had a high number of non-differentiated M0 attractors, because the mixed signals most likely inhibited each other, as reported by Munoz-Rojas et al. (2021), where LPS + IFN- γ , and IL-4 give rise to orthogonal global transcriptional programs. We observed only M0-M1 and M0-M2a transitions but no M1-M2a direct transitions. M1-M2a transitions are possible if they pass through an intermediate M0-like state with no cytokine production and require more than one perturbation. Such a phenomenon was indeed described by Tarique et al. (2015) for LPS + IFN- γ (M1), and IL-4 + IL-13 (M2) polarized macrophages, where the depletion of cytokines in the culture medium causes the cells to revert to the M0 phenotype, and by supplying the appropriate stimuli the macrophages can be re-polarized to the alternative phenotype. This could be a mechanism to warrant stability in the different cell types while allowing for plasticity if the environment changes past a certain time threshold. It also shows once more the power of experimental recapitulation of our network.

Traditionally, differentiation of hematopoietic cells has been considered a hierarchical process with clear differentiation pathways and well-defined cellular types, for example, M1 and M2 macrophages. However, as our understanding of macrophages in particular, and immune cells in general, has advanced, it is becoming increasingly clear that this is a highly dynamic process. Attempts to classify macrophages in subpopulations have proven intricate, as they seem to be both a continuum and a heterogeneous mix of subpopulations that do not always coincide with the M1/M2 paradigm (Sica and Mantovani, 2012; Wang et al., 2013; Mendoza-Coronel and Ortega, 2017; Grosjean et al., 2021; Munoz-Rojas et al., 2021). The analysis of our regulatory network suggests that there are independent circuits composed of receptors, transcription factors, and cytokines that activate in response to the signals in the environment. Some of these circuits inhibit each other (IFN- γ and IL-10), others are mostly independent (VEGF and IL-10), and others have more complex relationships (IL-6). Furthermore, these circuits can have dynamically stable oscillations, which affect not only the production of downstream cytokines, but also the crosstalk with other pathways. We propose that, on the one hand, when the circuits inhibit each other, we can expect a clear separation in the expression levels of the molecules involved (IL-12 and IL-10) and almost no plasticity, which creates pseudo-populations for those specific markers. On the other hand, when the circuits are independent or modulate each other in context-specific ways, the result is a continuum of expression for those markers, as seen in the M2-like family of attractors. In this case, given that the circuits are mostly independent, we should expect a higher level of “plasticity” as the circuits are activated or inhibited depending on the environmental signals. These circuits are further modulated by the microenvironment, the initial state of the cell, and stochastic effects. Focusing on the active regulatory circuits could give us a framework to comprehend the biological functions of macrophages in specific conditions while considering the environment, heterogeneity, and plasticity of these cells. This could have a profound impact on our understanding of the pathogenic mechanisms in certain diseases. For example, in patients with Crohn’s disease there is a clear difference between macrophage populations from the intestinal mucosa and from the mesenteric fat tissue. In the former, TLR-4, IL-1b and IL-6 protein levels are higher compared to those in patients with non-inflammatory disease; while in the latter there is no such increase. The authors of the study attribute these differences to the micro-environment, which in the case of intestinal macrophages is largely determined by the interaction with the microbiota. As a consequence, there is an anomalous up-regulation of the signaling pathways that result in the production of inflammatory mediators. Hence, a network like the one we devised could be of great value to understand this type of heterogeneous scenarios, helping improve medical care towards the design of treatments with side effects noticeably reduced in comparison to the ones currently prescribed.

The model also allowed us to determine the key nodes of the network. When subjected to knock-out or over-expression

experiments, IL12_out, IL10_out, STAT1, STAT6, and IL-10 mediated STAT3 activation (STAT3*) had the most notable effect, especially IL-12 and IL-10, as they are cell type markers. Also, *in vivo* cells are subjected to transient changes in extrinsic cytokine levels or stochastic effects in signaling pathways and transcriptional regulation, which we simulated as transient perturbations. The nodes that caused more transitions between cell types were: STAT1, STAT3*, STAT6, IC_e, FCGR, and SOCS1. On the other hand, IL12_out, VEGF_out, NECA_e, IL1R, and TLR4 had the least effect. STAT1, STAT6, and STAT3* activation has a higher number of out-going edges and directly or indirectly modulates the activation and inhibition of different network circuits, which explains their key roles within the network dynamics.

While the Boolean nature of the model favors the study of how the structure of the regulatory network determines cellular behavior, it also limits the scope of the analysis. The model uses discrete values for the nodes, severely restricting our understanding of how the range of expression levels observed in macrophages is generated. Furthermore, the model uses discrete time steps and synchronous actualization for very different processes like signaling, which can take minutes, and transcription, which can take hours. Most cyclic attractors of size two or three were unstable, but we did recover asynchronous cyclic attractors of size six or bigger, however understanding their biological implications is still an open question. The model is also deterministic, and the perturbation analysis, while sufficient to determine the possible transitions, is not a true stochastic analysis. This is particularly important as the internal state of the cell and random noise probably have an important role in the emergence of heterogeneous populations. Further models using differential equations or stochastic methods are warranted.

The model also oversimplifies the NF- κ B pathway to a degree where the predicted and experimental mutants are not in accordance. Additionally, our network would benefit from the inclusion of multiple molecules like TNF- α , TGF- β , TLRs, NODs, and MyD88. In fact, it will be necessary to incorporate these molecules to integrate the model to other cell types and create integrative immune models. Finally, the number of environments used was limited to reported polarizing conditions and mixed environments (Sica and Mantovani, 2012; Wang et al., 2013; Mendoza-Coronel and Ortega, 2017; Grosjean et al., 2021;

Munoz-Rojas et al., 2021). Thus, it will be interesting to see if the model can be extended to study diseases with complex immune profiles, like cancer, *tuberculosis*, or COVID-19.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/mar-esther23/Macrophage_Differentiation.

AUTHOR CONTRIBUTIONS

Design of the research study: MM-S, YB-M, JZ. Verified the model: LD-A. Performed analyses of the data: AO-A. All authors wrote the manuscript, contributed to the article, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2022.807228/full#supplementary-material>

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