Multistability in Macrophage Activation Pathways

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Macrophages are innate immune cells with a dynamic range of reversible activation states including the classical pro-inflammatory (M1) and alternative anti-inflammatory (M2) states. Common regulatory motifs reported for macrophage transitions, such as positive or double-negative feedback loops, exhibit a switchlike behavior, suggesting the bistability of the system. There are evidence for multistability (including bistability) in macrophage activation pathways at four molecular levels.

Macrophages Multistability miRNA systems biology inflammation bistability

1. Introduction

Macrophages are essential components of the innate immune system with multiple functions in both inhibiting/promoting cell proliferation and tissue repair. Diversity and plasticity are hallmarks of macrophages, whereby the classical pro-inflammatory (hereafter referred to as M1) and alternative anti-inflammatory (referred to as M2) activation profiles are thought of as two extremes of their dynamic changing states. The typical characteristics of M1 macrophages include a high capacity for antigen presentation, the intensified production of interleukin (IL)-12 and IL-23, and the increased production of nitric oxide (NO) and reactive oxygen intermediates. M2-type responses are observed in healing-type circumstances without infections. These responses can be further amplified by IL-4, IL-10, or IL-13. An imbalance in M1/M2 macrophage activation is often associated with various diseases, such as cancer or inflammatory conditions. Therefore, M1/M2 activation is a tightly controlled process entailing a set of signaling pathways and transcriptional and post-transcriptional regulatory networks (for a review see [1]).

There is an ongoing controversy surrounding the definition of the activation programs that macrophages undergo during inflammation, immune response, and trained immunity [1][2][3]. However the emerging consensus says that the activation spectrum is a continuum with many different instances defined by pathways with more than one steady state or attractor [3]. The existence of more than one steady state in a system is called multistability and speaks for switchlike behavior. The latter is indeed observed in several important macrophage activation pathways (see below) and can be modeled using systems biology.

Usually, systems have at least one steady state that acts as an attractor, indicating that a time course of the system will bring the concentrations of the model species to a specific state where all the fluxes (production and degradation) are balanced, establishing a minimum in potential energy. Under these conditions, the rate of change

is zero until a perturbation moves the system away from that steady state, increasing the potential energy to move back to that attractor. Therefore, when the perturbation is removed, the system will again be conducted to the same steady state. However, under certain conditions, more than one attractor emerges (local minima in potential energy), establishing a multistable system with several possible steady states [4]. A specific case of multistability is bistability (two attractors), establishing a molecular switch. The state of the system can alternatively move from one attractor to the other by overcoming the potential energy required to get out of the region of attraction of one steady state in order to enter the region of attraction of the other steady state. Therefore, despite the existence of several attractors with steady-state properties, the molecular pathways in macrophages treated in vitro can overcome the attraction of a first steady state in the presence of the appropriate signals to continue the activation process over time.

These molecular switches have been reported to emerge in several important regulatory processes of macrophage activation, dependent on various inflammatory mediators, signaling molecules, and transcription factors (TFs). Frequently, the specialized or polarized T cells (Th1, Th2, and regulatory T cells) play an important role in macrophage activation [5], although there are macrophage activities without T- or B-cell influence [6]. At the cellular level, canonical pathways have been postulated to control macrophage activation, such as the interferon regulatory factor (IRF) or the signal transducer and activator of transcription (STAT) signaling pathway. In response to Th1 cytokines and inflammatory stimuli such as interferons (IFNs) and Toll-like receptor (TLR) signaling, stimulation of STAT-1/STAT-2 and IRF-5 primes activation to M1 cells. In contrast, the IL-4- and IL-13-mediated activation of IRF/STAT (via STAT-6) will skew macrophage functions towards the M2 phenotype, while STAT-3 activation by IL-10 is associated with another anti-inflammatory phenotype [7]. Therefore, the IRF/STAT canonical pathway is a potentially interesting candidate, showing at least three different steady states depending on the initial type of stimulation.

These differences in macrophage functions can also be appreciated at the metabolic level, which indicates that the system undergoes a metabolic switch that has significant implications in macrophage activities and the shaping of the metabolic microenvironment. This interplay between metabolism and immune functions is called immunometabolism [8]. As an example, IL-10 signaling has been shown to play a critical role in controlling inflammatory responses by modulating cellular metabolism in activated macrophages [9].

Macrophages are thus not only essential immune cells but also attractive therapeutic targets. The characterization of molecules associated with the dynamic changes of macrophage activation and a deeper understanding of their interactions are crucial for elucidating the molecular basis of disease progression and for designing novel macrophage-based therapeutic strategies. The identification of robust targets controlling the transitions between given steady states is a big challenge. As mentioned above, the M1/M2 transitions observed in vitro are part of a repertoire (or continuum) of activation states of the macrophage.

2. Multistability, Hysteresis, and Ultrasensitivity

The aim of systems biology is not only to quantify the interactions between molecules, but also to predict and describe (quantitatively and qualitatively) emergent properties on the higher level. Each of the proposed hierarchical levels of a system has certain emergent principles that do not appear in the lower level of the organization, and therefore it is impossible to explain the functioning of a biological system using only a reductionist view of the physicochemical principles of the individual components [10]. Of such emergent properties is multistability (including bistability), which is a crucial feature of dynamical systems and is used in various all-or-none kinds of decision-making processes, leading to more than one self-perpetuating steady state (or attractor) [11]. Under these circumstances, two or more steady states coexist in a given set of experimental conditions. The attractors are represented as valleys in a landscape of potential energy[4]. This phenomenon was observed early on in a number of biochemical systems [12][13] and has attracted much attention in recent years. Its importance in biological systems, including cell signaling [14], differentiation [15], and the cell cycle [16], is well-known.

A cell signaling network is assembled from frequently occurring motifs that are building blocks for any network [17]. These network motifs play an important role in the propagation of signals in a network and can influence the sensitivity, robustness, and trade-off of the input–output (I/O) relation in a signaling network. The I/O relation is the information processing required for the detection of the amplitude and duration of the incoming signal to generate an output signal of proper strength and duration for the activation of the effectors that alter subcellular processes. A crucial part of the I/O relation is the existence of bistability, where the output signal can attain any of the two stable-activity states that persist under identical parametric conditions in a switchlike behavior [18].

A molecular switch implies the emergent property of bistability in a system. This usually arises in biological systems that contain a positive feedback loop or a mutually inhibitory, double-negative feedback loop. Depending on the parameter values of their interactions, these loops create more than one attractor steady state in the system. It has been considered that at least one positive feedback loop is a necessary requirement for the existence of multiple steady states [19][20], while other researchers state that a negative feedback loop is required for stability and a positive feedback loop is necessary for multistability [21]. Indeed, positive feedback circuits cover a wide class of biologically relevant systems with multiple steady states [22]. Nevertheless, the existence of positive loops is far from being sufficient; a positive feedback loop does not guarantee bistability, and this property has to be quantitatively explored for each particular system in order to confirm the emergence of this property [23]. This switchlike behavior is recognized by bifurcation analysis (or phase plane analysis), namely, equilibrium point analysis, which includes studies related to the changes in the qualitative and quantitative structures of the equilibrium points depending on the changes in the model parameters [24]. At bifurcation points, a system's behavior may differ qualitatively depending on small changes in the bifurcation parameters (those model parameters that enable the system to switch from one steady state to the other) [25].

Due to this attraction of a steady state, a system with positive feedback loops could produce an actively maintained 'memory' of a transient inductive stimulus, known as hysteresis [26]. Hysteresis is the phenomenon whereby bistable switching is observed for different stimulus responses and the state of the system depends on its history. The trajectory of the system from the steady state A to the steady state B is different from the trajectory from B to A [27]. This property can enrich the adaptation of organisms extending from bacteria to mammals by storing the

cellular memory of past stimuli [28][29]. An example of hysteresis in macrophage activation is observed for the phenomenon of lipopolysaccharide (LPS)-induced tolerance. After LPS stimulation, macrophages exhibit memory-like features at the molecular level related to gene-specific chromatin modifications, the silencing of inflammatory molecules, and priming for other genes, modulating the responsiveness of macrophages to subsequent activation [30]. Another example of hysteresis is trained immunity, as it represents a modified steady state of innate immunity after infection [2].

The significance of bistability for the identification of robust therapeutic targets has already been explored. For instance, bistability could lead to another emerging property called ultrasensitivity, where a short perturbation (pulse input) of a signaling cascade leads to a change in a steady state that is self-perpetuated by the system. This means that the perturbation is strong enough to move the system from the attraction zone of one steady state to the attraction zone of another steady state. This has been described for the mitogen-activated protein kinase (MAPK) cascade that is positively regulated by the activated MAPK [31] and the self-perpetuated activation mechanisms for extracellular signal-regulated kinase (ERK) 1/2, related to bistability [32]. Halder et al. used global sensitivity analysis to identify sensitive parameters and their role in maintaining bistability; they also used bistable switching to explore the underlying principles of the motifs exhibiting bistability [18]. They applied their results for the motifs of protein–protein interactions to identify potential drug targets in cancer networks with potential ultrasensitivity.

The hysteresis of a bistable switch can be reversible or irreversible depending on the strength of the feedback parameter [15]. A bistable system can reverse back to its previous state if the system shows reversible hysteresis, which is not possible for irreversible hysteresis. In addition, an irreversible hysteresis can be changed to a reversible hysteresis by decreasing the strength of the feedback parameter, suggesting that the manipulation of bistable switches is possible. Halder et al. hypothesize that the circuits with a larger reversible hysteretic range could be better drug targets to make the system switch to a disease-free (OFF) state from a diseased (ON) state by reversing the input parameter, which is more difficult for irreversible hysteresis. They observed that proteins present in the motifs with a higher reversible feedback range tend to be associated with higher numbers of drugs, leading to the identification of ultrasensitive targets. One of the two targets of the drug lapatinib is the epidermal growth factor receptor (EGFR), which belongs to the bistable motif with a higher reversible range [18]. This suggests that the identification of bistable motifs with a high range of reversible hysteresis holds promise for the identification of ultrasensitive targets. These targets would be able to switch the system from a pathological steady state to a resolving steady state.

Multistability in biological systems emerges from the nonlinear behavior of quantitative interactions between the individual components of key molecular pathways (those related to macrophage activation). These pathways have several alternative steady states that work as attractors, robust to the effects of small perturbations. However, when a perturbation is strong enough, it will make the system swap its configuration towards another attractor or steady state. In this sense, a perturbation is self-perpetuated, leading to a switch in the activation of a signaling circuit. Therefore, the identification of ultrasensitive targets to control bistable switches represents the most robust

type of therapeutic intervention possible for a system, since the correct use of a pulse signal can switch a system to a different self-perpetuating steady state.

3. Current Evidence Pointing to Multistability in Macrophage Programs

IRF/STAT signaling is a central pathway in controlling macrophage M1/M2 activation with the potential for the emergence of multistability. The M1 regulators STAT1, STAT5, IRF5, SOCS3, NFKB-p50-p65, and HIF- 1α have antagonistic and counteracting interactions with the M2 regulators IRF4, SOCS1, STAT3, STAT6, NFKB-p50-p50, and HIF-2α. Several of these transcription factors, including NF-κB, AP-1, and STAT family members, also potentially participate in macrophage trained immunity, resulting in hundreds of times higher gene expression in a short window of time [1][2][7][33].

All these counteracting activities or inhibitory cross-talks between the M1/M2 pathways indicate that the M1/M2 transitions may be considered bistable, as multistable systems consisting of molecular switches of mutually exclusive regulations. To quantitatively decode the underlying principles governing macrophage phenotypic activation and to harness its therapeutic potential in human diseases, a systems-level approach is needed. This is due to the significant number of signaling pathways and intracellular regulatory networks involved.

The M1/M2 transitions have already been studied using mathematical modeling. Zhao et al. published a mechanistic integrative computational model based on the literature-data-driven description of macrophage activation. They calibrated their ‘virtual macrophage’ model against experimental data, and mechanistically elucidated several signature feedbacks behind the M1/M2 antagonism and investigated the dynamical shaping of macrophage phenotypes within the M1/M2 spectrum. Model sensitivity analysis also revealed key molecular nodes and interactions as targets with potential therapeutic value for the pathophysiology of peripheral arterial disease and cancer [33]. However, they did not perform stability analysis or look for multistability in their model.

The emergent property of bistability has already been reported for macrophage-related pathways and has been studied using mathematical models, such as that of the tyrosine protein kinase (JAK)/STAT signaling pathway. Berez et al. examined how the interaction of STAT, APT (apontic), and SLBO (slow border cells) creates bistability in the JAK/STAT signaling pathway using parameter bifurcation and phase portrait analyses, leading to a model reduction to identify a minimal three-variable quantitative model [34]. They started with a 15-variable model but performed an elegant model simplification to obtain a minimal motif of 3 variables able to present bistability in *Drosophila melanogaster*. The inhibition of STAT activity by APT and the cross-repression of APT and SLBO conforms to a molecular switch that determines if an epithelial cell becomes motile or remains stationary. Interestingly, they also observed the resulting two steady states of the model between migratory and stationary cell phenotypes. Although this work was performed on the *Drosophila* model, it illustrates the property of bistability in the JAK/STAT signaling pathway, which is one of the most important pathways in macrophage activation.

Nickaeen et al. developed an ordinary differential equation model to perform bifurcation analysis and revealed mechanisms of macrophage activation and phenotype pattern distribution. They found saddle-node bifurcations in the internal regulators STAT1, STAT6, and NFKB. LPS was a bifurcation parameter for NF-κB, STAT1, and STAT6 transitions and IFN-γ for STAT1 and STAT6 transitions, while IL-4 levels did not give rise to any bifurcations. These observations confirmed in silico the presence of five switches in macrophage phenotype transitions and enabled different combinations of steady state levels attributable to nine different fates, including M1, M2a, M2b, and other phenotypes, thus describing a multistable system [35]. Furthermore, they developed a model to describe the mutual interactions between a macrophage and its neighboring cells that could affect macrophage fate through cytokine production. Therefore, they implemented an agent-based model, in which each agent represents a cell that is able to interact with other agents. This approach accounts for the stochastic behavior of each single cell, thereby representing the continuous cell population communications. To model these communications, they included IL-4, IL-10, IL-12, and IFN-γ concentration dynamics while introducing a time-scale separation for model simplification to describe cytokine levels in the function of the steady state concentrations of STAT6, STAT1, and NF-κB. In this way, they described a single cell model where the expression levels of STAT1, STAT6, and NF-κB depend on external stimuli and the concentrations of the cytokines can be determined as they diffuse to neighbor cells (**Figure 2**C). Despite the simplicity of their model, they calibrated it against experimental data and proposed that a dynamic bifurcation is a crucial built-in mechanism of macrophage activation [35]. Since an agent-based model is characterized by stochasticity, the individual agents (macrophages) display different configurations of multistable switches dependent on the corresponding amounts of activating cytokines. Their observation suggests that the extracellular cytokine production influences the control of these multistable switches at the macrophage population level.

While the researchers of [35] showed the bistable dynamics of macrophage phenotypes when exposed to external signaling cues, Smith et al. showed that after initial differentiation into M1 and M2, the M2 phenotype was ultimately dominant. They examined macrophage population response to simultaneous or sequential M1 and M2 activation signals to generate a subpopulation dataset based on M1/M2 marker expression, using flow cytometry. They found that M1 treatment potentiates the response to a subsequent M2 treatment, while M2 pretreatment blocks the response to M1 treatment [36]. This is an elegant experimental demonstration of the hysteresis proper to bistable systems, where the state of the system depends on its history [37]. In addition, they observed a heterogeneous distribution of markers, suggesting that the macrophages do not exist in discrete polarized states at the population level.

In addition, their mathematical modeling of candidate regulatory networks indicated that a complex interdependence of M1- and M2-associated pathways underlies macrophage activation. They used six minimal regulatory models of CD86 and CD206 expression in response to the different costimulatory conditions, using ordinary differential equations with different topological motifs. All models were built using generic formulations of self-stimulation and mutual inhibition, which are common building blocks in immune cell differentiation models [38]. Specifically, the researchers found that a mutual inhibition motif was by itself not sufficient to reproduce the temporal marker expression data. An incoherent feedforward mechanism of M1 activation as well as both the inhibition and activation of M2 by M1 were required for bistability (**Figure 2**D). Indeed, they included an additional

node called Y to comprise feedback inhibition mechanisms, such as those mediated by SOCS and STAT3 or NF-κB and STAT6 [36].

A recent work by Frank et al. also employed bifurcation and sensitivity analysis to reveal the key drivers of multistability in a simple model of macrophage activation, specifically tracking STAT1 and STAT6 activation levels as proxies for M1 and M2 activation, respectively. The researchers used ordinary differential equations and included self-stimulation and mutual-inhibition circuits between STAT1 and STAT6. They justified this choice because the individual steps are unknown; therefore, they assumed that responses in self-stimulation and inhibition are sigmoidal and can be modeled using a Hill function [39]. Despite its simplicity, the model exhibits complex dynamics. Furthermore, the researchers showed that external signaling cues are necessary for macrophage commitment and emergence to a phenotype, but that the intrinsic macrophage pathways are equally important.

These reports provide experimental and computational evidence of the emergent property of multistability in macrophage phenotypes. They also revealed the underlying principles of those molecular switches that include mutual-activation or mutual-repression circuits. However, realistic biological networks generally encompass more proteins and variables, precluding the use of traditional phase plane analysis to identify bistability in networks with longer mutually inhibitory feedback loops [23]. Thus, current models are very much simplified, precluding the understanding of the exact mechanism of bistability and the identification of targets to interfere with the molecular switch. This explains why larger, more complex models such as that of Zhao et al. were not analyzed for bistability [33].

The reports mentioned above also highlight some of the implications of bistability, particularly hysteresis. For instance, an M2-stimulated macrophage requires much higher concentrations of LPS + IFN-γ to undergo an M1 program compared to a naive, nonstimulated macrophage [36]. They also support the hypothesis that M1/M2 transitions are regulated by multistable pathways and describe interesting properties of this multistability. Although the literature cited in this section tries to extrapolate the multistable behavior of these pathways to macrophage activation, it is obvious that the concept of multistability better applies to molecular pathways and that there is more than one molecular switch at work defining macrophage phenotypes. As was nicely illustrated by Nickaeen et al., five switches led to nine potential different states [35]. Therefore, the higher the number of switches, the higher the number of potential discrete states in macrophage activation, potentially explaining why macrophage activation is considered a continuum at the population level [3], although it is in fact discrete but with many instances defined at the molecular level.

4. miRNA Circuits as Possible Sources of Bistability in Gene Expression

The previous reports indicate that signaling modules involved in M1/M2 programs have the intrinsic potential to give rise to bistability at the signal transduction level. However, many of the molecules reported on above are transcription factors, thus participating in complex gene expression programs interacting with other molecules such as miRNAs, which are small endogenous RNA molecules that bind mRNAs and repress gene expression [40]. As a

matter of fact, in order to keep the models simple, no systems biology study of M1/M2 transition bistability has included miRNAs, although they are known to be key regulators in gene expression and gene expression noise [41], especially for low-expressed genes such as transcription factors [42]. A typical miRNA is processed from a long primary RNA sequence into a short mature functional transcript around 22 nucleotides in length. A common characteristic of an miRNA is its ability to pleiotropically target the expression of hundreds or even thousands of genes [43], and their target genes can also be regulated by several miRNAs [44]. Current estimates indicate that the human genome contains 1917 annotated hairpin precursors and 2654 mature sequences of miRNAs [45], estimated to directly regulate >60% of human mRNAs [46]. In consequence, miRNA-transcription networks have a high degree of complexity and there is a high probability that miRNA-transcription factor interactions regulate important targets in M1/M2 transitions.

An important role of miRNAs in modulating macrophage phenotypic activation is demonstrated by accumulating evidence in which an excessive or impaired inflammatory response of macrophages is found to be tightly linked to the deregulation of miRNAs [38]. For example, some functional miRNAs such as miR-146, miR-125b, miR-155, and miR-9 have been reported to be induced by inflammatory stimuli to attenuate TLR4/IL-1R signaling pathways in monocytes and macrophages [47][48][49]. A wide range of miRNAs regulating the inflammatory profile of macrophages has been identified, including M1-related miRNAs (miR-9, miR-17, miR-20a, miR-98, miR-106a, miR-125b, miR-127, miR-146, miR-147 miR-155, miR-181, miR-451, and miR-720) and M2-related miRNAs (let-7b, let-7c, miR-21, miR-23a, miR-23b, miR-27a, miR-34a, miR-92a, miR-124, miR-125a, miR-132, miR-142, miR-223, and miR-511).

In particular, the modulation of macrophage activation by miRNAs has gained a lot of attention. The overexpression or depletion of miR-155 drove macrophages to the M1 or M2 phenotype, respectively, confirming that miR-155 plays a central role in regulating the serine/threonine kinase (Akt)-dependent M1/M2 activation of macrophages [50], and tumor-associated macrophages were successfully reprogrammed into pro-inflammatory M1 macrophages by miR-155 overexpression [51][52]. The manipulation of miRNAs to regulate macrophage activation has been suggested for gliomas, specifically for miR-142 [53]. Additionally, miRNAs contribute to trained immunity in macrophages due to the long half-life of miRNAs and the limited proliferative ability of macrophages [54]. The upregulation of miR-155 in response to inflammatory signals is associated with macrophage hyperactivation, indicating that cells with sustained miR-155 remain primed in a hyper-sensitive state to increase the response to a secondary stimulus [55].

The list of miRNA targets on the pathways involved in M1/M2 transitions is expanding, indicating that they represent strong modulators of M1/M2 transitions, potentially participating in the assembly of a molecular switch circuit. Withstanding with the opposite activity of the M1 and M2 regulators (e.g., STATs) cited above, examples of opposite activities in the miRNAs have also been reported. However, the complexity of this regulatory network is such that the identification of the master key regulators requires the aid of computational approaches.

Lu et al. correlated miRNA and mRNA expression over time to elucidate the expression profiles of miRNAs and their potential targetomes during mouse macrophage activation. They hypothesized that miRNAs mediate the early

events of the M1/M2 phenotype switch through a complex and dynamic miRNA-targeted mRNA interactome network. Their bioinformatic analysis revealed 31 differentially expressed miRNAs, including four top M1 miRNAs (miR-155-3p, miR-155-5p, miR-147-3p, and miR-9-5p) and four top M2 miRNAs (miR-27a-5p, let-7c-1-3p, miR-23a-5p, and miR-23b-5p), which could be divided into an early and a late cluster of miRNA expression. Their integrative analysis of miRNAs and mRNAs demonstrates that the miRNAs regulate nearly 4000 differentially expressed genes and most of the biological pathways that are enriched in macrophage activation [56].

Taken together, the literature includes many potential regulators of M1/M2 transitions, including important transcription factors and many miRNAs, leading to a paramount complexity. In fact, the endogenous transcription networks of miRNA–TF interactions have been reported to assemble complex motifs, including negative feedback loops, positive feedback loops, coherent feedforward loops, incoherent feedforward loops, miRNA clusters, and target hubs leading to nonlinear, systems-level properties such as bistability, ultrasensitivity, and oscillations [57][58]. This means that a differential expression analysis of target genes or miRNAs or the study of the network topology could be insufficient to identify the underlying mechanisms of bistability. Indeed, Cinquin et al. showed ways to derive structural (related to the interaction graph) and numerical (related to the magnitudes of the interactions) constraints required for bistability [22].

Therefore, addition to bistability arising from the interactions of molecules in the signaling modules of M1/M2 transitions, these transcription factors also participate in complex circuits with miRNAs, leading to bistability at the gene-expression level. To explore this possibility, researchers constructed a network of experimentally validated interactions from several databases using the miRNAs and transcription factors cited above and reported to be involved in macrophage activation. researchers constructed the network using the recently published biocomputational platform BioNetUCR [59]. The resulting network is highly complex, including 148 transcription factors, 24 miRNAs, and 537 genes (not shown). Due to the recent advances in the role of immunometabolism for macrophage activation (see below), researchers focused on the potential effects of this gene expression network on macrophage metabolism. For this purpose, researchers filtered the target genes identified in the network through the list of 3696 genes of the human metabolism extracted from Recon3 [60]. The gene expression subnetwork controlling metabolism includes 148 transcription factors, 20 miRNAs, and 105 metabolic genes. This network shows a central regulatory core of complex interactions and future work is required to assess their potential for the emergence of bistability. In addition, researchers highlighted the interactions of the mutually-inhibiting regulators of M1/M2 macrophage transitions with the metabolic genes, including both common and exclusive regulatory interactions.

Thus, macrophage metabolism implies an additional layer of complexity and it is very likely that the bistability at the signaling and gene-expression levels directly impacts metabolic genes, explaining the sharp differences in several metabolic pathways for the macrophage phenotypes. Moreover, the differentially activated metabolic pathways could lead to the production of specific metabolites that can be sensed by the same macrophages (or neighboring cells) modulating gene expression (see below), thereby increasing the complexity of the system.

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