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Macrophage States: *there's a method in the madness*

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Abstract

Single-cell approaches have shone a spotlight on discrete context-specific tissue macrophage states, deconstructed to their most minute details. Machine learning approaches have recently challenged that dogma by revealing a context-agnostic continuum of states shared across tissues. Both approaches agree that 'brake' and 'accelerator' macrophage subpopulations must be balanced to achieve homeostasis. Both approaches also highlight the importance of ensemble fluidity as subpopulations switch between wide ranges of accelerator and brake phenotypes to mount the most optimal wholistic response to any threat. A full comprehension of the rules that govern these brake and accelerator states is a promising avenue because it can help formulate precise macrophage re-education therapeutic strategies that can selectively boost or suppress the disease-associated states and phenotypes across various tissue.

Keywords

Macrophage; Polarization; Artificial Intelligence/Machine Learning; Single-cell biology

The chaotic (and often contentious) panoply of macrophage states

Macrophage represent one of the most functionally versatile cell types in our body [1]. In addition to recognizing and neutralizing foreign threats (e.g., pathogens), macrophages remove damaged, senescent, and exhausted cells, facilitate wound healing, and help achieve and maintain homeostasis [1,2]. Macrophages accomplish all this via responses that include wide ranges of reactivity, tolerance, proliferation and priming; they are also capable of **trained innate immunity** (such as during repeated exposure to microbes) [3–7]. Given the vastly complex nature of these cells, it is unsurprising that predicting their population level behavior upon perturbation, or defining their immunophenotypes in ways that are actionable and/or translatable (e.g., as in the development of therapeutics or biomarkers) remains a challenge [2]. In this article, we summarize the efforts to generate formal definitions and/or understand the rules that govern macrophage responses that span over ~3 decades; these efforts chronicle the swing of the pendulum from the extremes of

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Declaration of interests

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'oversimplification' to the extremes of 'deconstructed complexity', to the most recent years of artificial intelligence (AI)-guided identification of universally conserved patterns within such complexity [8–11]. If one were to look past the obvious chaos and the contention (surrounding the divergent terminologies, approaches, model systems, species, tissues, and disease states), the revelations of all efforts point in the same direction. The most recent AI-enabled revelations have helped reinterpret the results of the past decades and converge on a unifying concept and hypothesis, one that offers some sort of rationale and/or framework in the seemingly chaotic world of ever-expanding macrophage states in both health and disease. The major conceptual hypothesis that emerges, is that while mounting an immune response, macrophages finetune inflammation by splitting the whole population into two major **antagonistic** subpopulations: some become reactive (or inflammatory, i.e., accelerators) while others become tolerant (or non-inflammatory, i.e., brakes). These major subpopulations enable macrophages to multitask (i.e., neutralize threats, restore homeostasis, replenish pool) through optimal population and/or phenotypic drifts that are mediated *via* **autocrine** and/or **paracrine** signals and are highly contextual, dynamic, and finite. This conceptual hypothesis—of brakes and accelerators--is extended with surprising ease and sophistication to also explain the observed imbalances in the functional subpopulations and the basis for the same in diseased tissues. Finally, we discuss how such population and phenotypic changes could be interpreted immediately to extract actionable insights, interrogated further to answer exciting questions, and even optimized or manipulated for therapeutic purposes to re-educate macrophages.

The era of oversimplification

In 2000, Mills [12] and colleagues put forth the so-called 'M1/M2' macrophage polarization paradigm which was built on the observation that **lipopolysaccharides** (LPS; see Glossary) and interferon gamma (IFNγ) elicit divergent effects on murine macrophages isolated from different strains of mice, namely C57BL/6 and Balb/c. This M1/M2 paradigm was confused with the concept of classical vs. alternative macrophage polarization that was proposed earlier by Gordon in 1992 [13]. Gordon showed that IL-4 augments the expression of the mannose receptor on murine peritoneal macrophages without inducing TNFα production. This "alternative" activation of less inflammatory macrophages became, over time, synonymous with "M2" macrophages. In 2014, Murray and colleagues [14] proposed a nomenclature, calling for M(LPS) and M(IL-4), instead of M1 and M2, respectively, without tinkering with the M1/M2 paradigm. Conceptually, the M1/M2 paradigm continues to represent a highly reproducible response of murine macrophages *in vitro* to either LPS and/or IFNγ (M1) or Interleukin (IL)-4 (M2). Most agree that upon stimulation with a defined set of factors *in vitro*, these cultured macrophage states (i.e., taken out of their native environments) constitute an oversimplification of a highly complex and dynamic **continuum** (See Glossary) of functional states *in vivo*. Thus, The M1/M2 paradigm is currently believed to have stifled the discovery of new states, paradigms, or conceptual advances for nearly a decade.

The era of deconstructed complexity

With rapid advances in single-cell technologies (multiomic approaches), the past decade (2010-2020) has witnessed an explosive growth in our understanding of macrophage states, each dissected at an unprecedented level of detail. We learned of the existence of macrophage populations that perform diverse context-, threat-, and tissue-specific functions [15,16]. Such diversity is imprinted by their ontogenic origin, organ context, activation, or deactivation in response to signals stemming from microbial invasion, tissue damage, and metabolic derangement, as well as by their polarization, influencing adaptive T cell responses. Analyses on healthy and pathological tissues in humans and mice have helped build a near-complete catalog of these discrete macrophage functional states (see Figure 1A; [17–25]). It is akin to zooming in on a puzzle piece (discrete macrophage states) to understand the bigger picture (at tissue level). single-cell endeavors are helping to provide insight into Key puzzles related to macrophage states, including **immune quorum sensing** [26,27], homo- and heterotypic cell-cell contacts/interactions [28–33], rare functional states associated with specific disease phenotypes [34,35], and cellular and molecular mechanisms that support these discrete states [28–34], perhaps even spatially restricted at times, such as those demonstrated in the gut and the brain recently (summarized in Figure 1B) [35–37]. Consequently, we now know more about the epigenetic landscapes, transcription factors, and microRNA networks that enable macrophages to adapt to diverse environmental cues in a tissue-specific manner [38].

We have also learned that the balancing act of inflammation and immunity--in any tissue--relies upon the delicate crosstalk between many discrete macrophage populations with themselves (homotypic) or with other cell types (heterotypic). Besides a few instances where the macrophage subtypes are either yet to be fully characterized or represent naïve or dendritic cell (DC)-like states (Figure 1A), in most other instances, they might be lumped into two broader subsets: (i) ‘inflammatory’ or ‘i’ and (ii) non-inflammatory or ‘ni’, a.k.a. regenerative or tolerogenic (Figure 1A)[24]. Although the proportion of the two broad subsets remain balanced in health, their disbalance has been consistently encountered in the diseased tissues[24]. For example, in the case of the non-inflammatory macrophage subpopulation, their selective reduction or loss has been observed in specific tissues and associated with certain diseases. For example, a non-inflammatory macrophage subtype was found to be associated with neurodegenerative diseases; it was identified during the creation of a comprehensive map of all immune cell populations in wild-type and Alzheimer’s disease (AD)-transgenic (Tg-AD) mouse brains using single-cell sequencing and validated in human brain tissue of AD disease using histology [36]. A decrease in this non-inflammatory macrophage subpopulation was found to be consistently associated with progressive neurodegeneration in Alzheimer’s disease. Such association was not only in experimental mouse models but also in humans [36]. Similarly, single-cell RNA sequencing also revealed a reduction or loss of these non-inflammatory macrophages in human and murine livers identified using in the context of non-alcoholic steatohepatitis (NASH; a disease that is characterized by inflammation in response to fat accumulation, contributing to progressive liver fibrosis [17, 25]) and in human lungs afflicted byidiopathic pulmonary fibrosis (IPF; a chronic lung disease characterized by scarring and impaired lung function

[39,40]). The expansion of these non-inflammatory macrophage populations, on the other hand, has been observed using single cell analysis in human lung and breast tumors and are believed to favor tumor progression [19,41–44] and or therapeutic resistance [45].

In the case of the inflammatory macrophage subtype, their expansion in the lamina propria of the afflicted intestine in patients with inflammatory bowel disease and in the synovium of arthritic joints is a consistent observation (Figure 1A) [20,22]. Thus, the gathering consensus from these studies is that a balance between non-inflammatory macrophage and inflammatory subsets is key to achieving homeostasis. Here, non-inflammatory subsets might be likened to a ‘brake’ which opposes the inflammatory macrophages; the latter might be likened to an ‘accelerator’ that is mostly dispensable unless an inflammatory response is desired (Figure 1C). Diseased tissues show evidence of ‘broken’ (too few, or constitutively ‘off’) or ‘jammed’ (too many, or constitutively ‘on’) states of either the ‘brake’ or the ‘accelerator’ populations (Figure 1C) [28–34,46], thereby accounting for runaway responses.

Despite the knowledge gained, the true impact of single cell-based insights into discrete macrophage states remains uncertain. While we know of their existence, we are yet to understand where these discrete states fit into the compendium of continuum states across a spectrum of activation and tolerance. We are yet to translate the knowhow into actionable biomarkers or therapeutic targets for clinical use. Nevertheless, the hope is that these insights might help to predictably restore balance between subtypes in diseased tissues. Consequently, we argue that deconstructing the complexity of macrophage states down to minute details may yield an overwhelming amount of information on tiny details that can obscure the big picture, and thereby, risking our ability to see the forest for the trees.

The era of simplified complexity through pattern recognition

In 2023, using a **machine learning (ML)**-guided network transcriptomics-based approach, researchers [46][9,10,46–48] explored a large number of highly diverse macrophage bulk and single-cell datasets (which included circulating monocytes and macrophages from diverse tissues of murine and human origin, as well as murine and human macrophage cell lines; Figure 2A) with a singular intent: to find patterns within a seemingly chaotic process. The approach (i.e., **Boolean implication networks[49]**) that was prioritized had a decade-long track record [8–11] of being able to identify fundamental universally conserved changes in gene expression (or ‘invariant’ events) by blurring the thunderous noise that arises from other variables, e.g., the heterogeneity of tissues, circadian rhythms, metabolic states, species, and even perturbations (stimuli, disease states or conditions) [8–11]. Although they trained their initial model on a diverse pooled dataset comprised of isolated human monocytes and tissue macrophages, rigorous validation studies including human and disease analyzed by single cell-sequencing showed that the resultant model was bipartite; it accurately and independently identified both physiologic and pathologic spectra of “reactivity” and “tolerance” [46]. Specifically, A cluster of 42 genes on one side of this bipartite model were found to be primarily associated with the wide ranges of functions encountered in activated or inflammatory macrophages (‘accelerator’ function); two clusters of 296 genes on the other side carried similar information on the functions encountered in tolerant or non-inflammatory macrophages (‘brake’ function) (Figure 2B) [46]. When

used as gene signatures to interrogate >12,500 diverse datasets, both sides of the bipartite model were able to objectively detect and quantify the two seemingly opposing--reactive and tolerant--functional states, independently of each other. Notably, the model was able to accurately pick up these states across tissues, organs, species, and, surprisingly, even other immune cells [46]. This relatively simple model could identify macrophage polarization states even at single-cell resolution, i.e., on single-cell sequencing datasets, suggesting that simplification did not sacrifice the real-world complexity in tissues. Moreover, the model could identify dysregulated 'brakes' or 'accelerators' across diverse human diseases such as cancer, atherosclerosis, inflammatory bowel disease and liver fibrosis (Figure 2C), as well as murine pre-clinical disease models [46]. In many ways, this model captured the big picture (i.e., inflammation at tissue level) of most of the key puzzle pieces (discrete antagonistic macrophage states), all in the right place, and confirmed that dysregulated "brakes" or "accelerators" is consistently observed across diverse human diseases and murine pre-clinical disease models.

There are several key advantages of this model (summarized in Figure 2D), the most important one being that it provided a formal and universally relevant definition of macrophage states via an objective measure of expressed gene signatures in any sample, encompassing both circulating monocytes and tissue macrophages. The nature of the connectivity between the gene clusters in the model provided a predictive framework; tugging at the network at one end, could trigger changes elsewhere, exactly as predicted by machine learning. For example, using **crowd-sourced studies,** the authors showed that the predicted impacts due to network perturbations (genetic or pharmacologic) consistently matched the observed fates of both the reactive and tolerant macrophage states [46]. Consistent with the dysregulated 'brake' vs 'accelerator' states as putative drivers of disease (Figure 1C), the reactive and tolerant gene signatures derived from the model could detect broken (too little) and/or jammed (too much) brakes and accelerators in diseased tissues; they could also use such objectively quantifiable dysfunctions to prognosticate outcomes across diverse acute and chronic diseases, e.g., sepsis, liver fibrosis, aging, and cancers. Overall, this model provided some sort of framework for the scientific community to begin developing macrophage-targeted precision diagnostics and therapeutics. A notable limitation of this model, however, is that its predictive potential is yet to be rigorously tested through prospective studies employing diverse network-level perturbations, such as gene editing, infectious and non-infectious stimuli, and drugs/toxins.

It may be unsurprising that the network transcriptomics approach worked so well because the abundance of transcripts likely reflected the culmination of the actions of all other factors enabling macrophages to adapt to diverse environmental cues. These factors include epigenetic landscapes, which are modifications on DNA and proteins that can influence how genes are expressed, thereby shaping macrophage responses and determining their functional states [50]. Transcription factors are another key class of molecules that are also known to regulate macrophage polarization states, e.g., the role of STAT6 in curtailing the tolerant macrophage states in the tumor microenvironment [51]. Finally, microRNAs, which are small RNA molecules that modulate gene expression by binding to and inhibiting the translation of mRNA into proteins, also shape macrophage states, as shown in the

setting of atherosclerosis [52,53] and cancers [54]. What came as a surprise, however, was that the gene clusters identified through the network transcriptomics approach consistently exhibited a higher degree of overlap between transcriptome and proteome data compared to conventional macrophage signatures. Such transcriptome-proteome overlap raises hope that several candidate genes in the reactive and tolerant network-derived signatures could serve as high-value protein targets for therapeutic interventions with small molecules and/or biologics. Further rigorous examination through pre-clinical and clinical trials is required to validate the feasibility and effectiveness of these putative interventions.

Building a consensus through systems-level thinking.

Now that we have explored macrophage states through *in vitro* reductionist approaches (the M1/M2-like paradigm), *in vivo* single-cell technologies, and by a set of ML-derived unifying formal definitions (i.e. objective and quantifiable) that can identify a compendium of continuum states in which macrophages are capable of existing, two questions loom large: (i) How much agreement exists among these approaches, and where do they differ? This question seeks to understand the extent of consensus and discrepancies between the different methods in defining macrophage states. (ii) What comes step?

For starters, all three approaches – *in vitro* reductionist approaches focusing on the M1/M2-like paradigm, *in vivo* single-cell technologies, and by a set of ML-derived unifying formal – aiming to define macrophage states---despite spanning ~3 decades---probably saw the same ‘accelerator’- and ‘brake’-like functional populations from their own perspectives. For example, a third of the ‘accelerator’ genes in the ML-derived network model were overlapped with Murray’s definition of the M1 state; the ‘brake’ genes, however, were notably absent in the M2 state [46]. There were significant overlaps between ‘brake’ genes identified by single-cell studies and the ML-derived network model; the ‘accelerator’ genes however were notably distinct between these approaches [46]. Here, the analogy to the parable of the 5 blind men and an elephant is quite fitting [55], in which each blind man encounters an elephant for the first time and forms a distinct perspective based on their limited perception. Similarly, different approaches to study macrophage subpopulations provide unique insights into each subpopulation while lacking a comprehensive understanding of all delicate balances within them all within the entire ensemble.

As for what is next, further refinement of the model, perhaps to tissue/disease states with or without perturbation is an immediate priority on the computational side. Testing the performance of model-rationalized therapeutics and diagnostics in animal and human preclinical models is a high priority before one can truly estimate the translational potential of the model.

On a foundational level, however, solving individual versus collective macrophage behavior is a challenging problem, and likely to require transdisciplinary approaches. In fact, the need to navigate through complex and exhaustive catalogs of cell states resulting from single-cell studies is not a problem that is unique to macrophage biology. Most agree that cellular states often exist in a continuum rather than in distinct phases [56], and that understanding,

predicting, and manipulating those cell states is a cornerstone goal in any field of biomedical sciences. There is also a consensus that the continuum of states is a direct consequence of continuum gene expression (influenced by genetics and epigenetics), translating into continuum protein expression, and ultimately responsible for the multi-tasking behavior of that cell type [57–60]. The fields of developmental and regenerative biology [57] have set the precedent of using single-cell multi-omics and lineage tracing to dissect decision-making for switching cell states during development and differentiation [61]. In immunology, the recent revelation of the existence of shared continuum states among macrophages upon perturbation (Figure 3A–C) [9,46] presents an opportunity to infuse systems-level thinking when exploring complex paracrine interactions among these states (Figure 3D) [9,46]. We now know that a single perturbation (e.g., *Salmonella* sp. infection in mouse bone marrow derived macrophage [62] or LPS in human monocyte derived macrophage [63]) is sufficient to trigger dose-dependent multi-tasking behavior (such as reactive, tolerant, regenerative, cycling, etc.; Figure 3C), among a population of macrophages in ways that each subpopulation influences the functions of the other via a complex network of auto-/paracrine communication (e.g., TNFα, IFNβ, IFNγ, reactive oxygen species [ROS], IL-, Nitric Oxide, etc.) (see legend, Figure 3D), and consequently, that of the whole ensemble in inflammatory homeostasis.

We argue that a deeper understanding of cooperation or competition among macrophage populations and the threats they seek to neutralize may require methods and concepts from evolutionary **game theory** [64–67]; the latter is the study of strategic interactions where everyone's **payoff** not just depends on their own traits, but also on the traits of others (as illustrated in Figure 3D). In other words, game theory is the study of **optimization** problems in which fitness of individuals depends not only on the environment (dose or nature of the stimuli) but also on the changing frequencies of other phenotypes in the population, which might include competitors (e.g., macrophages vs pathogens) and cooperators (e.g., homotypic interactions between macrophage subpopulations, and heterotypic interactions with other immune and non-immune cells). Such problems are called 'games', the individuals, or groups of individuals in subpopulations are called 'players', and the phenotypic behavior each player adopts, is a '**strategy**' [64–67]. Evolutionary game theory has been used successfully in other areas of biology (e.g., cancers [65]) but has been underutilized in immunology. To our knowledge, the only examples that exist include studies evaluating the best strategy employed by macrophages to switch from a proliferating state to that of attacking the fungal cells (simulated using *Candida albicans* to stimulate J774 murine macrophage cell line) [68,69], and another study evaluating the best strategy employed by human polymorphonuclear neutrophils (PMNs) for their preferred mode of death upon LPS stimulation (simulating infections/injury) [70]. In the case of macrophages, it is tempting to speculate that brake and accelerator populations can cooperate to accomplish the most optimal outcome (see legend, Figure 3E) – a hypothesis that remains to be further explored.

Concluding remarks

The surprising new revelation that diverse macrophage states that were thought to be unique to each tissue niche, line up along the same shared continuum path of reactivity and tolerance suggests that we may be looking at some core fundamental rules that govern

how opposing populations of macrophages work together to respond to and neutralize any threat. While the full understanding of those rules is lacking currently (see Outstanding questions) it is beginning to get clearer that **ensemble fluidity** in macrophage functions and phenotypes and **temporal drift** in those functional populations may be crucial to support flexibility in mounting an inflammatory response while maintaining homeostatic stability. It is likely that the pursuit of these foundational questions will face the same challenges and limitations that come with any monumental cross-disciplinary effort, i.e., careful translation of the languages of each discipline (immunobiology, medicine, systems, and computation) while making cautious interpretations.

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Glossary:

Trained Innate Immunity
This refers to the ability of the innate immune system to form immune memory and provide long-lasting protection against foreign invaders.

Antagonistic
Actively opposing each other, in this case, conducting opposing functions regarding mounting an inflammatory response.

Autocrine
A form of signaling in which a cell secretes a hormone or a chemical substance that binds to the receptors on the same cell, leading to functional changes in the cell.

Paracrine
A form of cell signaling in which the target cell is near the signal-releasing cell.

Lipopolysaccharides (LPS)
outer membrane components of gram-negative bacteria.

Continuum
a continuous sequence in which adjacent elements are not perceptibly different from each other, although the extremes are quite distinct.

Immune quorum sensing
This refers to mechanisms that allow immune cells to regulate their activity concurrently in a spatial and temporal manner via autocrine or paracrine signals, which in turn favor the emergence of population-level behaviors and synchronized responses, two features that make the immune system robust and resilient to external perturbations.

Machine learning

A branch of artificial intelligence (AI) and computer science which focuses on the use of data and algorithms to find patterns that humans cannot find.

Boolean implication network
Is a pair-wise gene expression relationship between two genes with respect to their gene expression values and a Boolean implication network is simply a collection of Boolean implication relationships.

Crowd-sourced studies
Crowdsourcing is a beneficial research method that allows aggregating efforts of members of the public to advance studies to solve a problem, in this case, numerous perturbation studies on macrophages from laboratories around the world.

Game theory
The science of strategy, or at least the optimal decision-making of independent and competing actors in a strategic setting.

Payoff
The reward from the outcome of the interaction (for macrophages, this refers to its evolutionary fitness).

Optimization
The choice of the best set of actions to maximize a payoff function.

Strategy
The decision or type adopted by a player (in this case, macrophage phenotype or functional state).

Perturb-Seq
A method that combines perturbation by CRISPR-based genetic screening with information-rich, single-cell RNA-sequencing phenotypes.

DRUG-Seq
A cost-effective, target-agnostic, high-throughput RNA-seq method for drug discovery.

Ensemble fluidity
A collection of diverse states that are unstable or constantly changing viewed as whole rather than individuals.

Temporal drift
This refers to the problem of data changing over time, in this case, the macrophage states and the fraction of population that is at any state.

Equilibrium
A stable state to which a population converges over time.

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HIGHLIGHTS

- The era of single-cell biology has witnessed an explosive growth in our inventory of macrophage states, contributing to the belief that diverse macrophage states are unique to each tissue niche.
- This tenet has recently been challenged by machine learning (ML) approaches that are geared to identify what is common (shared), while ignoring what differs. Macrophages anywhere and everywhere, were found to share a universally conserved continuum of inflammatory and non-inflammatory states, regardless of tissues, species, or stimuli.
- Both approaches underscore the importance of maintaining a population-level equilibrium between inflammatory (accelerators) and non-inflammatory (brake) macrophages to mount a physiologic immune response.
- Both approaches conclude that diseases are a consequence of either population imbalance (too many/too few) and/or inability to switch states (‘jammed’ brakes/accelerators).

Significance Box

Macrophages are known to assume countless number of states in diverse tissue niches. Landmark studies using single cell technologies and, more recently, machine learning approaches establish that these states represent two opposing populations of macrophages: inflammatory macrophages that respond to neutralize threats, and non-inflammatory macrophages that promote healing and maintain homeostasis. Understanding how these two opposing populations originate or influence each-other's fitness, and what population-level strategy triggers them to switch states is a must before we can decode macrophage states.

OUTSTANDING QUESTIONS

- Can the ML-derived continuum model (Figure 2A–B) be refined to incorporate tissue-specific and disease-specific states, taking into consideration the types of perturbations that are unique to such disease?

Any model that is optimal for all macrophages is expected to be suboptimal for a specific kind of tissue macrophage. Therefore, model refinement should be a priority and can be done on datasets of either human diseased tissues, or macrophages isolated from such tissues or perturbed with the relevant stressor(s). Such refinement is expected to enhance the model's translational potential.

- What is the predictive accuracy of model-rationalized perturbations (genetic or drugs), and how should that be tested?

Testing the effectiveness of model-based therapeutics was only partially evaluated using with retrospectively curated crowd-sourced datasets and requires to be prospectively tested using RNA Seq technologies such as Perturb-Seq (which uses gene editing approach) or Drug-seq (which uses drugs/chemicals). Concordance between the two will ensures reliable performance of drug targets in real-world scenarios. Successful vetting of network-prioritized targets is expected to usher in a new therapeutic strategy, i.e., macrophage re-education therapy.

- How can we further our understanding of individual versus collective macrophage behavior to answer key questions, e.g., what triggers state-switching and/or temporal population drifts, and how does the population level behavior differ in response to various doses and types of threats?

Solving the puzzle of how macrophage populations cooperate or compete as they neutralized threats may require us to apply methods and concepts from evolutionary game theory. Application of this theory has the potential to reveal strategic interactions between accelerator and brake-like populations (and others, shown in Figure 3) where everyone's **payoff** not just depends on their own traits, but also on the traits of others.

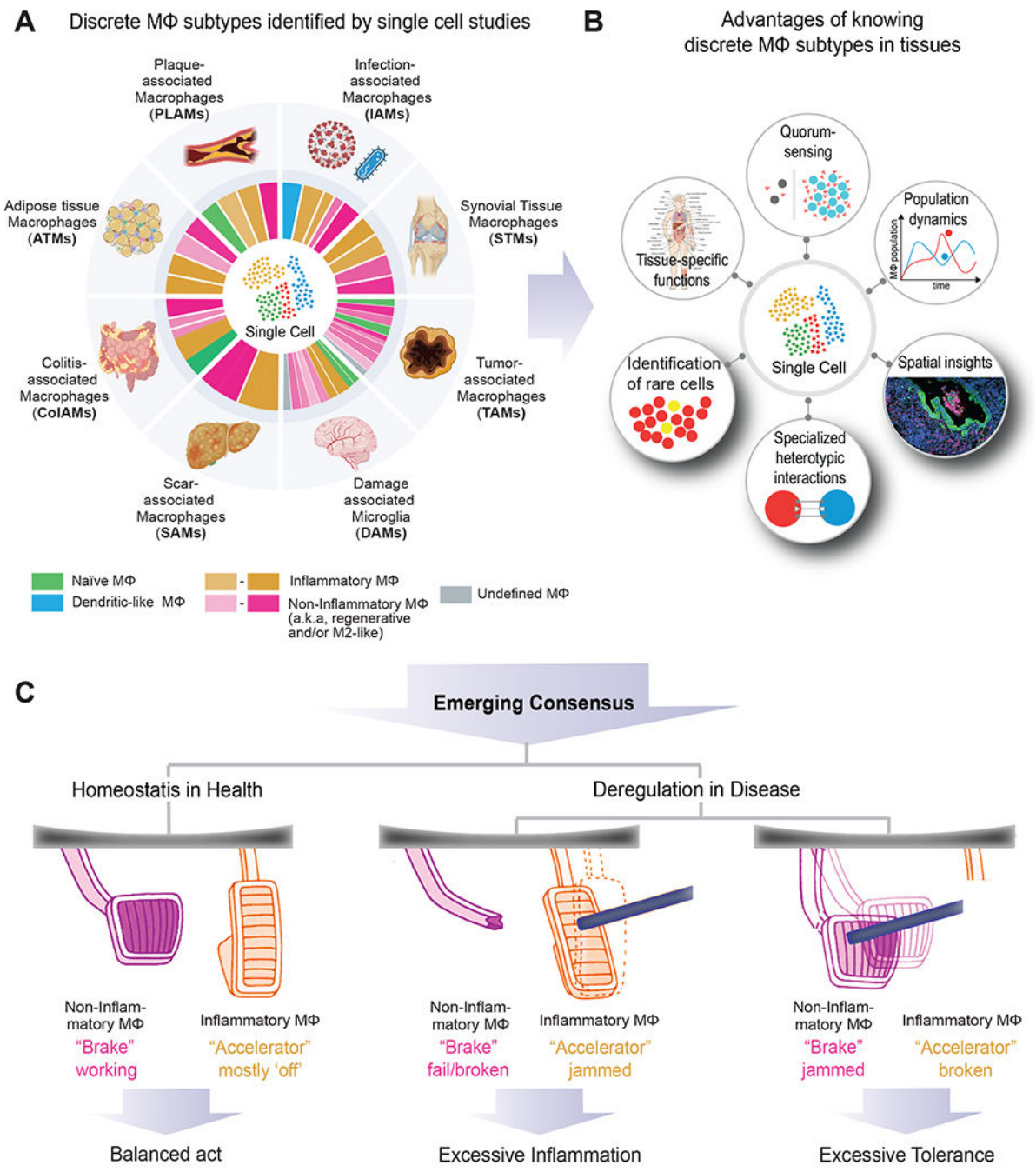

Figure 1. Macrophage diversity in health and disease, as revealed by single-cell studies [17–25].

A. A catalog of discrete macrophage states in diverse tissues can be identified using single-cell-based approaches, represented here as 5 broad subtypes (see color key below).

B. Added value of knowledge of discrete cell states that enabled a deeper understanding of the existence and characteristics of diverse macrophage populations and how they interact among themselves and other cell types in diverse tissue and disease contexts.

C. An equilibrium between non-inflammatory ('brake') and inflammatory ('accelerator') macrophage subsets can maintain homeostasis in health (*left*). Deregulation in diseased

tissues may present in two ways; excessive inflammation is often accompanied by a broken ‘brake’ and/or a jammed ‘accelerator’ (*middle*), whereas those characterized by inadequate inflammation (excessive tolerance) is often accompanied by a jammed ‘brake’ and/or a broken ‘accelerator’ (*right*). Macrophage (MΦ).

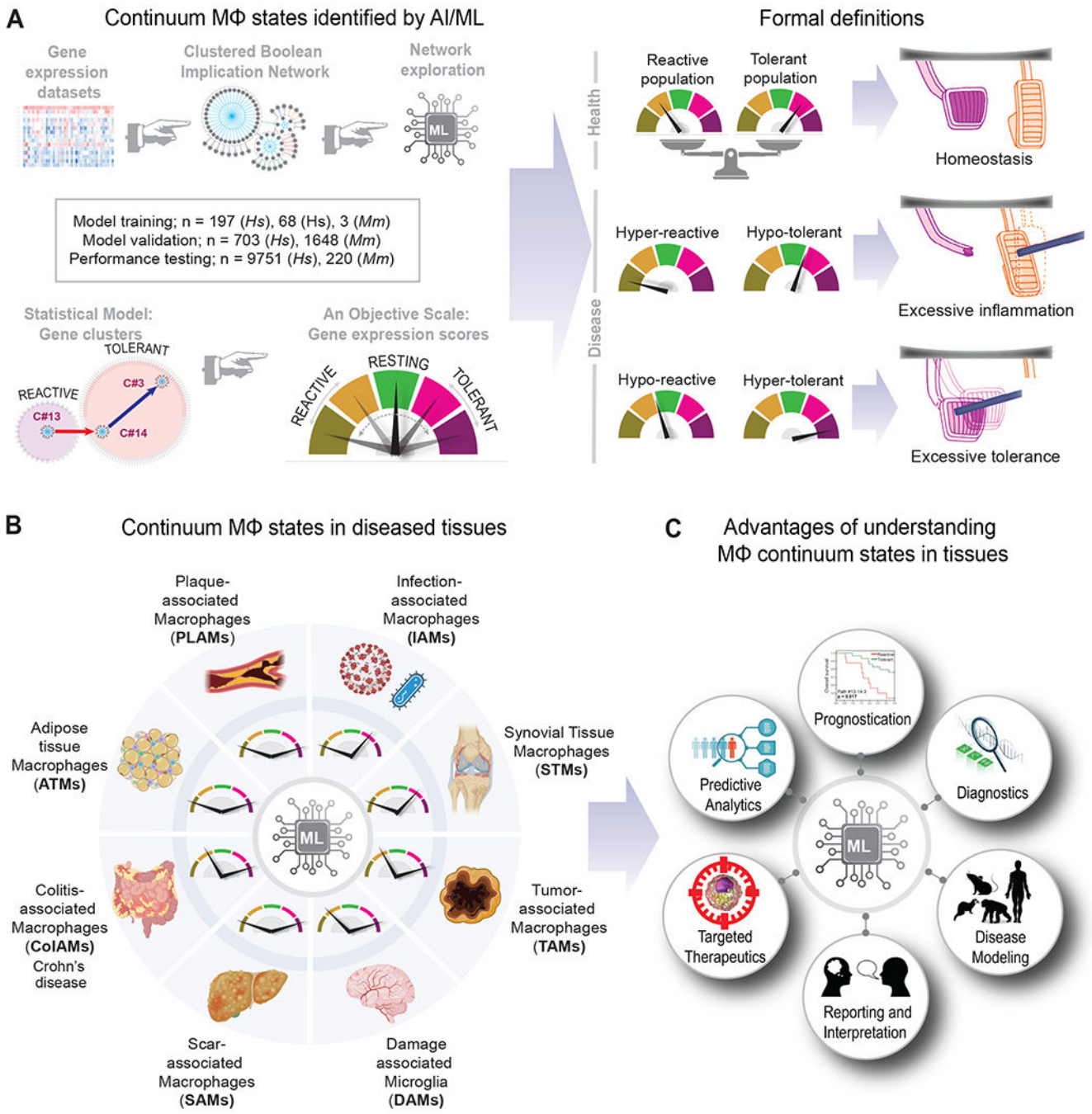

Figure 2: An universally conserved model of monocyte/macrophage states, as identified by machine learning [46].

A. An overview of steps (*top left*) involved in the derivation of formal definitions for macrophage reactivity and tolerance using network-transcriptomics and machine learning (ML). The resultant bipartite model (*bottom left*) could independently and accurately indicate the degrees of reactivity and tolerance. Numbers (n) in the box indicate the number and diversity of samples used for model training, validation and for testing its performance.

Hs, Homo sapiens; *Mm*, Mus musculus. Derivation of formal definitions of 'brake' and 'accelerator' states in health (*top-right*) and disease (*bottom-right*).

B. The altered balance of reactive and tolerant macrophage states that is observed in different diseased states.

C. Added value of a bipartite network model of macrophage states that enables objective assessment and quantification of macrophage states in bulk tissue.

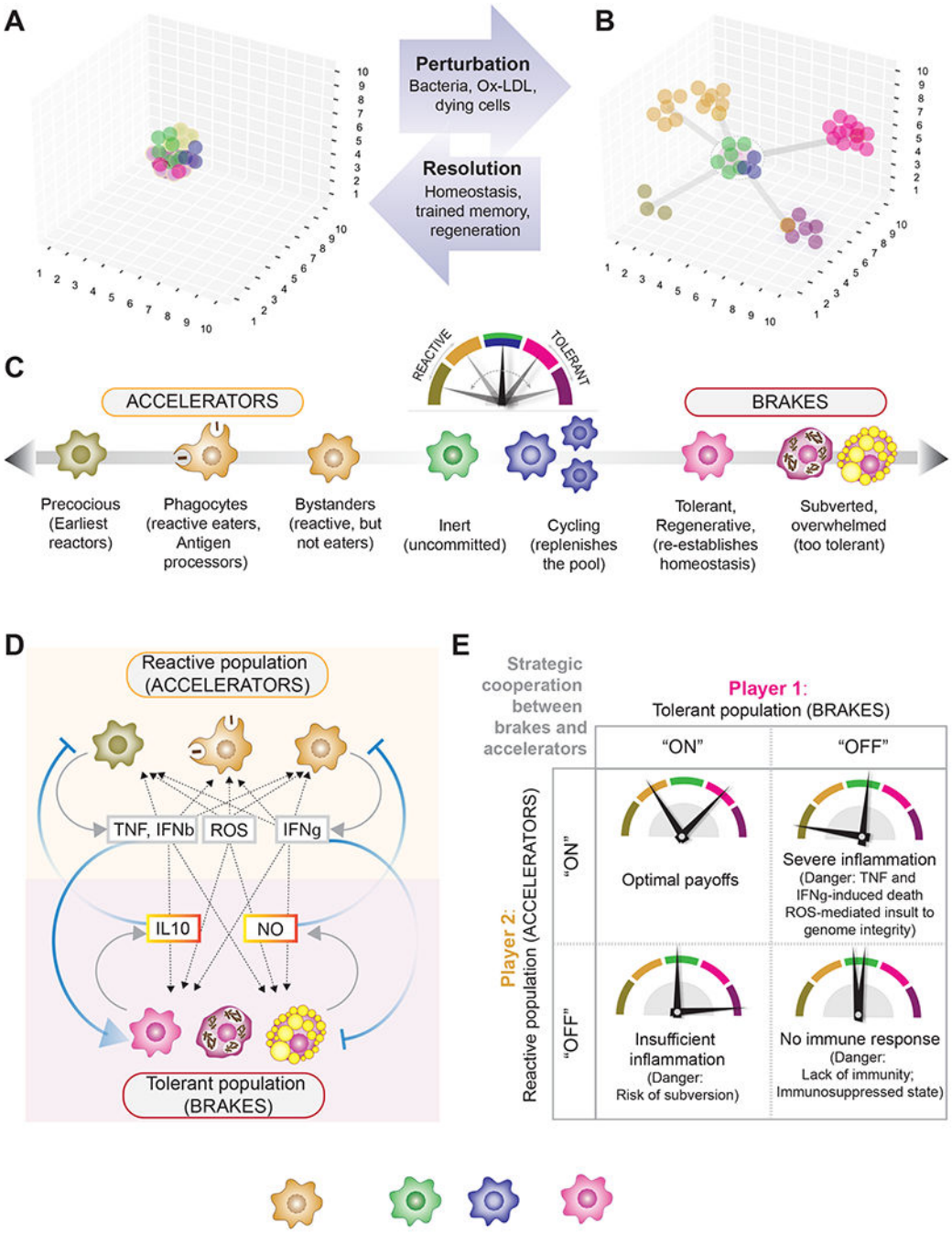

Figure 3. A proposed framework for comprehending macrophage states and their strategic interaction(s) in health and disease.

A-C. A schematic showing macrophage states (each colored sphere) distributed in three-dimensional data space before (A) and after (B) any perturbation. The colors of the sphere represent macrophage subpopulations multi-tasking along a reactivity↔tolerance spectrum (C). Although reactive (accelerators) and tolerant (brakes) cell states that occupy the ends of the spectrum are the most consequential when mounting a balanced inflammatory response, other supportive states may exist. For example, inert cells on standby awaiting signals to join

either the reactive or the tolerant group as needed, or cycling cells that replenish the dying cells. Colors in panel A indicate that the potential of any macrophage to reach any state is pre-determined by genetic and epigenetic heterogeneity in the resting population.

D. Complex auto- and paracrine crosstalk between brake and accelerator populations that regulate their own and each other's behaviors. Continuous grey arrows indicate key pro-inflammatory (top) and anti-inflammatory (bottom) factors produced by each subpopulation. Interrupted black arrows indicate pro-apoptotic and genomic stress inducing effects of the proinflammatory factors on the entire population. Blue lines show suppressive or stimulative effects of one population over the other. While too much of either TNFα [71] or IFNγ [72] (via TLRs) [73] can trigger macrophage death, they have opposing effect on IL10 production [63]. IL10 [63,74] and NO [27], both are known to antagonize the reactive populations and the production of proinflammatory cytokines to restrict inflammation and/or promote resolution; their actions are also highly dependent on cell density. Cell density also impacts the levels of production of TNFα [26].

E. Characterization of macrophage states by Evolutionary Game Theory. Upon perturbation with any inflammatory agent, strategic interactions between brake and accelerator subpopulations (via crosstalk, as in D) is essential to have a most optimal outcome that is dependent on the type of injury, cell density, and perhaps tissue niche.