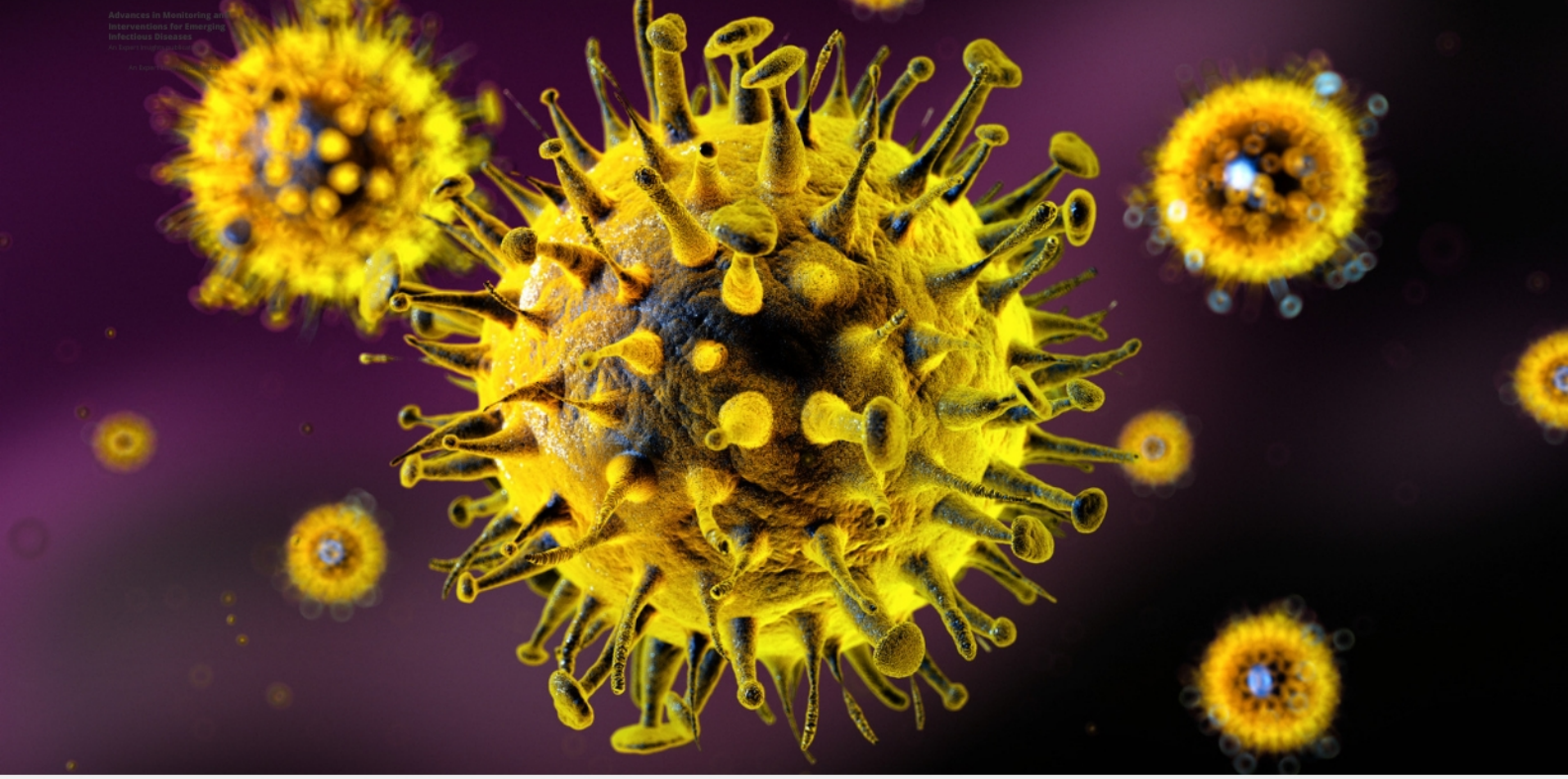
Revising immune cell coordination



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REVIEW

Revising immune cell coordination: Origins and importance of single-cell variation

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Moving from the optimalization of single-cell technologies to the interpretation of the multi-complex single-cell data, the field of immunoengineering is granted with numerous important insights into the coordination of immune cell activation and how to modulate it for therapeutic purposes. However, insights come with additional follow-up questions that challenge our perception on how immune responses are generated and fine-tuned to fight a wide array of pathogens in ever-changing and often unpredictable microenvironments. Are immune responses really either being tightly regulated by molecular determinants, or highly flexible attributed to stochasticity? What exactly makes up the basic rules by which single cells cooperate to establish tissue-level immunity? Taking the type I IFN system and its newest insights as a main example throughout this review, we revise the basic concepts of (single) immune cell coordination, redefine the concepts of noise, stochasticity and determinism, and highlight the importance of single-cell variation in immunology and beyond.

Keywords: heterogeneity · stochasticity · cellular decision-making · interferons

Introduction

Immune signaling systems are generally perceived as being either tightly regulated by molecular determinants (e.g., IL-4 and IL-13 expression by invariant natural killer T cells during inflammation [1]), or being highly flexible attributed to stochasticity (e.g., IL-12 production by macrophages and T cells in response to probiotic bacteria [2]). However, can the lawful regulatory actions observed in deterministic immune signaling systems really be distinguished from the seemingly lawless actions originating from stochastic processes? Are seemingly random events considered stochastic simply because a lack of information on deterministic determinants, or because they truly rely on stochastic principles? While the dualism between determinism and stochasticity is often perceived as black and white, obviously, they are not mutually exclusive. In developmental biology, deterministic control can suppress fluctuating gene expression noise on one hand [3], and on the other hand, gene expression noise can be exploited for cell type differentiation and diversification [4], which are considered to be deterministic processes. Even at the most extreme end of the spectrum, noise can be susceptible to deterministic bias and can be amplified to promote a particular outcome, described as noise propagation in gene networks [5, 6]. Immune signaling systems rely on similar principles, which translate into the basic principles of (single-) cellular decision-making whether to participate in immune responses. Besides, and importantly, immune signaling systems need to be carefully controlled, as every unit of power to kill pathogens (e.g., pro-inflammatory cytokines, such as type I IFN (IFN-I), etc.) also possesses power to kill its own cells [7].

To understand immune signaling systems, a tremendous effort has been put into the optimization and utilization of single-cell technologies, such as single-cell RNA sequencing, single-molecule RNA fluorescence in situ hybridization, single-cell

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quantitative PCR, and microfluidic approaches allowing single-cell activation. These technological advances allowed scientists to study (immune) cells at their greatest detail, to a point where the question arises whether we need such high resolution. In other words, the challenge no longer lies in zooming into cellular behaviors, but instead in how to interpret this gigantic wave of multidimensional, multi-complex single-cell data, and how to correlate certain inputs (encoding) with their subsequent outputs (decoding). In this review, we revise the coordination of immune responses at the single-cell level, as well as population-wide collective immune responses, based on their different modes of communication affected by noise, stochasticity, and determinism. We mainly focus on the conceptualization of the observed phenomena, rather than the technology that made this conceptualization possible, as these are broadly reviewed elsewhere (e.g., in [8] and [9]). The type I IFN system will be used as the main example for additional elaboration, however, the principles discussed in this review are applicable to a wide variety of immune (signaling) systems that go beyond the regulation of IFN-Is, such as the regulation of NF-κB-meditated pro-inflammatory cytokine production, and plasma cell fate programming, etc. [5, 8, 10–13].

Fundamentals of cellular decision-making

Whether considering single-cell behaviors or population-wide collective cellular behaviors, immune responses are generated by single cells making their own decisions. As the field is moving from optimizing single-cell technologies towards the interpretation of multi-complex readouts, a basic conceptualization of cellular decision-making is starting to become defined. Accordingly, cells are confronted with the fundamental problem of their biochemical decision-making machinery being intracellular, whereas they have to process signals being present in the extracellular environment. Therefore, the signals detected on the cell surface have to be processed intracellularly, during which cells have to distinguish from noise and anticipate the future state of their environment. For example, on the one hand, IFN-Is are constitutively expressed to modulate homeostatic balance, whereas on the other hand, IFN-Is are well able to propagate population-wide IFN-I production and signaling upon infection [14, 15]. Besides, immune cells must weigh the costs and benefits of each possible outcome given that future and must decide in the presence of other, potentially competitive decision-makers [i.e., other (immune) cells able of producing soluble signaling molecules] [16]. Likewise, in practice, the optimal immune response balances out the tradeoffs posed by infection (harm caused by the infectious agent) and immunopathology (harm caused by the immune system) [7, 17]. For example, fighting a pathogen favors a rapid and potent immune response, however, proinflammatory cytokines can be harmful to uninfected cells. Theoretically, these tradeoffs are not necessarily symmetrical, resulting in natural selection to favor strong defenses, sometimes at the cost of catastrophic overshooting, as observed in autoimmune diseases [18–21].

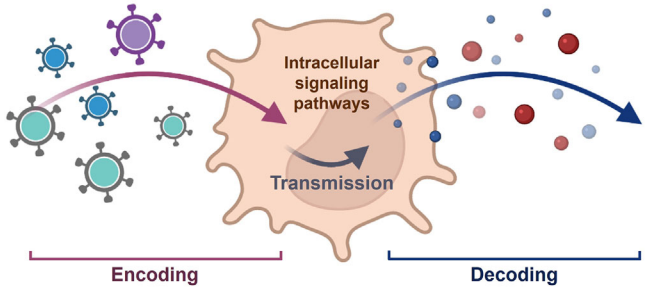

Figure 1. Encoding and decoding of immune cell dynamics. In the process of cellular decision-making, inputs (e.g., viral loads) are encoded by the individual cells. This information is transmitted via intracellular signaling pathways into functional outcomes (e.g., cytokine production), which is referred to as decoding. Figure created using BioRender (https://biorender.com/)

Another fundamental problem arises from the rapidly changing amounts and durations of inputs (e.g., cytokines, chemokines, juxtacrine interactions with cells, etc.), whereas cells must be able to generate reliable and robust outputs (e.g., production of additional cytokines, migration, apoptosis commitment, etc.), especially considering the slight delays introduced between the moment of detection and their actual functional response (e.g., gene expression, protein synthesis, etc.). Accordingly, cells have evolved a myriad of mechanisms to allow successful information-processing in fluctuating and noisy environments. In general, cells first encode the inputs they receive, by transmitting the inputs into intracellular signaling pathways (**Fig**. 1). Subsequently, cells decode these inputs into functional outcomes, which can vary from additional cytokine production, to the upregulation of (membrane) markers or receptors, etc. Advances in microfluidics have enabled a new level of precision in timing and dosage of stimulus, revealing crucial insights into the fundamentals of cellular decision-making, such as differences between analog and digital responses, of which the latter is often observed during immune signaling [8, 22]. For example, NF-κB signaling is considered digital, meaning that cells make binary decisions. However, cells also encode a set of analog parameters [i.e., NF-κB peak intensity, number of oscillations, etc.] to modulate their outcome, observed utilizing microfluidic chambers, real-time microscopy imaging, and lineage tracking [12, 23].

Dealing with noise

'Noise' is a physical manifestation of the concept of 'stochasticity' [24]. Stochasticity, synonymous with 'random', does not mean 'disorganized' or 'unpredictable', as often conceptualized. In fact, although random events cannot be predicted with absolute certainty, individually, they are statistically predictable at higher numbers, like throwing a die a hundred times. Hence, what defines noise, and how does it affect immune cell communication and cellular decision-making? In essence, fluctuations or variations in input signals that by itself do not cause different outcomes are called noise (**Fig**. 2). For example, as for most signaling

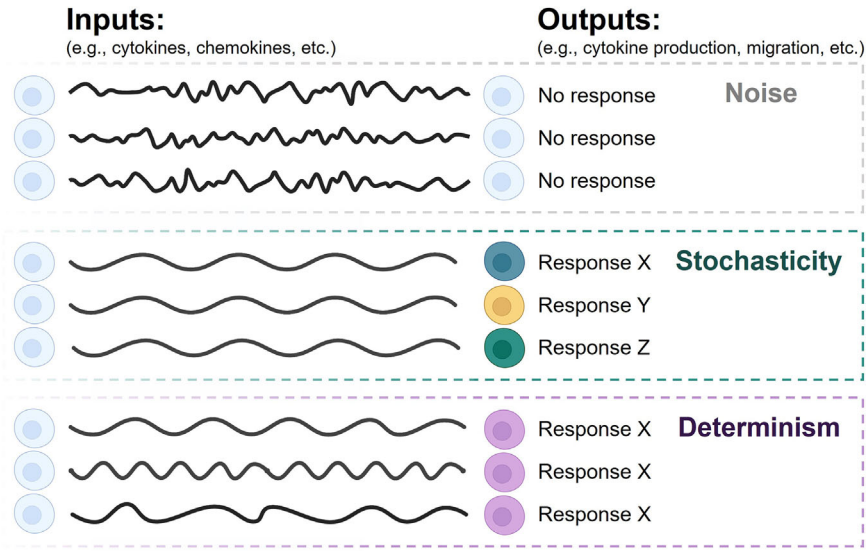

Figure 2. Cellular decision-making influenced by noise, stochasticity, and determinism. Noise is defined by fluctuations in inputs that do not initiate any different cellular outcome. In contrast, stochastic inputs lead to various cellular responses, despite the input can be exactly similar across cells. Determinism is characterized by similar cellular responses based on various inputs. Figure created using BioRender (https://biorender.com/)

systems in immunology and cell biology, an activation threshold needs to be reached before cells act accordingly. However, accumulations of noise can ultimately lead to cellular behaviors that are considered different, while it builds towards reaching the activation threshold, as it changes the so-called molecular potential landscape, thereby changing the likelihood of cells moving towards a certain fate [25]. In other words, noise seems to elicit an uncontrollable mess, however, cell signaling pathways perform with remarkable robustness [26], thereby challenging the dogma of noise being harmful to cellular information processing [27]. In fact, cellular outputs are favorably noisy, as noise reduction requires either high intracellular concentrations or costly negative feedback loops [25]. Taking again the IFN-I system as an example, after transcription of the IFN genes, the actual translation of IFN-I molecules is dependent on heterogeneous host-intrinsic factors, while strong negative feedback loops halt flexibility. Although still not completely understood, novel techniques for decoding single-cell dynamics provide crucial insights into robust cellular decision-making based on noisy inputs, and its actual importance [8, 24]. Accordingly, noise [i.e., intrinsic and extrinsic] appears to facilitate transcriptional control under dynamic inputs by synchronizing and entraining single cells, as observed in murine fibroblasts displaying synchronized NF-κB dynamics upon oscillating TNF inputs to allow for population robustness, observed utilizing valve-based microfluidic techniques [28].

Stochasticity governed by randomness

In many ways, stochasticity determines life at the cellular level, as both signal transduction [i.e., serving as cellular inputs], and gene expression [i.e., generating cellular outputs] involve biochemical reactions, which are stochastic by nature [29, 30]. Therefore, for the immune system to be able to generate predictable responses from stochastic events, cells must rely on numbers, thereby correcting for diverse life cycles and inherently varying molecular profiles. A main immune strategy to overcome this is by averaging high copy numbers of molecules between cells (e.g., plasmacytoid dendritic cells constitutively express high levels of IRF7 [31], macrophages constitutively maintain a pool of untranslated TNF mRNA [32], etc.) in order to generate coherent activity upon infection, reflected by cellular heterogeneity. Additionally, by averaging copy numbers over time, it allows for a sustained coherent activity on a longer timescale, also referred to as temporal averaging [33], and similar to the phenomenon describing transiently heritable fates/memory (e.g., transient heritability of rare gene expression programs associated with drug resistance in melanoma cells) [34, 35]. In other words, the immune system must maintain an arsenal of responsive cell states, at the right numbers and proportions, across individual cells and time. Regarding the IFN-I system, this comes down to the right amount of so-called first responders, as they are hypothesized to drive population-wide signaling, while their absence leads to a halting population-wide IFN-I response [36]. Upon infection, these cells must respond by generating a spectrum of effector functions that suits the nature and magnitude of the threat [33].

Despite the seeming randomness, stochasticity follows similar rules as to what has been described for heritable cell fates, at least to some extent. In fact, stochasticity is dictated by epigenetic switches that allow for the activation and silencing of response genes, which are uniquely heritable through cell division. Therefore, stochastic activation events can be maintained over the timescale of many cellular generations [37]. Accordingly, stochastic regulation of fate-specifying genes enables the expansion of response-driving precursor cells when the activation exceeds the time of a cell division [33, 34]. Additionally, and intuitively, assuming symmetrical cell division, parental cells with increased copy numbers of molecular determinants (e.g., MAVS, IRF3, TRIM25, etc., for IFN-I signaling [38]) will give rise to daughter cells with corresponding copy numbers.

At both levels of response initiation and response outcome, stochastic control allows for cellular heterogeneity that has benefits over hard-wired deterministic responses to specific

combinations of ever-varying inputs [11]. Especially considering the wide variety of threats the immune system can encounter, a deterministic strategy can easily become overly complex when many different cell fates are required and combinations of inputs are uncertain. As a pitfall, stochastic control may also lead to aberrant, sometimes even pathogenic activation and responses, as observed in autoimmunity [7, 20]. However, from an evolutionary point of view, such risks may necessitate the flexibility of fine-tuning stochastic rates for optimal immunity [7].

Determinism dictating responsiveness

Though theoretically stochasticity and determinism seem to cover the two ends of a spectrum, in reality, everything in between yields several reasons for potential misunderstanding. Firstly, multi-step processes with deterministic causation (e.g., gene expression) are often too complicated, therefore practically unpredictable with often a lack of information. The outcome may therefore seem, and be considered stochastic, but may not be lawless. Secondly, systems that seem random can follow fully deterministic principles that exhibit deterministic chaos, also referred to as the 'butterfly effect', such as during cell differentiation [5, 39]. Consequently, deterministic regulation dictated by interacting on–off elements can generate cellular heterogeneity indistinguishable from stochastic randomness [40].

Another reason for confusion, paradoxically, is the fact that deterministic features often, if not always, have a stochastic origin [5]. As currently conceptualized for cellular decision-making, the major difference between stochastic responses and deterministic responses boils down to whether cells are predispositioned to become a responder, and whether this predisposition can be transferred over multiple generations, thereby being heritable [34]. This predispositioning, manifested by epigenetics, in turn can be a stochastic process. As this almost seamlessly overlaps with the heritability of stochastic regulation described earlier, a rather new framework has been suggested to overcome the confusion hopefully once and forever [11]. According to this new framework, the difference between determinism and stochasticity comes down to differences between the effects of various inputs on cellular response outputs. Accordingly, a deterministic response is one in which a range of varying inputs (e.g., varying concentrations of transcriptional regulators, or varying viral loads) will always give the same output (e.g., target gene mRNA). In contrast, a stochastic response is one in which a given input yields different outputs (**Fig**. 2).

Rethinking cytokine-mediated immune cell communication

On top of the intrinsic features characterized by noise, stochasticity, and determinism, individual immune cells, being part of complex immune signaling systems, can communicate in various ways to elicit the appropriate immune response. Together with contact-mediated signaling, cytokine-mediated signaling underlies almost all known (immune) cell-cell communication in multicellular systems [41]. Although it seems rather simple, the diffusion of cytokines upon secretion can lead to rather complicated situations, in which cells have to deal with fluctuating levels, and distinguish noise from biological relevant levels [42]. By displaying different repertoires of receptors (e.g., IFN-I receptors, TNF receptors, etc.), cells sense different types and concentrations of cytokines that diffuse in their surroundings. Upon cognate cytokine-receptor binding, intracellular signaling cascades regulate a diverse set of processes, including apoptosis [43], differentiation [44], and cytokine secretion [15].

Typically, cytokine-mediated communication is categorized into two types, while a third type of cytokine-mediated communication is getting more and more established (**Fig**. 3). In autocrine signaling, cells secrete signaling molecules and simultaneously express the cognate receptor. In paracrine signaling, cells can either secrete signaling molecules without expressing the cognate receptor, or cells can express the receptor without secreting the molecule. Besides, paracrine signaling can be the result of the production of excessive levels of signaling molecules, those that exceed the saturation level of cognate receptor binding on the producer itself, resulting in the diffusion of signaling molecules to surrounding cells. For immune cells, the latter is often the case as in this way autocrine and paracrine signaling can easily be combined to allow for fast propagation of signaling and subsequent collective behaviors. Similarly, during IFN-I signaling, a dedicated fraction of first responders start to produce IFN-Is massively, thereby initiating autocrine feedback loops to sustain this production, but also eliciting cytokine production in neighboring cells as IFN-Is start to diffuse and activate neighboring cells in a paracrine signaling fashion, validated and characterized using droplet-based microfluidics [15, 45]. Although these modes of signaling have primarily been studied in mammalian systems, much progress has been made in relating them to microbial cell systems [e.g., bacterial biofilms], successfully [46]. Likewise, a well-known and ubiquitous form of microbial communication called quorum sensing is becoming part of the basic concepts of cellular decision-making in mammalian systems [16, 47].

Autocrine signaling

The ability to "talk" to yourself, in an immune cell context, has plenty of advantages. The primary purpose of autocrine signaling, accordingly to prevailing belief, has been that cells are able to prevent signaling molecules to diffuse to neighboring cells by massively expressing the cognate, high-affinity receptors [48]. A well-known example in immunology entails the production of IL-2 and the expression of high-affinity receptor component CD25 by activated T-cells [42, 49]. Additionally, computational approaches have revealed that autocrine signaling allows for a tight and well-desired autoregulation of cytokine production, which can either be negative or positive [50]. Negative autoregulation (NAR) occurs when autocrine signaling represses the production of addi-

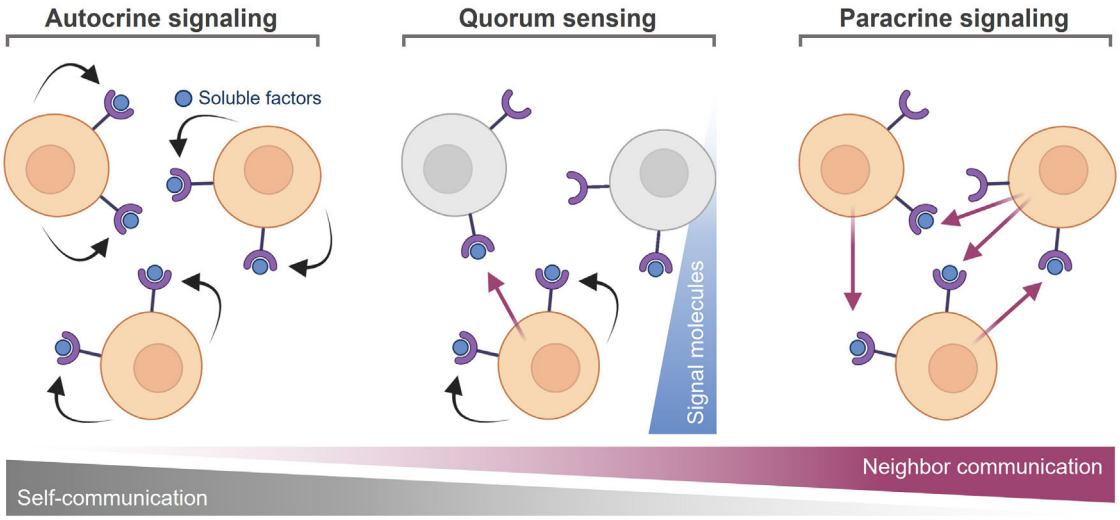

Figure 3. Revising modes of immune cell communication. Cytokine-mediated immune cell communication can occur in various ways. Autocrine signaling is characterized by self-communication, in which the signaling molecules activate cognate receptors expressed by the producing cell itself. On the other side of the spectrum is paracrine signaling, which is characterized by neighbor communication. This implies that signaling molecules diffuse to neighboring cells expressing the cognate receptors. Quorum sensing involves both autocrine and paracrine signaling, during which responsiveness is dictated by signal molecule density. Figure created using BioRender (https://biorender.com/)

tional cytokines, and serves two important functions. First, NAR shortens the response time of cytokine secretion, which is crucial considering the harmful effects of excessive levels [20]. When the concentration and subsequent autocrine signaling reaches the repression threshold of its own production, the production rates decreases, and is therefore always locked into a steady-state level that closely meets the repression threshold [50]. Secondly, and as a consequence of the steady-state, NAR reduces cell-cell variation in cytokine production. Positive autoregulation (PAR), on the other hand, occurs when the production of a cytokine gets enhanced upon autocrine signaling, thereby causing a positive feedback loop, giving opposite effects as compared to NAR with increased response times, as the production of cytokines is slow in the initial phase, when not much cytokines are part of the circuit yet. Subsequently, production picks up once more cytokines are being produced, in other words, when the activation threshold for autocrine signaling is met, which is prone to intrinsic variability in gene expression. Therefore, PAR, especially when its weak, tends to enhance variation, as observed in IFN-I production by plasmacytoid dendritic cells, and other cell types [36, 50]. In short, once cells start to produce IFN-Is, autocrine signaling is induced while IFN-Is bind to the IFN-I receptors expressed by the producing cell, thereby inducing the expression of so-called IFN-stimulated genes, which includes the expression of additional IRF7 transcription factors for IFN-I transcription [51].

Paracrine signaling

As indicated before, paracrine signaling covers multiple possible situations, including the situation in which cells express the cognate receptor without secreting the molecule at first. In the context of inflammation, the latter appeared to be crucial to control cellular heterogeneity and to establish complex dynamic responses, in which a 'core' module of antiviral genes is expressed very early by a few "precocious" cells, but is later activated in all cells, validated using single-cell RNA sequencing upon single-cell activation in microfluidic chambers [52]. Upon (homogeneous) infection, only a fraction of about 1–3% of the total population starts to produce IFN-Is, which has been observed across cell types and experimental settings (reviewed in [15]). In turn, IFN-Is prime surrounding cells via paracrine signaling, which in combination with viral recognition enhances IFN-I production in a much larger fraction of the total population. In another example, macrophages use paracrine communication networks to fine-tune their pro-inflammatory behaviors to avoid tissue damage due to hyperinflammation, with crucial roles for TNF and IFN-Is in inducing IL-10 production [53–55].

Quorum sensing

Quorum sensing is a form of signaling in which cells secrete signaling molecules depending on the density of the total cell population. The first described quorum-sensing example in bacteria entails the expression of genes required for bioluminescence, influenced by bacterial density [56]. In essence, quorum sensing is similar to paracrine signaling, as it is designed for cells to talk to others. By contrast, in terms of its molecular parts, quorum sensing is more similar to autocrine signaling, as cells produce both the signaling molecules and receptors. Therefore, quorum sensing can be considered a phenomenon in which autocrine

cells determine their population density due to cells engaging in neighbor communication, but without self-communication [57]. Typically, at least in bacterial quorum sensing, the receptors have low binding affinity and tend to be expressed in low abundance [58]. Therefore, the presence of signaling molecules can only be detected when there is a sufficiently high density of it, which only occurs when the total population of producing cells is sufficiently high. Subsequently, quorum sensing triggers collective and coordinated actions, as observed in dendritic cells activated in the draining lymph node in vivo [59]. Therefore, on the other hand, quorum sensing can be considered a phenomenon in which paracrine cells originate from the total population when a certain threshold of cell density is reached. Importantly, and in contrast to bacterial quorum sensing, immune cell quorum sensing is often, if not always, characterized by only fractions of activated cells, rather than the total population. For example, liposaccharide-induced activation of macrophages is dependent on cell density, thereby influenced by quorum sensing, and is considered bimodal in a sense that cells either become highly activated, or remain inactivated [55]. Not bimodal in a sense that either the whole population gets activated, or the whole population remains unactivated, as often observed in bacterial quorum sensing. Therefore, the term quorum licensing, as brought up by the authors in that same study, might be more suitable to describe this phenomenon for immune cells. Going back to the example, macrophages use quorum licensing to link the history of cell density to the fraction of cells that become highly activated, independent of canonical LPS-induced intracellular feedback induced upon TNF signaling [55].

Functional importance of single-cell differences

After decades of probing, measuring, and analyzing single-cell behavior, cellular heterogeneity turned out to be a hallmark of any cell population of seemingly identical cells, depending on the resolution of study rather than its biological relevance [60]. The gain of resolution is both literally, represented by high-resolution microscopy, and figuratively, when considering the movement from bulk averages towards single-cell, multi-dimensional data. Logically, the question arises whether the observed single-cell differences are relevant, and to what extent. While population-averaged assays are powerful tools to identify components and interactions within complex signaling networks, they easily mask the presence of rare, perhaps important subpopulations [36]. For example, using conventional bulk analyses (e.g., ELISA), the distinction between first, second, and non-responders during IFN-I signaling could not have been observed [15]. However, if single-cells behave ergodically (i.e., time-averaged and population-averaged, dictated by stochasticity only), a representative distribution of cellular behaviors could, in theory, still be estimated based on population studies [60]. In practice, ergodicity may be difficult to test rigorously and is expected to be overruled by both noise and determinism during immune response initiation [15, 61].

Today, numerous scenarios and hypotheses have been described explaining the functional importance of single-cell differences, of which three hypotheses and corresponding examples, which are most relatable to immune signaling systems, will be addressed [62] (**Fig**. 4). The response distribution hypothesis (1) entails cellular decision-making originating from intrinsic cellular heterogeneity (e.g., varying transcription factors levels, varying cell cycle states, etc.) to allow a fractional or dose-dependent population response, as observed in epithelial innate immunity in response to various stimuli [i.e., bacterial, viral, and cytokine-mediated signaling], validated using live-cell imaging [8, 12]. The fate plasticity and priming hypothesis (2) describes uncorrelated, sub-threshold fluctuations in regulators of cell fates, allowing subpopulations of cells to be primed to respond. Accordingly, response fates switch transiently and are prone to the effects of priming, as observed in the (de-)sensitization for IFNα signal transduction by both positive and negative feedback regulators [63]. Finally, the crowd control hypothesis (3) entails the presence of a rare subpopulation with the capacity to respond to perturbation, emitting local signals to coordinate population-wide responses. This hypothesis is similar to the one proposed to explain the crucial role of the fraction of first responding cells during IFN-I signaling in orchestrating population-wide responses [15, 36]. Overall, depending on what can be considered as an immune strategy, single-cell variation is not just a coincidence. Instead, it is hypothesized to be of curial importance.

Concluding remarks and future perspectives

Unlike the majority of cell types, most immune cells and their progenitors are constantly on the move and are broadly distributed throughout the body. Therefore, it seems logical that they depend less on predefined spatiotemporal cues and constraints, but instead rely on bottom-up self-organization orchestrated by noise, stochasticity, and determinism [33]. Especially stochastic principles appear crucial in guiding bottom-up differentiation of rare precursors, thereby offering flexibility and robustness in population-wide signaling control, which may be hard to achieve with deterministic principles. The importance of distinguishing between stochastic and deterministic regulations, and their relative contribution while not being mutually exclusive, comes down to the exploitation for new treatment strategies. In the context of immune cell behaviors, stochastic processes can be targeted according to the priming principles described by the fate plasticity and priming hypothesis, while desired cellular behaviors can be manipulated, thereby enforcing favorable outcomes, such as sensitizing cells for enhanced cytokine production. In contrast, deterministic processes can be targeted at the levels of progenitors, often located in the bone-marrow, where response fates are divided according to the crowd control hypothesis. In this case, potential responders could perhaps be regulated by gene therapy, which allows for the upregulation or silencing of gene expression

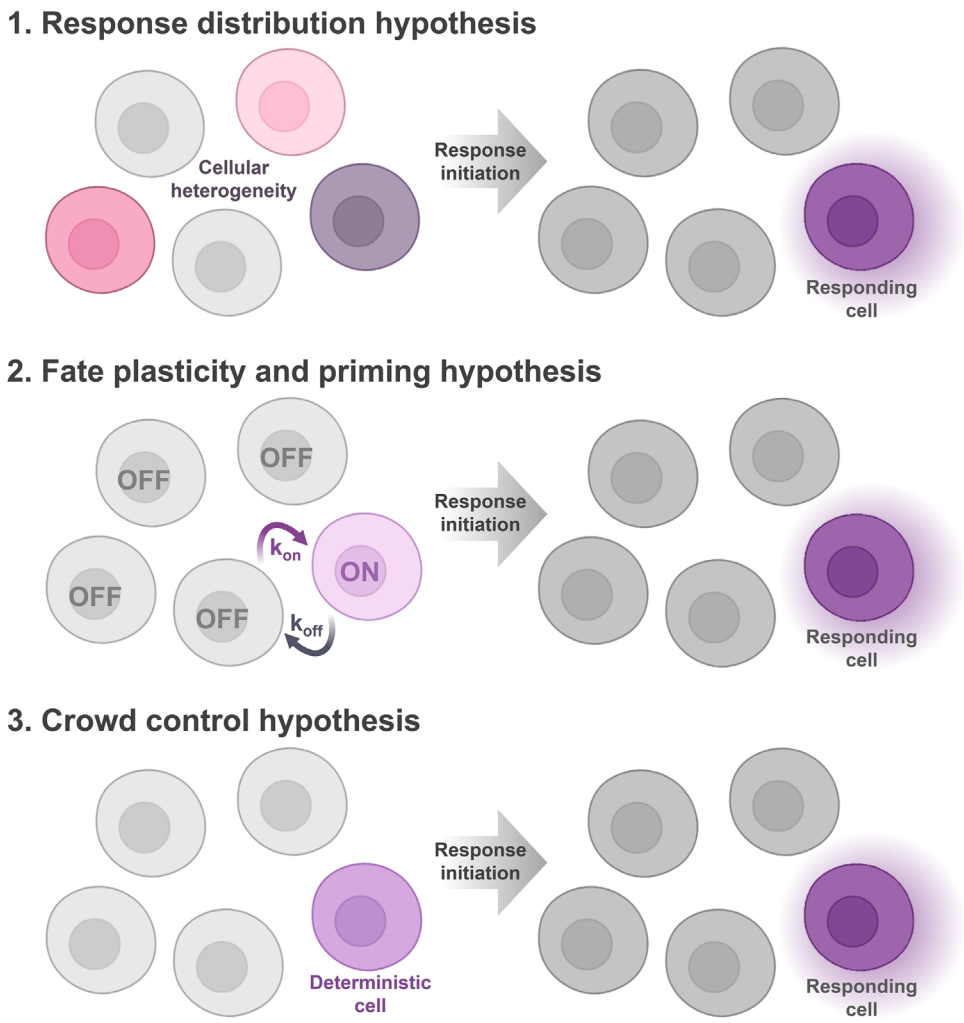

Figure 4. Hypotheses explaining the functional importance of single-cell differences. According to the response distribution hypothesis (1), cellular heterogeneity dictates binary cellular decision-making, while only a fraction of cells is able to elicit responses given its intrinsic (molecular) status. The fate plasticity and priming hypothesis (2) describes fluctuations in regulators of cell fates, allowing subpopulations of cells to be primed to respond (ON). Therefore, cell switch fate throughout (from ON to OFF, and vice versa), determined by kinetic parameters (k_{on} and k_{off}), which can be influenced by priming. Finally, the crowd control hypothesis (3) entails the presence of a rare subpopulation with a predetermined fate to become a responding cell upon response initiation. Figure created using BioRender (https://biorender.com/)

programs defining the responders' fate. Nevertheless, it is clear that the field of single-cell biology with its technological advances and its concepts are only at the start of its transformation, where mainly the tools to explore it keep on improving impressively. Let our conceptual understanding on the complexity, or perhaps simplicity of (immune) signaling systems join in this insightful transition.

Acknowledgments: This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (Grant agreement No. 802791). Furthermore, we acknowledge the generous support by the Eindhoven University of Technology.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

Author contributions: L.E. conceptualized and wrote the article. J.T. supervised the study, revised the manuscript, and acquired funding.

Data availability statement: Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Received: 29/6/2022
Revised: 15/8/2022
Accepted: 11/10/2022
Accepted article online: 17/10/2022