Decoding the dynamics of multilayered stochastic antiviral IFN-I responses

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Type I Interferon (IFN-I) responses were first recognized for their role in antiviral immunity, but it is now widely appreciated that IFN-Is have many immunomodulatory functions, influencing antitumor responses, autoimmune manifestations, and antimicrobial defenses. Given these pivotal roles, it may be surprising that multilayered stochastic events create highly heterogeneous, but tightly regulated, all-or-nothing cellular decisions. Recently, mathematical models have provided crucial insights into the stochastic nature of antiviral IFN-I responses, which we critically evaluate in this review. In this context, we emphasize the need for innovative single-cell technologies combined with mathematical models to further reveal, understand, and predict the complexity of the IFN-I system in physiological and pathological conditions that may be relevant to a plethora of diseases.

From population averages to single-cell resolution

Providing a robust first line of host defense, type I interferon (IFN-I; see Glossary) responses were first recognized for their role in antiviral immunity by Isaacs and Lindemann [1]. In addition, IFN-Is are known to have numerous immunomodulatory functions that go beyond the scope of antiviral immunity, with potent roles in antitumor responses, autoimmune diseases, and microbial infections [2–4]. The expression and regulation of IFN-I has been extensively studied in mammals, and important molecular regulators have been characterized (Box 1; reviewed in [4–6]). Population-averaged measurements have generated not only a wealth of knowledge on IFN-I responses and regulation, but also contradictory results that remain hard to interpret or understand [7]. Over the past few years, an overwhelming wave of single-cell data has changed the dogma on how systemic immune responses are generated, challenging the traditional assumption that all cells of a given lineage display nearly identical behaviors upon stimulation.

Improving technologies and experimental approaches, such as the use of fluorescently tagged cells, reporter mice, single-mRNA molecule in situ hybridization, single-cell quantitative PCR, single-cell RNA sequencing (scRNA-seq), and microfluidic approaches, have revealed a massive degree of mammalian cellular heterogeneity during IFN-I responses, along with multiple layers of stochasticity, which in turn yield cellular heterogeneity [7–17]. scRNA-seq has allowed the examination of virus–host interactions in a variety of in vitro systems, again highlighting the extreme cell–cell variability within IFN-I signaling pathways [9–11]. Given the pivotal roles of IFN-Is in multiple physiological and pathological conditions, it may be surprising that only small fractions of cells begin producing IFN-I upon viral infection, in vitro, ex vivo, and in vivo [7,13,16–19]. Of note, these fractions of first responders, also referred to as ‘precocious cells’ or ‘early responding cells’, are proposed to arise from all-or-nothing cellular decision-making, a phenomenon that has been observed for a variety of proinflammatory cytokines [7,20–22]. In turn, IFN-Is prime surrounding cells via paracrine signaling to enhance IFN-I production upon viral recognition, a process initiated by a much larger fraction of so-called cellular second responders;

Highlights

The rise of single-cell technologies has highlighted a massive degree of cellular heterogeneity during mammalian interferon (IFN-I) responses.

Multilayered stochasticity gives rise to all-or-nothing cellular decision-making, resulting in tightly regulated fractions of responding and nonresponding cells.

Mathematical modeling has provided crucial insights into the origins of cellular heterogeneity and the complexity of IFN-I responses.

Mathematical models can be considered as powerful tools to dissect and predict IFN-I-mediated physiological and pathological outcomes.

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Box 1. Basics of IFN-I responses

IFN-I-mediated immune responses provide a robust first line of host defense against viruses and other invading pathogens [5]. Given their long history of coevolution, these responses are hardwired within mammalian genomes and are complex. This complexity ensures that IFN responses can be tightly regulated, which is needed to avoid tissue damage and potential autoimmunity [103]. IFN-Is constitute the largest IFN class, comprising IFN-α, IFN-β, IFN-ε, IFN-ω, and IFN-κ in humans. Different subtypes display differential tissue expression and binding affinities to their receptors (reviewed in [101]). Consequently, various outcomes, involving antiviral, immunomodulatory, and antiproliferative activity, can occur [102–104]. IFN-I production can be elicited in almost every cell type, simply because viruses have evolved to successfully infect almost every cell type. Similarly, IFN-αR receptors (IFNARs) are constitutively expressed on almost all cells, allowing cells to acquire an antiviral state.

The prevalent pathway of IFN-I induction is initiated when viral, xenogenic, or autologous nucleic acids are recognized by the cytosolic receptors, RNA helicases, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5). Upon activation, these receptors, also known as RIG-I-like receptors (RLRs), initiate a signaling cascade leading to the translocation and activation of the transcription factor interferon regulatory factor 3 (IRF3), which induces the transcription of IFN-Is (reviewed in [4]). Via the expression of Toll-like receptors (TLRs3/7/8/9) in endosomes, phagocytic immune cells, such as macrophages, dendritic cells, and pDCs, can recognize endosomal viral nucleic acids, resulting in the activation of the transcription factor IFR7, which functions similarly to IRF3 [105]. IFN-I production is further enhanced via autocrine signaling of IFN-α and IFN-β predominantly, resulting in the upregulation IRF7 and other signaling components [106].

Upon vast production, IFN-Is diffuse and subsequently bind to IFNARs expressed on neighboring cells. Upon activation, the IFNAR1-associated protein tyrosine kinase 2 (Tyk2) and the IFNAR2-associated protein tyrosine kinases Janus kinase 1 (JAK1) regulate the phosphorylation and activation of different signal transducer and activator of transcription (STAT) proteins [5]. Activated STAT proteins homo- or heterodimerize and translocate to the nucleus, where they promote the expression of several target genes, such as IFN-stimulated genes (ISGs), including IRF7 [106]. In addition, IFN-Is can directly activate nuclear factor-XB (NF-XB) signaling pathways, resulting in the production of additional proinflammatory cytokines, thereby promoting the population-wide anti-viral responses in both infected and uninfected cells [105].

this, results in a second, more profound wave of IFN-I production [13–18,22–28]. Lastly, most cells, despite being infected and/or activated via paracrine signaling, will not start producing IFN-Is over the course of an infection or due to inflammation, and have been called nonresponders [17,18].

The IFN-I system is evidently complex and, therefore, the need to apply advanced technologies, innovative approaches, and different ways to interpret large amounts of single-cell and systems immunology data, has been magnified. Mathematical models enable scientists to dissect complex biological systems into smaller pieces that are easier to understand. Additionally, simulations that generate hypothetical outcomes can be validated with experimental data to improve the quality and the complexity of the models. Moreover, mathematical models can be used as powerful tools to predict certain experimental outcomes during interventional studies. For the IFN-I system, as for many other biological systems, mathematical models have provided crucial insights regarding the complexity of the system, which we address in this review. These models have enabled scientists to better understand the highly heterogenous cellular behaviors and cellular decisions made during the course of a viral infection. Here, we provide a brief overview of the multilayered stochasticity arising from virus-induced signaling, how it causes cellular heterogeneity, how it leads to the three aforementioned cell fates, and how mathematical models can aid in the interpretation, understanding, and prediction of outcomes. Altogether, these models can serve as powerful tools to reveal and understand the complexity of IFN-I signaling pathways, in a manner that may surpass knowledge of antiviral immune responses alone.

Stochasticity can dictate cellular heterogeneity

As cellular heterogeneity in the IFN-I system has become more evident, its origins are being studied more extensively. Cellular heterogeneity in the IFN-I system has been deemed to be the result of multiple different stochastic events, divided approximately into three distinct, but

**Glossary**

**Cellular decision-making**: process in which cells convert analog inputs into digital (all-or-nothing) responses by regulating their gene expression accordingly.

**Cellular heterogeneity**: variability between cells, often referred to in cells from a similar lineage or subset, which can involve numerous cellular characteristics (e.g., morphology, phenotype, gene expression, behavior, etc.).

**Epigenetic modifications**: alterations in the epigenome, involving chemical changes to the DNA and histone proteins; can alter transcriptional activation or silencing of genes.

**First responders**: considered the fraction of cells (1–3% of the total population) responding to virus-induced signaling by secreting type I IFNs via nucleic acid receptors and transcription mediated by IFN regulatory factors.

**Gene expression noise**: observed level of variation in gene expression among a population of supposedly identical cells.

**Immune quorum sensing**: phenomenon of nonlocal, population-level communication between cells; typically occurs upon the initiation of cytokine secretion and detection, followed by polarization of gene expression programs and cellular behavior in response to changes in population and cytokine densities.

**Interferon regulatory factors (IRFs)**: transcription factors, including IRF3 and IRF7, that regulate the expression of IFN-Is upon signaling via nucleic acid receptors inside the cell.

**Interferon-stimulated genes (ISGs)**: genes of which the expression is initiated upon stimulation by IFNs via IFN receptor signaling.

**Luria-Delbrück fluctuation test**: assay originally used to demonstrate the occurrence of genetic mutations in bacteria in the absence of selection, rather than being a response to selection, in which variability between different clonal populations is assessed.

**Nonresponders**: fraction of cells (usually most of the total population) that, despite the presence of first and second responders, do not start producing IFNs upon IFN signaling or nucleic acid detection.

**Nucleic acid sensors**: receptors, both cytosolic and endosomal, sensing viral, xenogeneic, or autologous nucleic
intertwined layers (Figure 1) [7,14]. Each layer of stochasticity originates chronologically over the course of a viral infection and involves different biological, biochemical, and biophysical processes. Consequently, these three layers of stochasticity can result in widely variable cellular outcomes in terms of infection onset time, viral load, IFN-I expression, IFN-I signaling, antiviral response, and so on. Even before the first cells become infected, the first layer of stochasticity may originate from the random distribution of viral particles that the individual cells encounter [29]. Additionally, genetic variability within the virus population may lead to even more variation, with some virus particles being more or less successful in establishing infection [30,31]. Subsequently, individual viral particles can vary in their replication efficiency inside the host, leading to differences in host cell signaling and subsequent IFN-I secretion [22,32]. Finally, a recent report showed that the recognition of live cytomegalovirus-infected cells mounted different IFN-I response dynamics in vitro in human primary plasmacytoid dendritic cells (pDCs) compared with the self-recognition of virus mimetics [33].

The second layer of stochasticity dictating cellular heterogeneity may involve the host cell state at the time of infection [34]. This layer arises from both deterministic processes (e.g., cell cycle) and acids, thereby inducing innate immune responses.

Ordinary differential equation (ODE) models: mathematical models often based on multiple ordinary differential equations, containing one or more functions of one independent variable and the derivatives of those functions.

Pathogen-associated molecular patterns (PAMPs): highly conserved small-molecular motifs on pathogens that can be recognized by TLRs and other pattern recognition receptors, inducing innate immune responses.

Post-translational modifications (PTMs): covalent and enzymatic alterations to proteins following biosynthesis.

Retinoic acid-inducible gene I (RIG-I): cytosolic pattern recognition receptor that recognizes viral double-stranded RNA and, once activated, is responsible for the downstream signaling leading to the production of IFNs.

Second responders: considered the fraction of cells (10–60% of the total population) that starts producing IFN-Is upon viral recognition only after IFN-induced signaling of IFN-Is is produced by first responders.

Stochasticity: (biological) systems of which the behavior is intrinsically nondeterministic, thus random and unpredictable.

Transcriptional bursts: fundamental property of gene expression resulting in ‘bursts’ or ‘pulses’ of RNA transcription.

Type I interferons (IFNs): one of the three classes of IFNs (type I, type II, and type III) that include cytokines mainly produced upon viral infection and secreted by nucleated cells.

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**Figure 1.** Cellular heterogeneity during antiviral interferon I (IFN-I) signaling originates from three distinct, but intertwined, layers of stochasticity. The cellular heterogeneity found in the mammalian IFN-I system is deemed to be the result of multiple different stochastic events, which can be divided approximately over three distinct, but intertwined layers [7]. Each layer of stochasticity originates chronologically over the course of a viral infection, starting with the stochastic elements introduced by the virus or other stimuli. These include the random distribution of viral particles and genetic variability that leads to differences in infectiousness, replication inside the host, and so on. Next, the second layer of stochasticity involves all stochastic elements introduced by the host cell state at the time of infection, comprising both deterministic and stochastic cellular processes. This leads to differences in susceptibility to the virus, IFN-I production, viral progeny release, and so on. Finally, different spatiotemporal diffusion gradients of both the release of viral progeny and IFN-Is comprise the third layer of stochasticity, thereby exposing individual cells to different gradients of viruses and IFN-Is, a process that is further enhanced by complex tissue structures [41]. Figure created using BioRender (https://biorender.com/).
stochastic processes, (e.g., gene expression noise) [35–37]. It is thought that intrinsic gene expression noise results from the stochastic nature of biochemical reactions, whereas extrinsic gene expression noise results from cell–cell fluctuations of components that are involved in generating the response [38]. Every step of IFN-I signaling involves limiting reactions and signaling intermediates; thus, every step is subject to the effects of gene expression noise [15]. For instance, in vitro, overexpression of individual components/molecules, such as retinoic acid-inducible gene I (RIG-I), interferon regulatory factor 3 (IRF3), and IRF7, significantly increased the percentage of murine fibroblasts expressing IFN-Is upon infection with Sendai virus, relative to control cells [15,39].

Once individual cells become infected, we argue that the third layer of stochasticity is deemed to arise upon the first expression of IFN-Is. Subsequently, the IFN-I-induced protective response can involve not only the infected cells, but also the surrounding, yet uninfected, cells [27]. Therefore, the third layer of stochasticity may no longer involve single cells evolving independently, but also populations of cells that comprise complex signaling networks. The heterogeneous expression of IFN-Is in infected cells may lead to nonuniform spatiotemporal diffusion gradients, which may depend on cell density, multiplicity of infection, and tissue architecture, as verified in mathematical models and in vitro settings approximating tissue conditions, as well as in murine in vivo studies [27,40,41]. Indeed, paracrine signaling enables neighboring cells to protect themselves from becoming infected by the expression of interferon-stimulated genes (ISGs) and the secretion of other protective cytokines [5]. Cells that are less able to protect themselves may allow higher rates of viral replication, leading to the release of higher numbers of viral progeny relative to more highly protected cells [7]. This introduces a new spatiotemporal diffusion gradient, which may further enhance the heterogeneous nature of the IFN-I system by exposing neighboring cells to different viral loads. Therefore, we posit that this final layer of stochasticity overlaps with the first layer of stochasticity, which drives a certain level of cellular heterogeneity due to exposure to variable viral loads.

Heterogeneity can drive cellular decision-making
In contrast to gene expression noise found in bacteria and yeast, causing protein amounts to fluctuate around mean values in individual cells, the stochastic nature of IFN-I responses (covering the heterogeneity observed in IFN-I production and the response to IFN-I sensing) can give rise to an all-or-nothing phenomenon in which cells either switch on IFN-I gene expression or not [7,14] (Figure 2). This switch can occur at widely variable time points, verified by time-lapse imaging of signal transduction and gene expression dynamics in murine reporter fibroblasts [14]. This all-or-nothing decision-making has been observed more broadly for other cytokines, in different contexts, across different species [21,42–47]. In fact, hundreds of key immune genes are bimodally expressed across cells, even for genes that are highly expressed among populations [20]. Of note, different types of cytokine and chemokine array, such as IFN-β, CXCL10, CXCL11, CCL2, and CCL5, tend to be co-expressed across species, including human, macaque, and mouse, with both positive and negative regulators of these cytokines being co-expressed [20,47]. These findings suggest that cytokine expression, as in the case of IFN-Is, is tightly controlled at the individual cell level.

Two mechanisms have been proposed to be important for driving cellular decision-making and, thus, are deemed essential for orchestrating IFN-I responses; these include epigenetic modifications of gene expression, including the genes encoding IFN-Is and ISGs, and post-translational modifications (PTMs) of signaling molecules [5]. Recent studies (e.g., in human macrophages) show that IFN-Is induce extensive remodeling of the epigenome by the modulation of histone marks, the creation and activation of new enhancers, and the disassembly of
enhancers, thereby reprogramming cellular responses to environmental cues [48] (reviewed in [49]). Moreover, chromatin remodeling is mediated by IFN-activated STAT proteins and newly induced transcription factors, such as IRFs, which recruit chromatin-remodeling enzymes and bind gene regulatory elements [48,50]. IFN-induced epigenomic changes can confer transcriptional memory, thereby sustaining the expression of ISGs and other sets of genes encoding inflammatory molecules, such as tumor necrosis factor (TNF) and interleukin (IL)6, which are involved in the priming of other immune cells and induce various immunological events, including tolerance [48,51]. Additionally, by targeting distinct signaling steps or components, conventional PTMs, such as phosphorylation, polyubiquitylation, acetylation, and methylation, can modulate...
IFN-I signaling transduction, with the phosphorylation of JAK1, TYK2, STAT1, and STAT2 identified as key events that drive IFN-I induction (reviewed in [52]). Finally, recent data interpretations (e.g., on scRNA-seq, single-molecule mRNA counting) and mathematical modeling suggest that seemingly noisy cellular decisions are defined by rather simple fundamental functional constraints, with variations in genome architecture being a major source of heterogeneity [38,53–55].

Fractions of first responders

Upon viral infection, multiple parameters affect the cellular decision to become one of the first IFN-I-producing cells, also referred to as ‘early responding’ or ‘precocious’ cells, later referred to as ‘first responders’ by the field. Despite the complexity of IFN-I signaling, the percentage of first responders found across multiple different studies in vitro, ex vivo, and in mice, often comprises only a fraction of the total population [7,13,16–19]. For instance, a microfluidic chip allowing single-cell activation and scRNA-seq supported this phenomenon ex vivo: in primary mouse bone marrow-derived dendritic cells (BMDCs) that were activated with various stimuli [i.e., Toll-like receptor (TLR) 2/3/4 ligands], scRNA-seq and RNA fluorescence in situ hybridization of Ifnb1 and Ift1 identified a frequency of only 0.8% of first responders among BMDCs [16]. Additionally, utilizing droplet-based microfluidics combined with single-cell RNA-seq, the phenomenon was verified in human primary pDCs, among which only 0.5–3% of first responders were detected upon TLR7/8/9 activation [17,18]. In addition, in vitro in human primary fibroblasts infected with herpes simplex virus 1 (HSV-1), only ~2–3% first responders were detected [7]. Elimination of the first responders upon single-cell activation in droplets showed that IFN-I responses were impaired without the presence of these cells, highlighting their importance in initiating population-wide response dynamics [18]. Recently, this finding was supported by a study reporting that patients with innate IRF7 deficiencies resulting in impaired IFN-I induction were more prone to developing life-threatening disease symptoms upon severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection compared with healthy individuals [56].

In general, first-responder cells exhibit a distinct transcriptome profile, with upregulated expression of genes encoding cytokines and chemokines [7,17,19]. However, it is unclear to what extent this profile is influenced by autocrine signaling. Moreover, the distinct characteristics of the early and potent IFN-I production observed in first responders and their quantity appear to be independent from an IFN-I-mediated feedback loop, as evidenced from paracrine signaling blockade in vitro using IFN-I-neutralizing antibodies or IFN receptor (IFNAR)-blocking antibodies, as well as IFNAR-knockout (Ifnar1−/− KO) mice [17,19]. Of note, during HSV-1 infection in primary human fibroblasts, the first responders were abortively infected, while most of the remaining cells became highly infected [7]. The implications of these findings are that first responders are key drivers of population-wide IFN-I dynamics; another interpretation may be that first responders could be epigenetically poised to enable better protection against the virus, possibly due to the immediate massive production of IFN-Is and subsequent autocrine signaling, although this remains conjectural [18]. Nevertheless, in favor of this hypothesis, a study in human primary pDCs reported that a fraction of first responders was not affected by different stimulus concentrations [17]. Therefore, we posit that becoming a first-responder cell might be viewed as a matter of cell fate, rather than a cellular decision, while by contrast, predisposition (e.g., regarding the abundance of IFN-I signaling intermediates) might be viewed as a stochastic process. Accordingly, one study showed strong correlations between inactivated sister cells transiently expressing RIG-I-like receptor signaling components [57]. Hypothetically, it may be reasonable to speculate that epigenetically regulated, transient expression of IFN-I signaling molecules might determine which cells become first responders, a possibility that warrants further investigation.
To establish the secretory phenotype of first responders, enhanced secretion of IFN-Is is promoted by multiple positive feedback mechanisms that lock cells into an autocrine signaling loop to sustain IFN-I signal transduction (e.g., by maintaining and increasing high basal amounts of IRF7 and other positive feedback regulators) [58,59]. After these cells have provided the first burst of IFN-Is to the system (e.g., as shown in pDCs), these cells either undergo apoptosis or enter an IFN-I-desensitized state that can last up to several days to avoid harmful effects to the host [60–63]. Several mechanisms underlying the IFN-I desensitized state have been proposed, such as IFNAR1/2 endocytosis and ISG-mediated desensitization, as described elsewhere [64,65]. Dysregulated mechanisms controlling the IFN-I desensitized state in humans can increase the chances of developing autoimmune disorders, such as systemic lupus erythematosus (SLE) and Sjögren’s syndrome (reviewed in [66]). Similar risks may occur if the IFN-I activation threshold is too low, with excessive concentrations of IFN-Is resulting in autoreactive cell activation [67]. By contrast, excessively stringent thresholds may limit rapid responses, thereby increasing the risk of chronic inflammation, as documented for rheumatoid arthritis or ulcerative colitis clinical findings [68]. Therefore, the regulation of first responders appears to be vital for avoiding pathological outcomes [18].

Waves of second responders

Upon IFN-I production by fractions of first-responder cells, IFN-Is begin to diffuse towards IFNARs on neighboring cells. Especially following early autocrine signaling, and enhancing the production of IFN-Is in first responders, paracrine signaling becomes increasingly abundant when the probability of diffusion increases and once IFNARs on first responders are bound to secreted IFN-Is [41]. At ~0.5–6 h post infection, depending on cell type and experimental conditions, the paracrine loops begins to prime surrounding cells, which, in combination with the viral input, can lead to a greater fraction of IFN-I-producing cells, ranging from 10% to 60%, as evidenced from a variety of in vitro, ex vivo, and in vivo experiments, in mainly mouse and human [13–18,22–28]. These cells, later referred to as second responders, can only start producing IFN-Is after having sensed the IFN-Is produced by first responders, a finding that was validated in vivo using Ifnar−/− mice [69]. Based on mathematical modeling, once the second responders become activated and IFN-I production ensues, the IFN-I system is dominated by paracrine signaling, and subsequently and coherently responds to viral infection dynamics [40].

The extent of the population-wide response may depend on the balance of induced feedback mechanisms. For example, positive feedback mechanisms augment the IFN-I signaling cascade by amplifying or maintaining the expression of signaling intermediates, such as the upregulation of IRF7 and nucleic acid sensors, whereas negative feedback mechanisms operate mostly at the IFN-I receptor level (reviewed in [5]). Currently, the three positive feedback proteins (IRF9, STAT1, and STAT2) and four negative feedback proteins (SOCS1, SOCS3, USP18, and IRF2) are considered to be most important in dictating the mammalian IFN-I system [70,71]. While autocrine signaling initiates JAK/STAT-mediated positive feedback mechanisms, thereby stabilizing nuclear IRF3/7 and nuclear factor κB (NF-κB) in first-responder cells, paracrine signaling sensitizes second-responder cells through a JAK/STAT-mediated positive feedforward mechanism that results in the upregulation of positive feedback components [63]. Moreover, the sensitization effects of paracrine signaling were recently shown to lead to the acquisition of histone H3 methylation in mouse embryonic fibroblasts and BDMCs, which accelerated the recruitment of RNA polymerase and other transcription factors to sustain IFN-I production, thereby revealing a potential mechanism underlying the fast and potent IFN-I production upon priming [51]. Additionally, many other positive and negative feedback mechanisms have been proposed, including IRF1 and IRF2, for positive and negative regulation, respectively; these putative mechanisms merit further investigation since they may all contribute to all-or-nothing cellular decisions within the IFN-I system (reviewed in [72,73]).
In theory, the effects of paracrine signaling or IFN-I priming that are elicited by first responders could result in three possible scenarios in the surrounding second-responder cells. First, we propose that IFN-I priming might lead to the desensitization of cells, resulting in lower cellular activation upon stimulation. Second, IFN-I priming might not change IFN-I signaling in primed cells. Third, priming might lead to the sensitization of cells, similarly to that of second responders, resulting in higher activation upon stimulation. A study in human hepatocyte-derived cellular carcinoma cells showed that their priming with a high IFN-I dose (1400 pM) resulted in desensitization of cells, and was mediated by the negative regulators SOCS1 and USP18, whereas priming with a low dose (2.8 pM) hypersensitized the pathway [71]. Similarly, IFN-I priming of HeLa cells for 24 h led to desensitization, whereas priming for only 2 h led to hypersensitization [37]. Therefore, in hypersensitized cells, the all-or-nothing cellular decision upon viral exposure might be much shorter and more potent than in desensitized cells, enabling second responders to rapidly and effectively elicit antiviral responses [71]. Altogether, whereas the fraction of first responders appears surprisingly stable among different experimental setups and different cell systems, the fraction of second responders appears to depend on the stochastic nature of IFN-I signaling dynamics, which may contribute to explain the reasonable range of IFN-I producing cells that has been documented in the field. This hypothesis is in agreement with the poor correlations observed between second-responder sister cells (murine fibroblasts) producing IFN-I upon Newcastle disease virus infection, emphasizing factors such as probability and randomness [14]. Therefore, it is reasonable to hypothesize that, in contrast to first responders, second responders are mainly stochastically regulated, in agreement with much of the literature describing this phenomenon.

The importance of nonresponders

As the general understanding of first and second responders increases, so too does our ignorance regarding so-called ‘nonresponders’. Even within populations of uniformly infected or activated cells, a rather large percentage of the total population will not produce IFN-I [17,18]. However, what drives these cells to become unresponsive remains elusive. Even by using droplet-based microfluidics, which allows for the precise administration of equal amounts of stimulus to single cells, most pDCs studied will not produce IFN-I upon activation with synthetic viral ligands, despite being properly activated, as measured from the production of other proinflammatory cytokines [16,17]. In vivo, individual cells will not all experience the exact same amount of stimulus. Instead, individual cells harbor complex signaling networks, surrounded by elaborate tissue architectures, which comprise different types of cell. Therefore, although pDCs are considered to be specialized IFN-I-producing cells, they are not always in the ‘driver’s seat’ regarding the initiation of IFN-I production, because they barely become infected by viruses in vivo (as shown in mouse and human) [62,74,75].

In contrast to the nonresponders mentioned earlier, especially during local infections, many cells will sense neither the virus nor the produced IFN-I. Therefore, another way of conceptualizing nonresponders in vivo is by studying immune homeostasis, which is aimed at spatiotemporally limiting viral replication as well as the excessive and potentially harmful amounts of IFN-I. Traditionally, local cellular competition, space limitations, and confluence restriction have been considered to be the main underlying factors shaping immune homeostasis [76]. Currently, immune quorum sensing is widely appreciated as a new major mechanism contributing to immune homeostasis [77,78]. Conceptually, it involves population-level communication on a scale that is larger than during paracrine signaling. It may typically occur when individual cells secrete and sense so-called ‘immune autoinducers’, in this case, IFNs, the concentration of which is usually too low for cells to respond to. However, as soon as enough cells produce IFNs, there might be correspondingly high enough concentrations of such autoinducers, thus leading to either the activation or repression of certain genes in individual cells, and to population-wide effects. Moreover, due to competition between cytokine diffusion and consumption, spatial
self-assembled niches of high cytokine concentrations with sharp boundaries may arise, according to the diffusion–consumption mechanisms of cytokine propagation [41,79]. Therefore, we argue that, together with the concept of immune quorum sensing, a hypothetical IFN-I landscape evolves, which is characterized by IFN-I hot spots and corresponds to niches of responding and nonresponding cells, thus allowing the tight regulation of IFN-I response dynamics (Figure 3).
Figure 4. Examples of different mathematical modeling approaches used to capture the complexity of the interferon I (IFN-I) system. Over the years, different modeling approaches have proven their purpose in understanding the complexity of the mammalian IFN-I system. The multilayered stochasticity present in the system involves not only single cells evolving independently, but also populations of cells being part of complex signaling networks. Therefore, the different levels, from molecular regulators (A) to individual cell behaviors (B) to complex spatiotemporal gradients (C), have been incorporated in numerous mathematical modeling approaches, as described in the literature. (A) Schematic
Insights from IFN-I modeling

The multilayered stochasticity and spatiotemporal dynamics of the IFN-I response have provided fertile grounds for applying a diverse set of mathematical modeling tools to understand its regulation, both at the population level and within individual cells (Figure 4). Since the beginning of IFN-I modeling, ordinary differential equation (ODE) models, comprising multiple differential equations, have dominated the field. Therefore, ODE models have had major success in modeling the intracelular regulatory circuits of IFN responses (Box 2). Furthermore, there has been a rich tradition of studying virus–host interactions using ODE frameworks that track the population dynamics of three species: target cells, infected cells, and viral titers [80]. Although highly informative, this approach is rather simplified, with the underlying assumption being that diffusion occurs sufficiently rapidly to create a spatial homogeneous or a well-mixed compartment, while the complexity of tissues often prevents that [41]. The simple structure of ODE models, compared with stochastic or spatial counterparts, is particularly advantageous in terms of the availability of standard toolboxes to simulate models, fitting models to data, discriminating between alternative model architectures, and leveraging well-developed analytical tools, such as stability analysis and sensitivity analysis, to systematically study dynamic cellular behaviors [80].

The first attempts to incorporate more complexity to ODE models, thereby better representing physiological conditions, are illustrated by several studies expanding these models to capture IFN-I dynamics and antiviral activity in uninfected cells that were later virally infected. Generally, in such approaches, infected cells secrete IFN-Is at a specific rate, which then functions to: (i) render uninfected target cells refractory to viral infections [81,82]; (ii) activate the innate immune response for cytolysis of infected cells [83]; and (iii) block viral replication in infected cells that are in the early stages of infection via autocrine and paracrine IFN-I signaling [84]. The fitting of these ODE models to in vivo data on influenza virus infection in horses showed a rapid decline in viral loads after their initial peak, due to cytolysis of infected cells via IFN-I-activated natural killer cells [83]. Similarly, application of ODE models to in vitro data in a study of Dengue virus infection in adenocarcinomic human alveolar basal epithelial cells, revealed rapid IFN-I induction in individual cells (within 2 h), which was amplified by autocrine feedback loops and, according to modeling, was key in driving IFN-I-mediated antiviral protection [84].

The next step in further enhancing ODE models can be achieved by incorporating heterogeneity in IFN-I responses, whereby only a fraction of infected cells synthesize IFN-Is [61]. Moreover, these models also consider feedback loops in which the rate of IFN-I production from infected cells is modulated by extracellular IFN-I concentrations. Fitting models to data from four different experimental systems, ranging from murine fibroblasts to human monocyte-derived DCs, not only revealed a significant fraction of nonresponders, but also supported a model in which the rate of IFN-I secretion decreased with increasing extracellular IFN-I concentrations [61]. These observations are consistent with the molecular mechanisms of IFN-I desensitization, mainly by inhibiting IFNAR signaling [71]. Model analysis further suggested that IFN-I feedback loops were subject to strong viral antagonism that blocked infected cells from synthesizing IFN-Is, but at the same time, cellular heterogeneity in IFN-I responses had an important functional role in protecting IFN-I feedbacks from viral antagonism [61]. The benefits of having a heterogeneous subpopulation of responders were recently investigated using spatial agent-based models of representation of the most prevalent signaling molecules during IFN-I responses (blue: main signaling in first responders; green: main positive regulators; red: main negative regulators). (B) Schematic representation of signaling networks in first and second responder cells, and nonresponder cells. (C) Schematic representation of spatiotemporal diffusion gradients of viral load and IFN-Is. Figure created using BioRender (https://biorender.com/). Abbreviations: IFNAR, IFN receptor; IRF, interferon regulatory factor; ISRE, interferon-sensitive response element; RIG-I, retinoic acid-inducible gene I; STAT, signal transducer and activator of transcription.
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BOX 2. Modeling intracellular IFN-I signaling

ODE models have had success in dissecting the intracellular regulatory circuits of IFN-I responses, many of them using a system of coupled biochemical reactions converted into ODE models (e.g., using chemical rate equations and Michaelis-Menten kinetics) [106–108]. One group developed a model of the JAK/STAT and RIG-I signaling pathways together with the positive feedback loop from secreted IFN-Is [107]. The model was tested using experimental data from human DCs that were first pretreated with IFN-α followed by influenza virus infection in vitro, capturing the intracellular dynamics of mRNA amounts of genes encoding IRF7, IFN-α, IFN-β, and SOCS1, as well as nuclear amounts of phosphorylated STAT and IRF7 proteins. The model provided important insights into the kinetics of IFN-I induction and predicted the saturation of IFN-I induction beyond a pretreatment threshold of dosage and time, a finding that was confirmed with experimental data [107].

Recent work revealed a comprehensive mechanistic model of the IFN-I signal transduction pathway in human hepatoma cells, including positive feedback loops mediated through IRF9 and STAT1/2, as well as negative feedback regulation via USP18 and SOCS1 [71]. The model was calibrated with temporal measurements of different signaling components and target genes in hepatocyte cell lines based on initial priming with IFN-α, followed by a second IFN-α treatment. Model analysis integrated with experiments revealed that the dynamics of the coupled positive-negative feedback circuit caused hypersensitization of the IFN-I pathway at low pretreatment dosages. By contrast, high pretreatment dosages led to desensitization of the pathway with reduced amounts of STAT1/2 phosphorylation [71]. Moreover, USP18 alone was not sufficient to drive desensitization, pointing to a concerted role of both negative feedback regulators (USP18 and SOCS1) in this process [71]. These desensitization results were also seen in HeLa cells, and captured via a simple ODE model that phenomenologically modeled a fast positive loop via IRF9 and a delayed negative loop via USP18 [37]. The model successfully predicted IRF9 induction for pulsatile versus sustained IFN-α inputs, with sustained treatments leading to reduced IRF9 induction. Of note, desensitization at high IFN-I pretreatment dosages in these recent studies was not reported in DCs, in which nuclear phosphorylated STAT amounts were saturated at high pretreatment dosages, suggesting that desensitization does not occur in immune cells [107]. However, it is unclear whether this difference in desensitization is related to cell type or experimental protocols, and further studies are warranted to dissect these discrepancies.

Advances in single-cell technologies have uncovered remarkable intercellular variation within an otherwise homogeneous and uniformly stimulated cell population. Therefore, the usage of stochastic models has become a necessity. While stochasticity has been attributed to low copy numbers of gene products in microbial cells, transcriptional bursts from individual promoters is considered a key driver of stochastic gene expression in mammalian cells [55,86–89]. Aided by stochastic modeling, expression variation can be harnessed to better understand interpromoter architectures. For example, cell-cell variation in Tnfa mRNA counts (encoding TNFα), as measured by single-molecule RNA counting upon TLR activation, revealed a two-state model in which the promoter randomly switched to a transcriptionally active state by synthesizing a burst of gene products before switching back to a transcriptionally inactive state [54]. By contrast, transcription of Il1b (encoding interleukin-1β) in murine macrophages cells, follows a more complex promoter topology, with additional regulatory steps, that results in enhanced, heterogeneous expression [54]. In this modeling analysis, quantification of intercellular variation by scRNA-seq showed that signaling-regulated transcription factors could modulate noisy expression of downstream genes by influencing the frequency and/or size of transcriptional bursts [54,90]. In addition, the concerted action of both autocrine and paracrine signaling and stochastic expression were investigated using spatially extended agent-based models [40].

Over the past decade, increasing evidence shows that stochastic gene expression can drive individual cells within an isoclonal population to distinct expression states that predisposes them to different cell fates upon stimulation. For example, stochastic expression of IFNARs in human breast cancer cell lines can determine cellular responses to IFN-α treatment, with only a
subpopulation of cells having high receptor expression showing antiproliferative activity [91]. In addition to stochastic expression, epigenetic promoter regulation can create multigenerational cell states. For example, epigenetic regulation at one of the TLR promoters showed the predisposition of a subpopulation of human epithelial cells to respond to pathogen-associated molecular patterns (PAMPs) [92]. Lineage tracing coupled with modeling revealed that multiple gene modules, including those important for IFN-I signaling, were expressed only in small fractions of the total cell population, implying that the corresponding epigenetic signatures might predict which cells would become responders, and which ones would not [92]. Of note, the expression of these gene modules was transiently heritable, while studies in vitro showed that, in most cases, these modules were transcriptionally silenced after several generations, suggesting a complicated underlying mechanism of determining cell fate [92]. Along these lines, in mouse embryonic stem cells and human melanoma cells, for example, several new modeling approaches are using cell fates at the end of a cellular lineage tree to determine whether single-cell responses are purely random or a deterministic function of an underlying heritable state [57,93–96]. Inspired by the classical Luria-Delbrück fluctuation test, such methods may be useful in providing novel insights regarding the heritability of cellular decision-making of first- or second-responder, and nonresponder cells.

Concluding remarks
Advancing single-cell technologies and improving experimental approaches have brought not only seemingly endless opportunities and large amounts of data, but also overwhelming complexity in interpreting the data. While IFN-Is are vital in initiating a plethora of immune responses, much progress has been made in identifying the molecular regulators mediating the IFN-I system. Improved mathematical models are aiming to dissect and explain one of many cytokine systems, with the goal of further improving the simulations and interpretation of the underlying biology. The phenomenon of first and second responders, and nonresponders has been observed for different immune responses involving the secretion of cytokines, both in vitro and in vivo, and may be further characterized. We posit that this immune response may be a strategy used by the host to ensure robust immunity, while simultaneously ensuring fine-tuned flexibility and precision in adjusting and adapting to different environmental scenarios within and around cells.

While transcriptional regulators have been a main focus of studying IFN-I dynamics for decades, information on additional types of regulator, such as epigenetic regulators, feedback loops, cellular interactions, and diffusion gradients, is shedding light on an already complex IFN-I system. Additionally, the crosstalk between different types of cytokine (e.g., IFN-I, IFN-II, and IFN-III) and cellular responses is becoming more apparent, thereby adding a new layer of complexity to in vivo outcomes of immunity [72,96]. One way to address some of this complexity is by decoding single-cell dynamics, in combination with mathematical models, and there are numerous examples in the field that are revealing the power of these approaches to move the field forward [97]. As knowledge of the IFN-I system improves, so too are the mathematical models that contribute to explain and predict experimental outcomes. In addition, further opportunities to apply these approaches are emerging (see Outstanding questions). For instance, model simulations are already providing crucial insights in viral immunology; for example, they have been applied to analyze SARS-CoV2 infections, aiming to improve clinical outcomes of patients with Coronavirus 2019 (COVID-19). Using such approaches, it has been suggested that repetitive short-term administration of IFN-Is to patients with hepatitis B virus or SARS-CoV2 could lead to less desensitization of IFN-I signaling dynamics compared with the conventional, prolonged treatment approaches currently used [98,99]. Finally, robustly designed experimental approaches, together with reliable data interpretation and proper validation of mathematical models with experimental data, will be necessary to continue unraveling the complexity of the IFN-I system in physiological contexts.

Outstanding questions
To which extent does each layer of stochasticity contribute to the introduced cellular heterogeneity observed during IFN-I responses? Although studies have concluded that the stochastic expression of IFN-I is exclusively a feature of the infecting virus, it is hard to explain the large amount of heterogeneity found in uniformly infected cells by only considering the variability introduced by specific viruses. Moreover, the lack of correlation between viral RNA and IFN-I mRNA expression in individual cells suggests that the heterogeneity observed in IFN-I induction is mostly of a host rather than viral origin.

What is the approximate ratio between stochasticity and predeterminism for becoming a first, second, or nonresponder? Increasing evidence for a vast fraction of first-responder heritability has been gathered over the past decade. By contrast, the concepts of immune quorum sensing and diffusion-consumption mechanisms of cytokine propagation, which are both stochastic in nature, may contribute to explain individual cells becoming second responders.

Can mathematical models contribute towards improved treatment strategies for certain pathologies? While some patients with IFN-I-associated disease (e.g., rheumatoid arthritis) thrive with IFN-I-based treatments, (e.g., anti-IFNAR therapies), others do not. Ultimately, the phenotyping of IFN-I status for different patients might inform aspects of disease pathogenesis and treatment responses in a variety of diseases.

What are the exact influences of other signaling systems (e.g., other types of IFNs) on the IFN-I system? While the IFN-I system is already complex when studied in isolation, potentially disturbing, noise-introducing systems can influence outcomes. Thus, it would be relevant to assess and incorporate multiple different signaling systems into mathematical models to bridge the gap between simplified in vitro assays and highly complicated signaling networks in vivo.
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