Stimulus-specificity in the responses of immune sentinel cells

Katherine M. Sheu[1], Stefanie Luecke[1] and
Alexander Hoffmann

Abstract
Innate immune sentinel cells must initiate and orchestrate appropriate immune responses for myriad pathogens. These stimulus-specific gene expression responses are mediated by combinatorial and temporal coding within a handful of immune response signaling pathways. We outline the scope of our current understanding and indicate pressing outstanding questions.

Addresses
Institute for Quantitative and Computational Biosciences and Department for Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA, 90095, USA

Corresponding author: Hoffmann, Alexander (ahoffmann@ucla.edu)
[1] contributed equally.

The innate immune response is a first-line defense against invading pathogens and coordinates the activation and recruitment of specialized immune cells, thereby initiating the adaptive immune response. While the adaptive immune system is capable of highly pathogen-specific immunity through the process of genetic recombination and clonal selection, innate immunity is frequently viewed as a catchall system that initiates general immune activation.

In this review, we are reexamining this view, as we are distinguishing between immune sentinel functions mediated by macrophages and dendritic cells and innate immune effector functions mediated by cells such as neutrophils, NK cells, etc. Given pathogen diversity, including modes of entry, replication cycles, and strategies of immune evasion and spread, all successive waves of the immune response ought to be tailored to the specific immune threat, leading us to postulate that immune sentinel functions by macrophages and dendritic cells ought to be highly stimulus-specific. Here we review the experimental evidence for stimulus-specific responses by immune sentinel cells which initiate and coordinate immune responses, as well as the mechanisms by which this specificity may be achieved.
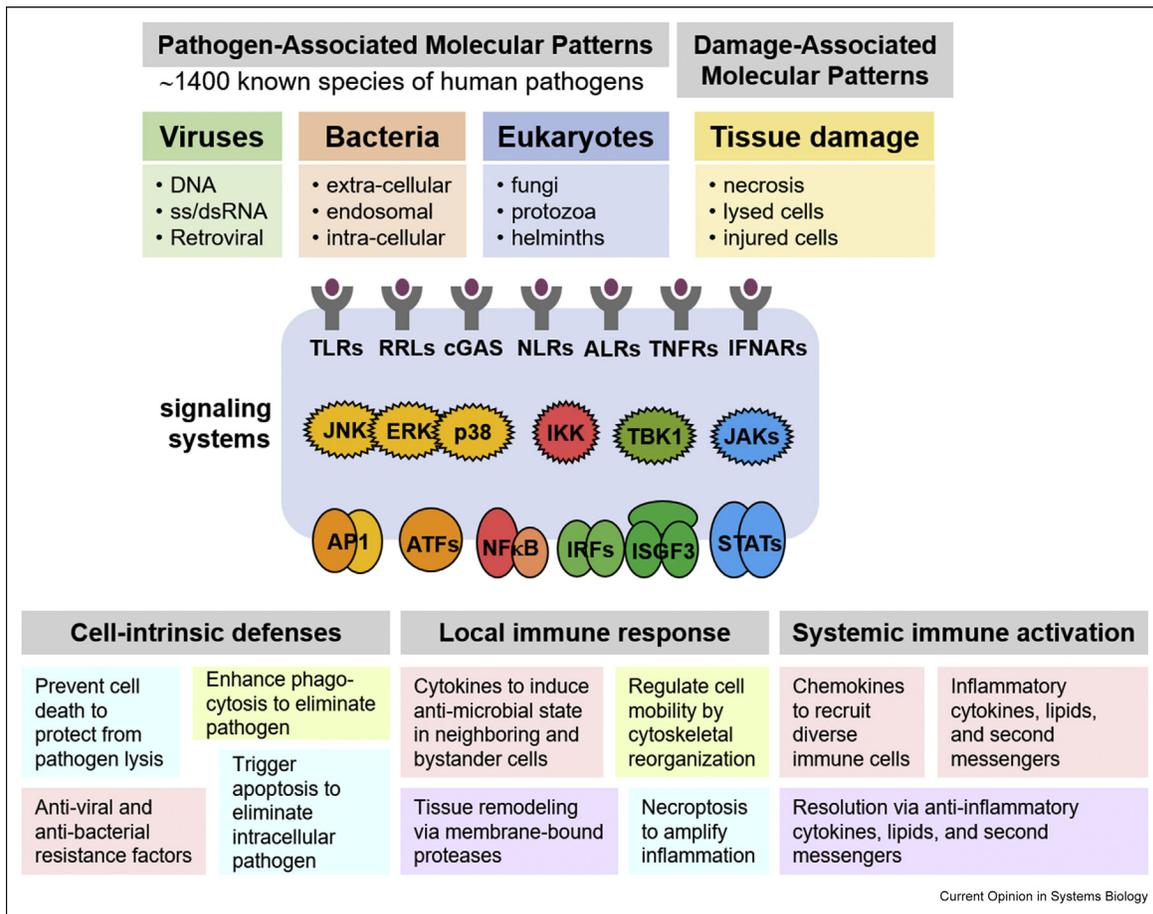
Functions of immune sentinel cells
Immune sentinel cells can sense a wide variety of molecules that derive from viruses, bacteria, fungi, or parasites, termed pathogen-associated molecular patterns (PAMPs), or that are indicative of tissue damage, termed damage-associated molecular patterns (DAMPs). They are recognized by dozens of diverse transmembrane and cytosolic pattern recognition receptors (PRRs) [1,2]. Cytokines produced by first responders, such as tumor necrosis factor (TNF) or interferons (IFNs), may be sensed through cytokine receptors. Both PRRs and cytokine receptors transmit information about the stimuli via signaling adaptors to a stimulus-responsive signaling network with overlapping downstream pathways consisting of signaling kinases and transcription factors to coordinate diverse immune sentinel functions (Figure 1) [1–3].

The functions of immune sentinel cells provide for both local antimicrobial activity and systemic immune activation, and they coordinate the resolution and tissue healing when the threat is eliminated [4]. On exposure to an immune threat, immune sentinel cells may induce resistance factors that may directly limit pathogen invasion, replication, or assembly [5]. Through secretion of inflammatory cytokines, phospholipids, and second messengers, immune sentinel cells communicate and spread this antimicrobial state to bystander cells within the tissue [4].

To limit pathogen spread, phagocytic immune sentinel cells, such as macrophages and dendritic cells, upregulate their ability to engulf pathogens through dramatic reorganization of their cytoskeletons [4]. Production of nitric oxide and reactive oxygen species

Figure 1

Functions of tissue-resident immune sentinel cells. Immune sentinel cells as such tissue resident macrophages or dendritic cells are capable of sensing diverse immune threats via dozens of different sensors and of responding in numerous distinct ways to regulate cell-intrinsic defenses, local immune responses, or systemic immune activation. Colors illustrate the diversity of functions but do not represent a color code.

contributes to pathogen killing [4]. In response to some intracellular pathogens, the induction of cell suicide may limit pathogen viability and may occur in the absence of inflammatory mediator release through apoptosis or through inflammation-inducing necroptosis [7].

Cell-intrinsic pathogen defenses are complemented by the induction of local inflammation and secretion of chemokines for the recruitment and activation of diverse immune effector cells such as neutrophils and NK cells. Secreted and membrane-bound proteases remodel the extracellular matrix and assist migration of immune sentinel cells to the infected site. Eventually, immune sentinel cells orchestrate the adaptive immune response through systemic inflammation and modulatory cytokines that increase antigen presentation, adaptive immune cell production, selection, maturation, and recruitment [4].

Finally, immune sentinel cells are also responsible for resolving inflammation and restoring tissue homeostasis through production of anti-inflammatory mediators, such as cytokines, and through tissue remodeling and repair [4].

In sum, innate immune activation has severe consequences at the level of tissue and organism homeostasis. Indeed, these functions are intrinsically toxic and may harm the physiology of the organism. In other words, the diverse functions of immune sentinel cells are to be deployed on an "only as-needed" basis. By this consideration immune sentinels should be expected to mount responses that are specific and appropriate for the particular immune threat.

Stimulus-specific gene expression
Many but not all functions of immune sentinel cells involve the *de novo* expression of gene products. To

examine pathogen responses of immune sentinel cells, transcriptome profiling studies have been carried out by a number of laboratories. A first report identified a core set of genes that are activated regardless of the pathogen [8], with subsequent studies revealing more diversity [9–11]. Interestingly, whereas immune sentinel cells such as fibroblasts and macrophages are capable of stimulus-specific gene expression when challenged by PRR ligands or cytokines, immune effector cells, such as B-cells, showed much less stimulus-specificity in parallel analyses of early gene expression studies (Figure 2a). This distinction may relate to the different physiological roles of innate versus adaptive immune cells. Unlike tissue-resident immune sentinel cells, B-cell specificity is encoded in the genome of each B-cell clone [12], but stimulation of its receptors triggers the activation of its immune effector functions, including a dramatic proliferative program.

However, the general impression of transcriptomic profiling studies is that there are just a few patterns of gene expression distinguished by the involvement of a handful of key signaling pathways [9,10]. A common approach in these analyses is the use of clustering methods which identify dominant patterns in complex datasets and the use of heatmaps for visualizing them. However, such methods may underestimate the degree to which individual genes do not actually match some of the dominant patterns [13] because they are either forced into a cluster or they are visually lost in the heatmap display of thousands of data points. The fact that polarizing cytokines found in distinct tissue microenvironments may alter pathogen-response gene expression in myriad ways [14] indicates a high degree of regulatory diversity.

To reexamine the stimulus-specificity of innate immune responses, we analyzed some of the available datasets encompassing immune response transcriptomes across a variety of cell types and stimulus conditions, using consistent analysis methods (Figure 2b). These datasets encompass cells participating in innate immunity, such as human and mouse macrophages, and dendritic cells. The results indicate that careful clustering and heatmap

Figure 2
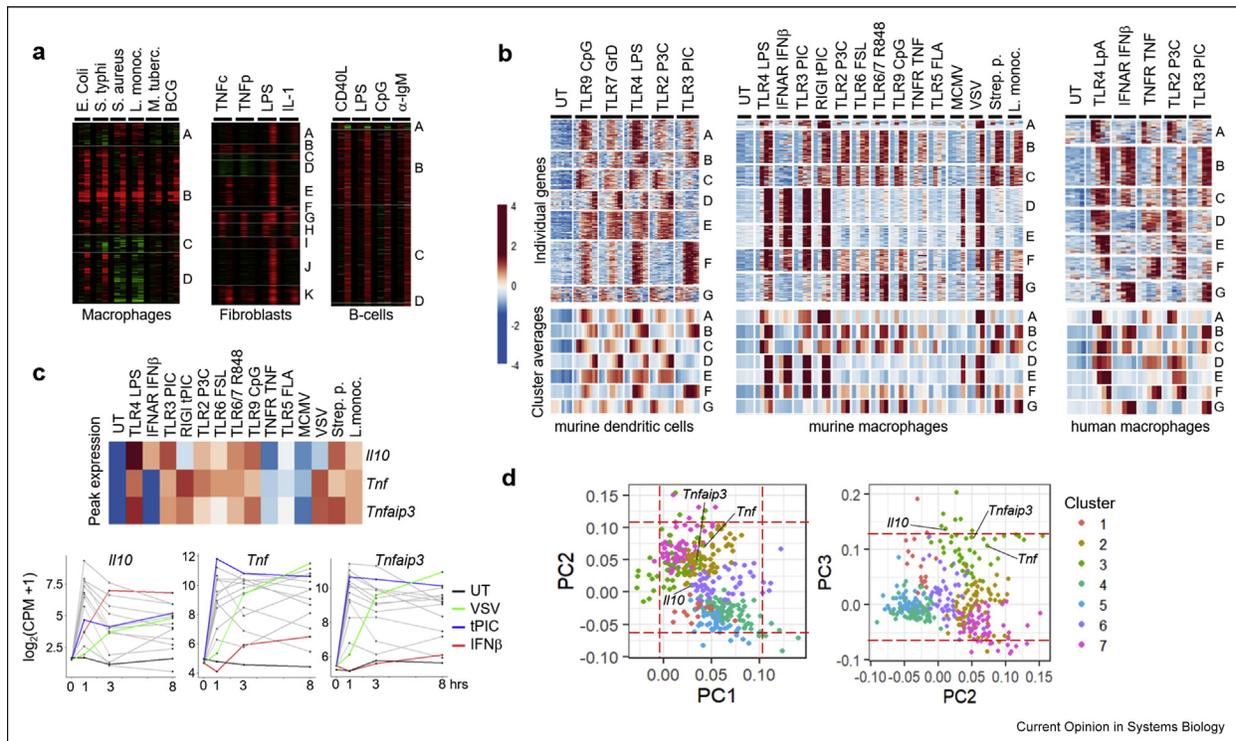
Stimulus-specific gene expression by immune sentinel cells as documented by prior transcriptome profiling studies. **(a)** Heatmaps of k-means clustered transcriptomic data from human macrophages (484 genes; 0, 2, 6 h) [8]; murine embryo fibroblasts (673 genes; 0, 1, 8 h) (Hoffmann lab ca. 2004), B-cells (433 genes; 0, 4, 12 h) [53]. **(b)** Murine dendritic cells (0, 0.5, 1, 2, 4, 8, 12, 16, 24 h) [9]; murine macrophages (0, 1, 3, 8 h) [10]; human macrophages (0, 1.5, 3, 5.5, 10 h) [14]. **(c)** Heatmap of peak expression and line graphs showing temporal trajectories for select genes from cluster C of murine macrophage dataset from (b) shows additional stimulus-specificity of individual genes that is hidden by clustering. **(d)** PCA captures genes contributing to stimulus-specificity. Murine macrophage gene expression data (as in (b)), with points colored by cluster number from the k-means heatmap. Dashed lines indicate genes with the largest weights for each component (~top 10 genes on each extreme).

visualization of induced genes reveals a large variety of expression patterns, but above-described concerns remain that the degree of stimulus-specificity is underrepresented by this analytical and visualization approach.

After all, considering just five different stimuli and categorizing gene expression in only three categories of low, medium and high, can theoretically yield hundreds of patterns. As hundreds of genes are responsive to innate immune stimuli, it seems likely that many patterns are in fact present. It is not surprising then that examining the peak expression value of individual genes in the timecourse reveals at least a dozen distinct patterns, even when not considering time of peak expression or their temporal patterning (Figure 2b). Increasing the number of clusters beyond the seven shown does not appreciably reduce the problem that genes with markedly different specificity patterns are grouped together. For example, in cluster C of the Cheng et al., 2017 heatmap (Figure 2b, 'murine macrophages'), the dominant pattern for those genes are medium to high expression in all stimuli except IFNβ and MCMV. However, the clustering hides the pattern of a gene such as Il10, which instead has a medium expression level in response to IFNβ and low expression to VSV, different from the average behavior of the cluster (Figure 2c).

Thus, other analytical approaches are required to ascertain the degree of stimulus-specificity of gene expression. We have, for example, explored principal component analysis, which focuses on the diversity present in a dataset to identify genes that drive this diversity and thus show stimulus-specific responses (Figure 2d). The top N genes of each principal component can be used to identify orthogonal gene programs. As each subsequent principal component captures the maximal variation in the dataset that is independent of the previous components, this approach can help select subsets of genes, including those in lower components that may be hidden in clustering approaches of all induced genes. As timecourse transcriptomic sequencing across multiple stimuli represents more degrees of freedom than can be represented in a matrix, tensor factorization may prove useful, such that subtensors may correspond to independent biological programs and the rankings of genes from significant subtensors may be used to identify individual genes that were specific to the conditions [15]. These analytical approaches, performed on bulk sequencing data, may reveal and illustrate stimulus-specific gene expression, but in order to truly quantify the stimulus-specificity of the responses of immune sentinel cells, which function as individuals in surveilling tissue health, transcriptomic data at the single cell level are required [16].

Given the present evidence for highly stimulus-specific gene expression responses by immune sentinel cells, a substantial literature addresses the underlying molecular mechanisms in gene expression control and in the signaling mechanisms that connect genes to extracellular stimuli. These mechanisms may be summarized within two complementary hypotheses about regulatory control: combinatorial coding and temporal coding.
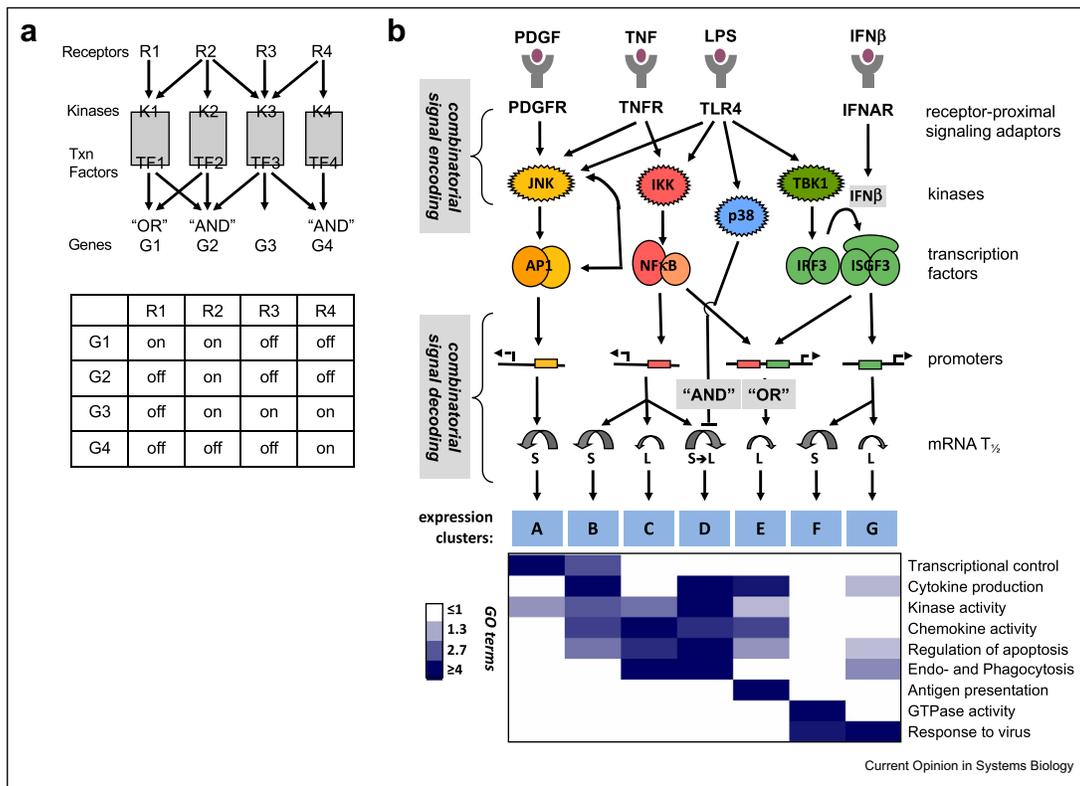
Combinatorial coding to produce stimulus-specific transcriptomes

The combinatorial code hypothesis proposes that stimulus-specific combinations of signaling pathway activity produce stimulus-specific responses [17]. Receptor-proximal mechanisms involving adaptor proteins mediate the activation of specific combinations of pathways (encoding). In turn, response genes contain combinations of response elements for stimulus-responsive transcription factors or mRNA processing or decay regulators that control gene expression (decoding). Theoretical considerations suggest that even with just three available pathways, seven potential transcriptomic patterns may be activated when only OR gates are available for decoding, 14 patterns when AND gates are also available, and many more with additional decoding logic gates [18] or when intermediate expression levels are distinguished. The input–output function of any combinatorial network can be related by a so-called "truth-table" (Figure 3a).

Within the innate immune signaling network there are numerous examples of combinatorial coding. For example, TLR2, TLR3, and TLR4 are pathogen sensors for distinct microbial products that are strong inducers of either NFκB only (TLR2) or both NFκB and IRF3 (TLR3 and TLR4) [1,19]. Whereas TLR2 and TLR3 activate distinct signaling adaptor proteins MyD88 and TRIF, TLR4 activates both (albeit sequentially). Although all TLRs effect signaling via a TIR domain, the small differences in the TIR domain structure along with plasma vs. endosomal membrane localization provide for specificity in engaging MyD88 vs TRIF adaptors [1,20]. This illustrates the principles of combinatorial encoding as distinct combinations of pathways are activated in a stimulus-specific manner.

Recent studies revealed that even the dose of a single ligand may be encoded by combinatorial coding [21]. Whereas low doses of LPS activate primarily NFκB and JNK pathways, high doses also activate MAPKp38, thus allowing — in principle — for a straightforward distinction of the LPS dose at the gene regulatory decoding step. Similarly, bacterially infected cells show both NFκB and MAPK/JNK activation, whereas many of the exposed but uninfected bystander cells will activate NFκB only or show no response [22]. Indeed, a few dozen genes were identified that respond fully only when both NFκB and MAPKp38 are activated, being regulated by a functional AND gate formed by NFκB-

Figure 3

Combinatorial Coding to produce stimulus-specific gene expression. **(a)** Top: Schematic of an imaginary stimulus-response network depicting combinatorial encoding by receptor proximal mechanisms and combinatorial decoding by gene-regulatory mechanisms. Bottom: The truth table of the network indicates that gene expression is stimulus-specific, with R1 and R3 providing more restricted gene expression programs than R2 and R4. **(b)** Schematic of prominent pathways within the signaling network governing the responses of immune sentinel cells. When the hundreds of immune response genes are grouped into 7 gene expression clusters A−G, which correlate with distinct physiological functions (as revealed by gene ontology analysis, bottom), the dominant combinatorial decoding mechanism could be identified for each cluster [10].

driven transcription and p38-driven transcript stabilization [10]. These genes, which contain many important inflammatory cytokines such as TNF or IL1, are induced substantially only by high doses of LPS, not low doses of LPS, and not TNF at any dose (Figure 3b).

The classic example for combinatorial decoding is IFNβ gene expression which requires activity of three transcription factors, AP1, NFκB, and IRF. Classic studies described the regulatory logic as an AND gate — only the presence of all three transcription factors would recruit chromatin remodeling machinery to move an inhibitory nucleosome off the transcription initiation site [23]. However, recent observations suggest that the logic may be more complex, as exposure to virus may lead to IFNβ activation even in the absence of NFκB [24], and the NFκB homodimer p50:p50 has the capacity to block IRF binding to the IFNβ enhancer [25]. That means that the combinatorial logic of the IFNβ enhancer remains to be elucidated more rigorously.

In all, although the principle of combinatorial coding is well established as a means of producing pathogen-specific responses in immune sentinel cells, for the vast majority of genes the operative combinatorial logic has yet to be described quantitatively, and the extent to which combinatorial coding may provide stimulus-specific or pathogen-appropriate immune sentinel responses has yet to be quantitatively determined.

Temporal coding to produce stimulus-specific transcriptomes

The temporal code hypothesis posits that the dynamical activity of even a single signaling protein (e.g. temporally varying kinase or TF activity) encodes information about the stimulus, such as the ligand identity and dose (Figure 4a). Furthermore, target genes of the transcription factor can decode these temporal profiles to result in appropriate stimulus-specific activation.

In innate immune signaling, much of the evidence for temporal coding stems from studies of the NFκB

Figure 4
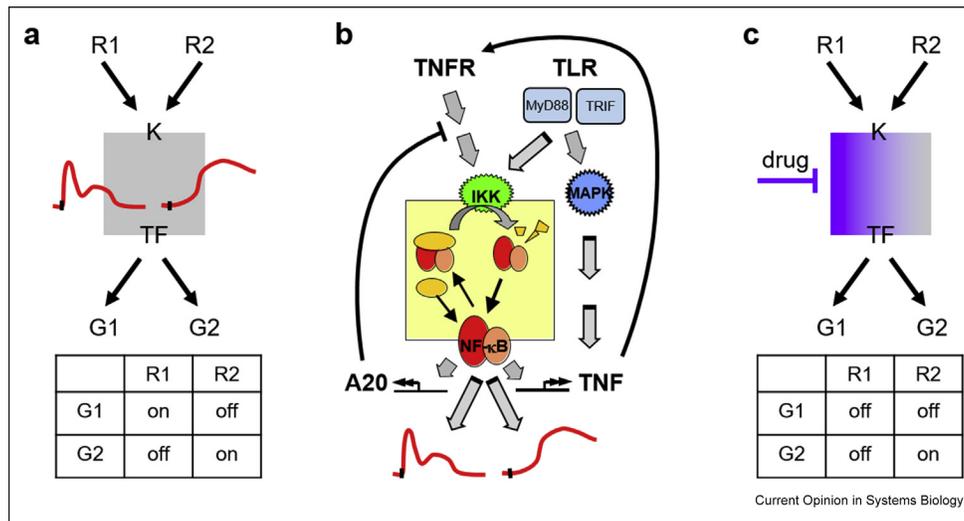
Temporal Coding to produce stimulus-specific gene expression. **(a)** Schematic of the Temporal Coding hypothesis. Two receptors elicit distinct gene expression programs via a single shared pathway; this is achieved via stimulus-specific temporal patterns of signaling activity. **(b)** Schematic of the network that allows for the encoding of TNFR vs TLR-specific temporal profiles of NFκB activity [27, 32, 37]. **(c)** When the regulatory mechanisms mediating stimulus-specific temporal control of signaling are elucidated, pharmacologic intervention within the shared pathway can be targeted to produce stimulus-specific inhibition [39].

pathway. Early population-level biochemical studies showed that the dynamics of NFκB activation are stimulus-specific when comparing the cytokine TNF and the bacterial endotoxin LPS [26,27] (Figure 4b). Furthermore, some NFκB target genes were found to be activated in an NFκB-dynamics-dependent manner [27,28]. Systems biology studies of mathematical modeling and experimentation uncovered a number of molecular mechanisms that allow for encoding of ligand- and dose-specific NFκB dynamics. These include four negative feedback loops to control NFκB nuclear translocation [29–33]; cyclical multistate enzyme control [34], positive feedback control [35], and recruitment scaffolds [36] to control the dynamics of the kinase IKK; a cytokine-mediated coherent feedforward loop that is deployed stimulus-specifically [37]; and a dose-sensing autoregulatory loop within a receptor proximal signaling module [38]. Based on the resulting mathematical models, it was shown that the dynamics of signaling could be targeted pharmacologically to achieve superior specificity in pleiotropic signaling pathways [39] (Figure 4c).

In parallel, single-cell studies using fluorescent protein–NFκB fusion proteins whose nuclear localization is monitored by live cell microscopy revealed complex dynamics in response to stimulation [40,41]. However, a high degree of cell-to-cell variability and seemingly ligand-independent oscillatory behavior [42] led to more questions than support of the notion of a temporal code. Still, innovative information theoretic analysis

found that more information is encoded in the time-course than any single timepoint [43]. However, a key limitation of present single-cell studies is that they involve overexpression of the fluorescent protein–RelA fusion in immortalized cells. Because this is not only a reporter but also an effector, it has the potential to alter the NFκB signaling system [44], and immortalized cell lines, which have been optimized for growth in cell culture, show diminished responsiveness to immune threats [45]. Primary fibroblasts from a knockin eGFP-RelA mouse yielded some timecourse data but signals proved too dim for a thorough analysis [46]. Improved primary cell experimental model systems will need to be established to determine to what extent NFκB dynamics are in fact ligand-specific, how much information may be conveyed, and what dynamical features convey stimulus-specific information.

How NFκB dynamics are decoded by target genes is the other key question — the complement to whether and how NFκB dynamics encode information about the ligand identity and dose. Early studies suggested that both the mRNA half-life and chromatin-mediated mechanisms may decode the duration NFκB dynamics [28], and recent same-cell NFκB dynamics and transcriptome measurements confirmed correlation of temporal profiles with distinct gene expression programs [47], but future studies ought to address this question in a quantitative, gene-specific manner. Alternative mechanisms include a coherent feedforward loop involving CEBPδ [48] but this awaits confirmation.

Another study proposed that fold change of NFκB activation may be decoded via an incoherent feedforward loop mediated by the repressive p50 homodimer which may be stimulus-induced [49]. NFκB oscillations have captured substantial research efforts, but to date there is little evidence that the oscillatory feature of NFκB dynamics affects gene expression [44], let alone what the molecular mechanisms might be that decode oscillatory versus nonoscillatory NFκB dynamics. In sum, while there is substantial literature on the temporal coding of NFκB and also other innate immune signaling pathways (e.g. MAPKp38 [50]), and how these may be modified by polarizing or conditioning cytokines [51], future studies ought to address to what extent these contribute to the capacity of immune sentinel cells to produce stimulus-specific gene expression programs.

Outlook

Past research has established that immune sentinel cells are capable of stimulus-specific gene expression programs and has provided strong evidence for the existence of two complementary coding schemes: signaling pathways that are triggered by sensors of the extra- and intracellular environment engage combinatorial and temporal coding to control the expression of nuclear target genes. Still, the extent to which immune sentinel cells are able to provide stimulus- or pathogen-specific responses to trigger and then orchestrate a pathogen-appropriate immune response ought to be addressed quantitatively at the single-cell level [52]. Similarly, while the mechanisms for combinatorial and temporal coding have begun to be delineated, quantitative insights will require the use of primary cells at single-cell resolution iterated with data-driven and knowledge-based computational modeling. Indeed, what remains entirely unchartered at this time is how temporal and combinatorial codes complement each other to improve information transmission, and how the mechanisms of encoding and decoding may be coordinated, independent, or interdependent. Furthermore, as immune sentinel cells function in diverse tissue microenvironments that affect their function through polarizing cytokines and are subject to priming and tolerizing mechanisms, it will be of interest to understand how these conditions affect the capacity for stimulus-specific gene expression and combinatorial and temporal coding within signaling pathways. This suggests that despite two decades of research into the responses of immune sentinel cells, the development of impactful signaling concepts, and an abundance of molecular mechanistic knowledge (captured in mathematical models), the field remains in its infancy. Further research is likely to lead to transformative insights about immune sentinel biology and the regulatory mechanisms that initiate and orchestrate pathogen-appropriate immune responses.

Conflict of interest statement

Nothing declared.

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