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Review

Stimulus-specific responses in innate immunity: Multilayered regulatory circuits

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SUMMARY

Immune sentinel cells initiate immune responses to pathogens and tissue injury and are capable of producing highly stimulus-specific responses. Insight into the mechanisms underlying such specificity has come from the identification of regulatory factors and biochemical pathways, as well as the definition of signaling circuits that enable combinatorial and temporal coding of information. Here, we review the multi-layered molecular mechanisms that underlie stimulus-specific gene expression in macrophages. We categorize components of inflammatory and anti-pathogenic signaling pathways into five layers of regulatory control and discuss unifying mechanisms determining signaling characteristics at each layer. In this context, we review mechanisms that enable combinatorial and temporal encoding of information, identify recurring regulatory motifs and principles, and present strategies for integrating experimental and computational approaches toward the understanding of signaling specificity in innate immunity.

INTRODUCTION

Immune sentinel cells such as macrophages and fibroblasts initiate and orchestrate immune responses to pathogen invaders or tissue injury. As first responders, they provide finely tuned immune activation to provide pathogen-appropriate protection, while avoiding excessive tissue damage and inflammation. Recent studies have revealed that immune sentinel cells produce gene expression responses that are remarkably more stimulus- and pathogen-specific (Amit et al., 2009; Cheng et al., 2017, 2019; Sheu et al., 2019) than previously thought (Nau et al., 2002). These support the theoretical consideration that because many immune-response gene products are intrinsically toxic to the host, they should only be expressed as needed.

Immune sentinel cells respond to hundreds of ligands, including PAMPs present on or shed by pathogens, DAMPs from damaged host cells, and secreted cytokines, using dozens of pattern recognition receptors (PRRs) and numerous cytokine receptors. They reside in diverse cellular locations, such as the cell surface (e.g., Toll-like receptors [TLRs] and cytokine receptors such as TNFR, IL-1R, and IFNAR), the endosome (TLRs), and the cytosol (e.g., the nucleic acid sensing cGAS and RIG-I like receptors [RLRs]) (Boraschi et al., 2018; Dostert et al., 2019; Janeway and Medzhitov, 2002; Takeuchi and Akira, 2010; de Weerd and Nguyen, 2012). The optimal response to each threat involves a distinct mixture of dozens of functional responses that comprise cell-intrinsic pathogen defenses, cell death control, recruitment of diverse immune effector cells, tissue remodeling, and activation of systemic and adaptive immune mechanisms (Sheu et al., 2019).

The premise of this review is that integrating these mechanisms identified by molecular immunology with principles of systems biology will lead to substantial advances in our understanding of how immune sentinel responses are regulated. We specifically review the signaling mechanisms described by the innate immunology literature and relate these mechanisms to regulatory motifs commonly described by systems biology. This review therefore aims to build bridges to connect the two fields to advance our understanding of innate immune response regulation.

CONNECTING MOLECULAR IMMUNOLOGY AND SYSTEMS BIOLOGY

Molecular immunologists have generated a wealth of knowledge about the molecular signaling network, which passes information from receptors to nuclear target genes, from identifying the components, to characterizing molecular interactions, and to understanding the quantitative dose-response relationship. These have been summarized well in recent reviews (Ablasser and Hur, 2020; Barrat et al., 2019; Brignall et al., 2019; Caruso et al., 2014; Hu and Sun, 2016; Kawai and Akira, 2011; Newton and Dixit, 2012).

In parallel, systems biologists have posited that the observed stimulus specificity may be understood in terms of two main coding strategies by which information about the extra-cellular stimulus is conveyed to nuclear target genes (Figure 1A). The combinatorial code produces stimulus-specific gene expression when different stimuli activate distinct combinations of gene expression regulators that are differentially utilized by immune response genes (Buchler et al., 2003; Cheng et al., 2017; Doyle et al., 2002; Gottschalk et al., 2016; Hoffmann, 2016; Junkin et al.,

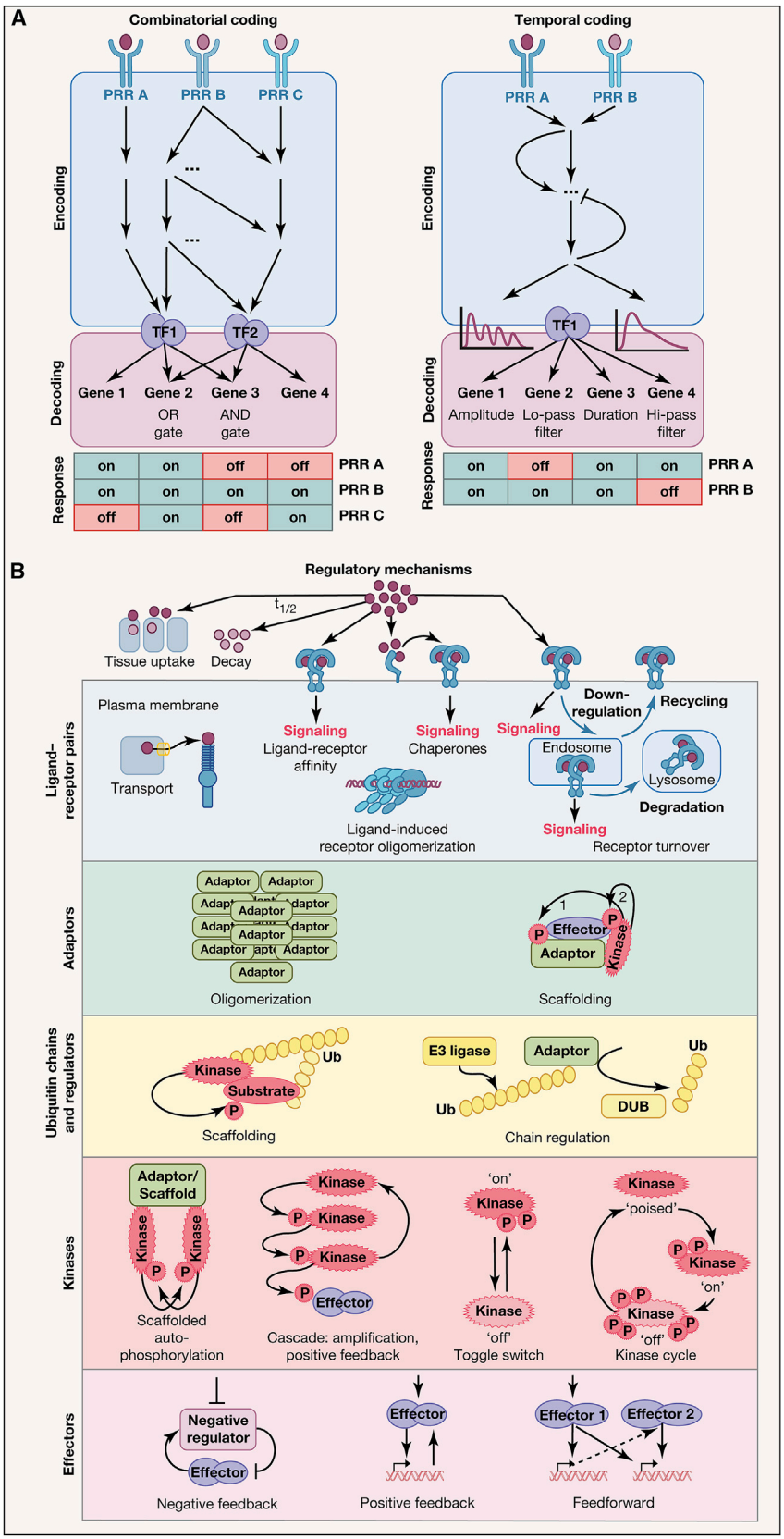

Figure 1. Principles of combinatorial and temporal coding for stimulus-specific gene expression and the layers of the stimulus-responsive network enabling it

(A) In combinatorial encoding, receptor proximal mechanisms activate stimulus-specific effector combinations. Response genes decode this through Boolean logic gate-based regulation. Stimulus-specific activity profiles of single effector modules allow temporal encoding of information, which is decoded by gene-specific mechanisms. PRR, pattern recognition receptor; Lo-pass filter, low pass filter; Hi-pass filter, high pass filter.

(B) Regulatory strategies controlling immune sentinel cell responses in the signaling network. The stimulus-responsive network consists of multiple functionally distinguishable signaling layers: (1) ligand-receptor pairs, (2) receptor-associated adaptors, (3) ubiquitin-signaling layer, (4) kinase hubs, and (5) SDTF modules and other effectors. Biochemical and cell biological mechanisms are shared between the diverse components of each signaling layer that together enable stimulus-specific coding. Ligand-receptor interactions in different subcellular locations are regulated through ligand stability, diffusion, transport, binding chaperones, and receptor transport and turnover. Adaptor proteins scaffold the assembly of larger signaling complexes. The kinetics of adaptor complex oligomerization are a key determinant of downstream signaling. Ubiquitin chains of different linkages pass on, fine-tune, and eventually downregulate signaling. They are regulated by the complex interplay of E3 ligases and DUBs. Signaling converges on three key kinase families, which are activated through scaffolded autophosphorylation or kinase cascades with toggle switch or kinase cycle mechanisms. The effector molecules activated by kinases are transcription factors inducing gene expression or post-transcriptional regulators. They are regulated through negative and positive feedback and feedforward loops. DUBs, deubiquitinases; Ub, ubiquitin; P, phosphoryl group.

Box 1. Toolboxes of molecular immunologists and systems immunologists

Molecular immunology: experimental methods

Molecular immunologists can interrogate all layers of the signaling network with a variety of experimental methods. In Note S1, we describe a selection of commonly used methods to measure:

- Protein abundance and turnover kinetics
- Protein-protein interactions
- Protein localization
- Kinase activity
- DNA-binding activity
- Chromatin state
- RNA abundance
- Metabolic state
- Cytokine secretion

For each, we describe assay characteristics pertaining to quantitation, sensitivity, resolution, and multiplexing, and data interpretation challenges.

Perturbation studies are key to understanding a biological system. We describe a few commonly used approaches:

- Pharmacological perturbation
- Knockdown by RNA interference
- Knockout by recombination and CRISPR
- Ectopic gene expression by transfection/retroviral transduction

For each, we describe its purpose and the advantages and caveats for interpretation.

Systems immunology: regulatory motifs

Systems biologists have described regulatory motifs with characteristic dynamical behavior. These are also are found in the innate immune signaling network. In Note S1, we describe some with the help of simple mathematical models:

(1) Toggle switch versus cycle switch
(2) Multistep reaction cascades
(3) Boolean logic gates
  - AND gates
  - OR gates
(4) Coherent feedforward loop
(5) Incoherent feedforward loop
  - pulse generator
  - fold-change detector
(6) Positive feedback loop
  - bistability
  - ultrasensitivity
(7) Negative feedback loops
  - track input duration
  - improve responsiveness
  - pulse generator
  - oscillation generator

For each, we provide a general diagram of the reactions, the ordinary differential equations that model the system, a demonstration of key behaviors that the motif allows for, and some examples of motifs in the immune response signaling network.

2016; Scholes et al., 2017). The temporal code allows stimulus specificity in gene activation through the action of a single effector, whose stimulus-specific temporal pattern of activity is decoded differentially by target genes (Behar and Hoffmann, 2010; Hoffmann, 2016; Purvis and Lahav, 2013; Sheu et al., 2019).

Yet, despite the significant advances made in both areas, a disconnect between the molecular immunology literature and the systems biology literature has slowed progress in understanding how immune sentinel cell responses are regulated. While early systems biology studies were focused on conceptual or proof-of-principle studies and therefore employed convenient experimental systems that do not always faithfully recapitulate complex biology, recent publications demonstrate that iteration between mathematical modeling and experimental studies can provide important biological insights when applied to primary cells, whether it be in embryonic stem cells (Heemskerk et al., 2019) or during gastrulation (Chhabra et al., 2019), epithelial cell oncogenesis (Aikin et al., 2019), cancer drug responses (Chopra et al., 2020), or epigenetic control of cell death (Vanaja et al., 2018).

However, in innate immunity research, integrated experimental and mathematical modeling studies remain rare. Though several seminal studies have been carried out in related fields, including chemosensing (Manes et al., 2015; Meier-Schellersheim et al., 2006; Xu et al., 2010), TRAIL signaling to apoptosis (Albeck et al., 2008a, 2008b; Spencer et al., 2009), T cell receptor signaling (Chakraborty and Weiss, 2014; Ganti et al., 2020), and T cell signaling (Feinerman et al., 2008a, 2008b; Voisinne et al., 2015), the first signaling study employing primary macrophages has only just been reported (Adelaja et al., 2021). Still, recent advances in technology such as CRISPR-mediated gene insertion (Kundert et al., 2019), single-molecule RNA *in situ* fluorescence (Eng et al., 2019; Foreman and Wollman, 2020; Xia et al., 2019), microfluidics (Junkin et al., 2016; Lin et al., 2019), or organoid culture (Zhang et al., 2019a) allow the generation of high-resolution, quantitative datasets that enable mathematical-model-aided systems biology studies of immune processes. These studies thereby illustrate how the wealth of molecular immunology knowledge may unravel how combinatorial and temporal codes control the stimulus specificity of innate immune responses. We offer a supplementary note describing experimental perturbation methods used by molecular immunologists to dissect the components of signaling pathways and the regulatory motifs used by systems biologists to study the behavior of signaling pathways (Box 1; Note S1).

LAYERS OF CONTROL IN THE STIMULUS-RESPONSIVE NETWORK

The signaling network that mediates inflammatory and anti-pathogenic gene expression consists of hundreds of proteins with diverse biochemical functions. We categorize them into five layers of regulatory control—receptors, signaling adaptors, ubiquitin-chain regulators, signaling kinases, and effectors—and briefly describe the unifying molecular mechanisms determining signaling characteristics at each layer (Figure 1B).

The first layer of control for initiating stimulus-specific responses comprises of the factors that govern ligand-receptor interactions. Ligands have distinct half-lives of bioactivity, differential diffusion,

transport, and cell uptake characteristics. Receptors are expressed at distinct levels and sorted to distinct subcellular locations via intracellular trafficking (Bagnall et al., 2018; Chen et al., 2017) including endocytosis trafficking (Leifer and Medvedev, 2016). These processes may be modulated by signals from the tissue microenvironment and by prior immune threat exposure, providing context specificity to the first control layer (Roberts et al., 2017). A variety of proteins (e.g., TIRAP and TRAM) regulate receptor localization and availability to bind ligands (Kagan et al., 2008; Leifer and Medvedev, 2016). Ligand-receptor interaction affinity may not solely be determined by structures of ligand and receptors, but also by co-factors (e.g., CD14 for LPS-TLR4 binding) that facilitate ligand-receptor interaction or receptor oligomerization (Park and Lee, 2013; Zanoni et al., 2011). Ligand binding induces di- or multimerization of PRRs and cytokine receptors, often a prerequisite for adaptor recruitment and signal transduction. For example, TLRs dimerize upon ligand binding, and RLRs multimerize into a filament on the RNA (Ablasser and Hur, 2020; Ohto and Shimizu, 2016). The cell-to-cell variability of receptor availability is an important determinant of phenotypic diversification. Systems biology modeling has proven very helpful in probing these relationships (Feinerman et al., 2008b; Gaudet et al., 2012; Martins et al., 2017).

Within the second control layer, signaling adaptors connect ligand-bound receptors with intra-cellular signaling cascades. Important adaptors in innate immune signaling include MyD88 for all TLRs except TLR3, TRIF for TLR3 and TLR4, MAVS for RLRs, and STING for cGAS. Among cytokine receptors, IL-1 signaling is also mediated by MyD88, but the large TNFR-superfamily has distinct adaptors (e.g., TRADD) (Ablasser and Hur, 2020; Boraschi et al., 2018; Chen and Jiang, 2013; Dostert et al., 2019; Luo et al., 2019). These adaptors generally lack enzymatic activity themselves but rather scaffold or nucleate the assembly of larger signaling complexes (e.g., the Myddosome). This often occurs through direct homotypic domain interactions with their receptors (e.g., CARD domains for RLR-MAVS interaction), but may also involve second messenger molecules (e.g., cGAMP for STING signaling) (Ablasser and Hur, 2020; Cadena et al., 2019; Monie et al., 2009; Wu et al., 2013). Therefore, the precise biochemical characteristics and kinetic rate constants of receptor-adaptor interactions, adaptor oligomerization, and second messenger production, half-life, and diffusion determine the signal transduction behavior of the second control layer. Interestingly, the biochemical characteristics may be further modulated by the subcellular location of the adaptor. MyD88 for example may function from both plasma membrane and endosomal compartments and may have distinct biochemical kinetic properties.

We define the third control layer by the complex network of E3 ubiquitin ligases and deubiquitinases (DUBs). These generate and degrade, respectively, K63-linked, K48-linked, and linear (M1-linked) ubiquitin chains. K63 and linear ubiquitin chains have scaffolding function to facilitate the interaction of downstream signaling kinases and substrates, while K48 ubiquitin chains induce proteasomal degradation of target proteins (Hu and Sun, 2016). Ubiquitin chains are attached either to the E3 ligases themselves (e.g., K63-linked ubiquitin on TRAF6 in MyD88 and MAVS signaling) or to upstream adaptors or bridge proteins (e.g., K63-linked chains on RIP1 kinase in TRIF and TRADD signaling) or may be unanchored (Hu and Sun, 2016; Xia et al., 2009). Regulatory specificity is achieved by the activation or recruitment of specific E3 ligases; the extent and duration of their activity may be modulated, and they also differ in the type of ubiquitin linkages that they generate (Hu and Sun, 2016; Parvatiyar et al., 2018). An interesting example is LUBAC, an E3 ligase that synthesizes linear ubiquitin chains, which contribute to activation of the IκB kinase (IKK) in response to TNF signaling but negatively affect RIG-I signaling (Hu and Sun, 2016; Tokunaga, 2013). Similarly, DUBs may have distinct roles in different pathways. CYLD, for example, attenuates RIG-I signaling by removing K63-linked chains but promotes STING signaling by removing K48-linked chains (Friedman et al., 2008; Zhang et al., 2018). Interestingly, the anti-inflammatory protein TNFAIP3/A20 was reported to act as both a DUB and an E3 ligase, negatively regulating NF-κB signaling by removing K63-linked chains and adding K48-linked chains to multiple targets (Catrysse et al., 2014; Wertz et al., 2004), though their physiological roles have been questioned (De et al., 2014; Skaug et al., 2011). Thus, the ubiquitin control layer regulates interactions, activity, and degradation of key signaling proteins and provides many levers for fine-tuning of responses, cross-regulation, and network memory.

The fourth control layer involves three central families of response kinases, namely MAPKs (JNK, p38, ERK), the IKK complex (consisting of IKKα, IKKβ, and IKKγ/NEMO), and TBK1 (Ablasser and Chen, 2019; Newton and Dixit, 2012). These kinases serve as the regulatory network hubs that directly or indirectly activate downstream effectors. Kinase activity in immune response pathways is generally initiated by trans-autophosphorylation when at least two kinase molecules are brought into proximity through oligomerized adaptors (e.g., activation of TBK1 downstream of STING) or ubiquitin chains (e.g., activation of TAK1 and IKK) (Emmerich et al., 2013; Häcker and Karin, 2006; Hu and Sun, 2016; Kensche et al., 2012; Ma et al., 2012; Polley et al., 2013; Shu et al., 2013; Xia et al., 2009; Zhang et al., 2019b)—in contrast, tyrosine kinase receptors are activated via ligand-induced conformational changes of receptor dimers (Livnah et al., 1998). Kinase activity may then be amplified through kinase cascades, the most prominent being MAPK cascades (Kholodenko and Birtwistle, 2009; Witzel et al., 2012). Scaffolds may also connect kinases to their substrates. For example, for IKK, NEMO, which connects the catalytic components to ubiquitin chains, also contains an IκBα recruitment domain that ensures efficient activation of NF-κB (Schröfelbauer et al., 2012). Kinase activity is then terminated via one of two mechanisms. In the classic toggle-switch model, inactivation occurs via dephosphorylation by phosphatases (Bardwell, 2008; Ferrell and Bhatt, 1997; Ferrell and Ha, 2014; Nolen et al., 2004; Zhao et al., 2012)—phosphatases may also target signaling molecules further upstream, such as signaling adaptors STING, TRIF, and MAVS (Ni et al., 2020; Xiang et al., 2016). However, IKK is thought to be regulated via a multi-state reaction cycle, in which an autophosphorylated inactive state must be recycled to a phosphorylation-free poised state before reactivation can occur (Behar and Hoffmann, 2013). The two inactivation modalities impart fundamentally different dynamic control to the kinase activity, and phosphatases play distinct roles. Their potential for inducible expression provides another level of regulatory control.

The fifth control layer comprises the signaling pathway effectors that directly control gene expression. There are two main

categories of effector proteins: transcription factors and proteins acting post-transcriptionally. Three prominent signal-dependent transcription factor (SDTF) families may be directly mapped to upstream kinases: MAPK-AP1/ATF, IKK-NF-κB, and TBK1-IRF (Cargnello and Roux, 2011; Hayden and Ghosh, 2004; Honda et al., 2006). In addition, NFAT is activated in dendritic cells in a Ca^{2+}-dependent manner (Fric et al., 2012; Zanoni and Granucci, 2012). Some kinases regulate post-transcriptional regulatory mechanisms. For example, IKK phosphorylates the mRNA decapping activator ECD4, affecting the levels of hundreds of mRNAs (Mikuda et al., 2018). Further, production of TNF involves not only NF-κB-dependent transcription, but MAPK p38/ERK-dependent mRNA splicing, mRNA stabilization via tristetraprolin (TTP) (Mahtani et al., 2001), and pro-protein processing via TACE (Caldwell et al., 2014), which explains why TNF secretion by single cells does not correlate well with NF-κB activity (Junkin et al., 2016). Effectors may be regulated in a simple toggle switch fashion with phosphatases terminating effector activity when kinase activity has diminished (e.g., IRFs and the MAPK substrates AP1 or TTP) (Gu et al., 2014; Long et al., 2014). Effectors may also be subject to regulatory feedback. NF-κB is subject to complex negative feedback regulation via IκB proteins, which inhibit NF-κB through direct binding in the nucleus and escort NF-κB back into the cytoplasm. This inhibition is reversible for some IκB isoforms (IκBα, IκBε) as their degradation is induced by IKK (Mitchell et al., 2016). In contrast, IRF induces the interferon-β (IFN-β) feedforward system to activate ISGF3-driven gene expression programs in both primary response and bystander cells (Ourthiague et al., 2015). In addition, MAPK phosphatases such as dual-specificity phosphatases (DUSPs) are strongly upregulated by MAPK-activating PRRs, contributing to negative feedback on MAPK pathways (Brondello et al., 1999; Kuwano et al., 2008; Salojin et al., 2006). The strength and time delays in these feedback systems are key to imparting specific activation dynamics to the effectors. Further quantitative studies are needed to enable reliable mathematical modeling studies.

It is the stepwise transmission and modulation of the signal by these biochemical and cell biological mechanisms through the network layers that mediates combinatorial and temporal coding of response specificity. While numerous molecular mechanisms have been described for key TLR pathways, how they function together to generate stimulus-specific responses is generally less well understood. As will be discussed below, stimuli activate combinations of effectors through the molecular mechanisms within receptor, adaptor, ubiquitin, and kinase layers, while stimulus-specific dynamic control is mediated by molecular mechanisms in all five control layers.

MECHANISMS THAT ENABLE COMBINATORIAL ENCODING

When stimuli activate different combinations of signaling pathways, they convey stimulus-information by combinatorial coding (Figure 1A). Hence, branching of the signal into two (or more) signaling pathways is key to combinatorial coding. Here, we first present the molecular mechanisms that allow for pathway branching and then describe how these may be deployed stimulus-specifically.

Pathway branching occurs at multiple control layers of the signaling network. Pathways usually branch when a regulator sequentially (not coincidently) engages two signaling complexes, often by moving from one sub-cellular compartment to another. A well-studied example of this in the receptor layer is how TLR4 engages two different adaptors at different subcellular locales, MyD88 at the cell surface (activating NF-κB and MAPKs) and TRIF in the endosomes (additionally activating IRF3) (Figure 2A). This adaptor switch is mediated by a switch of the bridge proteins TIRAP and TRAM, which facilitate the association of the adaptors with TLR4 within the plasma or endosomal membranes, respectively (Balka and De Nardo, 2019; Deguine and Barton, 2014). Thus, a key principle of combinatorial encoding by TLR4 is that adaptor engagement is sequential and may be regulated by regulating the transport of TLR4 from one compartment to the other (Leifer and Medvedev, 2016).

Signaling pathways may also branch at the adaptor layer (Figure 2B): TRIF-mediated activation of the TBK1-IRF3 axis and the IKK-NF-κB axis is a prominent example (Chen and Jiang, 2013; Jiang et al., 2004), with MAVS and STING functioning similarly (Abe and Barber, 2014; Dunphy et al., 2018; Fang et al., 2017a, 2017b; Liu et al., 2013; de Oliveira Mann et al., 2019; Yoboua et al., 2010; Zhang et al., 2019b). On the one hand, TRIF oligomerization leads to the recruitment of RIP1 kinase, which is ubiquitinated by E3 ligases (Cusson-Hermance et al., 2005). Those ubiquitin chains lead to IKK recruitment and trans-autophosphorylation, allowing IKK to activate NF-κB (Hu and Sun, 2016; Kensche et al., 2012; Polley et al., 2013). On the other hand, oligomerized TRIF also recruits TBK1, though the exact mechanism is not fully elucidated (Funami et al., 2008). Although initial TBK1 activation may require a priming phosphorylation through IKK activity (Abe et al., 2020), trans-autophosphorylation of TBK1 leads to its full activation (Ma et al., 2012; Shu et al., 2013). Activated TBK1 phosphorylates the adaptor, allowing IRF3 binding to the adaptor, which subsequently serves as a scaffold for TBK1-mediated IRF3 phosphorylation (Abe et al., 2020; Liu et al., 2015b). Thus, activation of two pathways by a single adaptor appears to occur sequentially, in which the second pathway activation requires a post-translational modification of the adaptor. Similarly, NF-κB and IRF3 activation seem spatially regulated downstream of STING, with NF-κB activation occurring from the endoplasmic reticulum (ER; in which STING is first activated), while IRF3 signaling requires STING trafficking to the Golgi (Ni et al., 2017; Stempel et al., 2019).

Ubiquitin chains constitute remarkably efficient signaling scaffolds that mediate pathway branching to the MAPK and NF-κB pathways (Figure 2C). For example, ubiquitin chains attached to adaptor TRAF6 provide scaffolds for the trans-autoactivation of kinases, such as TAK1, a MAPK kinase kinase (MAPKKK) (Ishitani et al., 2003; Kanayama et al., 2004; Scholz et al., 2010; Schröfelbauer and Hoffmann, 2011; Xia et al., 2009). In addition, the IKK complex trans-autophosphorylates when clustered on (preferentially linear) ubiquitin chains through its ubiquitin-binding subunit NEMO (Ea et al., 2006; Haas et al., 2009; Kensche et al., 2012; Polley et al., 2013; Rahighi et al., 2009; Tokunaga, 2013; Xia et al., 2009). Thus, the ubiquitin layer provides numerous opportunities for signaling crosstalk and pathway branching.

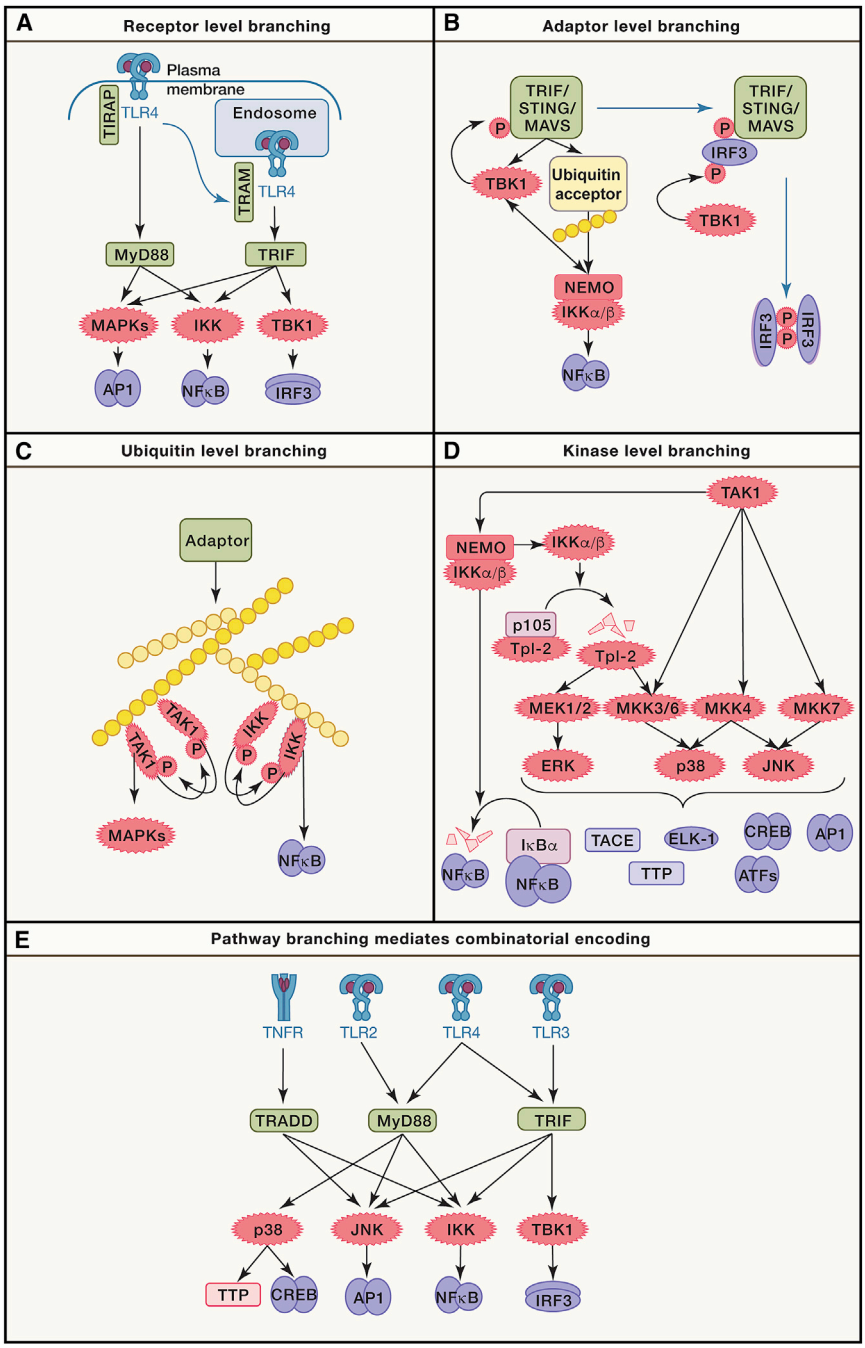

Figure 2. Molecular mechanisms for combinatorial encoding to achieve stimulus-specific responses

(A–D) Pathway branching, in which one upstream protein activates multiple downstream signaling mediators, can occur at several signaling layers. Examples include branching (A) at the level of receptors with TLR4 activating the MyD88 and TRIF pathway; (B) at the level of adaptors with TRIF, MAVS, and STING being capable of IRF3 and NF-κB activation; (C) at the level of ubiquitin chains, which scaffold activation of TAK1 for MAPK activation and of IKK for NF-κB activation; and (D) at the level of kinase cascades which branch to activate IKK and the MAPKs p38, JNK, and ERK. (E) Combinatorial coding in the innate immune network is mediated by receptor-specific utilization of adapters and pathway branching at the adaptor layer.

Given the enzymatic activity of kinases, they may phosphorylate several substrates and thus mediate pathway branching (Figure 2D): TAK1 mediates pathway branching to the MKK4/7-JNK axis and the IKK-NF-κB axis. TAK1 is the initiator of the MAPK cascade that leads to JNK activation (Cargnello and Roux, 2011). In addition, TAK1 may initiate IKK activation (Cohen and Strickson, 2017; Emmerich et al., 2013; Shim et al., 2005; Wang et al., 2001; Zhang et al., 2014), though this is stimulus-specific and may be cell-type dependent. It is not clear what determines TAK1's substrate specificity (Shim et al., 2005). Interestingly, IKK also engages in pathway branching by not only activating the IκB-NF-κB axis, but also the p105-TPL2-ERK axis (Beinke et al., 2004; Gantke et al., 2012). IKK mediated p105 degradation is essential for Tpl2 activation, which is the MAPKKK for ERK (Beinke et al., 2004; Gantke et al., 2012; Waterfield et al., 2003). Indeed, IKK may further phosphorylate Tpl2 (Roget et al., 2012). IKK's substrate specificity seems to be controlled by NEMO, which is essential for IKK's activation by inflammatory stimuli: NEMO directs IKK's substrate specificity to IκBα, but may then dissociate from the IKK dimer, broadening IKK's specificity to include p105 (Schröfelbauer and Hoffmann, 2011; Schröfelbauer et al., 2012). Furthermore, in pDCs, IKK was reported to activate IRF7 downstream of MyD88 mediating TLR7 and TLR9 signals (Honda et al., 2004; Hoshino et al., 2006; Kawai et al., 2004; Pauls et al., 2012; Uematsu et al., 2005). Spatial segregation between distinct endolysosomal compartments may contribute to the switch from NF-κB to IRF7 signaling (Honda et al., 2005; Sasai et al., 2010). This illustrates that pathway branching at the kinase control level also involves sequential mechanisms and may be cell-type specific.

To mediate stimulus-specific responses, pathway branching must be deployed stimulus specifically. The combination of the IKK-NF-κB and JNK-AP1 axes is activated by all adaptor proteins discussed here (i.e., MyD88, TRIF, STING, MAVS, and TRADD) ensured by pathway branching at the ubiquitin chain and the kinase layers, and thus generates a common core gene expression response (Amit et al., 2009; Cheng et al., 2017). However, originating at the receptor layer,

stimulus-dose and exposure route modulate JNK and NF-κB signaling to allow distinction of bacterial species, dose, and infection versus bystander status (Lane et al., 2019). More obviously, the TBK1-IRF3 and the MAPKp38 axes are deployed stimulus specifically by virtue of quantitative differences because of receptor-specific utilization of distinct adaptors, followed by pathway branching at the adaptor layer. Their stimulus-specific activation in conjunction with the common core pathways thus enables combinatorial coding (Figure 2E).

The TBK1-IRF3 axis mediates stimulus-specific induction of type I IFN (Kawai and Akira, 2011). In macrophages (and conventional dendritic cells), the IRF pathway is activated only in response to endosomal signaling by TLR4 and TLR3, and cytosolic signaling from cGAS and RLRs (Caruso et al., 2014; Kawai and Akira, 2011; Sparrer and Gack, 2015). This specificity is mediated by the ability of TLR3 and TLR4 to engage the TRIF adaptor in the endosome, while cGAS and RLRs engage STING and MAVS, respectively. TRIF, STING, and MAVS share an activation mechanism for the TBK1-IRF3 axis, while also being able to activate IKK-NF-κB and TAK1-JNK axes. Thus, for example, early studies showed TLR4 activation by lipopolysaccharide (LPS) from Gram-negative bacteria activates both NF κB and IRF3, while TLR2 activation by peptidoglycans from Gram-positive bacteria lacks the IRF3 component (Doyle et al., 2002). As IRF3 induces expression of the cytokine IFN-β, which activates the ISGF3 TF complex in an autocrine and paracrine manner, the effect of stimulus-specific activation of IRF3 is dramatically amplified both within the first-responder cell and its neighbors.

A second, less well-characterized example is the stimulus-specific activation of MAPKs p38 and ERK. Several lines of evidence indicate that functionally potent MAPKp38 activation is stimulus specific, even if some level of activity can be detected in response to many stimuli, such as TLR ligands, cytosolic nucleic acids, and TNF. First, transcriptome profiling of fibroblasts and macrophages stimulated with a diverse set of PAMP and cytokine ligands revealed a set of about 80 genes that required both NF-κB and MAPKp38 activity (Cheng et al., 2017). Second, MAPKs p38 and ERK have a key role in post-transcriptional processing, mRNA half-life, pro-protein processing, and secretion of TNF—cell stimulation with TNF failed to activate these processes (Caldwell et al., 2014). Finally, while stimulation with high concentrations of LPS led to TNF production, low concentrations activated NF-κB but failed to produce TNF (Gottschalk et al., 2016), in line with the conclusion that single-cell TNF secretion was better correlated with MAPK activation than NF-κB (Junkin et al., 2016). However, the mechanism of stimulus-specific activation of the MAPKp38 axis remains poorly understood. One hypothesis is that the MyD88 oligomerization into the Myddosome complex results in a thresholded dose response (Cheng et al., 2015) such that p38 is only activated at high ligand concentrations. In addition, the MAPK cascade itself is thought to have a thresholded dose response curve (Huang and Ferrell, 1996), dependent on the level of phosphatase activity (Altan-Bonnet and Germain, 2005). Interestingly, MAPKp38 activity may be induced through two different pathways, namely MKK4 downstream of TAK1 MAPKKK activity and MKK3/6 activated by Tpl-2 (Cohen and Strickson, 2017; Pattison et al., 2016). This may suggest that while TNF induces MAPKp38 via MKK3/6, LPS engages both pathways to generate a more potent p38 activity (Pattison et al., 2016).

In sum, combinatorial effector activation may encode information about both the molecular identity and the dose of a stimulus. Quantitative differences in the dose response curves of co-activated pathways increase the capacity for producing stimulus-specific responses via combinatorial coding. Future studies should address not only the mechanisms of pathway branching (e.g., the role of IKK in TBK1-IRF activation and the mechanisms of NF-κB and MAPK activation downstream of cytosolic nucleic acid sensing remain unclear), but also address dose-response behavior of each of the branched pathways and whether they are modulated by the microenvironment and the cell's exposure history. Importantly, during the infection process, pathogens stimulate several PRRs and autocrine cytokines (which further activate related pathways) that, in aggregate, characterize pathogen-specific responses *in vivo* (Gottschalk et al., 2019). Given that *in vitro* studies with multiple ligands revealed instances of synergy or antagonism between pathways (Gottschalk et al., 2019; Gutschow et al., 2019; Lin et al., 2017; Pandey et al., 2020; Tan et al., 2014), further study of cross-regulatory connections between co-activated pathways is warranted.

MECHANISMS THAT ENABLE TEMPORAL ENCODING

Signaling involves the transient perturbation of a homeostatic steady state. Hence, the activity of a signaling effector exhibits a temporal trajectory. The observation that two stimuli may trigger distinct temporal trajectories, and thereby differential gene expression, led to the hypothesis of a "temporal signaling code" (Figure 1A) (Hoffmann and Baltimore, 2006). Temporal trajectories may be quantitatively characterized by a set of features, such as peak amplitude, fold change, amplitude of the second signaling phase, total duration, or oscillations (Behar and Hoffmann, 2010; Levine et al., 2013; Li and Elowitz, 2019; Purvis and Lahav, 2013). Here, we describe how such dynamic features may be generated, or encoded, by the successive modulation of signaling activity by multiple mechanisms as the signal passes through successive control layers.

Within the first control layer, the first determinant of signaling dynamics is ligand availability as determined by ligand diffusion, endocytosis, and decay. Secreted TNF, for example, has a short half-life, whereas the endotoxin LPS persists. In tissue environments, diffusion rates and rates of uptake by other cells may also be important (Bagnall et al., 2018). Next, receptor clustering upon ligand binding (Caré and Soula, 2011) may threshold the dose-response curve preventing spurious activation in the absence of ligand (Bray et al., 1998). Following ligand binding, endocytosis of receptor-ligand complexes reduces the availability of receptors for further stimulation. The speed of receptor replenishment, through recycling or *de novo* synthesis, together with ligand half-life is a key determinant of the amplitude of second phase signaling (Becker et al., 2010) (Figure 3A).

In the next layer, the kinetic characteristics of adaptors are important determinants of signaling dynamics. For example, stepwise assembly of the Myddosome imparts the MyD88 pathway a thresholded, close-to-digital dose-response curve,

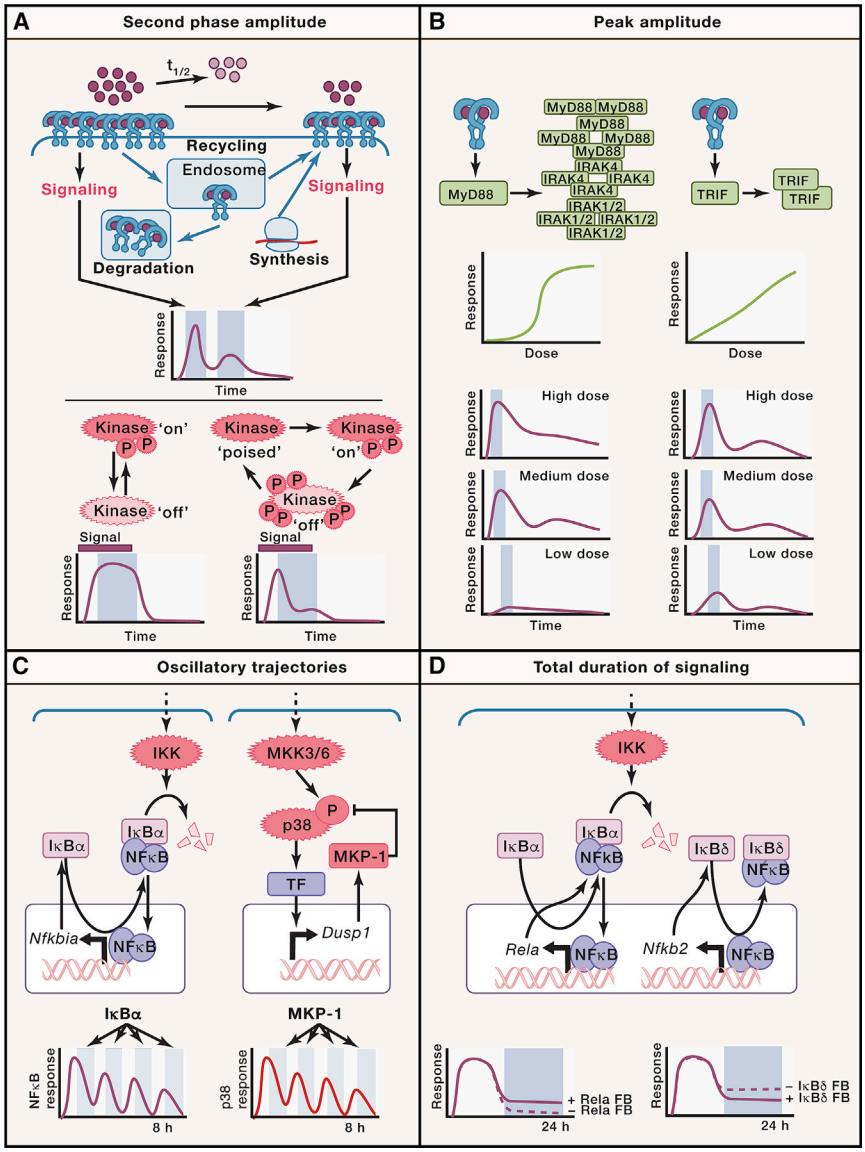

Figure 3. Molecular mechanisms for temporal encoding to achieve stimulus-specific responses

(A–D) Mechanisms contributing to important features of temporal signaling dynamics: (A) the second phase amplitude is controlled by ligand stability, receptor turnover dynamics, and kinase activity kinetics. (B) Dose-to-peak-amplitude-encoding is dependent on adaptor oligomerization properties, which differ between MyD88 and TRIF. (C) Oscillations in effector activity trajectories of e.g., NF-κB or MAPKp38 are introduced by negative feedback loops with delay. (D) Total duration of signaling is modulated by positive feedback or irreversible negative feedback, such as the induction of RelA and IκBδ expression, respectively.

whereas TRIF dimerization leads to a more linear dose-response curve and translates ligand concentration into effector amplitude more faithfully (Cheng et al., 2015) (Figure 3B). Interestingly, Myddosome-mediated signaling is transient and limited to a few hours, but how it is turned off remains unclear. In the case of IL-1 signaling, kinase mediator IRAK4 is proteolyzed after signaling (Ferrao et al., 2014), which may limit signal duration, but how Myd88 signaling is temporally limited demands further study. In the case of TRIF-signaling, the progressive acidification of the endosome appears to be a determinant of its duration, allowing for long-lasting and variable signal trajectories (Cheng et al., 2015; Husebye et al., 2006; McGettrick and O'Neill, 2010).

Within the ubiquitin layer, complex chain regulatory dynamics and their interplay with ubiquitin-binding proteins likely also control signaling; however, a quantitative, time-resolved understanding requires further study. Anti-inflammatory A20, one prominent regulator of ubiquitin-chain dynamics, blunts the dose response of TNF signaling and therefore limits the weaker signals of later time points. Both induced A20 feedback expression and constitutive A20 expression at certain levels can fulfill this function (Werner et al., 2008).

The kinase layer provides for interesting temporal modulation of signaling activity. The two-state toggle switch kinase transmits the incoming signal, but there is an inherent trade-off between faithful turn-off with cessation of the signal (requiring high phosphatase activity) and reduction of the peak amplitude dynamic range. This is resolved by kinase cascades (e.g., in the MAPK pathway), which amplify the signal's dynamic range without losing too much temporal control (Heinrich et al., 2002; Kholodenko and Birtwistle, 2009). An alternative topology is the kinase cycle (e.g., canonical IKK). This allows for a strong peak of activation, which triggers the kinase's transition to an inactive state (Delhase et al., 1999). From this, the kinase has to be recycled (via a phosphatase) to a poised state before reactivation. This results in a two-phased temporal trajectory with the duration of the first phase determined by the inactivation reaction and the amplitude of the second phase determined by the recycling reaction (Behar and Hoffmann, 2013). Thus, the distinction between first versus second signaling phases is enforced by kinase cycles or diminished by toggle kinase cascades (Behar and Hoffmann, 2013) (Figure 3A). When positive feedback is included in the kinase layer, an excitable dose-response curve may produce digital all-or-none responses (Shinohara et al., 2014).

Within the effector layer, feedback mechanisms are prominent, and they may reach back to upstream layers. The functional effects of feedback are diverse, determined by the speed, reach, and molecular mechanism of the regulator. Proximal negative feedback that is rapid and mediated by a directly binding but reversible inhibitor (e.g., IκBα) or a highly active inhibitory enzyme (e.g., MAPK phosphatase) can mediate oscillatory

effector activity if the input signal to the feedback loop persists (Hoffmann et al., 2002; Nelson et al., 2004; Tomida et al., 2015) (Figure 3C). The IKK-IκBα-NF-κB system generates particularly robust oscillations because of the rapid NF-κB-responsive IκBα synthesis (including an appropriate delay to the negative feedback), rapid nuclear import of IκBα, efficient removal NF-κB from the DNA through IκBα binding, rapid nuclear export of the complex, and responsiveness to IKK, which allows for efficient IκBα degradation after each cycle of re-synthesis (Mitchell et al., 2016). A delay in the negative feedback (e.g., IκBε) can diminish oscillatory propensity (Kearns et al., 2006). Irreversible negative feedback, as is mediated by A20 or by IκBδ (which, unlike other IκB isoforms, is not targeted for degradation by canonical IKK) can lead to a shutdown or attenuation of signaling that may impact both the amplitude of the second signaling phase, the total signal duration, and the response to a secondary signal (Shih et al., 2009; Werner et al., 2008) (Figure 3D). Hence, these proteins also mediate signaling crosstalk (Werner et al., 2008), as do the SOCS proteins regulating IRF/ISGF3 responses (Krebs and Hilton, 2001; Liu et al., 2015a).

Positive feedback and feedforward mechanisms mediated by (1) expression of signaling substrates or (2) induction of cytokines may extend the duration of effector activity. NF-κB-responsive expression of the main NF-κB dimer component, RelA, sustains long-term NF-κB activity (Sung et al., 2014) (Figure 3D), while ISGF3-inducible expression of its constituents (STAT1/2/IRF9) renders cells more responsive to subsequent IFN stimulation (Ivashkiv and Donlin, 2014). Importantly, induced cytokines not only amplify or prolong signaling in primary response cell, but also trigger signaling in previously un-exposed or un-responsive neighboring cells. For example, TNF was found to have an autocrine role in prolonging NF-κB signaling in response to synthetic TLR9 ligand CpG but primarily paracrine functions in response to LPS (Caldwell et al., 2014). Similarly, IRF3-dependent IFN-β induces amplification of type I IFN signaling in the primary response cell, but also initiates antiviral responses in neighboring cells. These cytokines are primarily controlled by a feedforward mechanism rather than positive feedback, as potent paracrine signaling coupled to positive feedback can lead to runaway inflammation (Ourthiague et al., 2015): In the case of TNF, MAPKp38 is critical for its expression, but is not strongly activated by it (Caldwell et al., 2014). In the case of IFN-β, IRF3 is critical for its expression but is not activated by it (Honda et al., 2006). That regulatory feature also allows these cytokines to extend the duration of signaling, but not indefinitely.

In order to mediate stimulus-specific responses, temporal trajectories must be stimulus specific. Thus far, this has been most extensively described for NF-κB, where the temporal trajectory may encode stimulus duration, dose, and molecular identity. Early studies examined how the duration of the TNF stimulus is encoded in the temporal profile of NF-κB. Persistent TNF stimulation leads to characteristic oscillatory NF-κB activity, while short TNF pulses lead to only a single peak whose duration of approximately 45 min is independent of stimulus pulse duration (as short as 1 min) (Hoffmann et al., 2002; Werner et al., 2008). Two temporal amplification steps explain this. First, the receptor-ligand complex and mechanisms in the ubiquitin layer provide for at least 10 min of IKK activity, and second, the IκBα feedback loop ensures 45 min of activity. When TNF pulses exceed 60 min, subsequent cycles of oscillations are activated allowing for faithful stimulus-duration to response-duration encoding (Hoffmann et al., 2002).

Stimulus dose can also be encoded in the temporal profile of the effector. For TNF, dose is encoded in amplitude and duration (Cheong et al., 2006, 2011; Tay et al., 2010; Turner et al., 2010). Amplitude provides only partial dose information as single-cell studies observed some degree of ultrasensitivity (Tay et al., 2010), and amplitude measurements at 30 min for doses over 3 orders of magnitude contained less than 1 bit of information (Cheong et al., 2011). Instead, the short half-life of TNF mediates dose-dependent control of NF-κB duration (Adelaja et al., 2021). For PAMPs, the MyD88 and TRIF pathways encode dose information differently (Cheng et al., 2015). MyD88-dependent PAMPs show an all-or-none response, with the threshold determined by receptor and MyD88 abundance. PAMPs engaging the TRIF pathway show a more graded dose-response with dose-dependent amplitudes. However, in both pathways the speed of response increases with dose.

The important question of whether molecular identity is represented in the temporal pattern of NF-κB activity had, until recently, received confusing answers. Early population level biochemical studies in primary fibroblasts showed that, in contrast to the oscillatory activity induced by TNF, the NF-κB response to LPS-TLR4 signaling is persistent and non-oscillatory (Covert et al., 2005; Werner et al., 2005). However, studies with immortalized cell lines did not provide confirmation at the single-cell level because NF-κB responses to several ligands showed noisy, largely oscillatory trajectories (Hughey et al., 2015). A recent study with primary macrophages provided clearer answers and identified six features within NF-κB temporal trajectories correlated with the molecular identity of the ligand (Adelaja et al., 2021). For example, whereas TNF, TLR2/1 ligand Pam3CSK4, and LPS activate NF-κB rapidly, the response to CpG and TLR3 ligand poly(I:C) was slower, due to a delay in endosomal ligand availability. Remarkably, oscillatory content was substantially higher in response to cytokine TNF than to PAMPs, largely because of the distinct amplitude of the second phase of IKK activity that either overrides IκBα negative feedback (in case of PAMPs) or allows it to generate oscillations (in case of TNF). Each ligand generated a unique combination of these six features of the temporal NF-κB code that may allow target genes to be expressed stimulus specifically.

Thus, the dynamic features in the temporal profile of an effector, modulated by biochemical and cell biological mechanisms at every signaling layer, can encode information about the stimulus such as molecular identity, dose, and duration. While two decades of research into temporal encoding in the NF-κB pathway have provided some mechanistic explanations for a handful of ligand receptor combinations, the temporal encoding of other effectors, such as the IRF and MAPKp38, remains underexplored. Indeed, how sequentially administered pulses of stimulation or multiple simultaneously administered stimuli are registered by the temporal activity profiles of effectors can provide insights about the intrinsic dynamic regulation of the signaling pathway (Ashall et al., 2009; Gutschow et al., 2019; Kellogg and Tay, 2015).

DECODING MECHANISMS THAT ENABLE STIMULUS-SPECIFIC GENE EXPRESSION

For immune sentinels to generate stimulus-specific gene expression, the gene regulatory network must interpret the combinatorial and temporal features of effector proteins. The gene regulatory network associated with each immune response gene involves DNA-associated mechanisms that move nucleosomes, establish enhancers, catalyze chromatin looping, or recruit components of the pre-initiation complex; RNA-associated mechanisms involving transcription initiation and RNA polymerase promoter clearance, transcription elongation, mRNA processing, splicing and export, or cytoplasmic decay; and translation-associated mechanisms such as pre-protein processing or activation.

Combinatorial decoding mediates stimulus-specific gene expression when immune response genes are differentially responsive to the various signaling effectors. Transcriptome profiling revealed that genes may contain AP1 binding sites, κB elements or ISRE sites, or combinations of these (Amit et al., 2009; Cheng et al., 2017). However, two binding sites being present does not indicate whether the gene is responsive to either pathway, or only when both pathways are active. The promiscuity of the former and specificity of the latter are aptly described by the conceptual framework of Boolean OR and AND logic gates, respectively (Buchler et al., 2003; Morris et al., 2010). Beyond combinatorial activation of signaling pathways, the number of combinatorial possibilities increases drastically when differential TF-promoter binding affinities and activation strengths are also considered (Brignall et al., 2019).

Within the pathogen-responsive transcriptome, a cluster of about 100 genes were identified as being activated by either NF-κB or ISGF3, their regulatory regions tend to contain both κB sites and ISREs and therefore classified as OR gate genes (Cheng et al., 2017). However, NF-κB and ISGF3 do not have redundant roles because they show distinct temporal control: only their combined action ensures that such genes are both rapidly induced (first by NF-κB) and expressed for extended times (then by ISGF3) (Figure 4A). Indeed, when such genes encode mRNAs with a long half-life, transient TF activity may be insufficient for full gene expression (Hao and Baltimore, 2009; Sen et al., 2020). In this regard, the OR gate may be thought of as providing an additional mechanism for temporal coding because it allows for the decoding of the combined temporal trajectory of both NF-κB and ISGF3.

In the established conceptual framework of Boolean logic gates, AND gate logic provides for high stimulus-selectivity, as both signaling pathway effectors must be active. It was thought that synergy between two TFs is achieved either by cooperative binding of DNA or by the cooperative recruitment of co-activators such as CBP/p300, which contains multiple TF interaction domains (Merika et al., 1998). However, in the pathogen-responsive transcriptome, there is little evidence for TFs needing to present at the same time or "coincident AND gates" at gene promoters. Rather, the sequential actions of signaling pathway effectors are required (Cheng et al., 2017). The discovery of sequential Boolean gates emphasizes the importance of integrating combinatorial and temporal signaling concepts for a full understanding of decoding.

One example of sequential AND gates are the small number of genes whose transcriptional initiation is regulated by both IRF3 and NF-κB (Freaney et al., 2013; Tong et al., 2016). While *Ifnb1* is the most well-known example, the molecular mechanism enabling NF-κB and IRF3 synergy is not clearly understood (Panne et al., 2007). In the case of *Ccl5*, recent studies suggest that IRF3 may facilitate a chromatin opening step that is required for the recruitment of NF-κB, which then activates transcription (Figure 4A) (Ramirez-Carrozzi et al., 2009; Tong et al., 2016). A second example of sequential AND gates pertains to a number of important cytokine genes, such as *Tnf*, *Il6*, and *Il1b*, that are dependent on both NF-κB and MAPKp38 activation downstream of the MyD88 adaptor (Cheng et al., 2017). While NF-κB is sufficient for transcriptional initiation, it appears that several post-transcriptional control steps, particularly mRNA stabilization, are p38-dependent (Figure 4A). In the case of TNF, the combined action of MAPKp38 and ERK enhance pre-mRNA splicing, cytoplasmic mRNA half-life, translation, and pro-protein processing (Caldwell et al., 2014). Thus, the sequential action of diverse biochemical processes in several different cellular compartments form a sequential AND gate in the production of secreted TNF. AND gate function may also control epigenomic reprogramming: a study on IFN-γ-induced gene repression showed that dissociation of active enhancers was a result of an AND gate at MAF-bound enhancers by IFN-γ-induced repression of MAF transcription and coordinate dissociation of PU.1 and C/EBP (Kang et al., 2017).

While combinatorial decoding interprets the activities of multiple TF pathways, temporal decoding distinguishes between distinct temporal trajectories of a single signaling effector and requires mechanisms that are kinetically regulated. For example, a kinetically slow mechanistic step not only transduces the instantaneous activity but also stores information about past activity (Behar and Hoffmann, 2010; Purvis and Lahav, 2013). Three molecular mechanisms have been identified: mechanisms controlling mRNA half-life, nucleosome displacement, and enhancer formation.

Long mRNA half-life has been shown by both mathematical modeling and experimental data to decode the duration of signaling effector activity. As short half-life transcripts achieve their half-maximal induction levels faster than long half-life transcripts, only long duration signals may allow full induction of long half-life mRNAs (Behar and Hoffmann, 2010; Hao and Baltimore, 2009). In the pathogen response, the long duration of IKK/NF-κB activity induced by LPS allows for induction genes that are poorly induced by transient TNF stimulation (Werner et al., 2005). A recent study quantified the contribution of long mRNA half-life to decoding differential duration of NF-κB activity for several dozen genes, but also showed that chromatin-associated mechanisms play important roles as well (Sen et al., 2020).

Nucleosome displacement also constitutes a kinetically "slow" step such that signal-dependent transcription factors (SDTF) binding to promoters or enhancers (Kaikkonen et al., 2013; Tong et al., 2016), co-activator recruitment, and pre-initiation complex (PIC) assembly and polymerase recruitment (Bhaumik, 2011) may, in principle, decode temporal dynamics of signaling. For example, LPS-induced NF-κB target genes fall into two categories: those with open chromatin immediately available for NF-κB binding and ready transcriptional induction, and those

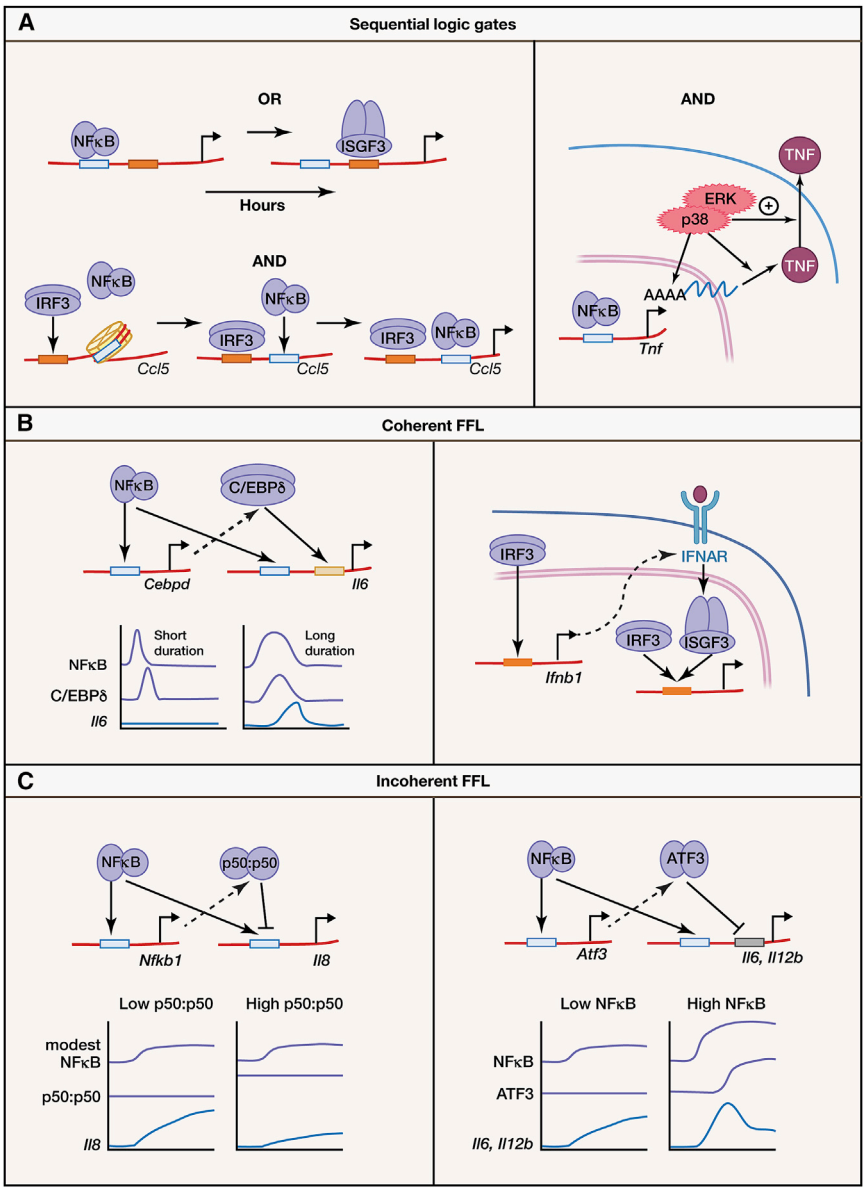

Figure 4. Mechanisms that decode combinatorial and temporal codes of pathway activity to achieve stimulus-specific responses.

(A) Decoding of pathway-specific effector combinations occurs through sequential logic gates that involve pre-transcriptional mechanisms, such as (left top) NF-κB and ISGF3 forming a sequential OR gate, allowing sustained responses; (left bottom) IRF3-mediated nucleosome movement for *Ccl5*, allowing NF-κB binding and forming a sequential AND gate; and (right) post-transcriptional mechanisms combining NF-κB-induced transcription AND MAPKp38 processing and stabilization of *Tnf* mRNA.

(B) Coherent feedforward loops, where one regulator positively controls a target and another positive regulator. Left: NF-κB induces transcription of *Cebpd*, which activates a second wave of response genes controlled by both NF-κB and CEPBδ. Here, coherent FFLs combined with AND gates allow duration decoding. Right: similarly, IRF3-induced *Ifnb1* generates a coherent feedforward loop through activation of IFNAR and subsequently ISGF3, which binds similar gene promoters as IRF3.

(C) Incoherent feedforward loops, where one regulator positively controls a target as well as a negative regulator. Together with combinatorial decoding principles they allow for fold-change detection and mediate biphasic responses. Left: NF-κB-induced expression of the competing p50:p50 homodimer provides memory of previous NF-κB abundances for fold-change detection on NF-κB target genes such as *Il8*. Right: NF-κB-induced transcription of Atf3, which in turn represses NF-κB target genes. This results in the diminishing of the second phase of response gene expression when NF-κB activity is high.

Corner arrow indicates active transcription.

requiring chromatin remodeling before NF-κB can bind (Kayama et al., 2008; Natoli, 2009; Ramirez-Carrozzi et al., 2009; Saccani et al., 2001). One recent study quantified the contribution of chromatin-associated mechanisms for differentiating between LPS- versus TNF-induced (2–3 h versus 1 h) NF-κB activity for 81 immune response genes (Sen et al., 2020). Interestingly, the chromatin-associated mechanisms provided a higher degree of specificity than the aforementioned long mRNA half-life mechanism and pertained particularly to immune effectors such as *Mmp3*, *Pfr1*, *Pilra*, and also *Ccl5*.

The formation of *de novo* enhancers is a third kinetically slow step that can decode temporal dynamics, but it is less reversible than the two previous examples. While poised target gene expression did not markedly differ in cells with oscillatory versus non-oscillatory NF-κB activity (Barken et al., 2005), a recent study demonstrated that the absence of oscillations in NF-κB activity determines whether stimulus-responsive *de novo* enhancers are established (Cheng et al., 2021). While the presence versus absence of oscillations in NF-κB dynamics is stimulus-specific, their frequency is not (Longo et al., 2013). The capacity to distinguish oscillatory and non-oscillatory NF-κB is based on the multi-step unwrapping and rewrapping dynamics of nucleosomal DNA-histone interactions, displacing the nucleosome and allowing the deposition of histone marks. A negative feedback mutant that converted normally oscillatory TNF-induced NF-κB activity to non-oscillatory activity allowed for more efficient nucleosome eviction and increased chromatin accessibility, and the subsequent formation of *de novo* enhancers enabled novel gene expression programs upon a secondary stimulation. SDTF dynamics may thus also be a critical determinant for epigenomic reprogramming that may allow immune sentinel cells to function in an exposure-history-dependent manner, potentially conferring innate immune memory (Netea et al., 2016).

A gene regulatory network where an effector positively controls a target both directly and indirectly via another positive regulator forms a coherent feedforward loop. Coherent

feedforward loops, which comprise of an AND logic gate, may decode differential TF durations (Figure 4B). For example, IL-6 expression was found to depend not only on NF-κB, but also the secondary TF C/EBPδ (Litvak et al., 2009). Because NF-κB activity induces the expression of C/EBPδ, they form a coherent feedforward loop to activate IL-6. However, a short exposure to LPS will not provide long-lasting NF-κB that can synergize with *de novo* C/EBPδ, thus preventing induction of secondary IL-6-mediated inflammation. The regulatory relationship of IRF3 and ISGF3 may also constitute a coherent feedforward loop on genes that require both IRF3 and ISGF3 for full activation. If, for example, IRF3 triggers a key chromatin remodeling step that then allows ISGF3 to bind and activate transcription, such target genes would primarily be induced in the infected (pathogen-exposed) cell rather than the bystander cells. A transcriptomic analysis yielded just a few candidates (e.g., *Cxcl10*, *Ifit3*) for such a regulatory mechanism (Ourthiague et al., 2015) and whether they differentiate IRF3-ISGF3 temporal features was not explored.

Another type of regulatory network motif, in which a signaling effector positively controls a target as well as a negative regulator of the same target, is referred to as an incoherent feedforward loop. Incoherent feedforward loops provide decoding mechanism for differences in fold changes (Figure 4C). For example, the decoding of fold changes of NF-κB activity on a set of target genes was reported to be governed by an incoherent feedforward loop involving the activation-domain lacking p50:p50 homodimer (Lee et al., 2014). A subset of TNF-induced genes was found to correlate particularly well with NF-κB fold changes in single cells. Interestingly, p50:p50 homodimers were also shown to bind a subset of ISREs and thus tune the responsiveness of ISGF3 target genes (Cheng et al., 2011). In an earlier example, an incoherent feedforward loop was shown to prevent run-away expression of inflammatory cytokines (Gilchrist et al., 2006). ATF3 was found to repress the NF-κB-induced expression of IL-6 and IL-12B. As ATF3 itself is induced by NF-κB, its repressive role on IL-6 and IL-12B becomes relevant when NF-κB activity is high and sustained, thereby preventing over-production of these potent inflammatory cytokines.

In further illustration of the precise temporal regulation of gene expression, incoherent feed forward loops may also involve post-transcriptional mechanisms. MicroRNAs known as inflammamIRs, mir155 and mir146, are NF-κB inducible and target immune response mRNAs (Rothchild et al., 2016; Testa et al., 2017). Mir146 targets regulators of the TLR signaling pathway and this repressive function serves to fine tune the innate immune response, whereas mir155 acts in an opposing and dominant function to further potentiate NF-κB response (Mann et al., 2017). Similarly, the RNA editing protein ADAR1 provides incoherent feedforward control because it is induced by IFN and then acts to repress RLR signaling, dampening the IFN response and preventing autoimmunity (Lamers et al., 2019; Mannion et al., 2014). While these regulatory circuits have the potential to decode dynamic features of the pathogen-responsive temporal code, their true functional impact will need to be addressed in future studies.

Decoding mechanisms are intricately tied to immune sentinel functions of context dependence and memory. Beyond generating the stimulus-specificity of immediate gene expression responses, decoding mechanisms that invoke chromatin remodeling are particularly important to the establishment of stimulus-specific innate immune memory. The eviction of nucleosomes and the deposition of new histone marks are a downstream consequence of stimulus-specific chromatin remodeling, though the functional and physiological consequences of this reprogramming, especially in disease contexts, remains to be explored.

CONCLUDING REMARKS

Recent work has begun to document the capacity of immune sentinel cells to mount pathogen-specific responses, as well as provide a set of mechanistic studies to support the notions of combinatorial and temporal coding. These insights may serve as a basis for addressing key questions through future studies.

First, while the mechanisms underlying temporal coding in the NF-κB signaling module are relatively well understood, the integration of combinatorial and temporal signaling via coordinated activities of IRF, JNK, and MAPKp38 pathways is a next big challenge in the immune signaling field. Temporal dynamics and pathway branching of signaling initiated by cytosolic PRRs such as cGAS, RLRs, and NLRs are also understudied.

Second, understanding how combinatorial and temporal signaling codes are interpreted to produce gene expression requires substantial effort, in part because every gene is controlled by a seemingly unique gene regulatory network. To study the connection between encoding and decoding of signaling activity, it will be necessary to develop efficient workflows that directly connect temporal effector activity profiles, ideally for multiple effectors, with gene expression or cytokine secretion measurements in the same cells (Gutschow et al., 2019; Lane et al., 2017).

Third, and related, at the single-cell level, both stimulus-responsive signaling activity and gene expression are highly heterogeneous, which may be essential for the breadth, robustness, and adaptability of the immune response but may also contribute to pathology (Satija and Shalek, 2014). While live cell imaging has been successful in characterizing the cell-to-cell variability of signaling activity, the stochasticity of decoding mechanisms at the gene promoter likely plays an especially important role in determining gene expression responses of a specific cell. This requires innovative integration of live cell imaging and necessarily destructive single-cell technologies, such as single-cell RNA sequencing (scRNA-seq), single-molecule fluorescence in-situ hybridization (smFISH), single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq), or single-cell chromatin immuno-precipitation followed by sequencing (scChIP-seq) to probe expression of native chromatin and endogenous genes (rather than ectopic reporters), via data driven and mechanistic modeling approaches.

Fourth, with such workflows in place, future work will be able to explore how encoding and decoding mechanisms are a function of microenvironmental context and the cell's exposure history. Cytokines in the microenvironment, which guide the functional specialization of immune sentinel cells, may alter the encoding of combinatorial and temporal signaling profiles, while exposure history that changes the cell's baseline epigenome affects how signals are decoded (Cheng et al., 2019; Kang et al., 2017; Park et al., 2017).

Detailed and predictive knowledge of how immune sentinel responses are regulated will undoubtedly impact our understanding of a range of immune-related disease, whether immunopathological responses to infectious disease (e.g., as seen during severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2] infections) or autoinflammatory and autoimmune disorders (Funes et al., 2018; Gustine and Jones, 2021). As pathogenesis may be triggered by small subsets of outlier cells, understanding the basis for the heterogeneity of responses at the single-cell level is key. By characterizing macrophages derived from a Sjögren's syndrome mouse model, a recent study revealed that a minority of cells show stimulus-confused NF-κB dynamics and gene expression (Adelaja et al., 2021). We therefore suggest that a quantitative and mechanistic understanding of immune sentinel responses will enable a better characterization and diagnosis of auto-inflammatory and auto-immune diseases and support the development of therapeutic strategies aimed at correcting disease-associated miscoding.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.immuni.2021.08.018.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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