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Functional Hallmarks of Healthy Macrophage Responses: Their Regulatory Basis and Disease Relevance

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Abstract

Macrophages are first responders for the immune system. In this role, they have both effector functions for neutralizing pathogens and sentinel functions for alerting other immune cells of diverse pathologic threats, thereby initiating and coordinating a multipronged immune response. Macrophages are distributed throughout the body—they circulate in the blood, line the mucosal membranes, reside within organs, and survey the connective tissue. Several reviews have summarized their diverse roles in different physiological scenarios and in the initiation or amplification of different pathologies. In this review, we propose that both the effector and the sentinel functions of healthy macrophages rely on three hallmark properties: response specificity, context dependence, and stimulus memory. When these hallmark properties are diminished, the macrophage's biological functions are impaired, which in turn results in increased risk for immune dysregulation, manifested by immune deficiency or autoimmunity. We review the evidence and the molecular mechanisms supporting these functional hallmarks.

1. PREFACE

One of the functions of macrophages was first described in the late nineteenth century, when Ilya Mechnikov looked into his microscope and made a striking observation: White corpuscles moved to surround a small splinter embedded in a starfish larva (1). This description of the phagocytes now known as macrophages launched investigations into innate immunity and the diverse functions of macrophages. Macrophages exist in almost all organs of the body but possess differences in development and function. A substantial proportion of tissue-resident macrophages are specified embryonically (2) and are derived from the yolk sac, fetal liver, or bone marrow, while macrophages that extravasate into tissues in response to injury or infection are recruited from the bone marrow later in life via differentiation of hematopoietic stem cells (3).

All macrophages detect tissue injury, pathogens, and antibody and respond with two broadly defined functions: (*a*) They have potent phagocytic effector functions, as their name implies, and (*b*) they also have potent sentinel functions to call up and direct innate and adaptive immune cells (**Figure 1*a***). As effectors, macrophages phagocytose pathogens and infected and dying cells, upregulate antimicrobial peptides, or trigger cell death to limit intracellular pathogens. As sentinels, macrophages initiate and coordinate local or systemic immune activation by secreting cytokines, chemokines, and growth factors and by presenting antigen to adaptive immune cells (4).

As first responders, macrophages are strategically placed. Almost every tissue in the body is populated with macrophages at a remarkably consistent density of ~5,000–10,000 per cubic millimeter (5–7). Tissue-resident alveolar macrophages, peritoneal macrophages, and Kupffer cells of the liver sense airborne pathogens or those from the digestive tract. In addition, bone marrow–derived

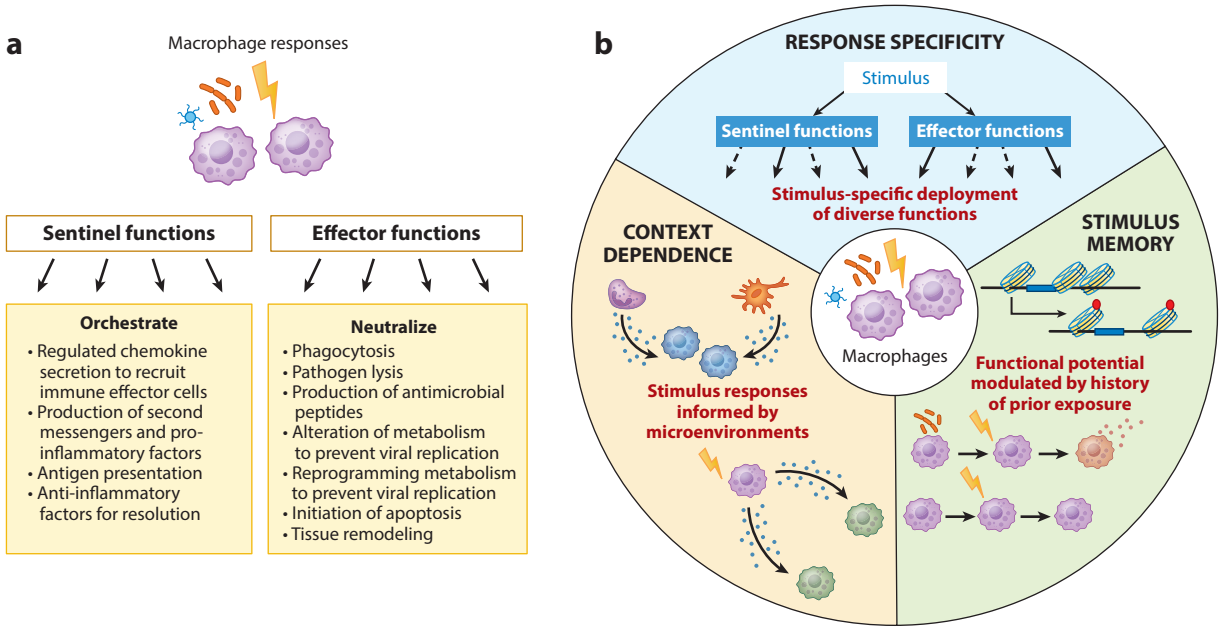

Figure 1

Functional hallmarks of macrophages. (*a*) Macrophages respond by performing a variety of functions. They can perform as sentinel cells of the immune system or as immune effector cells. (*b*) Macrophage responses exhibit three hallmarks central to immunological function: response specificity, context dependence, and stimulus memory. Dashed and solid arrows represent deployment of specific functions to different degrees or speeds.

SENTINEL AND EFFECTOR CELLS OF INNATE IMMUNITY

In comparison to macrophages, other cell types can exhibit effector and sentinel functions (9). Fibroblasts, which form part of the connective tissue, are tissue-resident sentinels that similarly express PAMP, DAMP, and cytokine receptors and activate stimulus-specific immune response genes upon ligand challenge (10–13). Endothelial cells, placed in a prime position to respond to circulating endotoxins (14, 15), are also sentinels that produce inflammatory cytokines to mobilize other immune cells (16–18). However, these structural sentinel cells do not exhibit the strong effector functions of macrophages. Neutrophils and natural killer cells also each share some of the functions of macrophages, such as the effector functions of phagocytosis and release of antipathogen lytic enzymes (19–21).

monocytes circulate in the blood and readily extravasate into tissues upon sensing chemoattractants secreted by the very first responding macrophages. Upon their sensing a pathogen-associated molecular pattern (PAMP), damage-associated molecular pattern (DAMP), or infected cell decorated with antibody, the differentiation of monocytes into macrophages is accelerated, and they rapidly contribute effector and/or sentinel functions at the site of infection or injury (see the sidebar titled Sentinel and Effector Cells of Innate Immunity).

Despite having disparate ontogenies and a wide variety of physiological roles, macrophages share certain functional characteristics. These characteristics are defined less by developmental origin or molecular markers [as seen in epigenomic or transcriptomic profiles (8)] and more by the functional properties macrophages exhibit when they respond to immune threats. While molecular hallmarks of macrophages defined by expression of cell type or ontological markers can be profiled at steady state, functional hallmarks are only evident upon stimulation by PAMPs, DAMPs, cytokines, or antibody. Here we propose three functional hallmarks of healthy macrophage responses (**Figure 1*b***): (*a*) response specificity, the capacity to mount threat-specific immune responses; (*b*) context dependence, the capacity to adapt threat-specific responses to the microenvironmental cytokine milieu; and (*c*) stimulus memory, the capacity to record prior exposure to stimuli and appropriately adapt subsequent threat-specific responses.

Does the misregulation of these functional hallmarks contribute to risk for disease? First, diminished response specificity may result in inappropriate immune activation. For example, autoimmune diseases, where symptoms often are sporadic and have unknown triggers, may involve the loss of healthy response specificity. Second, misregulation of context dependence in macrophage responses may contribute to altered immune function in diseases involving aberrant cytokine conditioning. For example, proper immune responses to infection or cancer may be adversely affected by microenvironment states associated with advanced age or obesity. Third, impaired immune memory resulting from improper stimulus-induced instruction of the epigenome may lead to detrimental hyper- or hypo-inflammatory disorders upon subsequent encounter with pathogen or stimulus. Understanding the molecular mechanisms that give rise to each functional hallmark may help delineate how the impairment or misregulation of them results in disease.

The mechanisms underlying signaling and epigenome regulation of macrophage responses have been closely studied and reviewed (22–25). However, it has been less obvious how these molecularly detailed biochemical and biophysical mechanisms give rise to the described functional hallmarks. Recent studies on the stimulus specificity of signaling dynamics (26), on feed-forward and feedback mechanisms (27, 28), or on context- or exposure-dependent epigenomic changes (29, 30) have begun to elucidate regulatory mechanisms. Furthermore, new technologies such as reliable live-cell microscopy, cost-effective single-cell RNA sequencing (scRNA-seq), and multidimensional flow cytometry, and algorithmic advances in analysis of the data, have enabled

Pathogen-associated molecular pattern (PAMP): pathogen-derived molecule recognized by macrophage receptors

Damage-associated molecular pattern (DAMP): host cell–derived molecule indicative of damage, e.g., DNA, histones, ATP, and other nuclear or cytosolic proteins

quantitative probing of the functional hallmarks. In light of these advances, in this review, we aim to describe the regulatory basis of the three functional hallmarks that characterize healthy macrophage responses.

Sentinel cell: a cell that conveys messages about the environment to activate other cells

2. RESPONSE SPECIFICITY

Macrophages express dozens of receptors and are capable of sensing hundreds of PAMPs, DAMPs, and cytokine ligands, as well as antibodies. These stimuli activate hundreds of immune response genes that are not expressed constitutively because they are in fact detrimental to the host. Hence, they must be deployed on an only-as-needed basis. Given that pathogens differ widely in their biology, different immune defenses are required to effectively counteract them. The only-as-needed rationale argues that healthy immune sentinel cell responses should be highly specific to the immune threat.

It is now appreciated that macrophages respond to ligands with stimulus-specific signaling profiles and stimulus-specific gene expression programs (11, 31, 32). Not only the ligand but also ligand dose and ligand exposure dynamics and duration generate distinctive responses (33–36). Yet, early transcriptomic studies of macrophages found a common core response, emphasizing the common phagocytic and antigen-presenting functions of macrophages (37). Only B cell– and T cell–mediated adaptive immune responses were thought to provide specificity. However, later studies revealed that macrophages, dendritic cells, and fibroblasts (38) produce gene expression programs that are in fact stimulus specific (11, 39), and they began to address the molecular mechanisms that allow for this response specificity.

As sentries, macrophages perform as individuals; each has the capacity to sense molecular patterns and activate signaling pathways to generate a response, and the immune response relies on the response of the individual cell. Yet, macrophages are not identical. Macrophages within tissue make up distinct cellular subsets (40), including subsets with distinct developmental origins (41). Furthermore, they are subject to molecular stochasticity that affects gene expression (and hence the abundances of pathogen sensors and signaling proteins) and the cells' spatial organization. The resulting cell-to-cell heterogeneity may affect stimulus-response programs, and thus the response specificity ascribed to the population. Therefore, quantitative studies of response specificity must involve measurements at single-cell resolution, and an analytical framework that compares distributions of responses to each stimulus.

2.1. Immune Response Signaling Network

Response specificity relies on molecular components and pathways that are activated in response to a specific stimulus. Given the large number of immune-activating stimuli but limited number of signaling factors, two principles have emerged that help to explain the stimulus-specific regulation of the molecular network: combinatorial and dynamic control of signaling pathways. These principles explain that stimulus-specific activation of immune response genes is dependent on (*a*) different stimuli activating different combinations of signaling regulators and (*b*) different stimuli activating the same regulator but with different dynamic patterns of activity.

Specific responses are initiated based on how cells sense ligands. Human macrophages sense PAMP and DAMP ligands with 10 Toll-like receptors (TLRs) (42) that reside in the plasma membrane and survey extracellular and endosomal environment, 22 cytosolic NOD-like receptors (NLRs) (43), 3 RIG-I-like-receptors (RLRs) (44), and the DNA sensor cyclic GMP–AMP synthase (cGAS) (45, 46). Immune-activating cytokines such as interferons, IL-1, TNF, and other TNF superfamily members are sensed by their cognate receptors in the plasma membrane. To

elicit stimulus-specific responses, the ligand-receptor interaction must be based on molecular specificity. Indeed, even low-complexity PAMPs are distinguished by different TLR family members, and the biophysical basis for this specificity has been studied (47). However, functional discrimination of ligands may also involve other mechanisms such as localization (48) or kinetic proofreading, as for example, in the case of RIG-I (49).

Pattern recognition receptor (PRR): sensor that detects molecules related to pathogens (PAMPs) or cell damage (DAMPs)

Despite the impressive number of pathogen sensors, the number of pathogens and potential PAMPs and DAMPs is even greater. Many receptors mediate sensing of distinct ligands. For example, TLR4, the known endotoxin [lipopolysaccharide (LPS)] sensor, also mediates sensing of DAMPs, such as HMGB1 (50), serum amyloid A (SAA) (51), and MRP8/MRP14 (52). The range of interactions may be expanded by soluble or membrane-bound cofactors such as CD14 (53). However, two ligands being recognized by the same receptor does not necessarily mean that they produce the same response. Ligand-receptor interactions are governed by specific kinetics of signaling adaptor recruitment, receptor internalization, and ligand degradation, which may produce not only stimulus-specific amplitudes of signaling but also stimulus-specific signaling dynamics. Such dynamic coding mechanisms also allow cells to mount dose-specific responses for the same stimulus via the same receptor.

Similar considerations govern how receptors activate downstream signaling pathways, which are mediated by a small number of primary signaling adaptors: MyD88, TRIF, TRAF2/6, MAVS, STING, and ASC (54). In fact, all TLRs except TLR3 utilize MyD88, as does the IL-1 receptor. TRIF is engaged by TLR3 and TLR4. TRAFs are engaged by TNFR family members, ASC by NLRs, and STING by cGAS (55). However, specificity is possible because the relative strengths and dynamics of adaptor recruitment and signaling pathway activation may differ between receptors. For example, in response to LPS, TLR4 dimerizes at the plasma membrane and initiates the oligomerization of the adapter MyD88, or it can be internalized to signal through the endosome, where it interacts with a different adapter TRIF to initiate TRIF-dependent signaling. LPS dose–response specificity is controlled at the adapter level by the different oligomerization dynamics of MyD88 and TRIF (56).

Adapters and associated ubiquitin chains ultimately activate a limited set of kinase–transcription factor modules. While interferons activate the JAK-STAT pathways without a dedicated signaling adaptor, four primary immune response signaling pathways may be mapped onto these adaptors: IRF3 is activated by TRIF, MAVS, and STING adaptors; MAPKp38 is strongly activated by MyD88; but NF-κB and JNK/ERK pathways are activated by all adapters (in the case of ASC via the IL-1β feedback loop) (55). Therefore, if response specificity solely relied on combinatorial coding, only four patterns would be observed: NF-κB and JNK/ERK (e.g., in response to TNF); NF-κB, JNK/ERK, and IRF3 [e.g., in response to poly(I:C)]; NF-κB, JNK/ERK, and MAPKp38 (e.g., in response to Pam3CSK4); and NF-κB, JNK/ERK, MAPKp38, and IRF3 (e.g., in response to LPS). However, stimulus-specific dynamic control of these pathways may allow for additional specificity (see Section 2.2).

Given that response specificity is a function of not merely biophysical interaction specificities that are genetically encoded in protein structures but also dynamics of signaling amplitude and time, it is subject to differences in the expression levels and localization of signal transducers. It is commonly appreciated that the pattern recognition receptor (PRR) repertoire that macrophages express determines their responsiveness. The above discussion suggests that the response specificity of macrophages is diminished by cell-to-cell heterogeneity in the expression of PRRs and other key signaling regulators (adaptors, kinases, etc.). When macrophages are stimulated with LPS, the primary driver of the heterogeneity of NF-κB activity was in fact found to be not the TLR4 abundance but the maturation time of the endosome, which determines the duration of TRIF signaling (56).

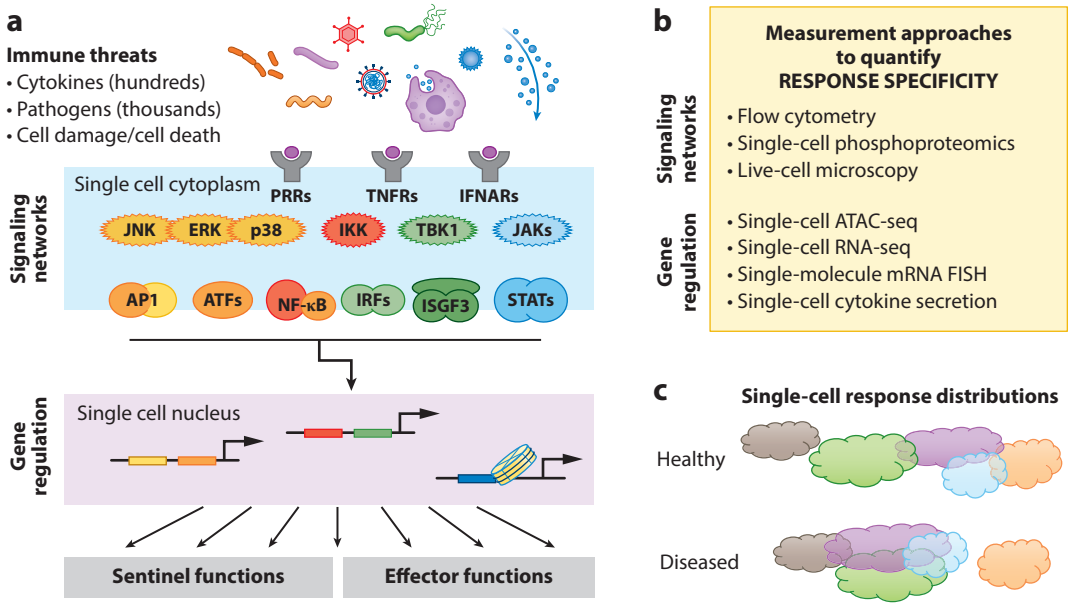

Figure 2

Mechanisms and measurement approaches of response specificity. (*a*) Response specificity relies on the ability of dozens of PRRs and cytokine receptors to recognize specific ligands. Ligand-receptor interactions activate specific signaling pathways with ligand-specific temporal and dose dynamics, which are recognized by gene regulatory mechanisms that decode the stimulus-specific combinations of temporally modulated transcription factor activities. Single-cell heterogeneity in signaling network activation and transcriptional regulation impacts response specificity. (*b*) Single-cell measurements of signaling or epigenetic events can be interrogated to quantify response specificity, (*c*) resulting in an understanding of ligand-response distributions in health versus disease. Abbreviations: AP1, activator protein 1; ATAC-seq, assay for transposase-accessible chromatin with sequencing; ATF, activating transcription factor; FISH, fluorescence in situ hybridization; IFNAR, IFN-α/β receptor; IRF, interferon response factor; ISGF3, interferon-stimulated gene factor 3; PRR, pattern recognition receptor; RNA-seq, RNA sequencing; TNFR, tumor necrosis factor receptor.

2.2. Immune Response Transcription Factors

Response specificity has been studied at the level of transcription factor activities, given the availability of quantitative assays. Biochemical studies performed in fibroblasts in the 2000s elucidated both the temporal and combinatorial regulation of kinase–transcription factor activities mediating stimulus-specific mean responses (38, 57, 58) (**Figure 2*a***). For example, NF-κB activation was shown to be persistent in response to LPS but oscillatory in response to TNF, mediated by negative-feedback regulation from IκBα (26, 35). Similarly, JNK activation showed two distinguishable phases of activity (59). Combinatorially, while the kinase–transcription factor modules IKK-NF-κB and JNK-AP1 are ubiquitous, adapter-specific activation of TBK1-IRF3 by TRIF and MAPKp38 by MyD88 further contributes to response specificity of transcription factor activity (11, 60, 61).

The cell-to-cell heterogeneity of macrophage responses has come into focus as a key element for assessing macrophage capacity to respond stimulus-specifically, as the distinction of mean responses indicated by population-level (bulk) assays does not reveal how well the distributions of responses across single cells may be distinguished. Not until the 2010s were appropriate single-cell technology and an analytical framework (based on information theory) developed to allow insight into the distinction of single-cell response distributions. Measuring NF-κB activity levels at a single time point in fibroblasts exposed to TNF, Cheong et al. (62) found that TNF

dose–response specificity was low; 12 different TNF doses spanning four orders of magnitude were measured, but cells were barely able to reliably distinguish two conditions, e.g., between the presence and absence of TNF. The temporal dynamics of signaling activity in macrophages exposed to different immune ligands and doses were subsequently studied by live-cell microscopy of fluorescently tagged proteins and found to provide information for stimulus distinction (26, 34, 63–65). Mechanisms of positive feedback from RelA were identified as one component enabling LPS dose specificity (66). Furthermore, across a range of doses and an array of immune stimuli targeting both TLRs and cytokine receptors, six NF-κB dynamical features, termed signaling codons, were shown to be key to maximally facilitating specificity to ligand dose and ligand identity (26). All six NF-κB signaling codons are determined by precise modulation of IKK activity over time: The IκBα negative-feedback loop amplifies small differences in IKK activity (low versus medium-low) by converting them into oscillatory versus nonoscillatory NF-κB trajectories. However, which sources of molecular noise are the primary drivers of the heterogeneous deployment of each NF-κB signaling codon remains unclear, and how such responses are modulated by microenvironmental or polarizing cytokines is still an active area of investigation.

Polarization: adoption by macrophages of specialized functions as adaptations to contextual cytokines or other microenvironmental signals

Single-cell studies have also begun to quantify to what extent the combinatorial activation of signaling pathways is a contributor to response specificity. Simultaneous measurement of NF-κB and MAPKp38 in single macrophages at a single time point revealed that dose-response curves for each pathway were distinct, with MAPKp38 being digitally activated above a ligand concentration threshold (34). Therefore, the combination of NF-κB and MAPK with differential dose-response curves may have a larger overall dose-discrimination capacity than either pathway alone. Another study that measured single-cell temporal dynamics for both NF-κB and JNK indicated that the two pathways combined were biologically informative and reflective of different levels of threat from pathogenic versus nonpathogenic microbes (63). The development of kinase translocation reporters for activity of the MAPKs ERK, p38, and JNK may allow evaluation of the combinatorial and temporal control of these signal transducers in single cells (59). Less is known at the single-cell level about the combinatorial and dynamic control and heterogeneity of IRF signaling.

2.3. Immune Response Gene Expression

Downstream of signaling pathways, response specificity is evident and may be measured at the level of immune response gene expression programs. Bulk transcriptomic studies identified sets of genes controlled by stimulus-specific combinatorial activation of signaling pathway activity (11). These gene sets could be mapped to the immune response transcription factors, identifying sets regulated by single transcription factors NF-κB, IRF, or AP1 but also those that required two pathways, such as a cytokine set whose transcriptional activation is mediated by NF-κB and whose posttranscriptional regulation of mRNA half-life is mediated by MAPKp38. *Ifnb1* and *Ccl5* are other well-known examples of genes requiring the combinatorial activation of NF-κB and IRF (67, 68).

Many studies relied on clustering algorithms of transcriptomic data to identify patterns of gene expression. These approaches readily identify a handful of distinct patterns when data from multiple ligands is available (11, 39), but closer inspection reveals many more patterns of expression, some of which may be exhibited by only a single gene (67, 69). Thus analytical approaches that have true single-gene resolution are critical. For example, the duration of NF-κB activity was shown to be decoded gene-specifically using a mechanistic modeling approach with differential equations for each gene rather than a statistical evaluation that requires considerations of sets of genes. As a result, the contribution of mRNA half-life or chromatin mechanisms to decoding the stimulus-specific duration of NF-κB activity could be quantified for each gene (13). A similar

mechanistic modeling approach has been developed to elucidate the combinatorial transcription factor control of gene expression at single-gene resolution (61), but its implementation requires a very large amount of data produced in different conditions.

While population-level gene expression studies showed that transcriptional responses are ligand specific and could elucidate regulatory mechanisms, the quantification of response specificity requires single-cell-resolution data, as cell-to-cell heterogeneity affects this quantity. Interestingly, the cell-to-cell variability in signaling pathway dynamics and activation levels may, in principle, be either buffered or amplified by the chromatin-associated or posttranscriptional regulatory mechanisms controlling the expression of each gene. Technological developments may be required to link signaling to gene expression in the same cell, such as advances in microfluidics, image analysis, single-molecule fluorescence in situ hybridization (smFISH), and scRNA-seq (70–72) (**Figure 2*b***). For example, in one study in macrophage-like RAW246.7 cells, after measuring NF-κB signaling dynamics in response to LPS and then profiling the transcriptome of each cell at the end point, Lane et al. (73) found that the expression of some cytokine and feedback regulator genes was correlated to the cell's NF-κB dynamics. In addition, certain pairs of genes possibly controlled by the same enhancer elements maintained correlated expression levels across single cells. However, a key limitation of some single-cell assays is the high degree of technical noise. Further work in profiling transcriptomic response specificity across multiple ligands and doses is needed to understand the extent to which decoding of signaling dynamics is stimulus specific. As with studies of signaling dynamics in sentinel cells, both statistical analysis and mechanistic modeling may elucidate mechanisms and sources of biological noise in single-cell gene regulation.

2.4. Cytokine Loops

One result of the primary signaling response is the secretion of immune response cytokines that may then be sensed in an autocrine or paracrine manner. They may function as feedback and feed-forward loops to contribute to response specificity.

Activation of the IRF pathway by PAMPs such as LPS and poly(I:C) induces the secretion of IFN-β, which acts in a feed-forward loop to produce ISGF3, which in turn reinforces IRF3-driven gene expression programs in primary response cells and produces an almost equivalent interferon-response program in neighboring bystander cells (74). A single-cell study of dendritic cells responding to LPS showed that early paracrine secretion of IFN-β in just a handful of cells was important for antiviral gene expression in the population; at later time points, *Ifnar*- and *Stat1*-dependent IFN-β paracrine signaling downregulated inflammatory genes not uniformly but in a fraction of the cell population (75). This presence or absence of negative feedback from type I interferons was shown to be biologically important for distinguishing gram-negative from gram-positive bacteria in bone marrow–derived macrophage responses. Bacterial class-specific production of key cytokines such as CXCL1 and TNF was diminished in IFNAR knockouts or when IFN-β was exogenously supplied (27, 76).

Like production of interferons, TNF production may further amplify or curtail response specificity. Single-cell studies of NF-κB signaling dynamics revealed that in the presence of TNFRII, a soluble TNF inhibitor, the responses to low-dose CpG or LPS stimulation become less variable due to a reduction of the oscillatory component from response trajectories (26). In another study, blocking TNF autocrine signaling decreased the heterogeneity of NF-κB signaling profiles in response to LPS, suggesting that cell-to-cell variability of signaling was in part affected by the heterogeneity of cellular secretion of TNF (73).

In the case of NLR signaling, the secretion of IL-1β and IL-18 is in fact critical for the activation of NF-κB and MAPK pathways (77, 78)—these are the pathways that allow for gene

expression responses in both primary responders and bystanders. Thus, the production, secretion, and responses to soluble cytokines, and the single-cell heterogeneity of these processes, may be features that can either expand or restrict sentinel ability to discriminate dose or identity of a pathogen or DAMP ligand.

2.5. Diseases of Impaired Response Specificity

By proposing that response specificity is a property of healthy macrophage function, we suggest that impaired response specificity contributes to disease. Healthy response specificity may be characterized by distributions of particular responses to each immune threat, and both increases and decreases in the heterogeneity may result in disease (**Figure 2*c***). As such, the behavior of outliers is critical in these diseases, as outliers in a responding population of macrophages may, for example, produce cytokines or second messengers that initiate an immune response. Rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis, and Sjögren syndrome all have remitting and relapsing characteristics (79–81). The intermittent nature of the presentation of multiple autoimmune diseases hints that low-probability outlier events may underlie their etiologies.

Indeed, aberrant TNF production and IFN-β production have been implicated as opposing sides of different autoimmune diseases (82). Excessive IFN-β production from dendritic cells was postulated to be an initiator of the autoimmune disease SLE (83). As IFN-β has both feed-forward and negative-feedback functions on neighboring cells, the improper production of IFN-β from even a subset of cells may have significant consequences on response specificity. On the other hand, TNF has been implicated in the pathogenesis of rheumatoid arthritis. Interestingly, patients undergoing anti-TNF therapy for juvenile rheumatoid arthritis can display an SLE-like syndrome and overexpression of IFN-α-stimulated genes (84). A recent single-cell study more directly couched the autoimmune disease Sjögren syndrome as involving loss of response specificity and pointed to cross-regulation of TNF and type I interferon pathways. Loss of the NF-κB negative-feedback regulator *Nfkbia* diminished the distinguishability of macrophage responses to TNF versus poly(I:C), interestingly through the increased expression of IRF target genes in a fraction of TNF-stimulated cells (26). TNF-induced IFN-β production through IRF1 has also been implicated in rheumatoid arthritis and could be corrected through JAK inhibitor drugs (85). Thus, the misregulation of the TNF versus type I interferon axes in autoimmune disease may provide clues into how to correct or control loss of response specificity (86).

3. CONTEXT DEPENDENCE

Macrophages populate all organ systems and are therefore exposed to different cytokine microenvironments that provide instructions for tailoring the function to the local physiological state. As a result, macrophage responses and functions have evolved to be highly adaptable. Interestingly, macrophages residing in the same organ system may have different developmental origins: Some of the resident population are longer-lived derivatives of the yolk sac or fetal liver, while others are monocyte derived and migrate to the tissues from the bloodstream (87). However, their immunological functionality is a product of both ontogeny and cytokine cues of the local environment, such that all tissue-resident macrophages within an organ adopt similar functions (88). Polarizing cytokines in the microenvironment can further shift the response repertoire of macrophages to allow them to carry out more specialized roles. This tuning of function is important in normal physiology in the contexts of inflammation, injury, or repair.

We thus propose that the second hallmark of healthy macrophage responses is context dependence. Context dependence allows for a subspecialization of macrophage functions, or to use Waddington's metaphor, a "canalization," such that both signaling networks and epigenetic

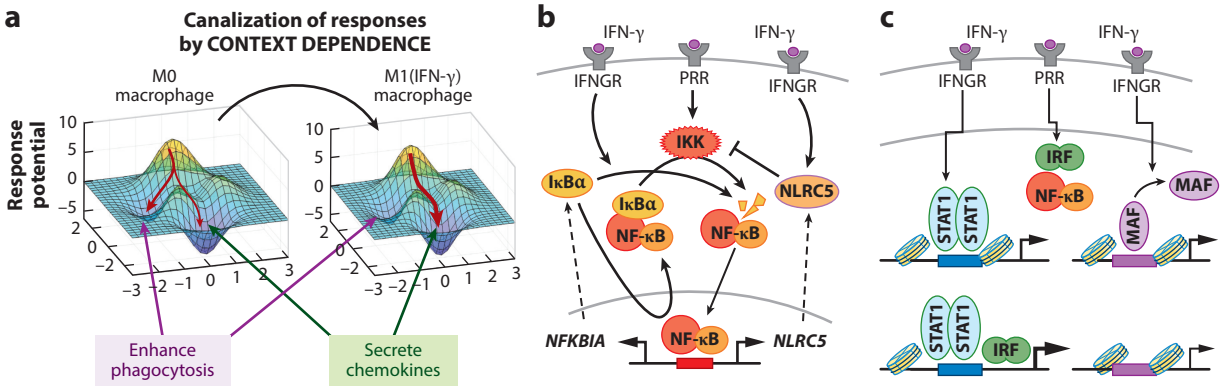

Figure 3

Context dependence is mediated by microenvironmental signals that may lead to a canalization of the diverse stimulus-specific macrophage responses. (*a*) Cytokine context results in specialization of function by reversibly altering the epigenetic states of signaling and gene regulatory networks. Arrows pointing to regions of the response landscape represent possible responses given an inflammatory stimulus. (*b*) Positive and negative regulation of signaling feedback regulators by polarizing cytokines may generate context-dependent signaling profiles. (*c*) Epigenetic mechanisms that either hold open promoters or disassemble enhancers allow for gene-specific regulation of context-dependent responses in macrophages. Abbreviations: PRR, pattern recognition receptor; IFNGR, interferon gamma receptor.

states are tuned to the current microenvironment to promote specialized functions while the tuning cytokine is present (**Figure 3*a***). Originally, for monocyte-derived macrophages, a simplified paradigm of context dependence existed within the framework of M1 and M2 macrophage polarization (89). A much more complex topology of macrophage subspecialization states is now known to exist with pro- and anti-inflammatory M1-IFN-γ and M2-IL-4 states generally thought to represent extremes (25, 90). M1 macrophages are canonically antimicrobial, producing proinflammatory cytokines and upregulating their phagocytosis ability; M2 macrophages are canonically associated with repair, producing anti-inflammatory cytokines or growth factors.

3.1. Signaling Mechanisms

What molecular mechanisms allow for context dependence? Both signaling cross talk and epigenetic mechanisms allow macrophages to respond in relation to their microenvironment. Generally, signaling networks that encode ligand-specific responses may be altered by polarizing cytokines that change the availability of signaling components (91). Such components may include receptors, adapters, transcription factors, feedback regulators, and even regulators of core machineries responsible for protein synthesis or decay.

M1/M2 macrophages: macrophages at the opposite extremes of polarization, often generated by IFN-γ or IL-4 cytokine contexts, respectively

Microenvironmental interferons can affect PAMP- or DAMP-induced signaling by altering expression levels of signaling pathway components: The type I interferon IFN-α upregulated the expression of TLR3, TLR4, and TLR7 (92), and type II interferon (IFN-γ) upregulated transcription factor IRF1 that augments TLR-dependent interferon signaling (93). Modulation of feedback regulator activity is also critical: IFN-β increases NF-κB activity by reducing the translation of IκBα, and in the late phase, by increasing IKK activity via expression of the viral RNA sensor RIG-I (94). IFN-γ was shown to elevate NF-κB activity through increased expression of proteasomal cap components that facilitate degradation of the feedback inhibitors of NF-κB, including IκBα and IκBε (94, 95). Another cross regulatory feedback protein has roles as a negative regulator of NF-κB. NLRC5, an NLR family member, is induced by IFN-γ (96) and decreases NF-κB activity via inhibition of IKKα/IKKβ phosphorylation. NLRC5 also negatively regulates

type I interferon signaling at the receptor level via inhibition of RLR-mediated type I interferon responses (97) (**Figure 3*b***).

Within the interferon pathway itself, polarizing cytokines induce negative regulators such as SOCS proteins that may also both enhance or suppress PAMP or DAMP responses. Low-dose IFN-α increases baseline STAT2 and IRF9 expression without strongly activating the negative-feedback regulators SOCS1, SOCS3, and USP18, thereby hypersensitizing cells to further interferon stimulation (28). However, higher levels of type I interferon increase the expression of these negative regulators. For example, recruitment of USP18 to the type I interferon receptor IFNAR (98, 99), as well as increased SOCS1 expression (100), desensitizes cells to any additional stimulation with interferon. Macrophage polarization that represents a wide spectrum of context-dependent canalization of macrophage functions is also achieved by regulation of feedback inhibitor proteins. IFN-γ was reported to upregulate the expression of SOCS3, inhibiting STAT3 activity and promoting M1 macrophage activation (101). In contrast, IL-4 was reported to upregulate SOCS1 but not SOCS3, thereby inhibiting STAT1 transcription factors and promoting the M2 macrophage phenotype (102). The strength of cross talk is modulated by the dose and duration of the polarizing cytokine cue, but it may also be subject to other cell properties, like cell cycle phase (103).

Priming: preparation of the epigenetic landscape by the microenvironment for stronger induction upon PAMP, DAMP, or cytokine stimuli, without return to baseline

Lineage-determining transcription factor (LDTF): transcription factor that establishes cell type by remodeling the enhancer landscape

3.2. Epigenetic Mechanisms

Epigenetic mechanisms are another layer of control critical to context-dependent canalization of macrophage functions. Importantly, in contrast to signaling mechanisms, epigenetic mechanisms result in gene-specific rather than pathway-specific alterations. IFN-γ-M1 and IL-4-M2 polarization are associated with chromatin alterations at STAT1/IRF1- and STAT6-enriched genomic regions, respectively (104). Interestingly, macrophages simultaneously exposed to multiple tuning cytokine signals, such as the opposing polarization cytokines IFN-γ and IL-4, as might occur in tissue or bloodstream microenvironments, did not show extensive antagonism at the level of signaling pathway activation; instead, cross talk occurred more prominently at the gene regulatory level through gene-specific binding of STAT1 or STAT6 (105).

Interferon priming can potentiate some subsets of genes while repressing others. In human macrophages, type II interferon, IFN-γ, synergistically enhanced TLR-induced transcription at inflammatory genes encoding TNF, IL-6, and IL-12B by recruiting STAT1 to enhancers and promoters to increase chromatin accessibility and prime genes without itself inducing transcription (93). Type I interferons IFN-α/β also impact chromatin. In cooperation with TNF, IFN-α generated increased chromatin accessibility at specific sites to potentiate the proinflammatory effect of LPS (106). Monocytes stimulated with LPS from patients with SLE displayed epigenomic similarity to those primed in vitro with IFN-α and TNF, suggesting that IFN-α exposure in vivo may alter chromatin to contribute to inflammatory symptoms of SLE (106, 107). In addition to enhancing the expression of inflammatory genes, IFN-γ was also found to repress M2-like genes by disassembling MAF and lineage-determining transcription factors (LDTFs) PU.1 and C/EBPβ bound at select enhancers (30) (**Figure 3*c***). At other genomic locations, IFN-γ also suppressed enhancers associated with STAT3 (108). Notably, the genes at MAF enhancers were also repressed in macrophages from rheumatoid arthritis patients, suggesting that both potentiation and repression at the chromatin level by IFN-γ priming may play roles in autoimmunity.

3.3. Specialized Physiological Functions via Polarization

By affecting signaling and epigenetic control mechanisms, contextual cytokines may alter macrophage functions to enhance protection of the host organism from pathogen threats or

minimize collateral damage of inflammation. The presence of both type I and type II interferons in the microenvironment may prime macrophages for an LPS challenge, enhancing the expression of innate immune genes and cytokines (109). Polarization can also resolve or more carefully regulate inflammatory processes. IFN-γ priming represses a portion of LPS-inducible genes, resulting in a reduction in the recruitment of neutrophils to the inflammatory site (110). These changes in the production of chemokines by macrophages can ultimately rewrite the script of systemic immune activation.

In addition to altering the level of expression of inflammatory gene programs, microenvironmental context may also tune cell-to-cell heterogeneity, which is a determinant of response specificity. A single-cell study measured propagation of variance in gene expression within gene regulatory networks in human macrophages and found that IFN-γ + TNF, IL-4, and IL-10 cytokine environments each generated distinct changes in biochemical parameters within the signaling network, altering patterns of cellular heterogeneity (111). For example, IL-10 signaling increased the phosphorylation and nuclear localization of ATF2, which in turn tuned the variability of ATF2 target gene expression. The adaptation of macrophage heterogeneity to cytokine microenvironments may be beneficial as a bet-hedging strategy that leverages an altered distribution in single-cell responses to immune stimuli (111).

Macrophage function depends on context such as organ system microenvironments (2, 112). Tissue-specific environments selectively activate transcription factors that collaborate with PU.1 to establish distinct sets of enhancers and superenhancers in the resident macrophage population (113, 114). This tissue-specific functional polarization of macrophages is reversible and held in place by transcription factors induced by the microenvironment. As for the inflammatory potential of the peritoneum, retinoic acid (vitamin A) from local tissues polarized peritoneal macrophages by inducing GATA6 (115). Interestingly, the expression of this peritoneal macrophage–specific gene was not required for peritoneal macrophage development and was decreased by depletion of vitamin A signal, pointing to the role of GATA6 as a peritoneal macrophage polarization gene rather than an LDTF. Thus, through constant surveillance of the contextual environment of the tissue, tissue-resident macrophages may be able to dynamically adjust their response potential (115).

Furthermore, the presence of polarizing cytokine contexts can also alter the macrophage's metabolism. For example, IFN-β triggers metabolic reprogramming. Exposing bone marrow–derived macrophages to live *Mycobacterium tuberculosis* restrained glycolysis and mitochondrial stress, a phenomenon recapitulated by IFN-β stimulation alone and abrogated in IFNAR knockout mice. However, whether such metabolic reprogramming is beneficial or detrimental to antimycobacterial or other immune responses remains unclear (116). Thus, even in the absence of acute infection, the presence of type I interferons in the environmental milieu has marked effects on signaling nodes central to pathogen or cytokine responses (117).

3.4. Pathology Due to Dysregulated Microenvironmental Contexts

While context dependence is a feature of healthy macrophage function, dysregulated microenvironments may also have detrimental effects on macrophage responses. Indeed, several chronic inflammatory diseases feature aberrant cytokine microenvironments, which alter macrophage responses. Examples common to humans include inflammaging, or chronic inflammation that occurs with aging (118), and obesity, which is a disease of chronic metabolic inflammation (119, 120).

Both old age and obesity have been linked to poorly regulated immune responses (121). For instance, a comparison of macrophages from old and young mice suggested that age impairs macrophage polarization, with significant decreases in M1 and M2 marker genes after exposure to polarizing ligands (122). Failure of macrophages to appropriately polarize may lead to less

effective context-dependent immune responses. Similarly, inflammation-associated aging may affect macrophage context-dependent responses through metabolic, signaling, and epigenetic mechanisms (123). This chronic, low-grade inflammation has been linked to immunosenescence and is marked by increases in circulating proinflammatory cytokines such as TNF and IL-6 (124–127). Furthermore, the context dependence of responses in aged macrophages is tissue specific. Within aging skeletal tissues, macrophages adopt a more M2-like phenotype (128), but the proinflammatory cytokine environment is hypothesized to produce age-associated proinflammatory M2-like macrophages (123). Further work may delineate the effect of age on the ligand responses of macrophages from different tissues and under a variety of polarization conditions.

Obesity is also associated with an inflammatory context that affects macrophage responses. Obesity causes inflammation of the adipose tissue, due to the release of IFN-γ by natural killer cells (129). In lean mice, tissue-resident adipose macrophages retained an anti-inflammatory M2-like state. However, with a high-fat diet, macrophages in the adipose tissue accumulate and adopt an M1-like proinflammatory phenotype marked by increased TNF and IL-6 expression (130). Interestingly, macrophages in obese IFN-γ knockout mice shifted toward an M2 phenotype, and organisms displayed improvements in insulin sensitivity (131). It remains to be seen whether the polarized states of macrophages due to metabolic inflammation influence the effectiveness of their responses to immune threats.

Pioneer factor: a transcription factor that contains a DNA anchoring α helix that allows direct binding to nucleosomal DNA

Signal-dependent transcription factor (SDTF): transcription factor activated upon an extracellular stress signal

4. STIMULUS MEMORY

The third functional hallmark of macrophages, stimulus memory, allows them to record past exposures within their epigenetic state to affect their responses long term. The identity of differentiated cells is defined by the epigenomic enhancer landscape, which is held in place by the stable expression of a set of cell type–specific transcription factors, termed lineage-determining transcription factors (LDTFs) (132, 133). These LDTFs are pioneer factors (134, 135) that have structural elements that enable them to bind to nucleosomal DNA and adjust the enhancer landscape during development (136). While the epigenetic landscape determines cell identity, epigenome plasticity allows differentiated cells to adapt their functions to environmental cues (**Figure 4*a***).

Stimulus memory concerns the malleability of the developmentally established epigenetic landscape, whereby signal-dependent transcription factors (SDTFs) are able to trigger the formation of new enhancers. When the SDTF activity ceases and those stimulus-specific changes to the chromatin epigenome are not reversed, epigenetic memory of the prior exposure event is formed. Stimulus memory is thus distinguished from context dependence in that the inducing signal need not persist and the signal is often triggered by exposure to PAMPs and DAMPs rather than polarizing cytokines. Thus, stimulus memory stores marks of previous exposure to influence sentinel cell responses to future stimuli.

4.1. Transcription Factors, Nucleosome Remodelers, Metabolites

Several classes of molecules in the nucleus mediate stimulus memory: transcription factors, nucleosome remodelers, and metabolites. For stimulus-induced epigenetic programming to occur, SDTFs, which bind to DNA, must be activated (137). Activation of SDTFs like AP1, NF-κB, and IRFs by immune threats is stimulus specific, but unlike LDTFs, they are not cell type specific. Because the combinations and dynamics of SDTF activities are stimulus specific, epigenetic memory may also be stimulus specific. Patterns of SDTF-DNA binding were shown to enable the stimulus-specific formation of de novo enhancers marked by H3K4me1 deposition, a covalent modification to the chromatin landscape (137). More recently, the mechanisms by

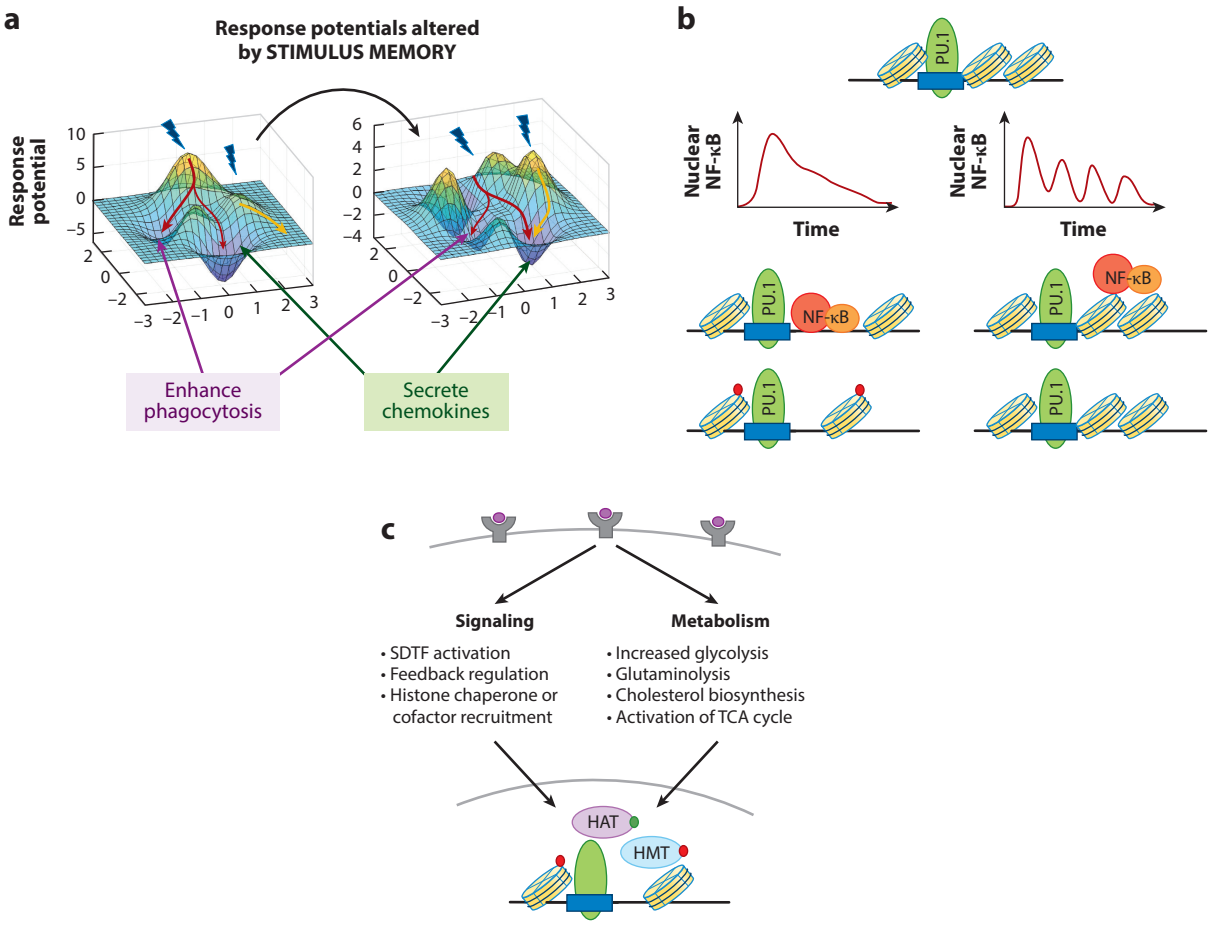

Figure 4

Stimulus memory involves prior exposure altering epigenetic states of signaling and gene regulatory networks. (*a*) Stimulus memory is mediated, for example, by changes to the chromatin enhancer landscape, altering response potential after the initial stimulus has subsided. Arrows pointing to regions of the response landscape represent possible responses given an inflammatory stimulus. (*b*) Stimulus-specific nonoscillatory activity of SDTFs opens chromatin in collaboration with cofactors and chromatin-remodeling enzymes. (*c*) Both signaling pathway activation and alterations to metabolic pathway activity are critical arms for generating innate immune memory. Abbreviations: HAT, histone acetyltransferase; HMT, histone methyltransferase; SDTF, signal-dependent transcription factor; TCA, tricarboxylic acid.

which the temporal dynamics of SDTF activity control de novo enhancer formation have also been elucidated. In macrophages, nonoscillatory NF-κB activity provides the continuous nuclear residence time necessary for nucleosome eviction and eventual H3K4me1 deposition (29). Because cytokines and viral PAMPS induced oscillatory NF-κB activity, while bacterial PAMPs induced nonoscillatory activity (26), the stimulus-specific dynamics of transcription factor activity determined the extent of epigenomic enhancer formation (29) (**Figure 4*b***).

Because SDTFs like NF-κB and IRF are not LDTFs, it seemed unlikely that they would impart long-term changes to the epigenome. However, biochemical and cryo–electron microscopy studies suggested that SDTFs like NF-κB could bind to nucleosomal DNA and potentially displace histone H1 (138, 139). The spontaneous unwrapping and rewrapping of DNA around the

histone octamer core, also referred to as DNA breathing, suggests that even SDTFs can invade the nucleosomal DNA–histone octamer complex (140, 141) by preventing rewrapping of the SDTF-bound sections of DNA (142). However, the rates of spontaneous rewrapping are rapid enough that NF-κB could bind to its cognate motif only when it was positioned at the edge of the nucleosome, and not close to the central dyad axis. Thus, while stimulus-specific epigenetic memory is mediated by the activation of SDTFs, only specific nucleosomes may be targeted, and cooperative mechanisms from other proteins may be required (143). For example, LDTFs such as PU.1 or C/EBPβ, which establish macrophage identity (144) and are pioneer factors capable of perturbing nucleosome structure (145), may facilitate SDTF-triggered de novo enhancer formation upon stimulation (137, 146–148). Histone chaperone proteins such as FACT (149) and ATP-dependent nucleosome remodelers such as SWI/SNF and RSC may catalyze unwrapping or nucleosome sliding (150, 151) and thus facilitate SDTF binding (152). Subsequent deposition of the H3K4me1 modification marks poised enhancers and persists even after the activate enhancer marks H3K27ac and H3K4me3 are lost (153).

Trained immunity: retainment of epigenetic histone modifications after a first exposure that generates hyperinflammatory response to a second

Beyond chromatin-modifying proteins, metabolites and alterations to metabolic flux are an integral component of epigenetic memory (154). These mechanisms are driven by the reliance of many epigenetic modifications on metabolic processes, such as one-carbon metabolism for histone and DNA methylation (155) and generation of acetate pools from acetyl-CoA for histone acetylation (156–158). Furthermore, mevalonate and cholesterol biosynthesis pathways are also downstream of acetyl-CoA production and influence the innate immune response through feed-forward mechanisms at the receptor that activate PI3K (phosphatidylinositol 3-kinase) signaling and mTOR (mammalian target of rapamycin) (159). Increased activity of catabolic processes including glycolysis and glutaminolysis also mediates trained immunity: In human monocytes stimulated with β-glucan, glutaminolysis and cholesterol metabolism resulted in the accumulation of fumarate, which inhibited KDM5 histone demethylases to promote epigenetic reprogramming (160). Fumarate treatment of monocytes itself also mimicked β-glucan treatment by increasing both H3K4me3 and H3K27ac deposition. In human macrophages exposed to IL-4, activity of the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate promoted demethylation of H3K27me3 in a manner dependent on Jmjd3, a histone demethylase (161). Therefore, rewiring of metabolic circuits is a key component for initiating and sustaining immune memory conveyed through histone modifications (162, 163) (**Figure 4*c***).

4.2. Memory of Prior Infection

Stimulus memory of past exposures serves the physiological purpose of changing future gene expression responses. Two main categories of innate immune memory, tolerance and trained immunity, are generated by different stimuli and alter responses in opposing directions (164). Tolerance was first described in mice surviving a lethal dose of endotoxin after having received a sublethal dose (165). It was also observed in macrophages that were exposed to a primary stimulus of high concentrations of LPS and, after a washout of up to five days, stimulated again with a secondary stimulus (166). The resulting blunted second response was accompanied by nucleosome repositioning and histone H3 lysine methyltransferase G9a, which generated heterochromatin assembly and epigenetic silencing (167). Interestingly, however, tolerance is dose dependent: When high doses of LPS, P3CSK, and poly(I:C) are diluted 100- to 10,000-fold, hyperresponsiveness rather than tolerance may result (164). Epigenetic changes resulting in tolerance are stimulus specific, but it remains to be seen to what extent and in what way the responses to heterologous secondary stimulation are also altered depending on the stimulus.

Tolerized: having retained epigenetic histone modifications after a first exposure that suppresses the response to a second

Trained immunity, involving an elevated response of key immunoregulatory genes upon stimulation, is a key outcome of stimulus-specific epigenetic memory. Monocytes responded to secondary stimulation with the fungal cell wall component β-glucan, or *Candida albicans*, with higher production of key cytokines like TNF and IL-6 (168, 169). Immune training was associated with increases in H3K4me3 and H3K4me1 enhancer marks, even after loss of H3K27ac (marking active promoters), suggesting that a stable epigenetic modification of enhancer regions helps maintain trained immune memory. IFN-γ secreted after initial challenge with *Cryptococcus neoformans* was also shown to generate innate immune memory for up to 70 days, resulting in excessive responses of proinflammatory cytokines upon a secondary challenge (170). Furthermore, dendritic cells also show stimulus-specific trained immune memory. Dendritic cells treated with the fungal pathogen *C. neoformans*, transplanted into naive mice, and challenged again showed increased interferon response gene expression as well as increased production of *C. neoformans* cytokines. This apparent memory was inhibited by treatment with histone methylase inhibitors (171).

Though studies of the epigenetic plasticity and memory of innate immune responses have focused on immune cells like macrophages, there is emerging evidence that fibroblasts, stromal cells, and hematopoietic stem cells may also be pliable to stimulus-specific epigenetic programming (172). These cells have longer lifespans than circulating monocytes, and may thus be well-positioned messengers to carry memory of past exposures (173, 174). Indeed, in fibroblasts, chromatin marks deposited after IFN-β stimulation led to faster and increased expression of interferon genes on a second stimulation (175). Epithelial stem cells were also shown to maintain memory of a primary response through sustained increase in chromatin accessibility at key inflammatory response genes, heightening responses to subsequent inflammatory stimuli (176).

4.3. Stimulus Memory via Vaccination

Given its role in physiology, several attempts have been made to harness stimulus memory and the training of innate immunity through vaccination. The tuberculosis vaccine BCG (*Bacillus* Calmette-Guérin) is a well-known example (177), where vaccination with this attenuated bacterium provides broad protection against multiple bacterial and fungal organisms through hyperresponse of key genes upon secondary stimulation (178). BCG-trained immunity not only affected monocytes via H3K27ac histone modifications (179), but also impacted the epigenetic landscape of hematopoietic stem cells (180, 181). BCG training of hematopoietic stem cells led to epigenetically modified monocytes and macrophages that had alterations in H3K4me1, H3K4me3, and H3K27ac and cleared tuberculosis infections more effectively than naive macrophages (181). Trained immunity of progenitor cells may explain the lasting effects of innate immune vaccination. Importantly, while programming the epigenetic landscape is specific to the stimulus, unlike vaccines targeted at adaptive immunity that aim to generate memory B and T cells, innate immune vaccination by BCG provides heterologous effects and protects individuals from many other bacterial, viral, and fungal pathogen threats (182, 183).

Tolerance or immune training via treatment with LPS or BCG, respectively, has also been suggested as a potential avenue for the modulation of autoimmune diseases like systemic sclerosis (184), which is marked by fibrosis as a result of chronic but sterile inflammation (185). Treatment of macrophages with LPS generated a tolerized phenotype that reduced inflammation-related fibrosis in a mouse model. On the other hand, BCG exposure generated a trained phenotype with increased production of proinflammatory cytokines, exacerbating the fibrotic process. LPS and BCG generated unique epigenomic changes, with gene-specific changes in chromatin marks, including H3K4me3 (184).

4.4. Diseases of Dysregulated Stimulus Memory

Severe pathology can result from dysregulated immune memory. Sepsis, which involves hyperactivation of the immune response as well as immune paralysis that prevents the clearance of bacteria in the bloodstream, affects millions of people yearly, and nearly one-third of hospital deaths are caused by sepsis. Both tolerance and trained immunity are relevant in this context. Tolerance eliminates excessive response on secondary stimulation, but misapplied regulation of tolerance results in poor host defense against secondary exposures to bacterial stimuli. Interestingly, the metabolic output of TCA cycle decarboxylation, itaconate, promoted tolerance in human monocytes, while β-glucan inhibited IRG1, the enzyme that promotes itaconate synthesis, leading instead to an enhanced secondary immune response (186). The ability of specific stimuli to generate trained immunity and revert disease-causing tolerance could lead to additional methods to modulate the immune system during or after infection.

Another disease of dysregulated immune memory is hyper-IgD syndrome (HIDS), an inborn error of metabolism where mevalonate kinase deficiency leads to accumulation of mevalonate (187). Monocytes and macrophages in these patients with HIDS produce higher amounts of TNF, IL-6, and IL-1β, and anti-TNF and anti-IL-1 therapies have been only partially effective (188). The metabolite mevalonate was shown to be critical in β-glucan- and oxLDL (oxidized low-density lipoprotein)-induced trained immunity by driving the mTOR pathway, activating the TCA cycle, and generating acetyl-CoA needed for altered H3K27ac at inflammatory genes (189). The chronic trained immunity of macrophages due to elevated mevalonate may be a cause of the sterile inflammatory phenotype seen in these patients, which includes febrile attacks, arthritis, and skin lesions (189). Importantly, administration of statins blocked the mevalonate-cholesterol synthesis pathway, attenuating trained immunity and reducing inflammatory attacks (190).

5. OUTLOOK

The physiological roles of macrophages are many (191, 192), and they rely on precise regulation. Here we propose that this enormous variety of physiological roles depend on a key set of three functional hallmarks. For each of these hallmarks, addressing outstanding questions will bring us closer to harnessing and controlling macrophage function, either for diagnostics or for treatment of disease. We discuss a few open questions below and outline further potential lines of inquiry in the sidebar titled Future Issues.

Might response specificity inform us about the health of the innate immune system and the risk for inflammatory disease? Response specificity, a property of macrophages that is affected by both cytokine context and immune memory, may prove a convenient metric for measuring health and disease states. In multiple immune diseases, noisy or ineffective recognition of an inflammatory threat leads to autoimmunity or faulty pathogen clearance. The functional health of the innate immune system, which is affected by context or prior exposures, could in the future be measured by perturbing monocytes isolated from peripheral blood and profiling the resulting transcriptome or epigenome. Diagnosis and prognosis of a wide variety of diseases, including autoimmune diseases, cancers, and neurodegenerative diseases, depend on having a measure of the patient's immune system functioning. It remains to be seen to what extent monocyte and macrophage response specificity reflects risk or stage of each of these diseases.

Will understanding context dependence allow us to predictably tune macrophage responses with microenvironmental cues? A central difficulty in understanding context dependence has been that of clarifying the effects of different ligands or ligand combinations, their doses, their duration, and the temporal order of exposure. In vitro studies have isolated the effects of particular combinations of contextual cytokines, often recapitulating context-dependent responses that arise

by exposure to real pathogens or from inflammatory diseases. Further work in uncovering the signaling and epigenetic mechanisms that canalize macrophage responses into subspecialized states may in the future allow us to use specific adjuvants to manipulate favorable microenvironment conditions for cancer, atherosclerosis, or metabolic disorders.

Might we harness stimulus memory to strengthen innate immunity and improve human health? A recent study reported that four weeks of aerobic exercise prior to surgery created a lasting phenotype of immune tolerance in Kupffer cells, improving ischemia-reperfusion injury outcomes (193). However, further study is needed to understand the physiological consequences of training innate immunity. For example, innate immune memory may play roles in post-COVID-19 (coronavirus disease 2019) immunity or inflammatory sequelae. A recent study on convalescing COVID-19 patients indicated that altered monocyte subsets are present after COVID-19, with increased chromatin accessibility at inflammatory genes, suggestive of trained immunity. $CD14^+$ and $CD16^+$ monocytes from convalescing patients maintained epigenetic modifications and had increased IL-6 and IL-1β production on subsequent stimulation with spike-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) pseudovirus (194). It remains to be determined whether this trained immunity has a similar effect as vaccination, protecting the individual from subsequent infection, or whether the subsequent hyperinflammatory responses predispose individuals to syndromes of long COVID-19.

SUMMARY POINTS

1. The health of the immune system depends on the health of macrophage function, as macrophages are the orchestrators of immune activation. There are three functional hallmarks of healthy macrophage responses: response specificity, context dependence, and stimulus memory.
2. Response specificity is the ability of single macrophages to selectively activate particular gene programs appropriate to the stimulus, whether pathogen, injury, or antibody, and it is evaluated by analyzing single-cell ligand-response distributions.
3. Context dependence refers to the canalization of macrophages into subspecialized states by polarizing cytokines or signals from the microenvironment.
4. Stimulus memory allows macrophages to store specific marks of prior exposures stably within the epigenome, fine-tuning future responses.
5. Immunological diseases involving macrophage responses arise from combinatorial dysregulation of these functional hallmark properties. Understanding mechanisms that generate these functions will allow us to measure them for diagnosis or manipulate them for treatment.

FUTURE ISSUES

1. What mechanisms of regulation of response specificity are the key sources of cell-to-cell heterogeneity, and do they cause pathology?
2. Which features of response specificity are predictive of innate immune health, and which are impaired when immune health is compromised by so-called preexisting conditions?

3. Context dependence is regulated by polarizing cytokines, but what are the other microenvironmental components, such as nutrients?
4. In understanding mechanisms of context dependence operating on signaling and epigenetic networks, might we be able to develop predictive interventions to improve macrophage function?
5. In encoding stimulus memory, what cofactors assist immune response transcription factors in evicting nucleosomes and establishing de novo enhancers?
6. Is stimulus memory sufficiently long and reliable such that trained immune precursor cells can produce trained differentiated macrophages that can provide health benefits?

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Errata

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