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Diversity, Mechanisms, and Significance of Macrophage Plasticity

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Abstract

Macrophages are a diverse set of cells present in all body compartments. This diversity is imprinted by their ontogenetic origin (embryonal versus adult bone marrow–derived cells); the organ context; by their activation or deactivation by various signals in the contexts of microbial invasion, tissue damage, and metabolic derangement; and by polarization of adaptive T cell responses. Classic adaptive responses of macrophages include tolerance, priming, and a wide spectrum of activation states, including M1, M2, or M2-like. Moreover, macrophages can retain long-term imprinting of microbial encounters (trained innate immunity). Single-cell analysis of mononuclear phagocytes in health and disease has added a new dimension to our understanding of the diversity of macrophage differentiation and activation. Epigenetic landscapes, transcription factors, and microRNA networks underlie the adaptability of macrophages to different environmental cues. Macrophage plasticity, an essential component of chronic inflammation, and its involvement in diverse human diseases, most notably cancer, is discussed here as a paradigm.

1. INTRODUCTION

Macrophages are a ubiquitous cellular component present in all tissues and body compartments under homeostatic physiological conditions (1–3). It has long been realized, based on gross morphological appearance, that components of what used to be called the mononuclear phagocyte system are diverse in appearance and functional properties. Microglial cells, osteoclasts, Kupffer cells, and bronchoalveolar macrophages illustrate the diversity of macrophages under resting homeostatic conditions (2–4).

The eponymous function of cells of the macrophage lineage is phagocytosis. Early on, it was realized that macrophages serve as the first line of defense against infection. Macrophages are an essential component of innate immunity. They express an unmatched repertoire of pattern recognition receptors, including Toll-like receptors (TLRs), inflammasomes, and lectin-like receptors, which are strategically located on the cell membrane, in the cytoplasm, and in the endosomal compartment. Macrophages engage in collaborative interactions with other cells of the innate immune system, including neutrophils and innate lymphoid cells, and natural killer (NK) cells in particular (5). Moreover, mononuclear phagocytes are a major source of components of the humoral arm of innate immunity, including complement and fluid-phase pattern recognition molecules (PRMs), such as PTX3 (6). In turn, humoral immunity components collaborate with macrophages in effector functions and regulate the activity of mononuclear phagocytes.

Macrophages are critical cells in the orchestration of chronic inflammation and related pathologies (7). Indeed, inflammation and its mediators represent a metanarrative of twenty-first-century medicine (8). However, chronic inflammatory reactions are extremely diverse, and adaptability to different tissue microenvironments and responses to different pathogenic insults are key features of mononuclear phagocytes.

This review focuses on the adaptability of macrophages in relation to their role in pathology. A concise overview of the origin and role of mononuclear phagocytes in development and homeostasis provides a background for understanding the plasticity of these cells in pathology, with an emphasis on cancer. The reader is referred to previous reviews for a framework through which to understand the present review (1–3, 7–11).

2. ORIGIN, DIVERSITY, AND HOMEOSTATIC FUNCTION

For almost half a century, the prevailing view in the literature and textbooks has been that tissue macrophages in health and disease originate from circulating monocytes (12). This long-held view has been challenged by evidence originating from cell-tracking, parabiosis, and genetic-tracing studies in the mouse and, to a lesser extent, from organ transplantation in humans (2, 13) In the mouse, macrophages located in many body compartments—including brain, skin, liver, kidney, lung, and heart—originate from the yolk sac or fetal liver, and their maintenance in adulthood in the absence of stressors is independent of circulating monocytic precursors. In other tissues, such as the gastrointestinal tract, monocytic precursors contribute to tissue macrophages. In many adult murine tissues, the resident population of mononuclear phagocytes is a mix of cells originating during development and circulating monocytic precursors (2, 14), as illustrated by heart macrophages (15). There is evidence for a dual origin of tissue macrophages, either self-sustaining local or monocyte-derived, also occurring in human mononuclear phagocytes (see, e.g., 15). Given the long life expectancy of humans compared with mice and the difficulty in inducing the proliferation of mature human macrophages compared with mouse macrophages using colony stimulating factor 1 (CSF-1), one can speculate that monocytes are a major contributor to macrophages in humans.

Macrophage populations are diverse among tissues, as exemplified by lung alveolar macrophages, microglia, and Kupffer cells. Moreover, single-cell analysis has added a new dimension to deciphering the diversity of mononuclear phagocytes, including that of tissue-resident cells (16). For instance, brain mononuclear phagocytes include in addition to microglia, perivascular, meningeal, and choroid plexus macrophages, and single-cell analysis has further dissected the diversity of brain phagocytes (17, 18).

Tissue macrophages play a wide range of fundamental physiological roles during development and in adult life, as summarized in **Figure 1**. The development of several organs requires mononuclear phagocytes, and branching morphogenesis in organs, such as in the mammary gland and pancreas, depends on macrophages (3, 18). In the cardiovascular system, mononuclear phagocytes contribute to functions ranging from construction of the vessel wall (19) to maintaining cardiac rhythm (20). In the liver, resident Kupffer cells engage in a bidirectional interaction with hepatocytes, for instance, in lipid metabolism, and are an essential component of fat (21). A major homeostatic function of macrophages is to provide a nurturing niche for stem cells (22).

The origin, diversity, and homeostatic functions of macrophages in different tissues have implications in pathology that, to a large extent, remain to be explored. Evidence suggests that, in general, tissue macrophages seeding tissues prenatally are born to subserve homeostatic functions, whereas monocyte-derived cells direct the response to pathological signals. However, the capacity of macrophages to respond and adapt to environmental cues defies a rigid division of labor.

3. MACROPHAGE PLASTICITY: PRIMING, POLARIZED ACTIVATION, TRAINING, AND TOLERANCE

It has long been known that exposure to microbes or to microbial components, such as bacterial lipopolysaccharides (LPSs), results in enhanced macrophage-mediated resistance and effector functions (23, 24). Building on these early observations about what used to be referred to as activation, a more refined view of the spectrum of responses elicited by different signals has been obtained (9, 10, 25) (**Figures 1** and **2**).

Following activation, in vitro and in vivo exposure to microbial components such as LPS can result in unresponsiveness to the same agent, a phenomenon referred to as tolerance. In vitro elicited LPS tolerance mirrors the immunosuppressive phenotype observed in patients with sepsis. It should be noted that tolerance does not affect the whole spectrum of macrophage responses (9). For instance, the production of interleukin (IL)-10 and chemokines that attract type 2 T helper cells (Th2) or T regulatory cells (Tregs), is retained. The evolutionary value of tolerance rests in its significance as a fundamental mechanism to limit tissue damage caused by inflammation (26) (**Figure 2**).

Interferon-γ (IFNγ) and other cytokines have long been known to prepare macrophages for enhanced responsiveness to microbial components or to synergize with them. This initial IFNγ response that counters microbial challenges is relatively short lived (**Figure 2**). Moreover, this antimicrobial resistance program is not unique to host-derived cytokines. It has recently been shown that the short-chain fatty acid butyrate, a bacterial metabolite, prepares macrophages with a set of antimicrobial molecules but not inflammatory cytokines (27). Microbial recognition can profoundly alter the repertoire of surface receptors and of the fluid-phase PRMs expressed by macrophages with upregulation of MARCO and dectin-1 (28); it can also alter the production of the humoral PRM PTX3 and complement components (29).

The Th2 cytokine IL-4 was shown to elicit an alternative form of macrophage activation (M2), including induction of a distinct set of surface receptors and effector molecules (25, 30). M1 and M2 activation reflect the main cellular sources of IFNγ and IL-4 and the nomenclature

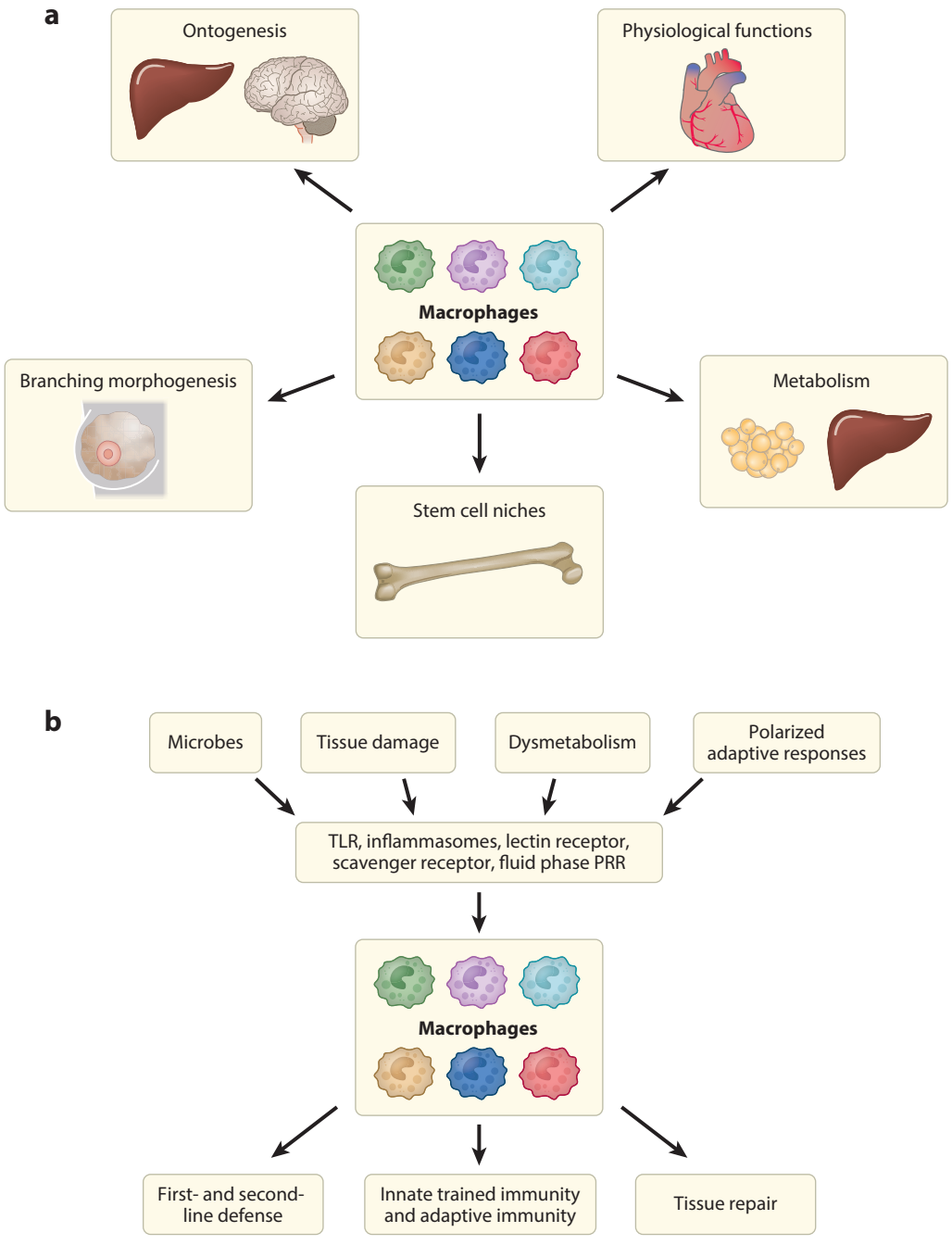

Figure 1

(*a*) The homeostatic functions of macrophages and (*b*) their response to environmental perturbations to restore homeostasis. Only selected organs, tissues, and homeostatic functions are presented. Abbreviations: PRR, pattern recognition receptor; TLR, Toll-like receptor.

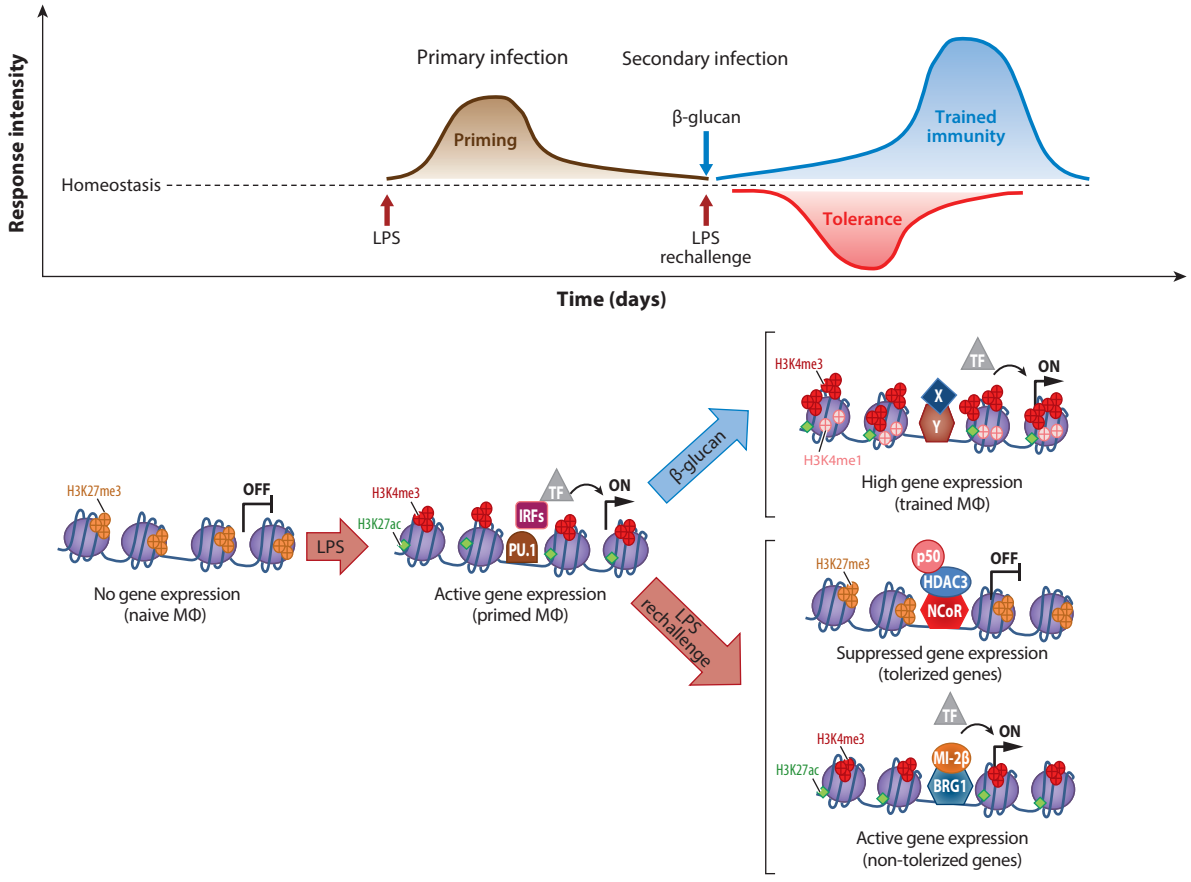

Figure 2

Chromatin modifications underlying priming, training, and tolerance and the main epigenetic events occurring after LPS challenge, followed by a second challenge under training or tolerizing settings. Abbreviations: LPS, lipopolysaccharide; Mϕ, macrophage; TF, transcription factor.

of polarized immune responses [such as, Th1 and Th2, innate lymphoid cell (ILC) 1 and ILC2, type 1 and type 2 immunity] (10, 25, 31). M1 and M2 polarized macrophages are extremes of a continuum of activation states in a universe of adaptive responses. M2-like has been used to refer to phenotypes with some generic relationship to IL-4-activated macrophages (see, e.g., 32). (For a discussion of the value and use of imperfect nomenclatures in immunology and related epistemological considerations, the reader is referred to Reference 11.)

A systematic effort to transcriptionally profile human macrophages exposed to a wide range of signals (33) has further extended the known spectrum of activation states beyond the original M1/M2 dichotomy. Single-cell analysis in pathology has further amplified the diversity of functional states of mononuclear phagocytes under physiological and pathological conditions.

Thus, the unmatched adaptability of macrophages in response to environmental signals goes beyond the original M1/M2 dichotomy, as is already evident in the early profiling of tumor-associated macrophages (TAMs) (34). However, macrophages largely mirroring the in vitro phenotypes of M1 and M2 polarized cells are present in pathological tissues (see, e.g., 10) and are part of type 1 and type 2 immune responses, which are driven by innate or adaptive lymphoid cells, or both, as discussed in Section 4.

Exposure to microbial moieties can also result in the long-term imprinting of innate immunity (35). Lymphoid cell–independent imprinting of phagocyte function was originally observed in invertebrates (36). The long-term imprinting of phagocytes has been referred to as memory (36), adaptive innate (37), or adaptive trained (35) immunity. Recent evidence suggests that the imprinting of myeloid precursors and neutrophils plays a major role in trained innate immunity (38). Interestingly, IL-1β has been shown to be a major driver of training at the level of myeloid precursors and differentiated monocytes (8, 38).

In summary, emerging evidence suggests that the cellular components of innate memory immunity or trained immunity are complex. The original views that these responses were macrophage cell–autonomous have evolved to recognize the involvement of myeloid progenitors and neutrophils (38). Moreover, recent data suggest that T cell help is required for induction of a trained phenotype in alveolar macrophages (39). Trained myeloid cell–mediated immunity may have broad significance, including for the development of adjuvants and vaccines. Dissection of the cellular and molecular mechanisms of T cell help may pave the way to translation. Exploration of the full spectrum of trained innate immunity will require further work.

4. MOLECULAR BASIS OF MACROPHAGE PLASTICITY

Dynamic regulation of the complex gene networks and signaling cascades that control macrophage polarization, priming, and plasticity is achieved through multilayered regulation of gene expression. Both transcription and translation are tightly regulated, sophisticated processes that strongly influence cell functions. The role of key transcription factor (TF) families is well established in defining macrophage identity and controlling functions through the induction and maintenance of specific transcriptional programs (40). Genome-wide studies profiling transcriptional and epigenetic modifications have identified differences in noncoding RNAs, histone modifications, and DNA methylation patterns that strongly affect the decision fate of macrophages (41) as well as the type and duration of macrophage-mediated inflammatory response (42).

In the following sections, we discuss how specific histone modifications, DNA methylation patterns, and regulatory RNA control macrophage identity, priming, polarization, and tissue-specific functions, and thus account for the heterogeneity and plasticity of macrophages (**Figure 2**).

4.1. Chromatin Remodeling in Primed Macrophages

Distinct epigenetic signatures are associated with specific macrophage differentiation states, suggesting that the relative epigenomic landscape is remarkably shaped by the integration of microenvironment- and stimulus-specific signals, resulting in a continuum of distinct transcriptional and functional outputs. Genome-wide studies profiling transcriptional epigenetic modifications occurring in differentiated macrophages have revealed profound dynamic changes in nucleosome positioning (43–45), histone modifications, and DNA methylation patterns (41, 46, 47).

Epigenetic regulation of chromatin activity through distinct histone-modifying enzymes controls multiple aspects of macrophage biology, including priming (42, 48). In the resting state, many inflammatory gene loci are in a repressed configuration, as inferred from low histone acetylation and the very low amount of RNA polymerase II loaded (48–50). Effective activation of proinflammatory genes by TLR signaling involves overcoming a rate-limiting chromatin barrier imposed by histone-containing nucleosomes that bind DNA (51). Mechanistically, this occurs through the recruitment of RNA polymerase II, histone acetylation to relax chromatin, and recruitment of ATP-dependent nucleosome remodeling of complexes for nucleosome repositioning or removal (50).

Genome-wide analyses strongly indicate that enhancers have important roles in signal-dependent transcriptional responses (46) In LPS-primed macrophages, a substantial and rapid reorganization of the epigenomic landscape occurs and mainly involves chromatin reorganization at enhancer regions. Depending on their activation state, enhancers can be generally classified as inactive, primed, or poised. Whereas inactive enhancers are located in heterochromatin regions and devoid of histone modifications and TF binding, primed enhancers (marked by H3K27ac) are located in nucleosome-free regions in close proximity to TF binding sites, and they become active in a signal-dependent manner after the recruitment of specific TFs and chromatin remodelers. Poised enhancers share most of the characteristics of primed enhancers, but they also contain repressive epigenetic marks. Furthermore, most macrophage-specific enhancers are premarked by the binding of the fate-determining TF PU.1 (46). PU.1 recruits chromatin remodelers that are able to displace or remodel nucleosomes, thus leading to the formation of small, accessible regions centered on the PU.1-binding site (41, 46). Some macrophage-specific enhancers contain binding sites for TFs activated by inflammatory stimuli [such as nuclear factor- κB (NF-κB), STATs, and AP-1], which are recruited in response to stimulation (46).

TLR4 signaling induces increased acetylation of activated enhancers, whereas poised enhancers are unaffected by LPS stimulation and retain basal H3K4me without acquiring the H3K27ac mark. In addition to the rapid reorganization of the preexisting enhancer landscape, LPS priming also induces activation of approximately 3,000 new enhancer regions, with the consequent formation of so-called super-enhancers. These are regions where enhancers are in close proximity to key regulatory genes and confer higher transcriptional activity and sensitivity to perturbations (52). Therefore, it has been suggested that the regulation of super-enhancer formation (operated by cooperative binding of NF-κB and BRD4) may represent a mechanism by which transcriptional and epigenetic regulators dynamically coordinate responses in primed macrophages.

As discussed above, IFNγ is a key activator of macrophages that enhances microbial killing and increases cytokine production in response to infectious or inflammatory challenges. Synergistic activation of inflammatory cytokine production by IFNγ and microbial products occurs through cooperation between epigenetic and signaling mechanisms, which create a primed chromatin environment to augment TLR-induced gene transcription (53). IFNγ increased chromatin accessibility by inducing acetylation of histone 4 and recruiting CBP–p300 as well as through stable and coordinated recruitment of STAT1 and IRF1 to enhancers and promoters of genes that are synergistically activated by IFNγ and LPS, such as tumor necrosis factor (TNF), IL-6, and IL-12B. This priming results in the removal of a rate-limiting chromatin barrier, thus greatly increasing and prolonging the recruitment of additional TFs and RNA polymerase II after TLR stimulation, and increasing transcription of inflammatory genes (53).

Irrespective of the specific underlying mechanism, evidence supports a model in which lineage-determining TFs collaborate to select and prime cell-specific enhancers, thereby enabling signal-dependent TFs to bind and function in a cell type–specific manner.

4.2. Epigenetic Marks of Macrophage Activation and Polarization

The plasticity of epigenetic modifications has been proposed to be a key molecular determinant of macrophage identity and heterogeneity. Dynamic and reversible epigenetic marks at enhancers and promoters of signal-responsive genes are important for rapid reprogramming of macrophage polarization and to tailor a response to a potentially hostile environment (46, 54). In contrast, long-term and more stable epigenetic marks contribute to defining macrophage cell identity (55) and to establishing the so-called epigenetic memory that influences macrophage responses to subsequent encounters with microbes (56). The macrophage epigenome is remodeled in response to acute stimulation and polarizing stimuli. Such remodeling involves changes in the expression of

chromatin-modifying enzymes during macrophage polarization, and these shape the epigenomic landscape, thus affecting the transcriptional output (49).

Several studies demonstrated the role of both histone methylation and acetylation in alternative macrophage polarization. The overexpression of DNA methyltransferase 3B or the loss of histone deacetylase 3 (HDAC3) renders macrophages hyperresponsive to IL-4, skewing differentiation toward the M2 phenotype (50, 51). Furthermore, the histone demethylase jumonji domain containing 3 (JMJD3) is induced by IL-4 in a STAT6-dependent manner and is required for macrophage alternative polarization through direct binding to M2 genes, such as *Arg1*, *Chi3l3*, and *Retnla*. Furthermore, JMJD3-mediated histone demethylation of the Irf4 promoter was shown to be necessary for the alternative activation-like response to helminths (50). By contrast, HDAC3 acts as a brake on IL-4-induced M2 polarization by restricting activating histone marks at a subset of PU.1-defined macrophage-specific enhancers. In macrophages that lack HDAC3 this brake is removed. These cells are hyperresponsive to IL-4 stimulation and display a polarization phenotype similar to IL-4-induced alternative activation (41). It has also been shown that IL-4 induces an epigenomic signature that selectively represses the macrophage inflammation program, thus favoring alternative macrophage polarization. This occurs via STAT6-mediated repression of a large set of inflammatory enhancers, characterized by reduced chromatin accessibility and reduced binding of p300, and of lineage-determining TFs (52). However, some aspects of the molecular mechanisms adopted by STAT6 to repress transcription are still unclear. In particular, it remains to be defined whether STAT6 acts as a transcriptional repressor by recognizing noncanonical binding motifs or whether repression occurs without direct DNA binding and, in that case, which DNA-bound factor interacts with STAT6. HDAC3 expression has been shown to be required for IL-4/STAT6-mediated repression only on a subset of genes. Reduced p300 binding at STAT6 repressed enhancers in IL-4-exposed macrophages, suggesting that this could be an important mechanism in the IL-4/STAT6-mediated transcriptional repression. A further example of the interplay between epigenetic and transcriptional regulation is represented by the key role played by IFNγ in establishing gene silencing at M2-related gene loci (47, 53, 57). IFNγ-induced macrophage activation is reinforced by a chromatin-based mechanism engaged by IFNγ to silence selected anti-inflammatory pathways in macrophages to achieve and stabilize an activated state (47, 53). The first mechanism of gene silencing that was described implied there was IFNγ-mediated recruitment of a repressor complex containing the histone methyltransferase EZH2 and associated deposition of the negative histone mark H3K27me3 into a small group of anti-inflammatory genes, including *Mertk* and *Pparg*. Gene repression is stabilized by the maintenance of H3K27me3 on gene promoters, which persists after termination of IFNγ stimulus. Moreover, these silenced genes are no longer responsive to glucocorticoids, IL-4, and macrophage colony–stimulating factor. Thus, cytokine-induced H3K27 trimethylation is a mechanism that stabilizes gene silencing in macrophages. A second mechanism by which IFNγ induces gene repression is by suppressing the function of enhancers associated with M2-like genes, which are enriched for binding sites for the TF MAF (57). Collectively, these findings strongly support the existence of underlying cross talk between transcriptional and epigenetic regulatory mechanisms in controlling macrophage plasticity.

4.3. Epigenetic Regulation of Endotoxin Tolerance

Combinatorial patterns of epigenetic changes confer highly specific regulation at genes and enhancers across several signaling pathways critical to the establishment of endotoxin tolerance (ET). Several studies both in murine sepsis models and in sepsis patients have demonstrated that rather than being inert in response to a second LPS exposure, tolerized macrophages show a shift in the

specific pathways that they activate, which is strictly associated with the dynamic establishment of distinct epigenetic marks (51, 58). Accordingly, a gradient in the response of tolerized macrophages to LPS rechallenge can be described, with some genes showing a tolerized pattern (no induction) and others showing a responsive pattern. Therefore, according to their responsiveness to LPS rechallenge, TLR-induced genes fall into two functional categories that are characterized by distinct epigenetic marks. The first class includes proinflammatory molecules, which are transiently silenced (tolerized genes), and the second class includes antimicrobial effectors, among which expression is not affected by LPS stimulus or is further upregulated (non-tolerized genes) (51). In tolerized macrophages, the recruitment of chromatin regulators, such as Mi-2β and BRG1, induced chromatin remodeling at non-tolerized genes, thus allowing the recruitment of LPS-induced TFs, such as NF-κB and C/EBPβ. Notably, the presence on a gene promoter of NF-κB binding motifs dictates its sensitivity to LPS tolerance. Transcriptional silencing of tolerized genes is generated through the formation of facultative heterochromatin, a process mostly controlled by NF-κB that selectively recruits the NcoR–HDAC3–deacetylated p50 repressosome to inflammatory genes, whereas non-tolerized genes maintain an open chromatin state, allowing the recruitment of LPS-induced TFs, and are not under the control of NF-κB (59). NF-κB-mediated recruitment of repressor complexes is not the sole mechanism responsible for specifying TLR-induced gene repression. Significant changes in the methylation and acetylation state of enhancers were detected in tolerized genes compared with responsive (or non-tolerized) genes. After initial LPS stimulation, both classes of genes are actively transcribed and show H3K27ac and H3K4me3 marks at their promoters. Upon LPS reexposure, tolerized genes maintain their basal promoter state and do not regain the H3K27ac or H3K4me3 mark, thus remaining silent and refractory to stimulation. Conversely, non-tolerized genes maintain the H3K4me3 mark, and their promoters are reacetylated in tolerant macrophages (51). This finding suggests that tolerant macrophages fail to accumulate H3K27ac at tolerized genes either through the absence of proinflammatory activators (e.g., IRFs and STATs) or through the presence of tolerance-inducing TFs (e.g., HIF1A) (60).

Different pathways and molecules are involved in controlling ET. A further layer of control of ET is exerted at the chromatin level by means of IFNγ, which can partially recover the expression of proinflammatory factors in tolerized monocytes and, thus, overcome ET (61). Mechanistically, IFNγ facilitates TLR-induced chromatin remodeling by recruiting ATP-dependent nucleosome remodeling complexes (such as BRG1) and restores the recruitment of TFs and RNA polymerase II at tolerized genes (e.g., *Tnf* and *Il-6*) (61). Finally, ex vivo β-glucan treatment of monocytes from volunteers with experimental endotoxemia partially reverses ET, restoring monocytes' capacity for cytokine production (60). Importantly, tolerance was reversed at both the promoters and enhancers of tolerized genes involved in metabolism and lipid biosynthesis, restoring LPS-repressed H3K27ac deposition at levels comparable to those observed in naive macrophages.

4.4. Trained Immunity

A distinguishing feature of trained macrophages is the ability to mount a stronger transcriptional response that is qualitatively and quantitatively different from that mounted by untrained cells. In one study, the expression of genes proximal to enhancers was induced in trained macrophages, peaking at 24 h postexposure, although they remained expressed at only low levels in LPS-exposed macrophages (60). In β-glucan-induced trained monocytes, modifications in H3K27ac, as well as increased deposition of H3K4me1 and H3K4me3 at gene promoters involved in trained immunity, resulted in transcriptionally active chromatin (60, 62). This led to transcriptional programs that rewired the intracellular signaling of innate immune cells and also induced a shift

of cellular metabolism from oxidative phosphorylation toward aerobic glycolysis, thus increasing macrophages' capacity to respond to stimulation (60). Importantly, a cross-link between metabolic pathways and chromatin remodeling has been documented in trained immunity. Emerging studies are reporting how specific metabolites can modulate the activity of DNA- or chromatin-modifying complexes, which, in turn, induce chromatin and DNA modifications, thus resulting in different trained immunity programs (62, 63). For instance, high levels of succinate have been shown to inhibit JMJD3 activity, leading to enhanced H3K27me3 of M2-like genes, thus suppressing their expression (62, 63).

4.5. The Role of Posttranscriptional Control of Macrophage Plasticity

The differential expression of microRNAs influences both the polarization status of macrophages and their capability to respond to infections (64, 65). In the following section, we provide an overview of current knowledge regarding the relative contribution of microRNAs to macrophage differentiation, polarization, and plasticity, pointing out the important role of microRNAs in immunomodulation and keeping the innate immune response in check through the reinforcement of positive or negative feedback circuits induced by proinflammatory and anti-inflammatory signals. We also discuss the impact of microRNA-mediated regulation of gene expression programs in tissue macrophage specialization in the context of chronic inflammatory diseases and tumors.

4.5.1. The role of microRNAs in activating macrophage polarization. The pivotal role of microRNAs in driving the development and maturation of immune cells is well established. The first evidence was provided by gene-targeting studies in which *Dicer*$^{lox/lox}$ mice were used to selectively deplete Dicer from the hematopoietic system. A significant reduction in all mature lineages, particularly myeloid cells, was observed together with a decrease in the frequency of the primitive LKS ($Lin^-/cKit^+/Sca\text{-}1^+$) progenitor population (66). Since then, several studies have described complex regulatory networks operating between microRNAs and key transcriptional regulators and their relevance for macrophage phenotype and function. Notably, specific subsets of microRNAs induced by different microenvironmental signals have been shown to modulate transcriptional output, thus resulting in the acquisition of distinct patterns of macrophage activation and polarization states, ranging from M1 to M2 phenotypes (**Figure 3**). For instance, miR-720 and miR-127 promote M1 polarization by respectively targeting GATA3 and BCL6, two TFs important in M2 macrophage polarization (67, 68). The overexpression of miR-720 resulted in the inhibition of M2 polarization (67). Consistently, ectopic expression of GATA3 restored the M2 phenotype in macrophages overexpressing miR-720, and enforced expression of miR-720 inhibits the promigratory behavior and phagocytic ability of M2-polarized macrophages (67).

miR-127-mediated inhibition of BCL6 led to increased phosphorylation of c-Jun N-terminal kinase, reduced expression of the phosphatase Dusp1, and increased levels of proinflammatory cytokines (68). Another microRNA targeting BCL6 and promoting M1 polarization is miR-155. Both gain-of-function and loss-of-function studies performed in vivo demonstrated that miR-155 is required for typical development of the macrophage inflammatory state (69). The enforced expression of miR-155 in M2 macrophages is sufficient to reprogram these cells toward a more proinflammatory phenotype (70), whereas deletion of miR-155 affects the expression of more than 650 genes. SOCS1 and IL-13RA1 are also miR-155 targets, and their deregulation is implicated in the promotion of the M2 phenotype (71–73). Increased levels of M2 marker genes (e.g., *CD206*, *ARG1*, *CCL22*, and *CCL17*) and a concomitant reduction in M1 phenotype markers (e.g., inducible nitric oxide synthase, IL-12, IL-6, TNF, and CD86) were observed in peritoneal macrophages overexpressing miR-146a, which has been shown to modulate macrophage polarization, at least

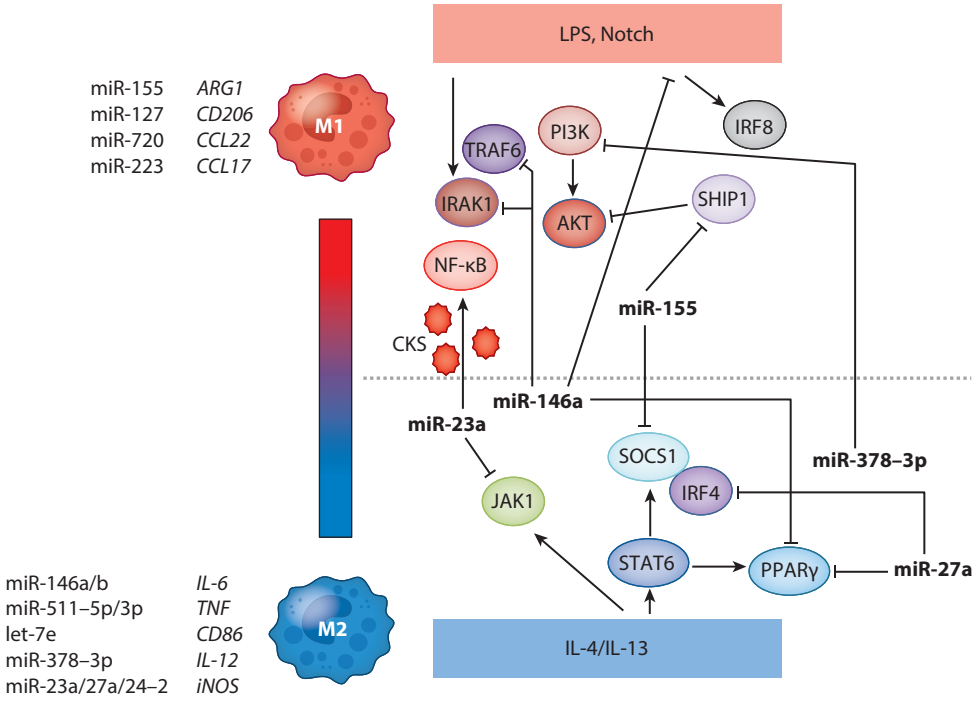

Figure 3

microRNAs in the regulation of macrophage plasticity. microRNAs engage in complex bidirectional interplay with transcription factors, tuning macrophage activation in response to different triggers. Abbreviations: CKS, cyclin-dependent kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator–activated receptor.

partly by targeting Notch1, peroxisome proliferator–activated receptor (PPAR)-γ, and the inhibin βA subunit of activin A (74, 75).

Another key transcription factor critical for normal macrophage function and sensitive to microRNA regulation is C/EBPβ, which is targeted by miR-223, a microRNA abundantly expressed in antigen-presenting cells, including dendritic cells, and macrophages resident in mouse intestine. By inhibiting C/EBPβ, miR-223 is able to limit the LPS-dependent release of IL-1β and IL-6 cytokines, thus impairing the proinflammatory activity of M1 macrophages and enhancing the alternative anti-inflammatory responses (76). The relevance of miR-223 in controlling alternative macrophage activation has been further supported by evidence demonstrating that in bone marrow–derived macrophages, miR-223 expression is transcriptionally regulated by PPARγ and the PPARγ/miR-223 regulatory axis drives M2 polarization through the targeting of *Rasa1* and *Nfat5* (77). Similarly, other studies identified regulatory feedback loop mechanisms in which microRNAs play an essential role in impairing the expression of M1 signature genes and, consequently, enhancing the production of M2-type cytokines. The expression of let-7c impaired the release of M1-related genes (i.e., *iNOS* and *IL12*) and increased levels of M2 markers (i.e., folate receptor-β) via the targeting of P21 activated kinase 1 (78) and C/EBPδ (79). In other instances, microRNAs function as molecular rheostats by participating in negative feedback loops that result in the attenuation of the alternative activation of macrophages. Among these, miR-378–3p and miR-511–3p are highly expressed in M2 macrophages in response to IL-4 stimulation. More precisely, miR-378–3p targets the PI3K/AKT1 pathway (80), whereas miR-511–3p downregulates

Rho-associated coiled-coil containing protein kinase 2 (ROCK2), a serine threonine kinase that phosphorylates IRF4, a TF that promotes M2 polarization (81).

The miR-23a/27a/24–2 cluster, also upregulated by M2-type stimuli, promoted the expression of proinflammatory cytokines and the concomitant inhibition of M2-type cytokines by acting on multiple signaling pathways (82). miR-23a activated the NF-κB pathway by targeting TNFAIP3, and targeting JAK1 and STAT6 directly suppressed the activity of the JAK/STAT pathway and reduced the production of M2-type cytokines; miR-27a showed the same phenotype by targeting IRF4 and PPARγ (82). Finally, the cross talk between miR-21 and the P13K/ERK/NF-κB axis, elicited by activation of the CSF-1R pathway, also has been identified as a further mechanism adopted by macrophages to suppress the inflammatory phenotype and promote the expression of M2 marker genes (83). Moreover, in mice, intraperitoneal injection of a miR-21 inhibitor increases the recruitment of inflammatory monocytes and enhances the peritoneal monocyte and macrophage response to LPS (84). Collectively, these findings strongly support the role of microRNAs as molecular determinants of macrophage plasticity via participation in key feedback loop mechanisms that sustain or impair the expression of M2 signature genes, which consequently bring about the redirection of macrophage polarization in accordance with the microenvironmental signals perceived.

4.5.2. Modulation of the primed and tolerant states of macrophages by microRNAs. As discussed above, establishment of macrophage priming and the tolerant state depends on the nature and intensity of the external stimuli. Major outstanding questions about the molecular mechanism responsible for the opposing effects of endotoxin priming and tolerance are still partially unresolved. However, evidence provided so far strongly supports a role for microRNAs in the broad reprogramming of macrophages, for which they act as molecular rheostats, able to switch from a pro- to an anti-inflammatory response (84, 85).

microRNAs have been described as active components of feedback loop regulatory mechanisms that significantly shape the inflammatory response through the modulation of key molecular pathways downstream of TLR signaling (86). The differential expression of NF-κB family members occurs in response to different doses of LPS, and dynamic regulation of the NF-κB pathway has been implicated in ET (87). Interestingly, NF-κB is known to control the expression of many endotoxin-responsive microRNAs. Among them, miR-146a and miR-155 were the first microRNAs characterized in LPS-primed macrophages, and their expression is regulated by NF-κB (88, 89). A pioneering study demonstrated the existence of a negative feedback circuit in which NF-κB induced the transcription of miR-146a, which, in turn, inhibited the NF-κB pathway by targeting two adaptor proteins: TRAF6 and IRAK1 (88). Further studies correlated the impairment of NF-κB activity and the observed decrease in the production of proinflammatory cytokines with a significant upregulation of miR-146a levels in tolerant THP-1 monocytic cells, thus suggesting the involvement of miR-146a in LPS desensitization (90, 91). Similar evidence has also been reported for miR-155, a proinflammatory microRNA that is rapidly upregulated by NF-κB in macrophages primed with several TLR ligands and type 1 interferons (89, 92, 93). Interestingly, miR-155 is the central component in multiple feedforward networks that are implicated in dictating the duration and the intensity of the inflammatory response, as well as macrophage sensitivity to the LPS response. More precisely, miR-155 expression initiates and amplifies the inflammatory response and antiviral innate immunity by directly inhibiting the expression of negative regulators of TLR signaling, including SOCS1 (94) and SHIP1 (95). SHIP1 is known to negatively regulate the PI3K/AKT1 pathway, which has an established role in controlling macrophage sensitivity to LPS. Moreover, AKT1 signaling inhibits the expression of miR-155, thus establishing a negative feedback loop that limits the proinflammatory response of macrophages and has a significant

impact on controlling ET, as reported in miR-155 knock-in mice that had high levels of TNF and increased susceptibility to LPS shock (93). Moreover, the proinflammatory activity of miR-155 is further regulated in a timely manner by IL-10 through the inhibition of miR-155 transcription by STAT3. This inhibitory effect is also sustained by miR-21, another microRNA expressed in LPS-primed macrophages that reduced activation of NF-κB and enhanced expression of IL-10 (96). Ultimately, the induction of IL-10 led to an increased expression of SHIP1 (97). Of note, in addition to miR-155, AKT1 controls the macrophage response to LPS also by regulating the expression levels of let-7e, miR-125b, and miR-181c (93). In particular, let-7e expression is positively induced by AKT1 and mediates LPS hyperresponsiveness in *AKT1*$^{-/-}$ macrophages by targeting TLR4. Further studies have confirmed the inhibition of TLR4 by let-7e and demonstrated the targeting of other components of the TLR signaling pathway, further supporting the anti-inflammatory role of let-7e and its importance in ET. Recently, the miR-125a/99b/let-7e cluster was found to be induced late by TLR agonists via the IL-10-dependent regulatory loop, and counterregulated by IFNγ (98). Interestingly, this is mirrored by the targeting of multiple different components of the TLR pathway and results in global downregulation of proinflammatory cytokines (98), indicating that this cluster of microRNAs is a potent tool used by macrophage cells to switch off the inflammatory response in a timely manner. Moreover, high levels of the miR-125a/99b/let-7e cluster were observed in LPS-tolerant monocytes, and enforced expression of this microRNA cluster impaired ET rescue exerted by IFNγ, thus suggesting that the inhibition of miR-125a/99b/let-7e expression is one of the mechanisms used by IFNγ to prevent the induction of LPS tolerance (98).

It is noteworthy that dualism in the expression or function, or both, of members of the same family of microRNA have been reported. The miR-125a and miR-125b family and miR-146a and miR-146b family represent two distinct examples of such dichotomy. miR-125a and miR-125b have been shown to be regulated in opposite directions by LPS stimulus in macrophages and also to exert opposing functions in the context of macrophage-mediated inflammatory responses. In contrast to miR-125a, levels of miR-125b decrease early in LPS-primed macrophages. The enhanced expression of miR-125b induces a greater IFNγ response and sustains proinflammatory cell activation by targeting IRF4, which promotes M2 macrophage polarization (99). In contrast, studies of miR-146a and miR-146b strongly suggest that they operate as a relay system to buffer the expression of proinflammatory genes induced by TLR4 triggering. This hypothesis is supported by the demonstration that miR-146a and miR-146b are induced by different transcription factors (i.e., NF-κB and STAT3, respectively) at different moments in the same cell type. Both miR-146a and miR-146b exert anti-inflammatory roles by downregulating the LPS receptor TLR4 and key adaptors and signaling molecules, including MyD88, TRAF6, and IRAK1 (85). Finally, the induction of miR-146b in monocytes tolerized by IL-10 and TGFβ and the functional role of miR-146b in ET have also been demonstrated (100).

Another interesting aspect of microRNA-mediated regulation of macrophage functions comes from a study published in 2017 investigating the role of miR-511 in ET (101). More specifically, the evidence demonstrated that, in contrast to what had been previously shown in murine macrophages, in which the miR-511–3p mature form is functional and important for M2 polarization, miR-511–5p is the most abundant strand of miR-511 and acts as an intracellular mediator of glucocorticoid (GC) and TGFβ (101). Indeed, the expression of miR-511–5p, which is inhibited by LPS and IFNγ, is significantly induced by anti-inflammatory stimuli, such as TGFβ and GC. Moreover, deregulated expression of miR-511–5p was involved in GC- and TGFβ-mediated inhibition of the production of proinflammatory cytokines observed in endotoxin-tolerant monocytes (101).

Altogether, this evidence demonstrates the capability of microRNAs to modulate the duration and the magnitude of the innate immune response, participating as an integral component of feedback loop regulatory mechanisms that significantly shape the inflammatory response and modulate sensitivity to endotoxin to prevent excessive inflammation in macrophages.

5. MACROPHAGE PLASTICITY IN PATHOLOGY: CANCER AS A PARADIGM

Macrophage infiltration is the hallmark of chronic inflammation. Macrophages adapt to the diversity of drivers of chronic inflammation, including type 1 and type 2 immune responses (10, 102) and tissue damage. They integrate multiple signals (102, 103) and orchestrate the function of other immunocompetent cells, stroma, and vascular cells. The adaptability of macrophages underlies their role in atherosclerosis and cardiovascular pathology (19, 20, 104, 105), neurodegeneration (18), autoimmunity and autoinflammation (see, e.g., 106), and cancer. As a paradigm for macrophage plasticity we focus on cancer, a disease characterized by dynamic evolution and a Darwinian microenvironment. Previous reviews on TAMs provide a framework for this section, which largely focuses on selected, more recent advances (32, 107–111). In general, macrophages in cancer are double-edged swords with the capacity to exert pro- and antitumor activity, depending on the balance of a number of signals, including those from cytokines, chemokines, antibodies, and myeloid checkpoints (**Figure 4**).

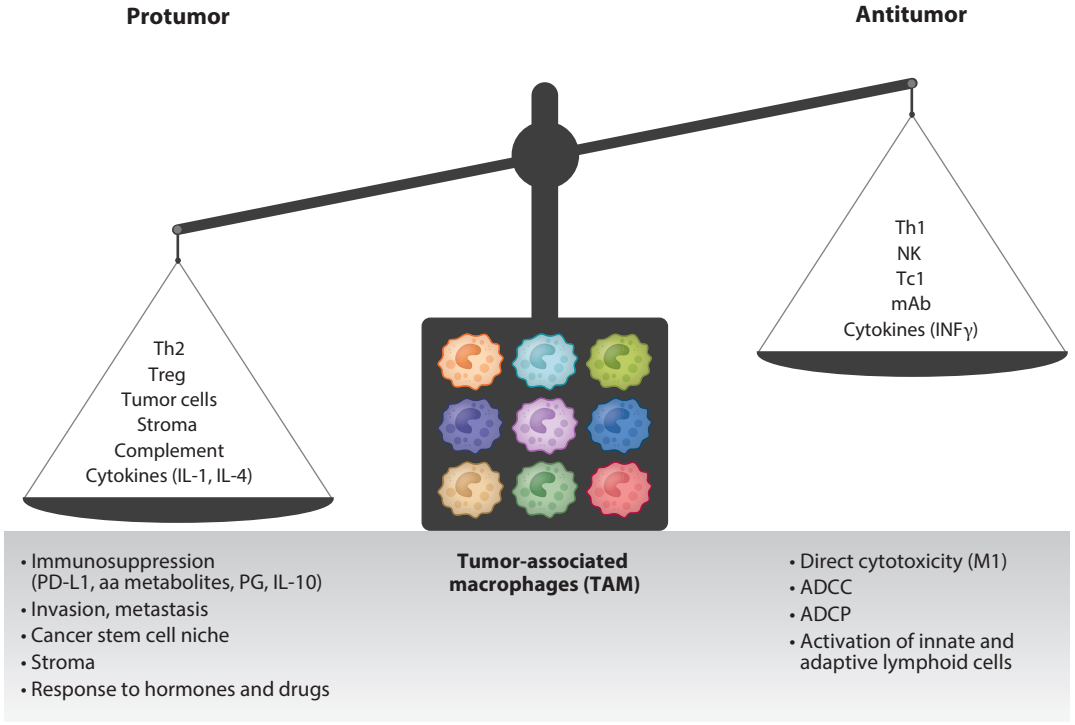

Figure 4

Macrophages act as double-edged swords in regulating tumor progression and response and regulating general response to perturbation. For brevity, only some cytokines and proteins are shown. Abbreviations: aa, amino acid; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; IFN, interferon; mAb, monoclonal antibody; NK, natural killer; PG, prostaglandins; Th, T helper cell; Treg, T regulatory cell.

5.1. Origin

It has long been held that TAMs originate from circulating monocytes (32, 112). As discussed above, evidence in mice and humans suggests a dichotomous origin for tissue macrophages: from embryonic and from adult circulating myeloid precursors (113). This fundamental paradigm shift raises the general issue of the embryonal versus hematopoietic origin of TAMs and of its functional relevance. In a mouse mammary carcinoma model, tumor growth was associated with the loss of tissue-resident cells and replenishment of the TAM component by monocytes (114). As may have been expected on the basis of ontogeny (see above), microglia persisted in murine gliomas (115), resulting in the infiltration of macrophages of mixed origin. In a recent interesting study, macrophages of embryonic origin persisted in pancreatic ductal adenocarcinoma (PDAC) and played a key role in fibrosis and progression (113).

Macrophages are an essential component of remodeling of the extracellular matrix. In different contexts—including tissue repair and response to pathogens mediated by type 1 or type 2 immunity—macrophages activated by IL-4 or IL-13, in concert with other environmental cues, orchestrate repair, remodeling, and fibrosis (102, 103, 116, 117). In early pancreatic adenocarcinoma in situ, IL-13 released by tuft cells led to the accumulation of M2-like cells that were shown to promote fibrosis and progression (118). While macrophages have generally been shown to promote fibrosis by acting on fibroblasts, in PDAC (113) and colon cancer (119) embryo- or bone marrow–derived macrophages directly deposited the extracellular matrix.

In a number of mouse tumor models, circulating monocytes were the main precursors of TAMs (32, 109, 114, 119). In the human context of bone marrow transplantation, lymphoma-associated macrophages were found to originate from bone marrow precursors (32). The lack of reliable biomarkers and molecular signatures has so far prevented an in-depth systematic analysis of the relative contribution to the accumulation of TAMs of macrophages of different origin in different human tumors. At this stage, one could assume that in at least some human cancers, mononuclear phagocytes of different origin coexist and that based on results obtained under physiological conditions, the tumor tissue microenvironment is a dominant determinant of the education of TAM populations.

5.2. From Tumor Initiation to Metastasis

There is now evidence that macrophages play a role in the whole spectrum of tumor evolution, from initiation to metastasis. Inflammation is a major driver of liver carcinogenesis in mice and humans, and macrophages are central cells in liver inflammation and carcinogenesis. Interestingly, single tumor-initiating cells were found to recruit polarized M2-like macrophages, and these help such cells evade immune clearance. The Hippo pathway was found to underlie macrophage recruitment to the tumor-initiating cell niche (120).

Genetic instability is a hallmark of cancer. Recent evidence is consistent with the view that myeloid cells contribute to genetic instability by producing reactive oxygen species (121). These and previous results on interactions with cancer stem cells (32) suggest that macrophages are involved in the early steps of carcinogenesis and in providing a nurturing niche for cancer stem cells, extending the classic concept that macrophages promote invasion and metastasis (32, 109, 122). Therefore, evidence suggests that macrophages contribute to the various stages of cancer progression, from initiation to the formation of distant metastasis.

5.3. Adaptation to the Tumor Microenvironment

In the tumor microenvironment (TME), signals originating from tumor cells, fibroblasts, stroma, and immunocompetent cells drive recruitment and orchestrate the function of mononuclear

phagocytes (32). Mediators responsible for shaping macrophage function in the TME include type 2 cytokines (IL-4 and IL-13) produced by Th2 cells and basophils, immunosuppressive cytokines produced by Treg cells, chemokines, tumor cell products, osteopontin, receptor tyrosine kinase (Ros1), and exosomes (123–125). Metabolism is a key component of macrophage function (126). Recent evidence suggested that tumor cells induced itaconic acid production in macrophages and that this, in turn, potentiated tumor growth (127). The TME is characterized by an acidic pH, which has recently been shown to induce macrophages with regulatory function that promotes immune evasion. Thus, intrinsic and extrinsic metabolic characteristics of mononuclear phagocytes contribute to shaping TAM function (128).

In selected tumors, such as mammary carcinomas, the type 2 cytokines IL-4 and IL-13 are major drivers of M2 or M2-like polarization (32). However, type 2 immunity can also be protective in a yin–yang relationship (see, e.g., 129), although the clinical significance of this observation remains to be defined. Dectin-1 is a macrophage receptor augmented by IL-4. The tetraspanin MS4A4A molecule was recently shown to be expressed by a subset of TAMs and to interact with dectin-1. MS4A4A was found to be essential for dectin-1-dependent macrophage-triggered NK cell–mediated resistance to metastasis early in progression (130).

Fluid-phase PRMs and complement are components of the TME (131–133). Complement activation has been shown to drive recruitment and functional skewing of mononuclear phagocytes (132, 134, 135). In murine sarcomagenesis, complement C3 was upstream of macrophage recruitment and functional orientation (132), whereas in squamous carcinogenesis, macrophage-derived urokinase plasminogen activator was responsible for C3-independent C5a generation (134). The complement regulator *PTX3* (29) was found to act as an extrinsic oncosuppressor gene in selected human tumors (132), thus pointing to its importance in human carcinogenesis. It will be important to assess the presence and pathways of complement activation in different cancers in humans.

Macrophages are endowed with an impressive armamentarium of immunosuppressive mediators including cytokines (e.g., IL-10); products related to iron metabolism (e.g., CO); enzymes involved in amino acid metabolism (e.g., indoleamine 2,3-dioxygenase, or IDO, an arginase); prostaglandins; and triggers of checkpoint blockade in T cells and NK cells, such as PD-L1 and VISTA (32, 111). Triggers of the immunosuppressive function of TAMs include cytokines produced by Th2 cells, Treg cells, and tumor cells. Moreover, C5a has recently been shown to drive TAM-mediated suppression of effective CD8 cell–mediated antitumor resistance (134). In transplanted tumors, including PDACs, the immunosuppressive function of TAMs has been reported to be driven by phagocytosis of dying tumor cells associated with the LC3 autophagy pathway and, unexpectedly, by antibodies in antibody-dependent cellular phagocytosis (ADCP) (136–140). Collectively, macrophages are a major component of the immunosuppressive milieu of different murine and human tumors and major drivers of checkpoint blockade, even in tumors such as Hodgkin's lymphoma, in which PD-L1 amplification occurs in neoplastic cells (141).

5.4. Tumor-Associated Macrophage Diversity

Early transcriptional profiling studies investigated TAMs as whole populations without taking diversity into account (34). Subsequent investigations revealed that macrophages with different phenotypes coexist within the same mouse or human TAM population (142–144). Hypoxia was identified as one determinant of regional differences in TAM phenotypes. High-dimensional single-cell analysis using mass cytometry and RNA sequencing has added a new dimension to the dissection of myeloid cell diversity in tumors (136, 145–147). In particular, in non–small cell lung cancer, high-dimensional single-cell analyses revealed differences between TAMs and normal tissue macrophages and the presence of several cell clusters (145, 147). Interestingly, in

a large-scale analysis, the different phenotypes of TAMs in lung cancer appeared to be part of a continuum (147), and the overall picture was consistent with M2 and M2-like polarization (147). Therefore, these results suggest there is a diversity of TAMs and a common theme of regulatory and immunosuppressive functions (136, 145–147).

5.5. Macrophage Reeducation

In established, progressing tumors, macrophages are a component of cancer-enhancing inflammation, but mononuclear phagocytes also have the potential to mediate anticancer activity (32, 148) (**Figure 4**). Rewiring macrophage function using a variety of classic activation signals has been shown to result in antitumor activity in preclinical models (149–153). Macrophages are potent effectors of antibody-dependent cellular cytotoxicity (ADCC) (32, 148). There is evidence that ADCC mediated by macrophages is an important determinant of the antitumor activity of the monoclonal antibodies (mAbs) that are in clinical use, such as rituximab and trastuzumab (32, 154).

The function of myeloid cells is under tight control by negative regulators acting at different levels (see, e.g., 149, 155–158). Myeloid cells in tumors are a major source of triggers of checkpoint blockade expression, such as PD-L1 and VISTA. In addition to triggering checkpoint blockade in T cells and NK cells by interacting with PD1, PD-L1 expressed in TAMs has recently been shown to act as a negative signaling molecule in mononuclear phagocytes (149). Blocking the PD-L1 pathway resulted in activation of the antitumor potential of TAMs (149).

Disease hyperprogression has been described in a fraction of patients treated with PD1 or PD-L1 checkpoint blockade immunotherapy. Circumstantial clinical and experimental evidence suggested that Fcγ receptor engagement and TAM reprogramming were responsible for hyperprogression (159). It will be important to further explore cellular and molecular determinants and candidate myeloid biomarkers to limit the occurrence of this paradoxical reaction to checkpoint blockade immunotherapy.

CD47 is a "don't eat me" signal expressed by many cell types. It interacts with SIRPα, which is present on the surface of macrophages, and CD47 plays a homeostatic role in disposing of aged cells, erythrocytes in particular (155). *C-myc*, an oncogene involved in many cancers, amplifies CD47 and PD-L1 (160). In a Phase I study, an anti-CD47 mAb together with anti-CD20 had impressive antitumor activity in patients with diffuse large B cell lymphoma that was refractory to treatments including anti-CD20 alone (161). These clinical results are consistent with preclinical data showing that blocking the CD49/SIRPα axis activates macrophage-mediated ADCP and acts in concert with ADCC elicited by anti-CD20 mAb (155).

Preclinical evidence suggests that targeting CD47 and other macrophage checkpoints, such as Clever-1, results in activation of adaptive T cell responses (155, 157). Moreover, macrophages can interact with NK cells and drive NK cell–mediated protection against primary carcinogenesis and metastasis (see, e.g., 130, 162). It will be important to assess the actual clinical relevance of lymphoid cell activation in the context of the emerging field of myeloid checkpoint immunotherapy.

6. CONCLUDING REMARKS

Mononuclear phagocytes are versatile cells of the innate immune system capable of adapting to microenvironmental signals under physiological and pathological conditions. Substantial progress has been made in defining the molecular basis responsible for the differentiation and specialization of macrophages in tissues, their diversity, and their short- and long-term functional regulation. As fundamental mediators and orchestrators of chronic inflammation in its diverse forms

and manifestations, macrophages are major players in a wide range of diseases, from autoimmunity to cardiovascular pathology to neurodegenerative disorders to cancer. Cancer has served as a paradigm of macrophage plasticity and was chosen for analysis in this review. The realization that mononuclear phagocytes have different ontogenetic origins raises the still largely unanswered question of their actual differential role as embryo-derived versus bone marrow–derived cells in human pathology. High-dimensional profiling of macrophages has added a new aspect to the understanding of macrophage diversity. Translating the understanding of this diversity into clinically useful signatures and biomarkers remains a challenge.

Under many conditions, macrophage infiltration and functional biomarkers have prognostic significance, as illustrated by the response of TAMs and myeloid cells to checkpoint blockade. In this case, the challenge is to move from prognosis to predicting responses to conventional therapies and immunotherapy. Targeting macrophages in cancer and chronic inflammation based on restricted surface molecules (130) or on understanding of the molecular basis of reprogramming may pave the way for innovative therapeutic approaches. The encouraging initial results with CD47-blocking mAbs may herald a new era of myeloid checkpoint immunotherapy with broad transdisease significance (148).

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