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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. Diversity is imprinted by their different ontogenetic origin (embryonal versus adult bone marrow-derived cells), by the organ context and by activation or deactivation signals from microbial invasion, tissue damage, dismetabolism and polarized adaptive T cell responses. Classic adaptive responses of macrophages include tolerance, priming and a wide spectrum of activation states including M1 or M2 or M2-like. Moreover, macrophages can retain more 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 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 is an essential component of the diversity of chronic inflammation and its involvement in diverse human diseases, cancer in particular discussed here as a paradigm.

Keywords

Macrophages; Plasticity; miRNA; Epigenetics; Cancer; Checkpoints

1 Introduction

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

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The eponimous function of cells of the macrophage lineage is phagocytosis. Early on it was realized that macrophages serve as first line of defence against infection. Macrophages are an essential component of innate immunity. They express an unmatched repertoir of innate immunity receptors, such as 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 innate immunity cells including neutrophils and innate lymphoid cells, 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 (PRM) such as PTX3 (6). In turn humoral immunity components collaborate with macrophages in effector function and regulate the activity of mononunclear phagocytes.

Macrophages are critical cells in the orchestration of chronic inflammation and related pathologies (7). Indeed, inflammation and its mediators represent a metanarrative of 21st century medicine (8). However chronic inflammatory reactions are extremely diverse and adaptability to different tissue microenvironments and responses to different pathogenic insults are a key feature of mononuclear phagocytes.

The focus of this review will be 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 will provide a background for the plasticity of these cells in pathology with emphasis on cancer. The reader is referred to previous reviews for a framework of the present essay (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 adult life, in many 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 that a dual origin of tissue macrophages (self sustaining local versus monocyte-derived) also applies to human mononuclear phagocytes (e.g. (15). Given the long life expectancy of humans compared to mice and the difficulty in inducing proliferation of mature human versus mouse macrophages with colony stimulating factor 1 (CSF-1) one could speculate of a relative major contribution of monocytes in man.

Macrophage populations are considerably diverse among tissues as exemplified by lung alveolar macrophages, microglia, and Kupffer cells. Moreover, single cell analysis has added a new dimension in decifering 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 Fig. 1. Development of several organs requires mononuclear phagocytes, and branching morphogenesis in the organs such as 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 rythm (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 which remain to a large extent to be explored. Evidence suggests that in general tissue macrophages seeding tissues prenatally are born to subserve homeostatic functions and monocyte-derived cells are involved in respose to pathological signals . However, the capacity of macrophages to respond and adapt to environmental cues defies a rigid division of labour.

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 lipopolisaccharides (LPS) results in enhanced macrophage-mediated resistance and effector function (23; 24). Building on these early observations on what used to be generally referred to as “activation”, a more refined view of the spectrum of responses elicited by different signals was obtained (9; 10; 25) (Fig. 1 and 2).

Following activation, i*n 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, production of IL-10 and Th2 - or T regulatory cells (Treg) - attracting chemokines is retained. The evolutionary value of tolerance rests in its significance as a fundamental mechanism to limit inflammation-caused tissue damage (26) (Fig. 2).

Interferon γ (IFNγ) as well as other cytokines have long been known to prepare macrophages for enhanced responsiveness to microbial components or to synergize with them. This getting ready to counter microbial challenges is relatively short lied (Fig. 2). Imprinting an antimicrobial resistance program is not unique to host derived cytokines. It has recently been shown that the short chain fatty acid butyrrate, 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

fluid phase pattern recognition molecules (PRM) expressed by macrophages with upregulation of MARCO and dectin 1 (28) and production of the humoral PRM PTX3 and Complement components (29).

The Th2 cytokine IL-4 was shown to elicit an alternative (M2) form of macrophage activation, with induction of a distinct set of surface receptors and effector molecules (25; 30). M1 and M2 activation reflectied the main cellular sources of IFNγ and IL-4 and the nomenclature of polarized immune responses (Th1 and Th2; ILC1 and ILC2; type 1 and type 2 immunity, etc.) (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 phenotype with some generic relationship to IL-4 activated macrophages (e.g. (32)[1].

A systematic trasncriptional profiling effort of human macrophages exposed to== a wide range of signals (33) has further extended the spectrum of activation states well 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 orginal M1/M2 dichotomy, as already evident in early profiling of TAM (34). However macrophages largely mirroring the *in vitro* phenotypes of M1/M2 polarized cells are present in pathological tissues (e.g. (10)) and are part of type 1 and type 2 immune responses driven by innate and/or adaptive lymphoid cells as discussed below.

Exposure to microbial moyeties can result in long term imprinting of innate immunity (35). Lymphoid cell-independent imprinting of phagocyte function was originally observed in invertebrates (36). Long term imprinting of phagocytes has been referred to as memory (36), adaptive innate (37) or trained (35). Recent evidence suggests that imprinting myeloid precursors and neutrophils play 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 or trained immunity are complex. From an original macrophage-autonomous view, the field moved to 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 adjuvant and vaccine development. Dissection of its cellular and molecular mechanisms may pave the way to translation. Exploration of the full spectrum of trained innate immunity will require further work.

[1]For a discussion of the value of use of imperfect nomenclatures in immunology and related epistemological consideration the reader is referred to 11. Mantovani A. 2016. Reflections on immunological nomenclature: in praise of imperfection. *Nat. Immunol.* 17:215-6.

4 Molecular basis of macrophage plasticity

Dynamic regulation of complex gene networks and signalling cascades that control macrophage polarization, priming and plasticity is achieved through multi-layers of gene expression regulation. Both transcription and translation are tightly regulated sophisticated processes that strongly influence cells function. The role of key transcription factor (TF) families in defining macrophage identity and controlling their functions through the induction and maintenance of specific transcriptional programs is well established (40). Genome-wide studies profiling transcriptional and epigenetic modifications identified differences in non-coding RNAs (ncRNAs), 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 will discuss how specific histone modifications, DNA methylation patterns and regulatory RNA regulate macrophage identity, priming, polarization and tissue-specific functions, and thus account for the heterogeneity and plasticity of macrophages (Fig. 2).

4.1 Chromatin remodelling in primed macrophages

Distinct epigenetic signatures are associated to specific differentiation states of macrophages, suggesting that the relative epigenome landscape is remarkably shaped by the integration of microenvironment- and stimulus-specific signals, resulting into a continuum of distinct transcriptional and functional outputs. Genome wide studies profiling transcriptional epigenetic modifications occurring in differentiated macrophages reveal 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 their priming (42; 48). In resting state, many inflammatory gene loci are in a repressed configuration, as inferred by the low histone acetylation and the very low amount of RNA polymerase II loaded (48–50). Effective activation of proinflammatory genes by TLR signalling involves overcoming a rate-limiting chromatin barrier imposed by histone-containing nucleosomes that bind DNA (51) Mechanistically, this occurs through recruitment of RNA polymerase II, histone acetylation to relax chromatin, and recruitment of ATP-dependent nucleosome remodelling complexes for nucleosome repositioning or removal (50).

Genome wide analyses strongly indicate important roles of enhancers in signal-dependent transcriptional responses (46) In LPS-primed macrophages a substantial and rapid reorganization of the epigenome landscape occurs and mainly involves chromatin reorganization at enhancer regions. According to their activation state, enhancers can be generally classified in inactive, primed, or poised. Differently from inactive enhancers, which are located in heterochromatin regions, devoid of histone modifications and TF binding, primed enhancers (marked by H3K27ac) are located in nucleosome-free regions, in close proximity to TFs binding sites and 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 also contain repressive epigenetic marks. TLR4 signalling induces increased acetylation of activated enhancers, whereas poised enhancers are unaffected by LPS stimulation and keep basal H3K4me without acquiring H3K27ac mark. Furthermore, most of macrophage specific enhancers are premarked by the binding of the fate-determining TF Pu.1 (46). Pu.1 recruits chromatin remodelers able to displace or remodel nucleosomes, thus leading to the formation of small accessible regions centered on the Pu.1-binding site (41; 46). A fraction of the macrophage-specific enhancers contains binding sites for TFs activated by inflammatory stimuli (like NFκB, STATs and AP-1), which are recruited in response to stimulation (46).

In addition to the rapid reorganization of pre-existing enhancer landscape, LPS priming also induces activation of about 3000 new enhancer regions, with the consequent formation of so-called “super-enhancers”. These are regions where enhancers are in closed proximity to key regulatory genes and confer higher transcriptional activity and sensitivity to perturbations (52). Therefore, it has been suggested that 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 by cooperation between epigenetic and signalling mechanisms, that creates a primed chromatin environment to augment TLR-induced gene transcription (53). IFNγ increased chromatin accessibility by inducing acetylation of histone 4 acetylation and CBP/p300 recruitment as well as stable and coordinated recruitment of STAT1 and IRF1 to enhancers and promoters of genes that are synergistically activated by IFNγ and LPS, such as Tnf, Il6, and Il12b. This priming of chromatin results in the removal of a rate-limiting chromatin barrier that greatly increases and prolongs recruitment of additional TFs and RNA polymerase II after TLR stimulation and increased transcription of inflammatory genes (53).

Irrespective of the specific underlying mechanism, evidence supports a model where lineage-determining TFs act in a collaborative manner 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

Plasticity of epigenetic modifications has been proposed as a key molecular determinant of macrophage identity and heterogeneity. Dynamic and reversible epigenomic marks at enhancers and promoters of signal responsive genes are important for rapid reprogram of macrophage polarization and to tailor the response to a potentially hostile environment (46; 54). On the other side, long-term and more stable epigenetic marks contribute to define macrophage cell identity (55) and to the establishment of the so-called “epigenetic memory”, that influences macrophage response to subsequent microbe encounters (56). Macrophage epigenome is remodelled in response to acute stimulation and polarizing stimuli. Such remodelling involves changes in the expression of chromatin-modifying

enzymes during macrophage polarization, which shape the epigenome landscape and thus affect the transcriptional output (49).

Several studies demonstrated the role of both histone methylation and acetylation in alternative macrophage polarization. Overexpression of DNA methyltransferase 3B (DNMT3B) or loss of HDAC3 renders macrophages hyper-responsive to IL-4, skewing differentiation towards the M2 phenotype (50; 51). Furthermore, the histone demethylase JMJD3 is induced by IL-4 in a STAT6-dependent manner and is required for macrophage alternative polarization, by 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. Consistently, loss of HDAC3 removes this brake and thereby promotes the IL-4-induced M2 polarization, as also observed in macrophages lacking HDAC3, which display a polarization phenotype similar to IL-4-induced alternative activation and are hyper-responsive to IL-4 stimulation (41). It has also been shown that IL-4 induces an epigenomic signature which selectively represses the macrophage inflammation program, thus favouring 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 transcriptional repressor by recognizing non-canonical binding motifs or if 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 was 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 described implies IFNγ-mediated recruitment of a repressor complex containing the histone methylase EZH2 and the associated deposition of the negative histone mark H3K27me3 to a small group of anti-inflammatory genes, including Mertk and Pparg. Gene repression is stabilized by maintenance of H3K27me3 on gene promoters, persisting after termination of IFNγ stimulus. Moreover, these silenced genes are no longer responsive to glucocorticoids, IL-4, and M-CSF. 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, enriched for binding by transcription factor MAF (57). Collectively, these findings strongly support the existence of underlying cross talks between transcriptional and epigenetic regulatory mechanisms in controlling macrophage plasticity.

4.3 Epigenetic regulation of ET

Combinatorial patterns of epigenetic changes confer highly specific regulation at genes and enhancers across several signalling pathways critical to the establishment of ET. Several studies in both murine and human sepsis models 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 and this is strictly associated with the dynamic establishment of distinct epigenetic marks (51; 58). Accordingly, a gradient in the response of tolerized macrophage 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 three functional categories, characterized by distinct epigenetic marks. The first class includes pro-inflammatory molecules, which are transiently silenced ("tolerized" genes), whereas the second class includes antimicrobial effectors, which expression is not affected by LPS stimulus or is further upregulated ("non tolerized" genes) (51). In tolerized macrophages, recruitment of chromatin regulators, such as Mi-2β and BRG1, induced chromatin remodeling at non tolerized genes, thus allowing 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 NcoR–HDAC3–deacetylated-p50 repressosome to inflammatory genes, whereas non tolerized genes maintain an open chromatin state, allowing 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 solely mechanism responsible for specifying TLR-induced gene repression. Significant changes in the methylation and acetylation state of enhancers were detected in tolerized genes compared to 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 re-exposure, tolerized genes maintain their basal promoter state, and do not regain nor H3K27ac or H3K4me3 mark, thus remaining silent and refractory to stimulation. Conversely, non tolerized genes maintain H3K4me3 mark and their promoters are re-acetylated in tolerant macrophages (51). This finding suggests that tolerant macrophage fail to accumulate H3K27ac at tolerized genes either through absence of pro-inflammatory activators (*e.g.* IRF and STATs), or through presence of tolerance inducing TFs (e.g. HIF1A) (60).

Different pathways and molecules are involved in the control of 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 overcome ET (61). Mechanistically, IFNγ facilitates TLR-induced chromatin remodelling 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 Il6) (61). Finally, ex vivo β-glucan treatment of monocytes from volunteers with experimental endotoxemia partially reverses ET, restoring their capacity for cytokine production (60). Importantly, tolerance was reversed at both promoters and enhancers of tolerized genes was 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 distinguish feature of trained immune macrophages is the ability to mount a stronger transcriptional response, qualitatively and quantitatively different compared with untrained cells. Expression of genes proximal to enhancers was induced in trained macrophages, peaking at 24 h post-exposure, while they remained lowly expressed 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 signalling of innate immune cells but 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 remodelling has been documented in trained immunity. Few studies reporting how some 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. 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 Role of post-transcriptional control of macrophage plasticity

MicroRNA differential expression influences both the polarization status of macrophages as well as their capability to respond to infections (64; 65). In the following section we provide an overview of current knowledge regarding the relative contribution of microRNA in macrophage differentiation, polarization and plasticity pointing out at the role of microRNAs as important immunomodulators, that keep the innate immune response in check through the reinforcement of positive or negative feedback circuits induced by inflammatory 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 MicroRNA in macrophage polarized activation (Fig. 3)— A pivotal role of microRNA in driving development and maturation of immune cells is well established. The first evidence has been provided by condition gene targeting studies in which $Dicer^{lox/lox}$ mice were used to selectively deplete Dicer from the hematopoietic system. A significant reduction of all mature lineages, particularly myeloid cells was observed together with decrease in the frequency of primitive LKS ($Lineage^{-}/cKit^{+}/Sca\text{-}1^{+}$) progenitor population (66). Since then, several studies described complex regulatory networks between microRNA and key transcriptional regulators, which control the phenotype and function of macrophages. Notably, specific subsets of microRNA induced by different microenvironmental signals have been shown to modulate the transcriptional output, thus resulting in the acquisition of distinct pattern of macrophage activation/polarization states, ranging from M1 to M2 phenotypes. For instance, miR-720 and miR-127 promote M1 polarization by targeting GATA3 and BCL6, two transcription factors important in M2

macrophage polarization. Overexpression of miR-720, resulted in the inhibition of M2 polarization. Consistently, ectopic expression of GATA3 restored the M2 phenotype in miR-720 overexpressing macrophages and enforced expression of miR-720 inhibits pro-migration behavior and phagocytic ability of M2-polarized macrophages (67). miR-127-mediated inhibition of BCL6 led to increased phosphorylation of JNK, reduced expression of the phosphatase Dusp1 and increased levels of pro-inflammatory 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 macrophage inflammatory state (69). Enforced expression of miR-155 in M2 macrophages is sufficient to reprogram these cells towards a more pro-inflammatory phenotype (70), whereas its deletion 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 M2 phenotype (71–73). Increased levels of M2-marker genes (e.g. CD206, Arg1, CCL22 and CCL17) and concomitant reduction of M1-phentoype markers (e.g. iNOS, IL-12, IL-6, TNF, CD86) were observed in peritoneal macrophages overexpressing miR-146a, which has been shown to modulate macrophage polarization at least in part by targeting Notch1, PPARγ, and 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, that is targeted by miR-223, a microRNA abundantly expressed in APCs, including DCs and macrophages resident in mouse intestine. Through the inhibition of 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 importance of miR-223 role in controlling macrophage alternative activation was further supported by evidence demonstrating that in bone marrow-derived macrophages miR-223 expression is transcriptionally regulated by PPARγ and that 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 microRNA play an essential role in impairing the expression of M1 signature genes and consequently enhancing the production of M2-type cytokines. Expression of let-7c impaired the release of M1-related genes (i.e. iNOs and IL-12) and increased levels of M2 markers (i.e., FR-β), via targeting of P21 activated kinase 1 (78) and C/EBP-δ (79). In other instances, microRNA 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-511-3, 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 ROCK2 (Rho-associated coiled-coil containing protein kinase 2), a serine threonine kinase that phosphorylates IRF4, a transcription factor that promotes M2 polarization (81).

miR-23a/27a/24-2 cluster, also upregulated by M2-type stimuli, promoted the expression of pro-inflammatory cytokines and the concomitant inhibition of M2-type cytokines by acting on multiple signalling pathways (82). MiR-23a activated the NF-κB pathway by targeting TNFα-inducing protein 3 (TNFAIP3), and by targeting JAK1 and STAT6 directly suppressed the activity of JAK/STAT pathway and reduced the production of M2 type cytokines while miR-27a showed the same phenotype by targeting interferon regulatory

factor 4 (IRF4) and peroxisome proliferator-activated receptor gamma (PPARγ) (82). Finally, the crosstalk between miR-21 and the P13K/ERK/NF-κB axis, elicited by activation of CSF-1R pathway, has been also identified as a further mechanism adopted by macrophage to suppress the inflammatory phenotype and promote the expression of M2 marker genes (83)CSF1-ETS2-induced microRNA. Moreover, intraperitoneal injection of mice with a microRNA-21 inhibitor increases the recruitment of inflammatory monocytes and enhances the peritoneal monocyte/macrophage response to LPS (84). Collectively, these findings strongly support the role of microRNA as molecular determinant of macrophage plasticity, by participating in key feedback loop mechanisms to sustain or impair the expression of M2 signature genes, with the consequent redirection of macrophage polarization in accordance with the microenvironmental signals perceived.

4.5.2 Modulation of primed and tolerance state of macrophages by microRNA—

As we already discussed, establishment of macrophage priming and tolerance state is strictly dependent on the nature and intensities of external stimulants. Major outstanding questions about the molecular mechanism responsible for the opposing effects of endotoxin priming and tolerance are still partially unsolved. However, evidence provided so far strongly support a role for microRNA in the broad reprogramming of macrophages, where they act as molecular “rheostat”, able to switch from pro- to anti-inflammatory response (84; 85). They 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 signalling (86). Differential expression of NF-κB family members occurs in response to different doses of LPS and dynamic regulation of NF-κB pathway have 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, which expression is regulated by NF-κB (88; 89). In a pioneer study published in 2006, Baltimore’s group demonstrated the existence of a negative feedback circuit in which NFκB-induced the transcription of miR-146a, that in turn inhibited the NF-κB pathway, by targeting the two adaptor proteins: tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1) (88). Further studies correlated the impairment of NF-κB activity and the decreased production of proinflammatory cytokines observed 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, rapidly upregulated by NF-κB in macrophages primed with several TLR ligands and type I interferons (89; 92; 93). Interestingly, miR-155 is central component of multiple feed-forward networks that are implicating in dictating the duration and the intensity of the inflammatory response as well as macrophage sensitivity to LPS response. More precisely, miR-155 expression initiates and amplifies the inflammatory signal and the antiviral innate immunity by directly inhibiting the expression of negative regulators of the TLR signalling, including suppressor of cytokine signalling-1 (SOCS1) (94) and Src homology-2 domain-containing inositol 5-phosphatase 1 (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 signalling inhibits the

expression of miR-155, thus establishing a negative feedback loop mechanism that self-limit the proinflammatory response of macrophage and have a significant impact in controlling ET, as reported in miR-155 knock-in mice, showing high levels of TNF and 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, also sustained by miR-21, another microRNA expressed in LPS-primed macrophage, that reduced activation of NF-κB and enhanced expression of IL-10 (96). Ultimately, induction of IL-10, led to an increased expression of SHIP1 (97). Of note, in addition to miR-155, AKT1 controls 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 confirmed the inhibition of TLR4 by let-7e and demonstrated the targeting of other component of the TLR signalling 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 late induced by TLR agonists *via* the IL-10 dependent regulatory loop, and counter-regulated by IFNγ (98). Interestingly, this is mirrored by multiple targeting of different component of TLR pathway and results in global downregulation of pro-inflammatory cytokines (98), thus representing a potent tool, used by macrophage cells to switch off the inflammatory response in a timely manner. Moreover, high levels of 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 to point out that a dualism in the expression and/or function of members of the same family of microRNA have been reported. miR-125a/b and miR-146a/b families of microRNA represent two distinct examples of such dichotomy. In the case of miR-125a and miR-125b, they have been shown to be oppositely regulated by LPS stimulus in macrophages and also exert opposing function in the context of macrophage-mediated inflammatory response. Differently from miR-125a, miR-125b levels decrease early in LPS primed macrophages. Enhanced expression of miR-125b induces a greater IFNγ response and sustain proinflammatory cell activation, by targeting IRF4, which promotes M2 macrophage polarization (99). Studies on miR-146a and miR-146b, instead, strongly suggest that miR-146a/b might operate as a relay system to buffer the expression of pro-inflammatory genes induced by TLR4 triggering. This hypothesis is supported by the demonstration that miR-146a and miR-146b isoforms 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 an anti-inflammatory role by downregulating the LPS receptor TLR4 and key adaptors/signalling molecules, including MyD88, TRAF6, and IRAK1 (85). Finally, the induction of miR-146b in monocytes tolerized by IL-10 and TGFβ and a functional role of miR-146b in ET has also been demonstrated (100).

Another interesting aspect of microRNA-mediated regulation of macrophage functions comes from a recent study, published in 2017, investigating the role of miR-511 in ET (101). More exactly, the evidence provided demonstrated that, differently from what previously shown in murine macrophages, where the miR-511-3p mature form is functional and

important for M2 polarization, the miR-511-5p is the most abundant strand of miR-511 has been shown to act as intracellular mediator of GC and TGFβ (101). Indeed, its expression, 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 responsible for GC and TGFβ-mediated inhibition of pro-inflammatory cytokines production observed in endotoxin tolerant monocytes (101).

Altogether, this evidence demonstrates the capability of microRNA to modulate the duration and the magnitude of the innate immune response, participating as integral components of feedback loop regulatory mechanisms, which 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. Macrophage 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. Macrophage adaptability underlies their role in atherosclerosis and cardiovascular pathology (19; 20; 104; 105), neurodegeneration (18), autoimmunity and autoinflammation (e.g. (106) and cancer. Here we will focus on cancer because of our background and because it has served as a paradigm for macrophage plasticity in a disease characterized by dynamic evolution and a Darwinian microenvironment. Previous reviews on TAM will provide a framework for this section which will be largely focused on selected more recent advances (32; 107–111). In general, macrophages in cancer are double edged swords with the capacity to exert pro- and anti-tumor activity depending on the balance of a number of signals, including cytokines, chemokines, antibodies and myeloid checkpoints (Fig.4)

5.1 Origin

It has long been held that TAM originate from circulating monocytes (32; 112). As discussed above, evidence in mice and humans suggest a dichotomous origin of tissue macrophages, from embryonic and from adult circulating myeloid precursors (113). This fundamental paradigm shift raised the general issue of the embryonal versus hematopoietic origin of TAM and of its functionalal relevance. In a mouse mammary carcinoma model, tumor growth was associated with loss of tissue resident cells and replenishment of the TAM component by monocytes (114). As it may have been expected on the basis of ontogeny (see above), microglia persisted in murine gliomas (115) resulting in infiltration of macrophages of mixed origin. In a recent interesting study, macrophages of embryonic origin persisted in 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 type2 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 (PanIN) IL-13 released by Tuft cells and PanIN lead to the accumulation of M2-like cells which 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 in ECM.

In a number of mouse tumor models, circulating monocytes were the main precursors of TAM (32; 109; 114; 119). In humans, in the 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 TAM accumulation of macrophages of different origin in different human tumors. At this stage one could assume that at least in some human cancers mononuclear phagocytes of different origin coexist. However, based on results obtained under physiological conditions, it is tempting to speculate that 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 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 evasion from immune clearance (120). The Hippo pathway was found to underlie macrophage recruitment to the tumor-initiating cell niche. 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 interaction with cancer stem cells (CSC) (32) suggest that macrophages are involved in early steps of carcinogenesis and in providing a nurturing niche for CSC. In agreement with a classic concept (32; 109) macrophages promote invasion and metastasis (122). Therefore, evidence suggests that macrophages contribute to the various stages of progression, from initiation to formation of distant metastasis.

5.3 Adaptation to the TME

In the 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, 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 in turn this potentiates tumor growth (127). TME is characterized by an acid pH. Acidosis in the TME has recently been shown to induce macrophages with regulatory function which promote immune evasion. Thus, mononuclear phagocyte intrinsinc and extrinsic metabolic characteristics contribute to shaping TAM function (128).

In selected tumors, such as mammary carcinoma, 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 (e.g. (129), though the clinical significance of this

observation remains to be defined. Dectin-1 is a macrophage he macrophage receptor augmented by IL-4. The tetraspan MS4A4A molecule was recently shown to be expressed by a subset of TAM and to intercat 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 pattern recognition molecules 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 metabolicm (CO); enzymes involved in aminoacid metabolism (IDO an arginase); prostaglandins; triggers of checkpoint blockade in T cells and NK cells such as PD-L1 and VISTA (32; 111). Triggers of the immunosuppressive function of TAM 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 PDAC phagocytosis of dying tumor cells associated with the LC3 autophagy pathway and, unexpectedly, by antibodies in antibody-dependent cellular phagocytosis (ADCP) has been reported to drive the immunosuppressive function of TAM (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 TAM diversity

Early transcriptional profiling studies investigated TAM as a whole population without taking diversity into account (34). Subsequent investigations revealed that within the same mouse and human TAM population macrophages with different phenotypes coexist (142–144). Hypoxia was identified as one determinant of regional differences in TAM phenotypes. High dimensional single cell analysis using CyTOF and RNA sequencing has added a new dimension to dissection of myeloid cell diversity in tumors (136; 145–147). In particular in lung non-small cell lung cancer (NSCLC) single cell high dimensional analysis revealed differences between TAM and normal tissue macrophages and the presence of several cell clusters (145; 147). Interestingly these appeared part of a continuum in a large scale analysis (147) and the overall picture was consistent with M2/M2-like polarization (147). Therefore these results suggest diversity and a common theme of regulatory/immunosuppressive function (136; 145–147).

5.5 Macrophage reeducation

While in established progressing tumors macrophages are a component of cancer enhancing inflammation, mononuclear phagocytes have the potential to mediate anticancer activity (32; 148) (Fig.4). Rewiring of 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 mAb 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 (e.g. (149; 155–158)). Myeloid cells in tumors are a major source of triggers of checkpoint blockade 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 TAM has recently been shown to act as a negative signaling molecule in mononuclear phagocytes (149). Blocking the PD-L1 pathway of negative regulation resulted in activation of the antitumor potential of TAM (149).

Disease hyperprogression has been described in a few patients treated with PD1-PD-L1 checkpoint blockade immunotherapy. Circumstantial clinical and experimental evidence suggested that Fcg receptor engagement and TAM reprogramming was 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 signal regulatory protein α (SIRPα) present on the macrophage surface and it plays a homeostatic role in disposal 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 (DLBCL) refractory to treatments including anti-CD20 (161). These clinical results are consistent with preclinical data showing that blocking the CD49-SIRPα axis activates macrophage mediated ADCP and act 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 (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 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 manifestation macrophages are major players of a wide range of diseases ranging from autoimmunity to cardiovascular pathology to neurodegenerative disorders to cancer. The latter has served as a paradigm of macrophage plasticity and was chosen for analysis here. The realization of the different ontogenetic origin of mononuclear phagocytes raises the still largely unanswered question of their actual differential role embryo-derived versus bone marrow derived cells in human pathology. High dimensional profiling of macrophages has added a new dimension to understanding of macrophages diversity. Translation of diversity into clinically useful signatures and biomarkers remains a challenge.

Under many conditions, macrophage infiltration and functional biomarkers have prognostic significance, as illustrated by TAM and myeloid cells in response to checkpoint blockade. Here the challenge is moving from prognosis to prediction of response 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 to innovative therapeutic approaches. The encouraging initial results with CD47 blocking mAb may herald a new era of myeloid checkpoint immunotherapy with broad trans-disease significance (148).

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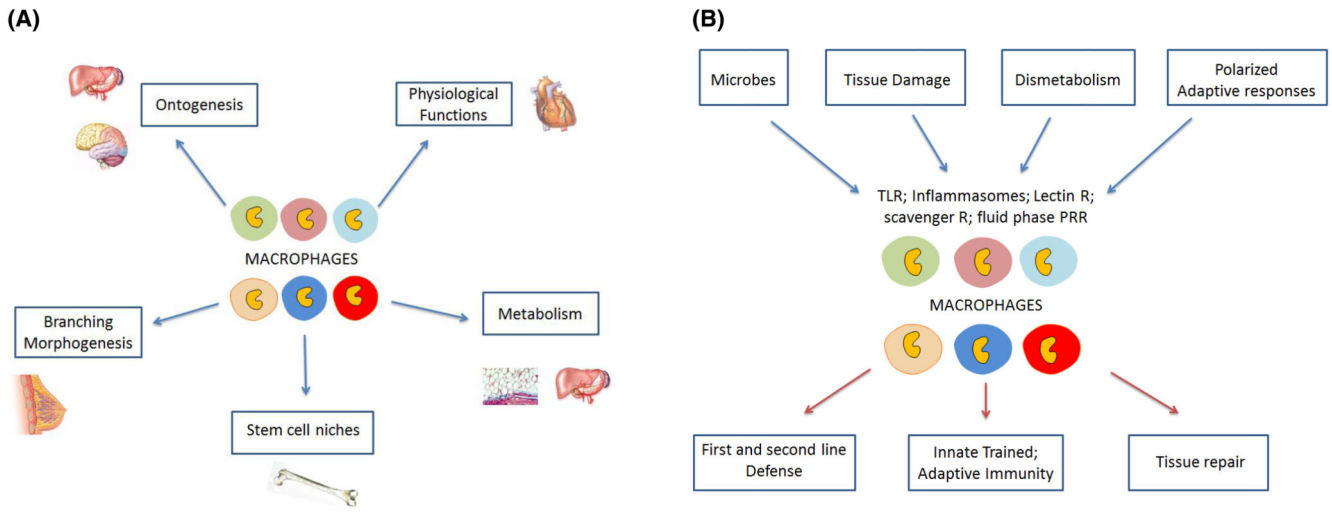

Figure 1.

Homeostatic functions of macrophages (A) and response to environmental perturbations (B) to restore homeostasis. Selected organs or tissues and homeostatic functions are presented. R stands for receptor.

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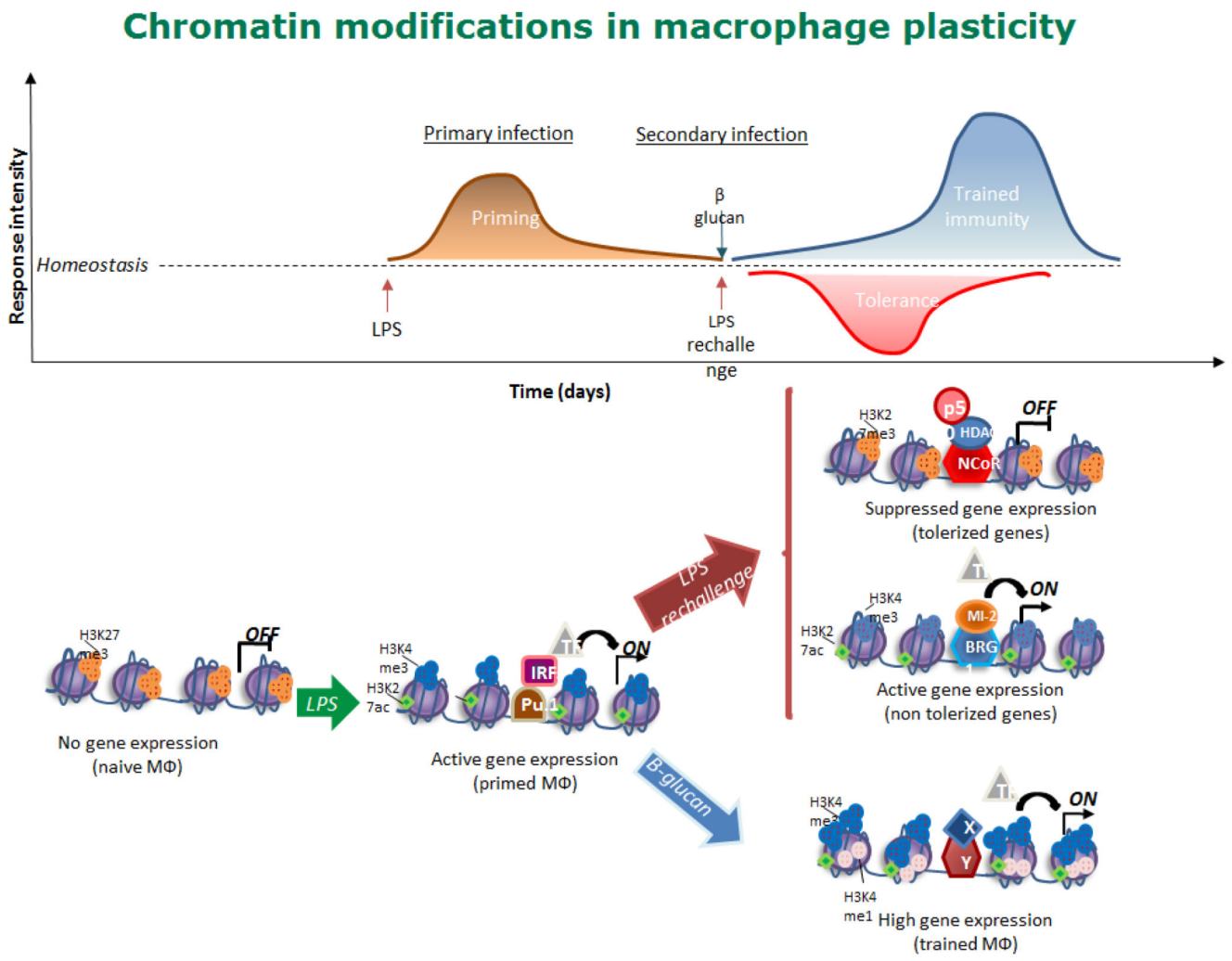

Figure 2.
Chromatin modifications underlying priming, tolerance and training.

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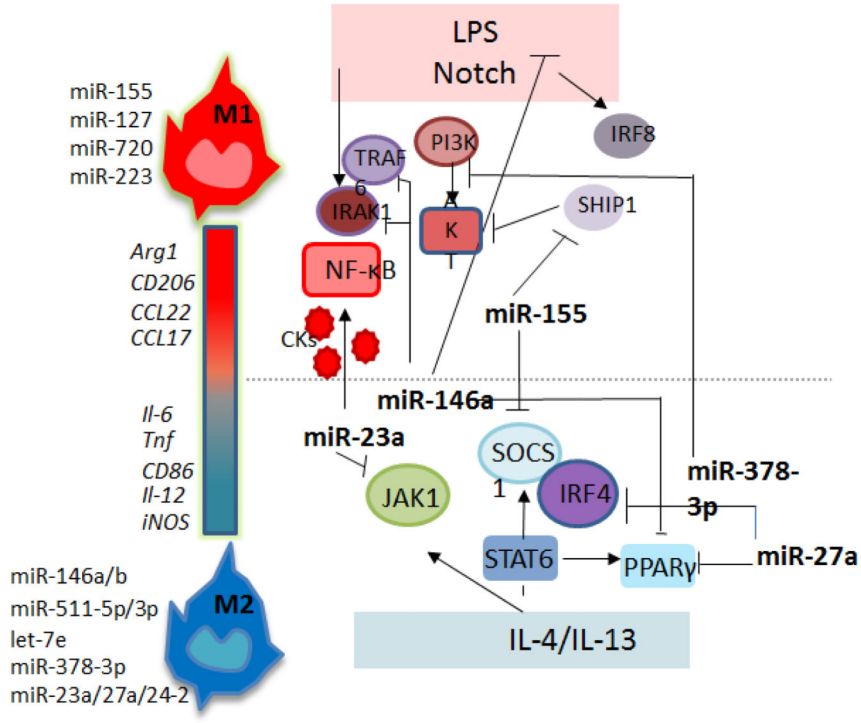

Figure 3.
MicroRNAs in the regulation of macrophage plasticity.

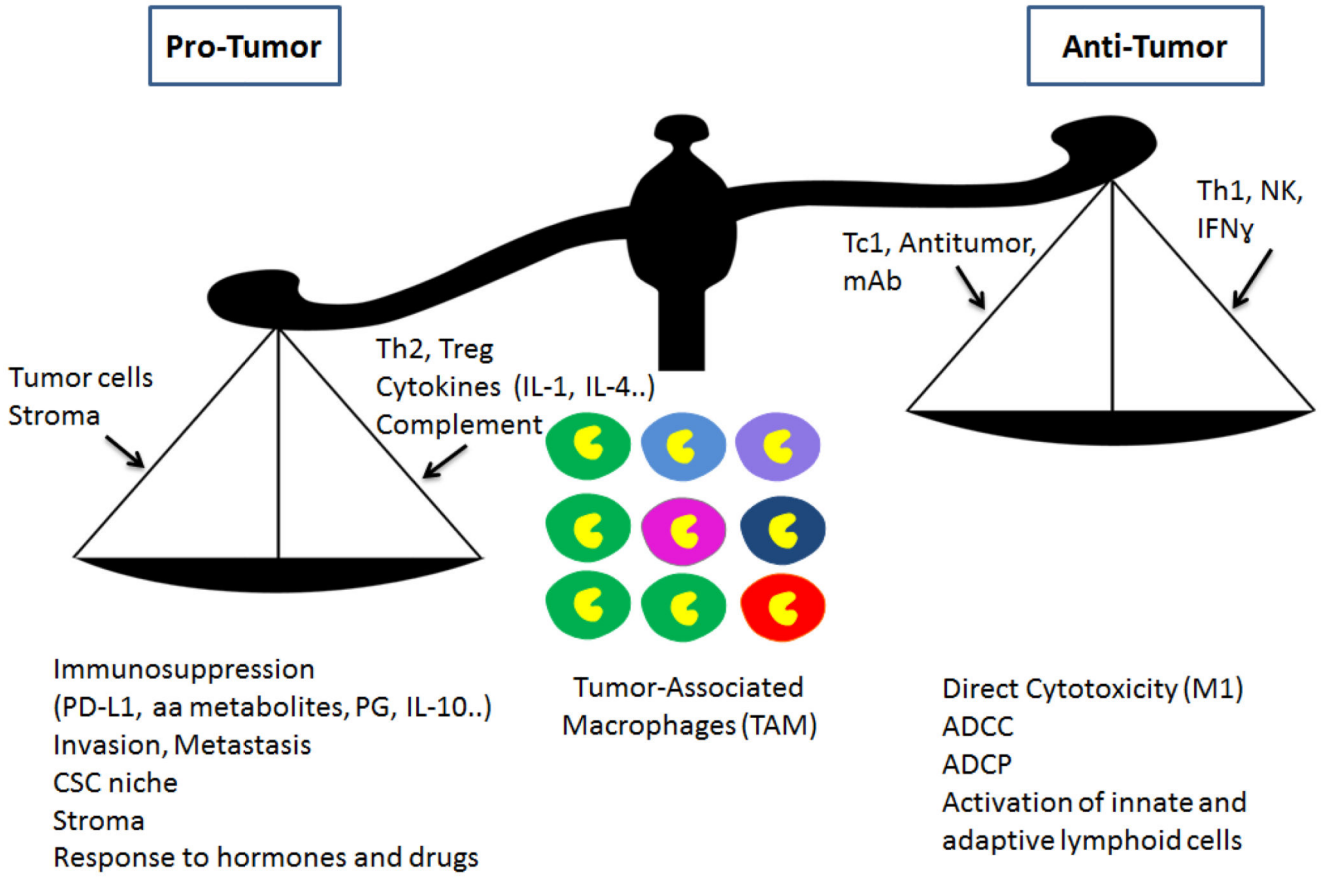

Figure 4.

Macrophages as double edged swords in the regulation of tumor progression and response aa, aminoacid, PG, prostaglandins (A) and general response to perturbation (B).

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