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The M1 and M2 paradigm of macrophage activation: time for reassessment

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Abstract

Macrophages are endowed with a variety of receptors for lineage-determining growth factors, T helper (Th) cell cytokines, and B cell, host, and microbial products. In tissues, macrophages mature and are activated in a dynamic response to combinations of these stimuli to acquire specialized functional phenotypes. As for the lymphocyte system, a dichotomy has been proposed for macrophage activation: classic vs. alternative, also M1 and M2, respectively. In view of recent research about macrophage functions and the increasing number of immune-relevant ligands, a revision of the model is needed. Here, we assess how cytokines and pathogen signals influence their functional phenotypes and the evidence for M1 and M2 functions and revisit a paradigm initially based on the role of a restricted set of selected ligands in the immune response.

Introduction

The concept of classic and alternative activation, also termed M1 and M2 to mimic Th cell nomenclature, has become increasingly broad and overinterpreted, hindering the understanding of pathogenesis and possible manipulation. Although there is evidence that many stimuli combine to determine the phenotype of macrophages, our view of this complex process has become too bipolar.

Macrophages evolved in simple multicellular organisms to perform phagocytic clearance of dying cells in development and adult life, and to protect the host through innate immunity, both as resident tissue macrophages and monocyte-derived recruited cells during inflammation. The development of acquired immunity with reciprocal interactions between macrophages and activated T and B lymphocytes provided novel levels of regulation and acquisition of enhanced antimicrobial resistance. The role of Th1-derived interferon-gamma (IFN-γ) in cell-mediated immunity to intracellular infection and of interleukin-4 (IL-4) (Th2) in extracellular parasitic infection gave rise to the concept of analogous M1 and M2 macrophages, now extended to a wider range of immunomodulatory agents and trophic functions.

In this review, we discuss signaling and genetic and functional signatures acquired during maturation and activation and consider how they fit the current M1/M2 model of macrophage polarization. Growing information indicates that recognition receptors, cytokines, and the signaling and genetic programs behind them control every aspect of cell activation, pointing to the need to recognize a broader functional repertoire for macrophages.

M1-M2 concept: background

Because macrophages are key modulator and effector cells in the immune response, their activation influences and responds to other arms of the immune system. In 1986, Mosmann, Coffman and colleagues put forward

the hypothesis that two subsets of helper T cells could be distinguished by the cytokines secreted after T lymphocyte activation, mediating distinct regulatory and effector functions [1]. Coffman recounts that the hypothesis derived from separate studies to answer the following questions: "are there T helper cells analogous to the classes of antibody made by B cells?" and "how are allergic responses, especially the immunoglobulin E (IgE) class of antibody, regulated?" [2]. These questions are implicitly relevant for infective diseases, in which intracellular and extracellular pathogens induce IgG vs. IgE responses, respectively, and macrophages deal with the infection, but also in type I and type II immune diseases in which macrophages contribute to tissue damage and pathology.

The term macrophage activation (classical activation) was introduced by Mackaness in the 1960s in an infection context to describe the antigen-dependent, but non-specific enhanced, microbicidal activity of macrophages toward *BCG* (bacillus Calmette-Guerin) and Listeria upon secondary exposure to the pathogens [3]. The enhancement was later linked with Th1 responses and IFN-γ production by antigen-activated immune cells [4] and extended to cytotoxic and antitumoral properties [5,6]. At the time, the effect on the macrophages of the Th2 arm of immunity leading to IgE and extracellular parasite protection and allergic responses remained unclear. The discovery that the mannose receptor was selectively enhanced by the Th2 IL-4 and IL-13 in murine macrophages, and induced high endocytic clearance of mannosylated ligands, increased major histocompatibility complex (MHC) class II antigen expression, and reduced pro-inflammatory cytokine secretion, led Stein, Doyle, and colleagues to propose that IL-4 and IL-13 induced an alternative activation phenotype, a state altogether different from IFN-γ activation but far from deactivation [7,8].

While investigating the factors that regulate macrophage arginine metabolism, Mills and colleagues found that macrophages activated in mouse strains with Th1 and Th2 backgrounds differed qualitatively in their ability to respond to the classic stimuli IFN-γ or lipopolysaccharide (LPS) or both and defined an important metabolic difference in the pathway: M1 macrophages made the toxic nitric oxide (NO), whereas M2 macrophages made the trophic polyamines [9]. They proposed that these be termed M1 and M2 macrophage responses, although this model dealt more with the predisposition of macrophages to develop specific phenotypes, it relied on the transforming growth factor-beta (TGF-β)-mediated inhibition of inducible nitric oxide synthase (iNOS) and was independent of T and B cells.

Bona fide evidence of *in vivo* macrophage alternative activation, equivalent to the observation of Mackaness for intracellular pathogens, came from work done by Allen, de Baetselier, Brombacher, and colleagues in parasite infection, which elicits a strong IgE and Th2 response; a recent review provides a more comprehensive functional perspective revealing heterogeneity in the response, depending on the nematode, the tissue, and type of macrophage [10].

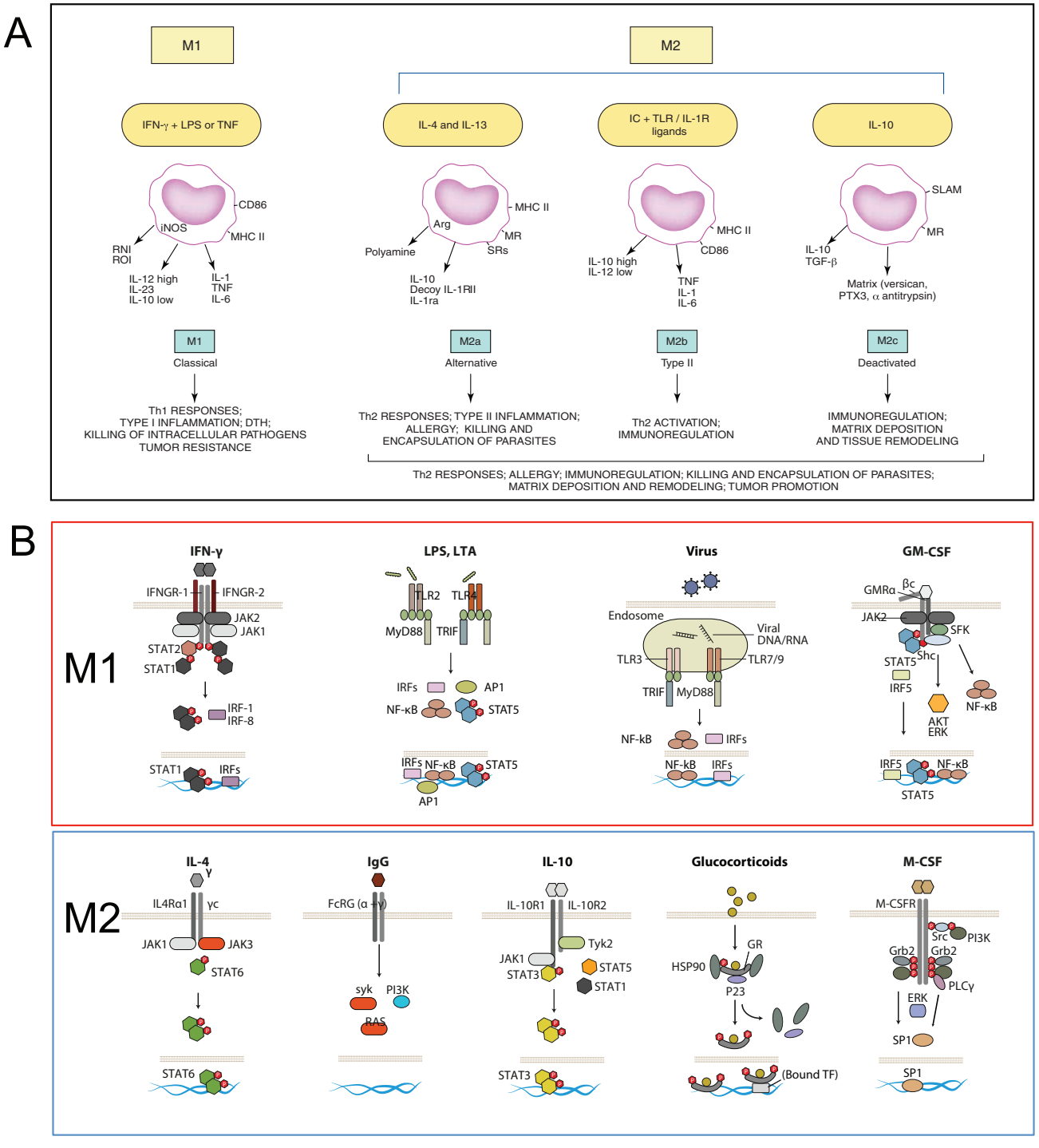
Until here, the questions were in tune with the Th1 and Th2 context. However, other cytokines and factors, such as IL-10, TGF-β (today recognized regulatory T [Treg] products), and glucocorticoids did not fit clearly in the context of the Th1 Th2 response and nonetheless seemed to elicit similar phenotypes in macrophages, with reports showing upregulation of mannose receptor, induction of IL-10 itself, and apparent antagonism to classic stimuli with downregulation of inflammatory cytokines and dampening of reactive nitrogen intermediate and reactive oxygen intermediate killing mechanisms. To integrate the phenotypic similarities and differences, Mantovani and colleagues grouped the stimuli in a continuum between two functionally polarized states, based on their effects on selected macrophage markers, termed M1 (IFN-γ combined with LPS or tumor necrosis factor [TNF]) and M2 (IL-4 [M2a], IL-10, and GCs [M2c]) (Figure 1A) [11]; activation induced by Fc receptors and immune-complexes, described by Mosser, was termed M2b. This careful categorization also made distinctions between M2 groups, such as "product of Th2 activation", "pro-Th2 activation", and "immunoregulation". Later, findings regarding granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) effects in macrophages led to the independent inclusion of these as M1 and M2 stimuli, respectively [12].

The current classification of macrophage immune activation is challenging because two very distinct aspects are considered: the *in vitro* effects of selected immune-related ligands on the phenotype of macrophages and *in vivo* evidence for distinct subsets of macrophages in disease, comparable to polarized B- and T-cell responses. The main limitations of the current view are, first, it ignores the source and context of the stimuli; second, the M1 and M2 stimuli do not exist alone in tissues; and, third, macrophages may not form clear-cut activation subsets nor expand clonally. Next, we discuss these and other aspects of the macrophage activation paradigm based on published evidence.

Grouping of M1 and M2 stimuli: need for immunological contextualization

The definition of M1 and M2 macrophage polarity derives from the pre-genomic era, when a few markers

Figure 1. The M1/M2 paradigm, origin, and molecular basis



(A) Mantovani and colleagues [11] proposed an M1-M2 macrophage model, in which M1 included interferon-gamma (IFN-γ) + lipopolysaccharide (LPS) or tumor necrosis factor (TNF) and M2 was subdivided to accommodate similarities and differences between interleukin-4 (IL-4) (M2a), immune complex + Toll-like receptor (TLR) ligands (M2b), and IL-10 and glucocorticoids (M2c). Diagram reproduced with the permission of Elsevier. **(B)** The signaling behind the effects of M1 and M2 stimuli in macrophages has gained clarity in recent years. Here, we highlight receptors and key signaling mediators in common and distinct pathways, explained in the text. The diagram includes granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) as M1 and M2 stimuli.

were considered to establish differences and similarities in macrophage responses to stimuli. The original definition took into account the possible context of the stimuli, but current generalized views lack this perspective. Furthermore, updated knowledge of cytokine signaling, the role of cytokines in the development of the hematopoietic system and in disease models with genetically modified mice and transcriptomic and proteomic analysis reveal a far more complex picture and challenge the current grouping. In the next section, we discuss the different role of stimuli considered in the M1 and M2 paradigm and highlight signaling pathways and gene expression singularities between the M1 and M2 stimuli.

M1 stimuli

The M1 stimuli are grouped according to their ability to induce prototypic inflammatory responses and markers, but their source, role, receptors, and signaling pathways differ substantially. We discuss, as examples, three of the main M1 stimuli recognized today. IFN-γ is the main cytokine associated with M1 activation and the main Th1 cell product. Other cells, such as natural killer (NK) cells and macrophages, themselves have been shown to produce the cytokine. The IFNGR-1 and IFNGR-2 chains form IFN-γ receptor (Figure 1B). The receptor recruits Janus kinase (Jak)1 and Jak2 adaptors that activate STAT1 (signal transducers and activators of transcription1) and interferon regulatory factors (IRF), such as IRF-1 and IRF-8; for a recent comprehensive review, see [13]. IFN-γ controls specific gene expression programs involving cytokine receptors (CSF2RB, IL15 receptor alpha [RA], IL2RA, and IL6R), cell activation markers (CD36, CD38, CD69, and CD97), and a number of cell adhesion molecules (intercellular adhesion molecule 1 [ICAM1], integrin alpha L [ITGAL], ITGA4, ITGbeta-7 [B7], mucin 1 [MUC1], and ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 [SIAT1]). The major mediators of IFN-γ-induced signaling, STAT1, JAK2, and IRF1, and regulators cytokine inducible SH2-containing protein (CISH), N-myc-interactor (NMI), protein tyrosine phosphatase, receptor type, C PTPRC, protein tyrosine phosphatase, receptor type, O (PTPRO), and suppressor of cytokine signaling 1 (SOCS1) are also under the control of the cytokine [14]. IFN-γ is included in combination with LPS in the M1/M2 paradigm, and gene expression profiles of the combination are different from LPS or IFN-γ profiles alone [15,16]. Mice lacking IFN-γ or its receptors are viable and fertile and their steady-state macrophage numbers are normal [17,18]. Macrophages, however, show impaired production of antimicrobial products, and mice are susceptible to *Mycobacterium bovis* and *Listeria monocytogenes*. This defect is not important for prototypical Th1/M1 responses only: knockout (KO) mice are susceptible to protozoa, such as *Trypanosoma cruzi* [19], *Leishmania amazonensis* and *Leishmania major* [20], and *Cryptosporidium parvum* [21], as well as defense against some nematodes (e.g. *Litomosoides sigmodontis* [22], *Schistosoma mansoni* [23], and *Schistosoma japonicum* [24]). In humans, mutations resulting in the lack of expression of the receptor drive severe immunodeficiency (e.g. susceptibility to mycobacteria *M. avium; M. kansasii; M. chelonei, Salmonella typhimurium* and *S. paratyphi*) in patients with familial disseminated atypical mycobacterial disease [25].

Pathogens are recognized by pattern recognition receptors. The activation induced is part of the M1 group and also defined as "innate" activation [26]. Full bacteria induce gene programs similar to those of isolated Toll-like receptors (TLRs), and major parts of the pathogen profiles can be ascribed to TLR ligands, such as LPS, muramyl dipeptide, and lipoteichoic acid [16]. LPS is the best-studied M1 macrophage signal and is recognized by TLR4 (Figure 1B), although recent evidence shows that LPS can also be recognized by TLR4-independent mechanisms leading to inflammasome activation [27,28]. Conventionally, TLR4 activation induces MyD88 and MaL/Tirap (Toll-interleukin 1 receptor domain containing adaptor protein)-dependent pathways that lead to strong pro-inflammatory cytokine profiles (e.g. IFN-β, IL-12, TNF, IL-6, and IL-1β), chemokines (e.g. chemokine [C-C motif] ligand 2 CCL2, chemokine [C-X-C motif] ligand 10 [CXCL10], and CXCL11), and antigen presentation molecules, such as MHC members, co-stimulatory molecules, and antigen-processing peptidases. The profiles are controlled by nuclear factor of kappa light polypeptide gene enhancer (NF-κB), activator protein 1 (AP-1), IRFs, STAT1, and EGR (early growth response) family members, many of which participate in the IFN response [13]. Although there is a degree of overlap between LPS and IFN-γ gene profiles, similarities are not enough to consider the stimuli to be homologous. As for IFN-γ, the numbers of macrophages in TLR KO animals are normal, but their activation is defective and therefore survival to infection is severely impaired; for seminal and recent views, see [29-32]. In humans, genetic mutations in the TLR family have gained clarity, and as for mice, there is evidence for susceptibility to infection with mycobacteria, pneumococci, meningococci, malaria, and susceptibility to develop bacteremia [33].

Granulocyte macrophage colony-stimulating factor (GM-CSF) is the latest addition to the M1 category of stimuli. GM-CSF is produced by a variety of cells, including macrophages and parenchyma cells. The GM-CSF receptor

forms a dodecamer structure [34] and recruits JAK2, leading to the activation of STAT5, extracellular signal-regulated kinase (ERK), and V-Akt murine thymoma viral oncogene homolog 1 (AKT) as well as the nuclear translocation of NF-κB and IRF5 upon binding (Figure 1B) [35]. Many of these regulators are part of the IFN-γ and TLR signaling pathways. GM-CSF enhances antigen presentation, complement- and antibody-mediated phagocytosis, microbicidal capacity, leukocyte chemotaxis, and adhesion. GM-CSF induces monocyte and macrophage cytokine production of IL-6, IL-8, G-CSF, M-CSF, TNF, and IL-1β, but less than, for example, LPS. GM-CSF transcriptome analysis shows that GM-CSF regulates several known cell surface molecules (e.g. CD14, Fc fragment of IgG, high affinity Ia (FCγR1A), CD163, and nuclear receptor subfamily 1, group H, member 3 [NR1H3]) [36]. The GM-CSF KO has normal numbers of macrophages in some tissues but has defects in the maturation of alveolar macrophages and develops pulmonary alveolar proteinosis [37]. In humans, mutations in the GM-CSF receptor, especially in the common beta chain, lead to alveolar macrophage defects and proteinosis but also to malignancy [38,39]. As such, the main functions proposed for GM-CSF include regulation of hematopoietic cell proliferation and differentiation, and modulation of the function of mature hematopoietic cells. Other stimuli that share pro-inflammatory properties have been termed M1 (e.g. TNF, IL-1ß, and IL-6). This adds further heterogeneity to a group that already comprises T cells, bacterial products, and a lineage-determining cytokine.

M2 stimuli

The M2 group of stimuli arose from the initial IL-4 observations, and they are grouped mainly due to their ability to antagonize prototypic inflammatory responses and markers; however, as for M1 stimuli, their source, role, receptors, and signaling pathways differ. We discuss, as examples, five of the main M2 stimuli. IL-4 is produced by the Th2 cells, eosinophils, basophils, or macrophages themselves and is recognized by three different receptor pairs. IL-4Rα1 can pair with the common gamma chain (γc), enabling IL-4 binding, and with the IL13Rα1 chain, enabling IL-4 or IL-13 binding (Figure 1B). In addition, IL-13 binds to the IL13Rα2 chain, a controversial signaling receptor. Receptor binding of IL-4 activates JAK1 and JAK3. JAK activation leads to STAT6 activation and translocation. Other transcription factors involved include c-Myc and IRF4. IL-4 induces macrophage fusion and decreases phagocytosis. The IL-4 multispecies transcriptome includes transglutaminase 2 (TGM2), mannose receptor (MRC1), cholesterol hydroxylase CH25H, and the prostaglandin-endoperoxide synthase PTGS1 (prostaglandin G/H synthase 1), the transcription factors IRF4, Krüppel-like factor 4 (KLF4), and the signaling modulators CISH and SOCS1 [40]. IL-13 signatures are similar to IL-4 signatures but are not totally overlapping [41]. In IL-4 KO animals, the numbers of macrophages and maturation are normal, and defects appear in the immune response against nematodes and some viral infections; for recent reviews, see [42,43]. In humans, polymorphisms in the IL-4R have been associated with the development of asthma and atopy [44,45].

Another M2 category is the type II-activated macrophage defined by Mosser and classified M2b by Mantovani and colleagues. This represents the only example of crosstalk with the B cell. This is another combined state, similar to the M1 combination of IFN-γ + LPS, in which ligation of FcγRs on LPS-activated macrophages turns off IL-12 and induces IL-10 secretion in addition to upregulating antigen presentation and, importantly, promoting Th2 responses [46,47]. IgGs are recognized by the Fc gamma receptor family that includes the activatory FcγRI (CD64), the inhibitory FcγRIIA (CD32), FcγRIIB (CD32), and the activatory FcγRIIIA (CD16a) and FcγRIIIB (CD16b). CD32 seems to be crucial for the type II activation in human monocytes and macrophages [48]. FcR signaling involves spleen tyrosine kinase (Syk) and phosphoinositide 3-kinase (PI3K) activation [49], but details in macrophages and its interplay with Myd88 pathways need further study. Type II-activated macrophages are distinct from IL-4-activated macrophages and their gene expression profiles overlap only partially. FcR KO animals have normal macrophage numbers, but their opsonic phagocytic capacity is highly impaired [50]. In humans, genetic differences in FcRs contribute to autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis [50]. Curiously, IgE, more relevant for Th2 and antiparasitic responses, has not been implicated in this phenotype.

Glucocorticoids and IL-10 are included in the current M2 category, although they represent a very different type of stimulus. Glucocorticoid hormones secreted by the adrenal glands are metabolized by cellular enzymes in macrophages. Active glucocorticoids are lipophilic and diffuse through the membrane to bind the glucocorticoid receptor (GCR) alpha, leading to nuclear translocation of the complex (Figure 1B). The GCR complex binds DNA directly to promote/repress gene transcription or indirectly by interacting with transcription factors, such as NF-κB or AP-1. Expression analysis of glucocorticoid-stimulated monocytes showed induction of complement component 1 subunit A (C1QA), TSC22 domain family, member 3 (DSIPI), MRC1, thrombospondin 1 (THBS1), IL-10, IL1R2, and CD163 [51]. Long-term exposure drives different gene expression programs that interact with LPS and IFN-γ pathways in a complex and non-exclusively

antagonistic manner [52]. The profiles are altogether different from those induced by IL-4. Glucocorticoids affect monocyte adherence, spreading, phagocytosis, and apoptosis. Mice with GCR deficiency do not survive long after birth, because of respiratory failure, and alterations in receptor dimerization induce susceptibility to sepsis [53]. In humans, GCR polymorphisms in the genes are pleiotropic and have been involved in a variety of malignancies and inflammatory and autoimmune disorders [54].

IL-10 binds the IL-10 receptor, a dimer of IL10R1 and IL10R2 (Figure 1B). Receptor autophosphorylation leads to the activation of the transcription factor STAT3 and its binding mediates inhibition of pro-inflammatory cytokine expression. IL-10 is a Th2 product and potent inhibitor of Th1 cells [55]. IL-10 is produced by virtually all leukocytes. In macrophages, IL-10 is elicited in response to TLR activation, glucocorticoids, and C-type lectin signaling (e.g. DC-SIGN [CD209 molecule] and dectin 1 ligation). The macrophage transcriptome induced by IL-10 includes selected Fc receptors, the chemoattractants CXCL13 and CXCL4, and the recognition receptors formyl peptide receptor 1 (FPR1), TLR1, TLR8, and macrophage receptor with collagenous domain (MARCO) [56]. IL-10-deficient mice have normal macrophage numbers but develop inflammatory bowel disease following colonization of the gut with resident enteric bacteria [57] and show exaggerated inflammatory responses to parasites [58]. In humans, defects in the cytokine receptors are similar and involve colitis and exacerbated inflammation [59].

M-CSF, like GM-CSF, is a late addition to the paradigm and has been classified as an M2 stimulus. The M-CSF receptor is a tyrosine kinase transmembrane receptor (Figure 1B). M-CSF binding leads to receptor dimerization, autophosphorylation, activation of ERK, phosphatidylinositol 3-kinase, phospholipase C, and eventually Sp1 transcription factor nuclear localization. The transcriptional response to M-CSF includes transient gene clusters with overrepresentation of cell cycle genes (e.g. cyclins A2, B1, D1, and E1) and downregulation of human leukocyte antigen (HLA) members and stable gene clusters, including TLR7 and the complement C1QA/B/C subunits [15].

Reports generally focus on the differences between M-CSF and GM-CSF; a recent and comprehensive comparison of the response to GM-CSF and M-CSF by human and mouse macrophages shows 530 genes regulated in the same direction in both human and murine models [60,61]. M-CSF mutant mice show reduced levels of monocytes and selected macrophages and osteopetrosis [62]. Mutations in the M-CSF receptor in humans lead to myelodysplastic syndromes or acute myeloid leukemia [63], and the mutations have been associated with hereditary diffuse leukoencephalopathy [64], but no human patients with osteopetrosis secondary to M-CSF deficiency have been identified.

The M2 group includes very different stimuli that span four levels of recognition/response: a level in which the macrophage acquires matured phenotypes, a level in which the macrophage interacts with immune cells (eosinophils, basophils, and Th2 cells), a level in which the macrophage actually deals with the pathogen, and a resolution level.

We discussed signaling cascades elicited by current M1 and M2 stimuli, which are complex and include transient and stable gene signatures. Importantly, activation of macrophages is controlled not only by intracellular kinases and transcription factors. Other mechanisms, such as microRNAs (miRNAs) [65-69], enhancer RNAs [70-72], and epigenetic enzymes, control the activation landscape [73-78]. These are beginning to be elucidated in macrophages, and more information exists for the mouse. The research done on epigenetics does not cover the full spectrum of macrophage activation but does provide messages similar to the signaling pathways discussed above (i.e. specific mechanisms control different forms of activation, and these mechanisms are not fixed; for every acetylase or methylase, there is a counterpart ready to be activated). Before discussing our view of macrophage activation, we briefly discuss the evidence for M1/M2 activation in disease.

M1 and M2 in disease: lack of defined subsets

In vitro studies have contributed to our understanding of macrophage activation, and as discussed, KO animals for key cytokines and receptors have established a role for some of these in the development and maturation of macrophages, whereas others regulate activation and the tuning of the response. The activation signatures defined *in vitro* are highly influenced by factors that are often overlooked but important *in vivo* (e.g. maturation of the cell, adhesion, extracellular matrix composition, and chemoattractants). Translating *in vitro* results to disease poses a major problem because of the complexity of *in vivo* systems and the failure to mimic these conditions *in vitro* [79]. Defining specific M1 and M2 functions with cytokines or receptor KO is difficult because the genes are pleiotropic and expressed at different stages of macrophage development or in other cell types; development of conditional, macrophage-specific KOs will help to illuminate these functions. Whole genome studies have shown substantial differences between M1 and M2 activation programs in humans and mice, indicative of evolutionary plasticity among macrophages, yet adding difficulty to translation.

When it comes to infection, it is clear that macrophage responses to different pathogens are affected by virulence and evasion mechanisms. Non-infectious diseases in humans are not as homogeneous as in mouse models, and thus we are often looking at a collection of tissue and systemic conditions that lead to a common syndrome but with different macrophage phenotypes.

Because of the relationship with the Th1 and Th2 paradigm, macrophage M1 and M2 markers have been investigated in prototypic diseases (e.g. Th2 Asthma [80] and Th1 chronic obstructive pulmonary disease (COPD) [81]). Another area where macrophage profiles have been investigated is atherosclerosis [82] and tumors [83]. For a recent review, see [84]. The emphasis has always been to fit the profile of tissue macrophages in diseases to *in vitro* predictions, but the message is clear: *in vitro* models are unable to mimic the complex profiles observed in disease and, as such, the numbers of genes that can be confirmed is limited. Our feeling is that when it comes to tissue macrophages, we need to start with a fresh view. Importantly, because macrophages can develop mixed M1/M2 phenotypes in pathological conditions, we need to focus not only on populations but also at the single-cell level [85,86].

From a functional view, the main properties of macrophages are phagocytosis, endocytosis, secretion, and microbial killing, but chemotaxis, adhesion, and trophic functions are an integral part of their activation [11]. Because macrophages are able to perform all these activities in the steady state, M1 and M2 contribution to disease is, for the most part, modulation and tuning. As before with markers, functions are complex (e.g. phagocytosis involves a collaboration of multiple receptors as well as interactions with different particles). The discrimination and killing of microbes and host target cells are also incompletely understood. To date, M1- or M2-specific functions beyond M1-enhanced microbicidal and M2 antiparasitic defense have expanded to encompass metabolic, thermoregulatory, healing, and antiviral effects [10,42,43,87-90]. Recent evidence shows that M1 and M2 activation display differences but also overlapping effects that need clarification and a more dynamic appreciation of the activation process. In Table 1, we have summarized a few findings that exemplify the increasing complexity of the M1/M2 landscape.

Detailing M1 and M2 marker studies in particular human disease processes is beyond the scope of this review; we highlight some considerations:

(a) Rather than distinct macrophage populations, M1 and M2 signatures do not necessarily exclude each other and often coexist; the resultant mixed phenotype then

Table 1. Selection of M1 and M2 effects in macrophages

	M1 (IFN-γ)	M2 (IL-4/IL-13)
Functions elicited in macrophages		
Phagocytosis / endocytosis	-Increases phagocytosis of C. albicans [95] -Decreases Fc-mediated phagocytosis [96] -Decreases complement-mediated phagocytosis [97]	-Decreases phagocytosis of particles while increasing inflammatory cytokine production [98]
Autophagy	-Induces autophagy in TB infection [99]	-Decreases autophagy in TB infection [100]
Macrophage Fusion	-Increases fusion in combination with concanavalin A [101] -Induces fusion in alveolar macrophages [102]	-Induces fusion [103] -Inhibits IFN-γ-induced fusion [101]
Nitric Oxide	-Induces Mycobacteria killing via NO [104]	-Favours Arginase-1 vs. i-NOS, Arg1+ macrophages suppress Th2 inflammation and fibrosis [105]
Parasite killing and expulsion	-Mediates parasite killing via NO [106, 107]	-Although the cytokine is important for worm expulsion, the effect does not depend on macrophages [108]
Virus replication	-Inhibits replication of HIV at early pre-integration steps [109]	-Inhibits HIV replication at post-integration level [109]
Markers		
Human	CD64, IDO, SOCS1, CXCL10	MRC1, TGM2, CD23, CCL22
Mouse	CXCL9, CXCL10, CXCL11, NOS2	Mrc1, tgm2, Fizz1, Ym1/2, Arg1

The current M1 and M2 paradigm includes a variety of stimuli of different natures. This complicates the understanding of the contribution of adaptive immunity to the innate response, and the specialized functions that arise with activation. Here, we focus on IFN-γ and IL-4 effects. This table is not comprehensive, nor does it include other stimuli currently part of the M1-M2 paradigm. With the table we wish to highlight the distinction between M1 and M2 functions, whose features are not polar but are reflections of a more subtle process.

C. albicans, Candida albicans; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand ;iNOS, inducible nitric oxide synthase; IFN-β, interferon beta; IFN-γ, interferon gamma; MRC1, mannose receptor; NO, nitric oxide; NOS2, nitric oxide synthase 2; SOCS1, suppressor of cytokine signaling 1; TB, tuberculosis; TGM2, transglutaminase 2.

depends on the balance of activatory and inhibitory activities and the tissue environment.

(b) Pleiotropism of stimuli and lack of cell specificity of markers indicate that macrophage specialization rests in part on their ability to migrate and deliver specific functions where barriers have failed to stop infection.

(c) The role of M1 or M2 stimuli needs to be considered in their dynamic complexity, beyond the current bipolar dogma of IFN-γ as exclusively important for intracellular pathogens or IL-4 for allergy and extracellular parasitic defense. The multipolar interplay between these and many other signals requires further studies *in vivo* and *in vitro*.

Figure 2. A multipolar view of the macrophage activation paradigm from an immunological perspective

Growth and survival factors	**Lineage determining cytokines (CONVENTIONAL MATURATION)**		**Survival, recruitment and retention**	**Other**
	M-CSF R	GM-CSF R	Adhesion molecules Chemokines	VitD3, Retinoic acid, PPRgamma ligands
Lymphoid and myeloid Cytokines	**Classical and Alternative activation**		**Pro and antinflammatory**	
	IFN-γ	IL-4, IL-13	TNF, IL-6, IL-1ß	IL-10, TGF-ß,
Interaction with Pathogens	**Direct interaction**		**Humoral**	
	TLRs, NODs, NLRs, RLRs, Nucleic acid sensors		IgG, IgE, IgA	Complement, Lectins, Ficolins
Resolution	**Systemic mechanisms**		**Local mechanisms**	
	Glucocorticoids		ECM Proteoglycans, ATP and sugar nucleoCdes, Resolvins, Maresins, etc	

The cytokines and stimuli that we call M1 and M2 play different roles in the development, maturation, and activation of macrophages. Integration of these isolated stimuli is necessary to represent the complex changes that macrophages undergo during full activation. Lessons from basic immunology suggest that many of the mechanisms that affect innate and acquired immunity are underappreciated (for example, humoral pattern recognition receptors, inflammatory cytokines other than interferon-gamma (IFN-γ) and tumor necrosis factor (TNF)). Here, we focus on the levels of activation imposed by the immune system rather than proposing a new classification of macrophage phenotypes, which is currently under discussion internationally. We propose that the stimuli governing macrophage activation should be organized according to their role in the immune response, rather than in groups that overlook their individual differences, and highlight the fact that complex combinations should be assessed to understand the full repertoire of macrophages. We identify at least four levels: growth and survival factors, interaction with lymphoid and myeloid cytokines, interaction with pathogens, and resolution. In the first level, in addition to prototypic maturation signals, we add stimuli known to promote survival of monocytes. A second level is interaction with cytokines. Here, we place interleukin-4 (IL-4), and IFN-γ and other cytokines produced by lymphoid and myeloid cells. However, the contribution of non-hematopoietic cells cannot be ignored. The next level is that of interaction with pathogens directly or through humoral recognition receptors, such as lectins, ficolins, and the B cell-derived immunoglobulins. A final level of resolution we classify as systemic (e.g. glucocorticoids) or local, such as ATP, resolvins, and other mediators with general anti-inflammatory properties. The combinations of stimuli commonly associated with extreme phenotypes of cells are IFN-γ + lipopolysaccharide (LPS) or TNF, immune complexes + Myd88, and granulocyte macrophage colony-stimulating factor (GM-CSF) + IL-4, which in monocytes induce a dendritic cell-like phenotype. However, there are many other possible combinations that are not considered special cases. We plan to extend the potential of macrophages, taking into consideration other functionally relevant combinations.

Conclusions

Today, we know that, in addition to Th1 and Th2 cells, Treg cells and Th17 cells participate in the pathogenesis and resolution of disease and that these cells are able to display shades of activation within the general categories [91]. The Th1/Th2 paradigm has remained valuable in T lymphocyte heterogeneity and, to an extent, in the field of macrophage activation, but a reassessment is required to accommodate current findings.

We do not here propose a revised model or nomenclature for macrophage activation but present a view on what needs to be taken into account (Figure 2). Our opinion is that macrophages do not form stable subsets but respond to a combination of factors present in the tissue; we have, rather than subsets of macrophages, pathways that interact to form a complex, even mixed, phenotypes. A satisfactory paradigm needs to take into account at least four levels of recognition/response: first, one in which the monocyte survives and acquires matured phenotypes; second, a level in which the macrophage interacts with immune cells (NK and Th cells, eosinophils, and basophils); third, one in which the macrophage deals with the pathogen, and a final level, of resolution. Also, the M1-M2 paradigm is commonly associated with properties of mature macrophages, but activation takes place in the extended macrophage family, including monocytes [41,48,51,92], myeloid-derived dendritic cells [93,94] and multinucleated giant cells. In tissues, all these events combine to produce a resultant phenotype, and, though useful for the sake of understanding, any sort of hierarchy or order does not represent the biology of the cells. We require a dynamic view of this process to take into account the multiple elements in their systemic and local milieu and to define the kinetics, plasticity, reversibility, and memory of their responses in order to encompass the full functional range of activated macrophages. The process is highly complex, and for an improved understanding, considerably more information is required about macrophages *in vivo* and at the population level as well as at the single-cell level. Epigenetic, gene expression, and functional studies will help to elucidate these matters.

At present, the M1/M2 paradigm has provided a useful framework, especially for selected immune responses, but a more comprehensive classification is clearly required. This should guide an iterative research strategy from *in vitro* to *in vivo* studies and back to disease models in genetically defined mice, for example, to establish mechanisms and possible therapeutic targets for manipulation in human disease.

Abbreviations

AP-1, activator protein 1; C1QA/B/C, complement component 1 subunit A/B/C; CISH, cytokine inducible SH2-containing protein; c-Myc, V-Myc avian myelocytomatosis viral oncogene homolog; CXCL, chemokine (C-X-C motif) ligand; EGR, early growth response; ERK, extracellular signal-regulated kinase; FCγR, Fc fragment of IgG, high affinity; GCR, glucocorticoid receptor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IFNGR, interferon gamma receptor; Ig, immunoglobulin; IL, interleukin; IL-4Rα1, interleukin 4 receptor alpha 1; IRF, interferon regulatory factor; ITGB7, integrin beta-7; Jak, Janus kinase; KO, knockout; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; MRC1, mannose receptor; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor of kappa light polypeptide gene enhancer; NK, natural killer; SOCS1, suppressor of cytokine signaling 1; STAT signal transducers and activators of transcription; TGF-β, transforming growth factor-beta; Th, T-helper; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, T regulatory.

Disclosures

The authors declare that they have no disclosures.

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