

Macrophage activation and polarization

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1. ABSTRACT

Macrophages are widely distributed immune system cells that play an indispensable role in homeostasis and defense. They can be phenotypically polarized by the microenvironment to mount specific functional programs. Polarized macrophages can be broadly classified in two main groups: classically activated macrophages (or M1), whose prototypical activating stimuli are IFN γ and LPS, and alternatively activated macrophages (or M2), further subdivided in M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1 β or LPS) and M2c (IL-10, TGF β or glucocorticoids). M1 exhibit potent microbicidal properties and promote strong IL-12-mediated Th1 responses, whilst M2 support Th2-associated effector functions. Beyond infection M2 polarized macrophages play a role in resolution of inflammation through high endocytic clearance capacities and trophic factor synthesis, accompanied by reduced pro-inflammatory cytokine secretion. Similar functions are also exerted by tumor-associated macrophages (TAM), which also display an alternative-like activation phenotype and play a detrimental pro-tumoral role. Here we review the main functions of polarized macrophages and discuss the perspectives of this field.

2. INTRODUCTION

Macrophages play an indispensable role in the immune system with decisive functions in both innate and acquired immunity. In innate immunity, resident macrophages provide immediate defence against foreign pathogens and coordinate leukocyte infiltration (1). Macrophages contribute to the balance between antigen availability and clearance through phagocytosis and subsequent degradation of apoptotic cells, microbes and possibly neoplastic cells (1). Macrophages collaborate with T and B cells, through both cell-to-cell interactions and fluid phase-mediated mechanisms, based on the release of cytokines, chemokines, enzymes, arachidonic acid metabolites, and reactive radicals (1-3). Macrophage activation can be either pro-inflammatory or anti-inflammatory, contributing to tissue destruction or regeneration and wound healing. Thus, macrophages play an essential role in triggering, instructing and terminating the adaptive immune response, depending upon the functional phenotypes they acquire as a consequence of tissue-derived signals (4).

Historically T cells have been considered the main organizers of the immune response, while

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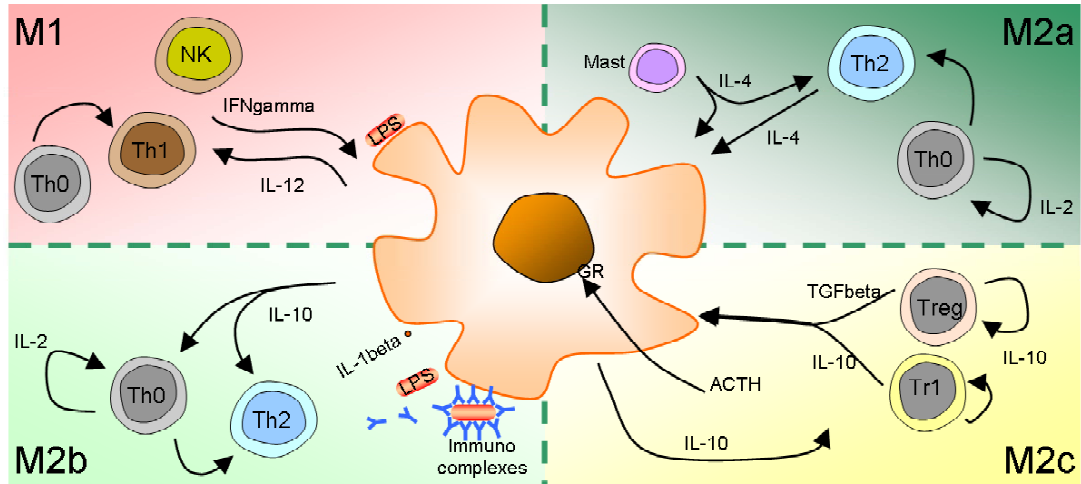


Figure 1. Macrophage polarization paradigm is in agreement with the type 1 type 2 response tenet. Macrophages are capable of displaying different functional phenotypes, many of which are antagonistic, under the influence of specific mediators. M1: Bacterial LPS in combination with IFN γ elicits the classical activation triggering an IL-12 IFN γ loop. M2a: IL-4 and IL-13 are both capable of eliciting alternative activated macrophages. M2b: Ligation of Fc receptors in the presence of Toll stimuli can induce a polarization antagonistic to M1 which promotes Th2 responses through an IL-10 circuit. M2c: Deactivation is required for the termination of inflammation and is elicited by IL-10, GC or TGF β .

macrophages have long been merely regarded as effector cells active during acute inflammatory responses and delayed type hypersensitivity reactions (5). In 1986 Mosmann, Coffman and collaborators reported that murine Th lymphocytes could be divided into Th1 and Th2 cells, based on their respective cytokine production profiles: IFN γ and IL-4 (6). In addition, they showed that these cytokines possess cross-regulatory properties and coordinate two fundamentally opposite immune response usually indicated as ‘type I - type II’ immune response (7, 8). Although excluded from this paradigm, the role of macrophages in this balance is being increasingly appreciated (5). In particular, it has been shown that macrophages are able to secrete either IL-12 or IL-10, crossregulatory cytokines crucial for the elicitation of IFN γ production and development of Th1 cells or IL-4/IL-13 secretion and Th2 cells proliferation, respectively (1, 5).

These preferential production of IL-12 or IL-10 set the basis for the M1/M2 polarization paradigm (3, 4, 9, 10), elsewhere defined as elicitation of functionally distinct macrophage populations, in response to the factors that dominate the inflammatory scene (11-13). The first clue for the existence of a macrophage polarization axis was the pioneering observation by Gordon and colleagues that macrophage exposure to IL-4 or IL-13 elicited an “alternative type of activation” with a distinctive phenotype different to the classical macrophage activation, known to depend on the Th1 and Natural Killer (NK) cells product IFN γ (1). Subsequently, Mosser and colleagues reported that ligation of Fc- γ receptors on IFN γ -primed macrophages blocked IL-12 synthesis and induced large amounts of IL-10, providing evidence for a third distinctive phenotype called “type II activation” (14, 15). In 1999, Goerdt and colleagues proposed a

classification of activation phenotypes based on grouping all activators other than IFN γ and LPS/microbes into a common alternative activation group (16). This classification overlooks important differences in the response to modulators such as IL-4, IL-13, IL-10, glucocorticoids (GC) and TGF β , and for this reason in 2002 we proposed an extended classification in which M1 polarization included the classical activation, obtained by stimulation with pathogen-derived LPS alone or in combination with IFN γ , while M2 polarization, mainly associated with antiparasitic and tissue repair programs, was subdivided in M2a or alternatively activated macrophages; M2b, corresponding to type II activated macrophages; and M2c, which includes heterogeneous macrophage deactivation stimuli (11).

3. MACROPHAGE CLASSICAL ACTIVATION

Classically activated or M1 macrophages develop in response to concomitant stimulation with IFN γ and microbial products such as LPS (12). IFN γ is the sole type II IFN and it is recognized by a IFN γ receptor (IFN γ R) comprised of two ligand-binding IFN γ R1 chains associated with two signal-transducing IFN γ R2 chains. (17). IFN γ is mainly secreted by Th1 and CD8 $^{+}$ cytotoxic lymphocytes, NK cells, and professional antigen-presenting cells, and to a less extent by B cells and NKT cells (Figure 1). LPS is the principal component of the outer membrane of Gram-negative bacteria, it is recognized by the plasmatic LPS-binding protein and delivered to a cell surface receptor complex constituted by CD14, the transmembrane signaling receptor toll-like receptor 4, and its accessory protein MD2 (18).

M1 macrophages display widespread morphology depending on their tissue location, but are accumulated by

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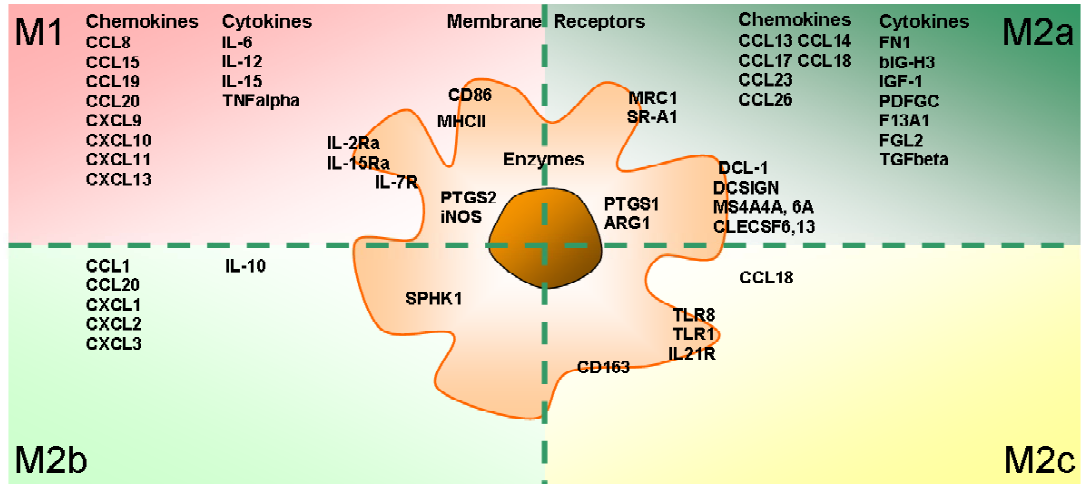


Figure 2. Macrophage polarization is associated with distinctive gene signatures. Macrophages exposed to the M1 or classic activation express cytokine receptors whereas M2a or alternative activated macrophages are characterized by abundant levels of non-opsonic receptors (e.g. the MRC1). M1 cells also have a higher ratio of CXCR3 ligands while M2a cells express CCR8 and CCR3 ligands. The less characterized populations M2b and M2c produce high amounts of CCL1 and CCL18, respectively.

their ability to secrete significant amount of proinflammatory cytokines, such as IL-1beta, IL-15, IL-18, TNFalpha, and IL-12, a covalently linked heterodimer formed by a light chain of approximately 35 kDa (p35 or IL-12alpha) and a heavy chain of approximately 40 kDa (p40 or IL-12beta) (19) (Figure 2). They also secrete the chemokines CCL15/HCC-2, CCL20/MIP-3alpha, and CXCL13/BCA-1(20), and the angiostatic INFgamma-responsive chemokines CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC, which coordinate NK and Th1 recruitment in type I immune responses through their activity on CXCR3 (4, 10, 21-24). In addition, M1 display elevated expression levels of MHC-II and costimulatory molecules CD80 and CD86, as well as low levels of MRC1 or Fcgamma RII (25). Functionally, M1 cells are characterized by enhanced endocytic functions and enhanced ability to kill intracellular pathogens (26, 27). This increase microbicidal activity is mediated by different mechanisms including restriction of iron and other nutrients for microorganisms, acidification of the phagosome, synthesis of ROI (28, 29), and release of nitric oxide (NO) from L-arginine by virtue of iNOS activity, a macrophage specific INFgamma-inducible isoform of NO synthase (30). Although well characterized in mouse, in humans NO detection has been difficult and reported only in very specific circumstances (30).

Macrophage can undergo activation supported by direct recognition of microbial Pathogen-Associated Molecular Patterns, such as LPS, through germ-line encoded Pattern Recognition Receptors (PRR) (31, 32), such as Toll-Like Receptors (TLRs) and Nucleotide Oligomerization Domain (NOD) receptors (33, 34). This activation phenotype, which has been referred to as innate activation, resemble to a certain extent the M1 activation, in that it is characterized by secretion of proinflammatory cytokines, such as IL-1beta, IL-6 and TNFalpha, and increased expression of costimulatory molecules. Different from M1 cells, innate activated macrophages do not show

overall increased phagocytic properties, although they express a different pattern of membrane receptors for the pathogen such as Marco receptor, a novel innate activation marker of macrophages (35). Innate activation is usually unable to fully develop into an M1 profile since typically TLR ligation induces expression of only low levels of p40 and is therefore insufficient to trigger production of the M1 biologically-active IL-12 (19). However, complete pathogens can induce functional IL-12 heterodimers, indicating that microbial stimulation can be considered a subgroup within the M1 polarization (36). Consequently, we envisage a sub-classification in M1a and M1b cells, to distinguish between classical and innate activated macrophages.

4. HETEROGENEITY OF MACROPHAGE ALTERNATIVE ACTIVATION

Originally described as macrophages activated by IL-4 (1) as opposite to the classical combination of INFgamma and LPS, alternative activated macrophages, now referred to as M2 cells, have then been described in a number of variants, depending upon the stimuli used to generate them. M2 cells are now regarded as a continuum of functionally and phenotypically related cells, with a critical role in type II inflammation and in the resolution and tissue repair phase, subdivided in M2a or alternatively activated macrophages, elicited by type II cytokines IL-4 or IL-13; M2b, corresponding to type II activated macrophages, obtained by triggering of Fcgamma receptors in the presence of a Toll receptor stimulus; and M2c which includes deactivation programs elicited by GC, IL-10 or TGFbeta (11).

M2a activation is obtained by stimulating macrophages with IL-4 or IL-13, which are mainly produced by Th2 cells, mast cells, and basophils (37) (Figure 1). IL-4 binds two receptor complexes: the high-affinity receptor complex constituted by the IL-4Ralpha

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chain and the IL-2R common gamma-chain, expressed to different degrees by T, B cells, mast cells and macrophages and the low affinity receptor complex constituted by a IL-4Ralpha/IL-13Ralpha1 heterodimer expressed by B cells, monocytes, endothelial cells, and fibroblasts. IL-13 instead binds to three receptor complexes: the low-affinity IL-13R-alpha1 monomer, the high-affinity IL-13Ralpha2 monomer, and IL-13Ralpha/IL-4Ralpha heterodimer(38). The biological actions of IL-4 and IL-13 on monocytes and macrophages were for long considered to be merely anti-inflammatory, based on the downregulation of proinflammatory mediators such as IL-1beta, TNFalpha, IL-6, IL-8, IL-12, GM-CSF, IFNgamma, CCL2/MCP-1, and superoxide anions production. In addition, IL-4 decreases the expression of important membrane molecules, i.e CD14 and CCR5 (39). In recent years a number of studies have shown that IL-4/IL-13 also regulate key molecules such as MHC-II, beta2 integrins, the chemokines CCL22/MDC (11) and CCL18/AMAC-1, tissue-type plasminogen activator and metalloproteinase 1 (11, 40, 41). In a recent genome-wide M1/M2a transcriptional comparison study we confirmed the upregulation induced by IL-4 of several scavenger receptors and C-type membrane lectins, including MRC1, SR-A, Dectin-1, DC-SIGN, DCIR (CLECSF6), DCL-1, and CLECSF13 (20) (Figure 2). M2a cells also express fibronectin 1 (FN-1) and matrix associated protein betaG-H3, which promote fibrogenesis (42), and the coagulation factor XIII and insulin like growth factor 1 (IGF-1), which provide signals for tissue repair and proliferation (43). IL-4/IL-13 also affect the IL-1beta system by enhancing the production of IL-1R receptor antagonist (IL-1ra) and the decoy IL-1beta type II receptor (IL-1RII)(44, 45) which synergistically interfere with the IL-1beta biological activity (46). Moreover, we recently reported that IL-4/IL-13 also downregulate caspase 1, which is responsible for the proteolytic cleavage of pro-IL-1beta into its active mature form (47, 48). The reduction in caspase 1 activity is expected to have similar effects on the processing of IL-18 (49). From mouse studies it is known that, differently from M1 cells, M2a cells do not express iNOS, but express high levels of arginase 1 (ARG1), which skews the metabolic pathway of NO to the production of proline. Consequently, these cells fail to produce NO and are significantly compromised in their microbicidal ability for intracellular pathogens (50, 51), but they synthesise polyamine and proline that stimulate cell growth, collagen formation, and tissue repair (51). In human *in vitro* polarized macrophages this metabolic signature is absent (52, 53). M2a macrophages express high levels of the chemokines CCL13/MCP-4, CCL8/MCP-2, and CCL26/eotaxin-3, which coordinate the recruitment of eosinophils, basophils and some polarized Th2 cells, through the activity on CCR3 (10) and are involved in proangiogenic networks (21-24). *In vitro* alternatively activated macrophages also express the extracellular matrix-remodeling enzyme metalloproteinase (MMP)-12, which has been demonstrated to be selectively expressed in the late, but not early stages of tuberculosis (54).

In 2002, Mosser and colleagues reported a macrophage phenotype clearly distinct from either M1 and

M2 cells, and termed it “type II” macrophages. (55). These cells, which we later renamed M2b cells, are elicited by stimulation with LPS or IL1beta, through TLR4 or IL-1R (both members of the IL-1R/Toll-like receptor superfamily) and immune complexes recognized by receptors for the Fc portion of immunoglobulin G (FcgammaR) (14, 56) (Figure 1). M2b cells are the functional converse of M1 cells, being characterized by low IL-12 and high IL-10 production, a cytokine profile which favours the development of type II adaptive immune responses (15). In terms of B cell responses, M2b cells efficiently sustain antibody production, the majority of which are of the IgG1 isotype, consistently with a type II IgG class switch (55). M2b are distinct from M2a since they produce much higher levels of IL-10, but also produce significant amounts of TNFalpha, IL-1beta, and IL-6, indicating that these cells are not anti-inflammatory *per se*. Interestingly, a selective production of CCL1/I-309, the sole CCR8 agonist, has been demonstrated, and might be of relevance for the recruitment of T regulatory cells (1, 10, 11, 16) (Figure 2). M2b are also clearly distinct from M2a cells in terms of the expression of the sphingosine kinase 1 (SPHK1) enzyme, which catalyzes the production of sphingosine-1 phosphate from sphingosine (57). SPHK1 is also highly expressed in M1 cells but downregulated in M2a cells (20). Although further evidence in support of these observations are required, this expression profile could translate in production of high levels of sphingosine-1 phosphate by M1 and M2b cells, but not M2a cells.

The M2c category include cells stimulated with IL-10, TGFbeta, or glucocorticoids, a functionally heterogeneous group of cells that once more testify for an over-simplification of this classification. All in one, M2c are usually regarded as deactivated macrophages in that their common hallmark is the downregulation of proinflammatory cytokines, the increased debris scavenging activity, and the carry over of a prohealing functional program (Figure 1). IL-10 is produced by macrophages, T cells, B cells, and a variety of other cell types including mast cells and keratinocytes, as part of the homeostatic response to infection and inflammation. It plays a critical role in limiting the duration and intensity of immune and inflammatory reactions. In macrophages, IL-10 inhibits production of proinflammatory cytokines such as TNFa, IL-6, IL-12 and antigen presentation by monocytes or macrophages via downregulation of MHC II and costimulatory molecules. TGFbeta is a pleiotropic cytokine which mediates a wide variety of effects on cellular differentiation, activation, and proliferation. On macrophages and monocytes TGFbeta regulates activation, cytokine production, host defense, and chemotaxis. TGFbeta acts as a negative regulator of CD163 macrophage expression and inhibits LPS induced macrophage production of the proinflammatory cytokines TNFalpha, IL-lalpha and IL-18 (58). Glucocorticoids are released in response to a variety of stressors (starvation, pain, trauma, infection) and are essential for maintenance of homeostatic functions (59). Their recognition takes place in the nucleus by the glucocorticoid receptor, which strongly represses proinflammatory cytokines such as TNFalpha, IL-4, IL-5, IL-1, IL-6, IL-8, IL-12, and proinflammatory mediators

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such as iNOS and cyclooxygenase 2 (59). GC also inhibit the inflammatory response by increasing the expression of IL10 and other molecules with anti-inflammatory functions such as the scavenger receptor CD163, and by interfering with the IL1 system, increasing expression of the decoy receptor IL-1RII (Figure 2). GC finally downregulate a great variety of genes known to be upregulated by IFN γ , such as the chemokines CXCL10/Ip-10, CXCL11/I-TAC, CCL5/RANTES and CCL24/eotaxin 2, and the chemokine receptor CX3CR1 (60).

5. MACROPHAGE POLARIZATION BEYOND INFECTION

Outside the context of type 1 and type 2 immune response paradigm macrophages have also been associated with important non pathogen-driven diseases, such as atherosclerosis (61), osteoporosis (62, 63) tissue injury and uncontrolled tissue remodeling (64-66). Moreover, certain resident macrophage populations are shaped by the local microenvironment to undertake polarized phenotypes (67). In particular, M-CSF-differentiated macrophages acquire a gene expression profile partially overlapping with IL-4-driven M2a macrophages. Since M-CSF is a homeostatic growth factor circulating at high levels in normal blood, the drift towards M2 may be a default pathway in macrophage differentiation (20).

6. TUMOR-ASSOCIATED MACROPHAGES

The capability of macrophages to express distinct functional phenotypes is typically manifested in pathological conditions, including cancer. Tumor microenvironmental signals have the capacity to pilot recruitment, maturation and differentiation of infiltrating leukocytes and play a central role in the activation of specific transcriptional programmes expressed by tumor-associated macrophages (TAM) (10, 11). The production of IL-10, TGF β and PGE2 by cancer (ovary) and TAM (68) cells contributes to a general suppression of antitumor activities. IL-10 promotes the differentiation of monocytes to mature macrophages and blocks their differentiation to dendritic cells (69). Thus, a gradient of tumor-derived IL-10 may account for differentiation along the dendritic cell versus the macrophage pathway in different microanatomical localizations in a tumor. IL-10 induce TAM to express M2c-related functions, such as scavenging debris, angiogenesis, tissue remodelling and repair (68). TAM are poor producers of NO (70). In ovarian cancer, macrophages positive for iNOS are a scanty population found in a low percentage of tumors and always confined at their periphery (71). Arginase expression in TAM has not been studied, however, it has been recently proposed that the carbohydrate-binding protein galectin-1, which is abundantly expressed by ovarian cancer (72) and shows specific anti-inflammatory effects, tunes the classic pathway of L-arginine resulting in a strong inhibition of the nitric oxide production by LPS-activated macrophages. In contrast to M1 cells, TAMs have been shown to be poor producers of ROIs, consistent with the hypothesis that these cells represent a skewed M2 population. Moreover, TAM express low levels of proinflammatory cytokines, such as

IL-12, IL-1 β , TNF α , and IL-6 (11). In agreement with the M2 signature, TAM also express high levels of both the scavenger receptor A (73) and the mannose receptor (Allavena P, and Sica A; unpublished observation). Furthermore, TAM are poor antigen presenting cells (11).

Angiogenesis is an M2-associated function which represents a key event in tumor growth and progression. In several studies TAM accumulation has been associated with angiogenesis and production of angiogenic factors such as VEGF and platelet-derived endothelial cell growth factor (11). More recently, in human cervical cancer, VEGF-C production by TAMs was proposed to play a role in peritumoral lymphoangiogenesis and subsequent dissemination of cancer cells with formation of lymphatic metastasis (74). Additionally, TAM participate in proangiogenic process by producing the angiogenic factor thymidine phosphorylase, which promotes endothelial cell migration *in vitro* and whose levels of expression are associated with tumor neovascularization (75). TAM contribute to tumor progression by also producing proangiogenic and tumor-inducing chemokines, such as CCL2/MCP-1 (76). TAM accumulate in hypoxic regions of tumors and hypoxia triggers a pro-angiogenic program in these cells. Therefore, macrophages recruited *in situ* represent an indirect pathway of amplification of angiogenesis, in concert with angiogenic molecules directly produced by tumor cells. On the anti-angiogenic side, in a murine model, GM-CSF released from a primary tumor upregulated TAM-derived MMPs and angiostatin production, thus suppressing tumor growth of metastases (77). Finally, TAM express molecules which affect tumor cell proliferation and dissolution of connective tissues. These include epidermal growth factor, members of the fibroblast growth factor family, TGF β , VEGF, and some chemokines. In lung cancer, TAM may favor tumor progression by contributing to stroma formation and angiogenesis through their release of PDGF, in conjunction with TGF β production by cancer cells (11). Macrophages can produce enzymes and inhibitors which regulate the digestion of the extracellular matrix, such as MMPs, plasmin, and urokinase-type plasminogen activator and its receptor. Direct evidence have been presented that MMP-9 derived from hematopoietic cells of host origin contributes to skin carcinogenesis (78). Evidence suggests that MMP-9 has complex effects beyond matrix degradation, including promotion of the angiogenic switch and release of growth factors (78). The mechanisms responsible for the M2 polarization of TAM have not been completely defined yet. Recent data point to tumor (ovarian, pancreatic) derived signals which promote M2 differentiation of mononuclear phagocytes (Marchesi F, Allavena A, and Sica A; unpublished data).

7. PERSPECTIVES

The concept of macrophage polarization has been increasingly appreciated. However many questions remain unsolved in this matter. Through years Mouse and Man macrophage polarization differences have been overlooked. Nevertheless, increasing evidence highlight the inadequacy

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of direct inter-species translation. As M1 macrophages are concerned, experimental evidence accumulated in our group and others demonstrate that the NO differential network is absent in human *in vitro* macrophages(52). In M2a, IL-4/IL-13 do not induce the human homologs of the mouse alternative activation markers arginase 1, Fizz1, MMP-1 and Ym1(52) (10), while human M2a are characterized by genes not present in mouse alternative activation, such as platelet-derived growth factor C. Further research on these differences may reveal instrumental for model comparisons.

The polarization paradigm is so far an *in vitro* paradigm, with few although strong evidence for a relevant role in *in vivo* systems. Gene expression screening is helping to put forward many new markers that may aid to track and better study the different phenotypes in disease and physiological condition. Macrophages are a key cells component of the inflammatory reactions expressed at various pathological sites. Understanding the pathogenetic role played by polarized functions may pave the way for the identification of novel therapeutic approaches. In cancer, established evidence ranging from adoptive transfer of cells to genetic manipulations, suggest that myelomonocytic cells can promote tumor invasion and metastasis, although under certain conditions they can express antitumor reactivity. Thus, therapeutic targeting of macrophages may represent a valuable strategy to complement established anticancer strategies. This may also have clinical implications, as both preclinical and clinical observations clearly demonstrate an association between macrophage number/density and prognosis in a variety of murine and human malignancies.

8. CONCLUSIONS

Polarization of mononuclear phagocytes is a useful simplified conceptual framework, describing a continuum of functional states classified according to their phenotypes (1, 4). A dichotomy that mimics the Th1-Th2 paradigm is currently in use, and distinguishes the elicited macrophage phenotypes in M1 and M2. The M1 and M2 phenotypes explain the diverse roles that these cells exert, both in the inflammatory and the resolution phase, with M1 displaying a strong microbicidal program and M2 linked to immunity against extracellular parasites, production of anti-inflammatory cytokines and elimination of tissue debris in the last phases of acute and chronic inflammation (79, 80). The paradigm still awaits for more biological data to acquire stronger significance.

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