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Colony-Stimulating Factor-1 Receptor in the Polarization of Macrophages: A Target for Turning Bad to Good Ones?

Elisabetta Rovida* **and Persio Dello Sbarba**

Department of Biomedical Sciences Experimental and Clinical "Mario Serio", Section of Pathology and Experimental Oncology, University of Florence and the Tuscan Cancer Institute, Italy

***Corresponding author:** Elisabetta Rovida, Department of Biomedical Sciences Experimental and Clinical "Mario Serio", Section of Pathology, University of Florence, Viale G.B. Morgagni 50, Firenze I-50134, Italy, Tel: 390552751320; Fax: 39055432431; E-mail: elisabetta.rovida@unifi.it

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Abstract

Macrophages, which are found in all tissues, are an essential component of the innate immune system, and they play important roles in host defense, inflammation, autoimmune diseases as well as cancer. Functionally, macrophages are classified into two types: classically-activated M1 macrophages and alternatively-activated M2 macrophages. The M1 macrophages typically produce high levels of proinflammatory cytokines and chemokines, whereas M2 macrophages show an efficient phagocytic and scavenging activity. Because the phenotypes of polarized M1 and M2 macrophages can be induced, and reversed to some extent, by various signals, different phases of many diseases are associated with dynamic changes in the balance between M1 and M2 macrophages. The colony-stimulating factor 1 receptor (CSF-1R), a class III receptor tyrosine kinase, sustains the survival, proliferation and differentiation of monocytes and macrophages. Drugging CSF-1R may be the only way to target macrophages within a pathological context. However, CSF-1R-dependent signals may be either positive or detrimental depending on the disease and even on the phase of disease. The role of CSF-1R and its ligands, the colony-stimulating factor-1 and interleukin-34, in macrophages with respect to the pathogenesis of several inflammatory or neoplastic diseases has been reviewed previously. This review will focus specifically on evidences obtained about the role of CSF-1R in macrophage polarization in the context of physiological as well as pathological conditions including inflammation and cancer. The possibility to target CSF-1R, using the several inhibitors already available, for the treatment of inflammatory diseases as well as cancer will be also discussed.

Keywords: c-Fms; Macrophage activation; M-CSF, macrophage colony-stimulating factor; M-CSFR, macrophage colony-stimulating factor receptor; Cancer; Inflammation

Abbreviations

AKI: Acute Kidney Injury; BM: Bone Marrow; BMDM: BM-Derived Macrophages; ccRCC: clear cell Renal Cell Carcinoma; CNS: Central Nervous System; CSF-1: Colony Stimulating Factor-1; CSF-1R: CSF-1 Receptor; DC: Dendritic Cell; DT: Diphtheria Toxin; ERK: Extracellular-signal-Regulated Kinase; FRβ: Folate Receptor β; GFAP: Glial Fibrillary Acidic Protein; GBM: Glioblastoma; GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor; HIV: Human Immunodeficiency Virus; HIVE: HIV-Induced Encephalitis; IFNγ: Interferon γ; IL: Interleukin; I/R: Ischemia/Reperfusion; LCCM: L929 Cell-Conditioned Medium; LPS: Lipopolysaccharide; mAb: Monoclonal Antibody; M-CSF: Macrophage Colony-Stimulating Factor; M-CSFR: M-CSF Receptor; MAPK: Mitogen-Activated Protein Kinase; MMTV/PyMT: Mouse Mammary Tumor Virus/Polyoma Middle T; PBMC: Peripheral Blood Mononuclear Cells; PI3K: Phosphatidyl Inositol 3-Kinase; PTX: Paclitaxel; SAA: Serum Amyloid A; SBS: Sickness Behavior Syndrome; SCF: Stem Cell Factor; SIV: Simian Immunodeficiency Virus; SIVE: SIV-Induced Encephalitis; TGFβ: Transforming Growth Factor β; TLR: Toll-Like Receptors; TAM: Tumor-Associated Macrophages; TNFα: Tumor Necrosis Factor α

Macrophage Polarization

Monocytes are released from the bone marrow (BM) into the circulation and migrate into almost every tissue of the body, where they differentiate into mature macrophages. Macrophages include liver Kupffer cells, brain microglia, lung alveolar macrophages, peritoneal macrophages, adipose tissue macrophages and bone osteoclasts [1]. Monocytes/macrophages are critical effectors and regulators of a number of patho-physiological processes within innate and adaptive immunity, systemic metabolism, hematopoiesis, angiogenesis, reproduction and cancer [2-4]. Macrophages are highly plastic cells that change phenotype to acquire different skills depending on the microenvironmental stimuli to which they are exposed.

Microbial stimuli, including toll-like receptor (TLR) ligands, the prototype of which is lipopolysaccharide (LPS), and Th1 cytokines like interferon gamma (IFNγ) induce the "classical" macrophage activation (M1-like macrophage polarization). These cells show increased expression of inflammatory cytokines, chemokines, and reactive nitrogen/oxygen intermediates. M1 cells preferentially promote Th1 effector response, possess anti-microbial ability, protect against various types of bacteria and viruses, and display tumoricidal functions. On the other hand, an "alternative" activation (M2-like macrophage polarization) may be elicited by Interleukin (IL)-4 and IL-13 (M2a), immune complexes (M2b), the anti-inflammatory cytokine IL-10 (M2c), transforming growth factor β (TGFβ) and/or glucocorticoids. Alternatively activated macrophages typically show increased expression of scavenging receptors and scavenging activity, reduced

expression of inflammatory cytokines, and metabolize arginine to ornithine preferentially *via* arginase. Moreover, these cells are mainly devoted to tissue repair-remodeling functions and are important for the clearance of parasite infection. Additionally, M2 cells may also be involved in tumor promotion. M1/M2 polarization thus mirrors Th1/Th2 polarization and mediates Th1/Th2 immune responses. Nevertheless, phenotypic and functional abilities of M1 and M2 macrophages are not rigidly segregated, rather representing a continuum ranging from M1 to M2. Furthermore, mouse and human macrophages are distinct with respect to the expression levels of some markers of polarization [5-8].

The Colony-Stimulating Factor-1 Receptor in Macrophage Development and Differentiation

The colony-stimulating factor-1 receptor (CSF-1R, also known as macrophage colony-stimulating factor receptor, M-CSFR) is a homodimeric type III receptor tyrosine kinase that is encoded by the c-*fms* proto-oncogene. It contains an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain that contains a split kinase domain. CSF-1R regulates the differentiation of myeloid progenitors into heterogeneous populations of monocytes, macrophages, dendritic cells (DC) and bone-resorbing osteoclasts [9]. Moreover, activated CSF-1R promotes the survival, proliferation, differentiation and chemotaxis of differentiated macrophages. The known CSF-1R ligands are CSF-1 (also known as macrophage colony-stimulating factor, M-CSF) and IL-34 [10,11]. Although there are significant differences in their signaling through CSF-1R, it is primarily the different expression of CSF-1 and IL-34 *in vivo* that controls their different spatiotemporal action through CSF-1R. Moreover, CSF-1 exists in several isoforms, which show different, yet sometimes overlapping, roles: a secreted glycoprotein and a secreted proteoglycan, both of which circulate, and a membrane-spanning, cell-surface glycoprotein. The amount of circulating CSF-1 is humorally-regulated. Nevertheless, CSF-1 serum concentration is around 10 ng/ml in healthy people [12]. By contrast, IL-34 is not detectable in the circulation of healthy individuals, so that IL-34 action is likely restricted to the microenvironments where it is released [11]. CSF-1R appears to be the only receptor for CSF-1, because all of the CSF-1 deficiency phenotypes are exhibited by CSF-1R-deficient mice [13]. On the other hand, IL-34 acts also through the protein tyrosine phosphatase-z receptor [14].

The biological activities of CSF-1R have been extensively characterized, especially those following its engagement with CSF-1 in myeloid cells. CSF-1 binding leads to CSF-1R auto-phosphorylation on many tyrosine residues. Eight tyrosines are known to be phosphorylated in the intracellular domain of the activated murine CSF-1R proto-oncoprotein/oncoprotein: Tyr-559 and Tyr-544, in the juxtamembrane domain, Tyr-697, Tyr-706, and Tyr-721 in the kinase insert domain and Tyr-807, Tyr-921 and Tyr-974 in the carboxy-terminal tail [11]. Phosphorylation of individual CSF-1R tyrosines creates docking sites for several signaling molecules thus leading to the activation of signaling pathways. It is well established that phosphorylation of Tyr-559 determines the activation of Src-Family Kinases and of the mitogen-activated protein kinase (MAPK) extracellular-signal-regulated kinase (ERK) 5 (through Tyr-561 of human CSF-1R that corresponds to Tyr-559 in the murine CSF-1R), while phosphorylation of Tyr-721 activates phosphatidyl inositol 3-kinase (PI3K). On the other hand, phosphorylation at different sites including that of Tyr-807 activates the MAPK ERK1 and ERK2, while recruitment of Grb2 by phosphorylated Tyr-921 or Tyr-697 may lead to the activation of other signaling pathways [11].

Referring to the relationship of CSF-1R-elicited signals to macrophage polarization, it is known that the PI3K pathway, which is activated by CSF-1R, regulates M1/M2 activation programs in macrophages [15]. Along this line, it was recently reported that pTyr-721 signaling downregulates proinflammatory genes, including IL-1β (a cytokine typically expressed by M1 polarized macrophages), while upregulating the expression of the M2 genes coding for Arginase (*Arg1*) and IL-10 [16]. Moreover, miR-21 emerged as a CSF-1-induced pTyr-721- and PI3K-dependent product involved in the regulation of macrophage activation. Indeed, miR-21 inhibition impairs the CSF-1-dependent reduction of IL-1β expression. Besides suppressing the proinflammatory phenotype *in vitro*, miR-21 attenuates the recruitment of Ly6C^{high} (i.e., inflammatory) monocytes to the peritoneal cavity in response to LPS *in vivo*. Taken together, these results indicated that pTyr-721 signaling suppresses the macrophage proinflammatory phenotype [16].

Besides ligand-induced tyrosine phosphorylation, CSF-1R activity may be regulated by ligand-induced down-regulation or down-modulation that occurs by ectodomain shedding. In the latter scenario, CSF-1R protein degradation follows protein kinase C activation by TLR-activating molecules, such as LPS, or by IL including IL-4 and IL-2 [17-19]. Whether CSF-1R down-modulation affects macrophage plasticity and response to M1/M2-polarizing stimuli has not been addressed. However, it should be noted that either M1- (LPS, IL-2) and M2-like inducing agents (IL-4) are able to down-regulate CSF-1R [20]. Another important point that should be taken into consideration, especially when thinking about the possible targeting of CSF-1R, is the fact that CSF-1R signaling may be activated, in a ligand-independent manner, following transactivation by other stimuli including prostaglandin E2, a key mediator of immunity, inflammation and cancer, following engagement to its G-protein-coupled E-prostanoid receptors [21].

The essential role of CSF-1/CSF-1R axis in the development of most tissue macrophages has been well-established using naturally occurring *csf1*-deficient *op/op* mice [22,23] and *csf1r* knockout mice [13]. Moreover, during early myeloid differentiation, CSF-1 synergizes with other growth factors, including stem cell factor (SCF) and IL-3, to generate, from hematopoietic stem cells, mononuclear phagocyte progenitor cells [24,25]. The proliferation and differentiation of these cells to monocytes and macrophages is then regulated by CSF-1 itself, which also controls the survival, proliferation and function of fully differentiated macrophages [24].

The role of CSF-1R in tissue development and homeostasis has been extensively reviewed [26-29]. Recent reports identified a role for CSF-1R in macrophage activation/polarization under physiological conditions. Microglia, the macrophages resident in the central nervous system (CNS), is absent in *csf1r*-deficient mice [30], while *csf1*-deficient mice show decreased microglial numbers and *IL34*-deficient mice an even more severe lack [31,32]. Increased levels of CSF-1 together with increased microglial cell number and activation are found in many different CNS pathologies including tumors, degenerative diseases and injuries [33,34]. To test the hypothesis that increased CSF-1 levels impact on both microglial cell numbers and phenotype *in vivo*, a mice model was developed that overexpresses *csf1* in the glial fibrillary acidic protein (GFAP) compartment (i.e. astrocytes) [35]. Compared with controls, *csf1*-overexpressing mice harbor significantly greater numbers of microglia cells (i.e. CD11b+

cells) in brain and increased microglia proliferation. Pharmacological Inhibition of CSF-1R with PLX3397 (Table 1) promotes microglial apoptosis and reverses the microglial increase observed in *csf1*-overexpressing mice. CSF-1 overexpression does not promote basal activation *in vivo* but causes defects in response to LPS. Indeed, when several parameters linked to macrophage activation were measured *ex vivo* in microglia from mice systemically-treated with LPS, *csf1*-overexpressing mice were found to underexpress, when compared to control mice, M1-type molecules such as IL-1β and iNos/NOS2 but also the M2 marker IL-10. These results are at variance with several reports indicating that CSF-1 acts synergistically with LPS to induce the expression of several cytokines in monocytes/macrophages. In order to explain these differences, the authors proposed that microglia behaves differently from other macrophage populations on which most of the studies are based [35].

In keeping with a primary role of CSF-1 in monocyte/macrophage development, it was recently reported that endothelial cells that express CSF-1 are able to support the growth of murine BM hematopoietic cells [45]. Endothelial cells and macrophages are known to interact, thereby contributing to the modulation of vascular function [29]. The long-term co-culture of murine endothelial cells immortalized from different tissues with murine hematopoietic cells (in the presence of cytokines such as SCF, Flt3-L, IL-6, IL-3, thrombopoietin and vascular-endothelial growth factor A) allows the development of colonies of differentiated macrophages (F4/80+ and Mac1+ cells). Under the above-described conditions, immortalized endothelial cells express the membrane form of CSF-1, while cells of developed colonies express CSF-1R. The functional contribution of CSF1/CSF1R in the expansion, rather than the formation, of these colonies was established using the CSF-1R inhibitor GW2580. Colony macrophages express high levels of M2 markers including Arg1, CD206/Mrc1 and CD36, while no detectable M1 markers (IL-12, IL-1, and tumor necrosis factor α, TNFα) were found. However, whether CSF-1R was involved in M2 polarization was not addressed [45].

According to the fact that CSF-1 alone is able to sustain monocyte/macrophage differentiation, the experimental characterization of macrophage activation and functions has been performed mainly using macrophages obtained through CSF-1-induced differentiation of murine BM or human peripheral-blood monocytes (PBMC). In this respect it should be taken into consideration that the use of human recombinant CSF-1 (hrCSF-1) or of L929 cell-conditioned medium (LCCM) as a source of CSF-1 [46] may lead to different outcomes. Indeed, a different macrophage activation status may arise depending on the source of CSF-1 used, as described for the response to *A. fumigatus*, where hrCSF-1 exhibited a lower effect than LCCM in the predisposition to M1 phenotype [47]. These differences are likely due to the fact that LCCM contains, other than CSF-1, variable amounts of several growth factors and cytokines, including granulocyte macrophage colony-stimulating factor (GM-CSF) and type I interferons, that can cause additional effects in subsequent activation experiments [47,48].

Peritoneal macrophages (resident or elicited) from mice and GM-CSF-cultured macrophages from murine BM are also widely used. However, GM-CSF- and CSF-1-differentiated macrophages have different transcriptomes [49] and GM-CSF and CSF-1 may promote alone an M1 or an M2 phenotype, respectively [50,51]. In spite of this, to refer to GM-CSF and CSF-1 as the cytokines responsible for the prototypical M1 or M2 phenotype in not based on solid evidence [7]. Another important point is that CSF-1- and GM-CSF-differentiated macrophages may react similarly to a further polarization induced by IFNγ or IL-4+IL-13 [8,52]. In this respect, it has been recently shown that the most robust M2 activation phenotype is induced by IL-4/IL-10/TGFβ following differentiation with CSF-1 [53]. Indeed, flow cytometry showed that the expression of the M2-like CD206 mannose receptor as well as of other M2 markers (including the costimulatory receptor CD273, the gene CiiTA, the class B scavenger receptor SRB1, the mannose receptor CD206, the IL-1 receptor 2 (IL-1R2) and heme-oxygenase (HO)-1) were best induced following differentiation with CSF-1 rather than GM-CSF. On the other hand, CSF-1-differentiated macrophages are capable to undergo a later polarization towards the M1 phenotype [8]. While the costimulatory receptor CD86 was elevated following LPS/IFNγ-induced M1 activation, with highest expression on GM-CSF-differentiated macrophages, the expression of the costimulatory receptor CD274 was elevated following M1 activation, but, surprisingly, was highest in CSF-1-differentiated cells. Following IL-4/IL-10/TGFβ-induced M2 activation, the M1-associated markers IL-1 and IL-23 dominated in GM-CSF-differentiated cells, whereas CXCL10, CXCL11, IL-6, IL-12α and TNFα were higher in CSF-1-differentiated M1 cells. Furthermore, CSF-1-differentiated cells, that are often considered to be M2-polarized, expressed accordingly higher levels of M2-associated IL-10, CCL14 and CD206, which were retained following secondary M1 stimulation. These results indicate that, while M1 and M2 activation states can be induced irrespective of a rigid segregation of differentiation signals, it is the balance of these signals (growth factors) which defines the ultimate macrophage activation phenotype [53].

CSF-1R is also able to interfere with IL-32-induced macrophage polarization [54]. IL-32 promotes the differentiation of monocytes into macrophage-like cells and induces the production of various cytokines including TNFα and IL-8, so that it is considered an inducer of M1-like macrophages [55,56]. While investigating how the co-treatment with CSF-1 and IL-32 affects the M1/M2 ratio, it was found that, in CSF-1-differentiated PBMC cells, a further treatment with IL-32γ or CSF-1 activated MAPK such as p38, JNK and ERK1/2, while the IL-32γ/CSF-1 combination induced a more sustained MAPK activation and increased survival. Moreover, IL-32γ induced the production of IL-6, GROa and IL-8, as well as the expression of the costimulatory molecule CD80, at higher levels than CSF-1. These IL-32γ-induced M1-like characteristics were unaffected by the cotreatment with CSF-1. Moreover, IL-32γ–treated macrophages showed rather low expression levels of CD86, another M1 marker, compared with CSF-1-treated macrophages. On the other hand, M2-like characteristics, including phagocytic activity, and CD14 and CD163 expression were significantly upregulated in IL-32γ-treated compared with CSF-1-treated macrophages and CSF-1/IL-32γ cotreatment further upregulated the expression of CD14 and CD163. These results suggest that IL-32γ induces macrophages with both M1 and M2 phenotypes, and that CSF-1 preferentially accelerates the M2 polarization induced by IL-32γ. Thus, CSF-1 seems to have either additive or inhibitory effects on IL-32γ action as far as the M1 and M2 phenotypes are concerned [54].

Role of CSF-1R in the Polarization of Macrophages in Inflammatory and Autoimmune Diseases

CSF-1R signaling plays important roles in inflammatory/immunitary diseases, including systemic lupus erythematosus, arthritis, atherosclerosis and obesity [57-59]. Importantly, CSF-1 concentration in serum may be increased in several chronic diseases

[58] where an autocrine mechanism may also be involved [60]. Macrophage populations elicited by CSF-1R signaling are associated with, and may exacerbate, a broad spectrum of inflammatory and autoimmune diseases. On the other hand, macrophages can also contribute to immunosuppression, disease resolution and tissue repair [12,28].

Pro-inflammatory role of CSF-1R signaling

Besides inflammation-associated organ dysfunction, patients with infections and autoimmune diseases suffer from sickness behavior syndrome (SBS), which is characterized by fatigue, depression, weight loss and reduced social activities [61]. The interactions of CD40 with its ligand (CD40L) play a major role in the development of the host response to infectious pathogens and of chronic inflammatory diseases including autoimmune liver diseases. CD40 mediates T cell-dependent B cell responses, enhances the expression of MHC and costimulatory molecules on DC and macrophages and elicits the production of proinflammatory cytokines including TNFα, IL-1β, IL-6, typical M1 cytokines, and IL-8 and GM-CSF and CSF-1 [62]. Treatment of mice with the CSF-1R-blocking monoclonal antibody (mAb) M279 protects from CD40-induced SBS (i.e., prevents the decrease of motional activity and body weight) while increasing IL-10 (M2 cytokine) production rather than decreasing the amount of the proinflammatory cytokines such as the M1 cytokines TNFα and IL-6. However, CSF-1R blockade did not impair CD-40-induced splenomegaly and hepatitis. Protection from SBS upon CSF-1R inhibition is mediated by the induction of IL-10 expression in inflammatory monocytes, because anti-CSF-1R mAb failed to prevent the development of CD40-induced SBS in IL-10$^{-/-}$ mice. This study thus indicates that CSF-1R blocking might be a useful tool to prevent SBS, which impairs quality of life in patients, although the overall course of chronic inflammation would not be affected. Moreover, the above results indicate that targeting CSF-1R may lead to a conversion of inflammatory monocytes to a mixed pro-inflammation/pro-resolution cell type [63]. This is in keeping with the fact that the prolonged treatment of mice with the M279 mAb allows the depletion of CSF-1R-expressing resident tissue macrophages in liver, lung broncho-alveolar space, intestinal tract, pancreas and kidney without ablating the development of inflammatory monocytes [40].

Altered macrophage behavior is central to the pathogenesis of chronic obstructive pulmonary disease (COPD) [64]. Serum amyloid A (SAA) is expressed locally in chronic inflammatory conditions such as COPD, where macrophages that do not correspond to the classic M1/M2 paradigm also accumulate. Differentiation of human monocytes with CSF-1 and GM-CSF together with SAA stimulates the M1 cytokines IL-1 and IL-6 concurrently with the M2 markers CD163 and IL-10 more efficiently than in the absence of SAA. Experiments performed *in vivo* showed an increase of the inflammatory CD11c^{high}/CD11b^{high} population following SAA challenge. Inhibition of CSF-1R by intranasal pretreatment with the AFS98 anti-CSF-1R mAb markedly reduces the emergence of this population. These results indicate CSF-1R targeting as a possible tool to prevent this emergence and a novel approach to treat chronic inflammatory conditions associated with persistent SAA expression [65].

Many individuals with human immunodeficiency virus (HIV) infection develop varying degrees of cognitive impairment, collectively termed HIV-associated neurocognitive disorders. Although the pathogenesis of these disorders is not completely understood, activated microglia and macrophages infiltrating CNS are believed to play a prominent role in its development and/or progression [66]. CSF-1 is detectable in cerebrospinal fluid of HIV-infected patients and is believed to support virus production and disease progression [67]. Macrophages accumulate in the perivascular cuffs and within nodular lesions, either lesions being among the histological hallmarks of both HIV-induced and simian immunodeficiency virus (SIV)-induced encephalitis (HIVE and SIVE, respectively) [68]. A recent study identified macrophages as the main source of CSF-1 in SIV-infected rhesus macaques, a relevant animal model for HIV-associated neuropathogenesis [69]. In the same study, IL-34, the alternative ligand of CSF-1R, was found mostly associated with cells scattered in the parenchyma, rather than with cells accumulating around blood vessels and within nodular lesions [69]. In another paper, the same authors explored the potential relationship of CSF-1 and/or IL-34 with M2 activation in SIVE [70]. They found that CD163, a marker of a subclass of M2 macrophages [7], is upregulated in brain of SIV-infected rhesus macaques with SIVE. Moreover, in SIVE brain, accumulating CSF-1-positive macrophages are also CD163+, indicating that they are a source of CSF-1 in SIVE. By contrast, CD163 expression in parenchyma does not colocalize considerably with CSF-1, but does with IL-34. In addition, in the frontal grey matter, the neurons also express CSF-1 and IL-34, and CSF-1 expression is significantly decreased in SIV and even more in SIVE. *In vitro* experiments showed that treatment with the CSF-1R inhibitor GW2580 decreases the number of CD163+ cells, thus demonstrating that CSF-1R signaling, via either of its ligands, may contribute to sustained M2 activation in brain in SIV infection and SIVE [70]. On the basis of the above results, the authors conclude that, upon CSF-1 ligation, CSF-1R signaling plays an important role in the pathogenesis of HIV-associated neurodegenerative disease, while additional studies are needed to elucidate the potentially divergent roles of CSF-1 and IL-34. IL-34, indeed, may contribute to M2 activation/polarization in the brain, but may also promote neuronal survival.

Anti-inflammatory role of CSF-1R signaling

Renal tubule epithelia represent the primary site of damage in acute kidney injury (AKI), a process initiated and propagated by macrophage infiltration [71]. Using two murine models of AKI, based on ischemia/reperfusion (I/R)-induced injury or diphtheria toxin (DT)-induced selective proximal tubule injury, Zhang and colleagues examined the role of macrophages/DC (F4/80hi) in the recovery following AKI. In both models, macrophage/DC depletion during the recovery phase increased functional and histological injury and delayed regeneration. After I/R-induced AKI, there was an early increase of inflammatory (M1) monocytes (as detected based on iNOS expression), followed by the accumulation of renal macrophages/DC with a wound-healing (M2) phenotype (increase of Arg1). By contrast, DT-induced AKI only generated an increase of M2 cells. Genetic or pharmacologic (GW2580) inhibition of CSF-1R blocked macrophage/DC proliferation, decreased M2 polarization and inhibited recovery. These findings demonstrated that CSF-1R-mediated expansion and polarization of resident renal macrophages/DC is an important mechanism mediating renal tubule epithelial regeneration after AKI [72]. The same authors, in a very recent study, confirmed and further characterized the protective role of CSF-1R signaling following AKI. In both models of AKI, selective deletion of CSF-1 expression in the proximal tubule determined decreased M2 polarization, delayed functional and structural recovery and increased tubulointerstitial fibrosis [73]. Therefore, CSF-1, that is abundantly produced in the kidney and specifically at the proximal tubule, stimulates renal

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epithelial cell proliferation directly after AKI [74], but is also an important mediator of macrophage/DC polarization and recovery from AKI [73].

Role of CSF-1R in the Polarization of Macrophages in the Tumor Context

Tumor-associated macrophages (TAM) are the predominant leukocytes infiltrating solid tumors and can represent up to 50% of the tumor mass. TAM can exert dual influence of cancer depending on the activation state, with classically activated (M1) and alternatively activated (M2) cells generally exerting antitumor and pro-tumor functions, respectively [75,76]. These are extremes in a *continuum* of polarization states. It is now recognized that TAM, that are recruited by CSF-1 and MCP-1/CCL2, represent the most abundant immunosuppressive cell population in the tumor microenvironment [77]. A number of cell types within the tumor microenvironment as well as tumor cells produce CSF-1 [78] and circulating CSF-1 may increase in several cancer types including that of breast, endometrium and ovary [79-81].

Macrophage infiltration in tumors has been identified as an independent factor predictive of poor prognosis for several cancer entities, colorectal cancer (CRC) being probably the only exception because in this cancer TAM density is significantly associated with enhanced overall survival [76,82]. These diverse anti-tumoral or tumor-promoting activities of TAM are likely promoted by distinct TAM subpopulations, linked to different intratumoral microenvironments [76,83]. Indeed, molecularly and functionally distinct TAM subpopulations coexist in tumors, depending on cancer type, stage of tumor progression and location within the tumor tissue [4]. This TAM heterogeneity likely reflects the inherent plasticity of macrophages in response to microenvironmental triggers. The relevant role of CSF-1R in TAM with respect to cancer progression is well acknowledged [12, 27, 84-86]. More importantly, CSF-1/CSF-1R blocking may represent the only truly selective approach to manipulate macrophages in cancer patients. Accordingly, strong reductions of TAM number have been reported in various tumor models upon blocking of CSF-1/CSF-1R signaling using mAb targeting either CSF-1 or the extracellular domain of CSF-1R, or using chemical inhibitors of CSF-1R tyrosine kinase activity and thereby the downstream signaling [84].

Pro-tumoral activity of CSF-1R signaling in TAM

The presence of extensive TAM infiltration in clear cell renal cell carcinoma (ccRCC) microenvironment contributes to cancer progression and metastasis by stimulating angiogenesis, tumor growth, and cellular migration and invasion [87]. Immunostaining of human ccRCC specimens showed a correlation between the number of CD163+ M2 macrophages and age, sex, nuclear grade and TNM classification [88]. On the other hand, high levels of CD163+ cell infiltration were significantly associated with poor clinical prognosis in univariate but not multivariate analysis. Culture supernatants from ccRCC cell lines induced M2 polarization of macrophages, as indicated by the increased expression of CD163 and IL-10, two M2 typical markers, following LPS treatment. Stronger results were obtained when direct interaction of ccRCC cells and macrophages was allowed. Primary ccRCC cells consistently express surface CSF-1. Genetic or pharmacological inhibition of CSF-1R (obtained using specific siRNA or GW2580, respectively) or block of CSF-1 binding to CSF-1R suppressed macrophage IL-10 production induced by ccRCC cells. These results indicate that the signaling initiated by the cross-talk between ccRCC cells and macrophages is mediated by the CSF1/CSF1R pair. Furthermore, the block of CSF-1R signaling and the consequent reversion of immunosuppressive conditions emerges as a promising approach for anticancer therapy in patients with ccRCC [88].

Although macrophages are endowed with a high level of plasticity, within the tumor microenvironment they mostly facilitate tumor development rather than regression, mainly because they function as immunosuppressive cells. Along this line, skewing of monocyte differentiation from DC to macrophages has been proposed to contribute to immunosuppression [89]. In a study performed to investigate on the possibility that TAM could be redirected toward a DC-like phenotype, it was found that treatment *in vitro* with GM-CSF, which is able to induce maturation of myeloid cells toward a DC phenotype [90], does not change the expression of M1-type (IL-1β, TNFα, and CXCL10) or M2-type (CCL22, CCL17, IL-10, and CD206) markers in F4/80^{high} and CD11b^{high} TAM isolated from abdominal tumors established by inoculating the colon carcinoma cell line MCA38 [91]. Moreover, the combined treatment of TAM with GM-CSF and CSF-1R-targeting siRNA did not alter the expression pattern of M1/M2 marker but was able to induce the expression of STAT1, STAT5 and STAT6, which are usually expressed by DC [92]. These findings indicated that GM-CSF administration together with a CSF-1-inhibiting treatment could not redirect TAM to a monocyte-derived DC-like phenotype, as determined by M1/M2 marker expression, but was able to modify cell signaling pathways towards a DC-like pattern [91].

Glioblastoma (GBM), the most aggressive form of glioma, has an invariably unfavorable prognosis, as patients respond minimally to current therapies, including surgery, radiation and chemotherapy. TAM are associated with high tumor grade and poor prognosis in gliomas, possibly because, under the influence of glioma cells, microglia creates an immunosuppressive microenvironment which promotes glioma growth [93]. In a mouse model that recapitulates human proneural GBM, *in vivo* CSF-1R inhibition using the CSF-1R inhibitor BLZ945 significantly increased survival and induced regression of established tumors [94]. However, these effects were not associated with TAM depletion. Rather, GBM-secreted factors, including GM-CSF and IFNγ, facilitated TAM survival in the context of CSF-1R inhibition and resulted in a repolarization from pro-tumoral M2 to a highly phagocytic M1 phenotype. These results identify TAM as a promising therapeutic target for proneural gliomas and establish the translational potential of CSF-1R inhibition for GBM therapy [94].

Despite the fact that a role for CSF-1/CSF-1R signaling and TAM in breast cancer progression has been well established [27], attempts to obtain the therapeutic depletion of macrophages from tumors using as single agents mAb or chemical inhibitors targeting CSF-1 or CSF-1R were disappointing [36,95]. Nevertheless, the administration to mice of inhibitors of CSF-1R signaling in combination with standard-of-care chemotherapy slows down the growth of primary tumors significantly and reduces pulmonary metastasis [36]. On the basis of these data, phase 1 and 2 clinical studies combining the CSF-1R inhibitor PLX3397 with chemotherapy are currently under way. The mouse mammary tumor virus/polyoma middle T (MMTV/PyMT) transgenic model of luminal B-type mammary carcinoma was used to shed light on the mechanisms by which the CSF-1/CSF-1R pathway and macrophages repress response to cytotoxic therapy in mammary

carcinoma. It was found that the anti-CSF1 5A1 blocking mAb, alone or in combination with paclitaxel (PTX), depleted mammary tumors from $CD11b^+F4/80^+MHCII^+Ly6C^-$ macrophages [96]. Following anti-CSF-1 mAb treatment, IL-10 expression was markedly reduced and it turned out to be mostly produced by macrophage cells in MMTV/PyMT tumors. Moreover, the block of IL-10R by specific antibodies improves response to PTX, in terms of tumor volume in MMTV/PyMT mice, or of survival in mice following orthotopic transplantation of MMTV/PyMT-derived tumors, or lung metastasis following treatment with IgG1, anti-CSF-1 mAb, anti-IL-10R mAb, PTX, or a combination thereof and similarly to what observed by anti-CSF-1mAb/PTX. Improved response to chemotherapy was $CD8^+$Tcell-dependent, but IL-10 did not directly suppress $CD8^+$Tcells or alter macrophage polarization. Instead, IL-10R block increased in intratumoral DC the expression of IL-12, which was necessary for improved outcomes. These data indicate that macrophages infiltrating mammary carcinomas are a significant source of IL-10, which in turn suppresses IL-12 production by DC and thereby limits T-cytotoxic responses during chemotherapy. These data identify a CSF-1/IL-10/IL-12 axis which is suitable for therapeutic targeting [96].

In another study, it was identified a new mechanism that allows macrophages to maintain locally restrained and smoldering inflammation, which is required in angiogenesis and metastasis. Indeed, it was found that IL-34- and CSF-1-differentiated macrophages exhibit most of the phenotypic (*i.e.,* CD14high $CD163^{high}$ $IL\text{-}10^{high}$ $IL\text{-}12^{low}$ $CD86^{low}$) and functional characteristics (*i.e.,* low T-cell costimulatory properties, inhibition of activated effector T-cell functions) of TAM isolated from human ovarian cancer. Moreover, IL-34- and CSF-1-differentiated TAM in human ovarian cancer switch memory Tcells into Th17 cells *via* membrane IL-1α [97].

A number of papers from Corbí's group identified several markers possibly associated to CSF-1-, but not GM-CSF-, differentiated macrophages within the tumor context. Indeed, it was found that CSF-1-, but not GM-CSF-differentiated macrophages express folate receptor beta (FRβ, encoded for by *FOLR2*) and display folate binding and internalization ability. Therefore, CSF-1 promotes the expression of a functional FRβ protein, which was therefore proposed to be a further marker of CSF-1-differentiated M2 macrophages. Consistent with a possible role of FRβ *in vivo*, FRβ is functionally expressed in IL-10 mRNA-expressing $CD14^+$ $CD68^+$ $CD163^+$ melanoma TAM. Other cytokines commonly released by tumors, including IL-6 alone and IL-10 in combination with CSF-1, upregulate FOLR2 mRNA expression. Moreover, conditioned media from several cancer cell lines are able to induce the expression of FRβ. Incubation with an anti-CSF1 blocking antibody greatly reduced the upregulation of FOLR2 mRNA promoted by ascitic fluid from breast carcinoma or by conditioned medium from tumor-associated fibroblasts or JEG-3 tumor cells. Therefore, CSF-1 is a major determinant for FRβ expression on human macrophages and contributes, alone or in combination with other cytokines, to FRβ cell surface expression on TAM [98]. These results identified a new possible marker for TAM and, more importantly, provided a possible rationale for folate-conjugated drugs in cancer therapy approaches [99]. In another paper, the same authors showed that the heme regulatory molecules CD163 and HO-1 are preferentially expressed by CSF-1-differentiated (M2) macrophages with respect to GM-CSF-differentiated (M1) macrophages [100]. They also showed that M1 macrophages secreted large amounts of functional activin A that promotes the expression of M1 (GM-CSF-differentiated) markers, impairs the acquisition of M2 (CSF-1-differentiated) markers, and down-regulates the production of IL-10. These results indicate that activin A contributes to macrophage polarization and shapes the inflammatory behavior of macrophages [101]. More recently, it was also found that CSF-1-differentiated macrophages obtained from human PBMC express and produce CCL2, while GM-CSF-differentiated macrophages express CCL2R. $CD14^+$ TAM isolated from ascitic fluid of different types of cancer express CCL2 at an even higher extent that CSF-1-differentiated macrophages [102]. Taken together, the above-described results support further a protumoral activity of CSF-1R signaling.

Anti-tumoral activity of CSF-1R signaling in TAM

Despite the abundant literature on the many pro-tumoral functions of TAM in several types of cancer, their role in CRC is controversial. Some studies indicated that macrophages in CRC appear to have antitumor activity and are associated with improved disease-free survival. On the contrary, other studies provided evidence that a massive macrophage infiltration is correlated with tumor progression, growth and disease aggressiveness [103]. Nevertheless, we could not find any literature evidence of a role of CSF-1R in macrophage polarization in the context of CRC or other cancer types.

Conclusive Remarks in View of Drugging CSF-1R to Target Macrophage Polarization

Targeting CSF-1R signal could be undoubtedly beneficial in many conditions, including several types of cancer and chronic inflammations [12,28,86]. The block of CSF-1R may represent a truly selective approach and several CSF-1R signaling inhibitors have been developed [86,104]. However, several points are worth to be discussed, especially with respect to the role of CSF-1R signaling in macrophage polarization.

First, CSF-1R-elicited signals are important for several homeostatic mechanisms, so that their prolonged inhibition might be generally detrimental, although necessary for the treatment of specific diseases such as cancers or chronic inflammations. For example, CSF-1 is a main regulator of osteoclast differentiation, as evidenced by the osteopetrotic phenotypes of CSF-1- or CSF-1R-deficient mice [13,22]. Therefore, different ways to inhibit CSF-1R-signalling should be used to achieve different outcomes. In this respect, using antibodies that specifically prevent the binding to CSF-1R of CSF-1 but not IL-34, or *vice versa*, would block only specific effects. In this respect, it is worth pointing out that anti-CSF-1R mAb that impair CSF-1 but not IL-34 binding to CSF-1R have been identified [44]. These differences have been explained by supposing that IL-34 and CSF-1 bind overlapping yet different domains of CSF-1R. For example, it has been reported that either CSF-1- or IL-34-differentiated macrophages exhibit most of the phenotypic and functional characteristics of TAM isolated from human ovarian cancer [105]. However, IL-34 and CSF-1-differentiated macrophages may have different biological activities [44]. Another approach to avoid extreme toxicities due to systemic and persistent CSF-1R inhibition led to the development of the new H27K15 anti-CSF-1R monoclonal antibody, that, differently from other anti-CSF-1R mAb, does not compete with ligand binding and exhibits different effects on signal transduction and cellular trafficking [41]. First, the H27K15 mAb downregulates osteoclast differentiation and activity, which could block metastasis-induced bone degradation. Second, it inhibits monocyte differentiation into $CD163^+CD64^+$ M2-polarized suppressor macrophages, rather driving their differentiation towards $CD14–CD1a^+$ DC. Third, this antibody differs from other anti-CSF-1R mAb by affecting monocyte survival only marginally. Therefore, the

H27K15 mAb shows interesting properties that may make it suitable for clinical uses in cancer therapy [41].

On the other hand, in some specific context, the inhibiting strategy (antibodies *versus* chemical inhibitors) could be chosen based on the kind of molecule preferred in relation to the tissue to be targeted. Importantly, small molecule inhibitors that are able to cross the blood-brain barrier have been developed (Table 1). Referring to small molecule inhibitors that act at the level of CSF-1R kinase activity, they would also prevent ligand-independent CSF-1R activation, which may be induced via the crosstalk with other signaling pathways [21]. However, mAb that are able to prevent CSF-1R dimerization have also been developed; these mAb are also able to prevent ligand-independent CSF-1R activation if it involves receptor dimerization [106]. Another important fact that should be taken into account when choosing the inhibiting treatment is that CSF-1 is cleared from serum by receptor-mediated endocytosis [60], so that the use of antibodies which prevent CSF-1 to CSF-1R binding causes massive elevation in circulating CSF-1. A similar elevation may also result from the killing of CSF-1R-expressing cells. On the other hand, the increase in circulating CSF-1 is not supposed to occur if kinase inhibitors are added, unless they are deadly (see above), because receptor-mediated internalization does not require CSF-1R kinase activity.

Another possible approach to target disease-associated macrophages would be to shift a macrophage population towards a more appropriate phenotype. Indeed, skewing of monocyte differentiation from DC to macrophages has been proposed to contribute to tumor-induced immunosuppression [89]. For example, it has been reported that pharmacological [107] or genetic [91] inhibition of CSF-1R, as well as that based on anti-CSF-1R blocking mAb [41], can change the function or morphology of macrophages and induce DC-like characteristics. Moreover, mounting evidence from a number of different studies *in vivo* and *in vitro* has generally indicated that identifying the activated states of macrophages and targeting the macrophage polarization from M1 to M2 or vice versa might represent a novel diagnostic or therapeutic strategy for a number of diseases [15]. In these respect, several examples of modulation of macrophage plasticity have been described in this review.

Inhibitor	Type of molecule	Target	Other targets (IC50, nM)	Notes	Reference
PLX3397	Small-molecule inhibitor	CSF-1R	CSF-1R (20) KIT (10)	Crosses blood-brain barrier	[36]
GW2580	Small-molecule inhibitor	CSF-1R			[37]
BLZ945	Small-molecule inhibitor	CSF-1R		Crosses blood-brain barrier	[38]
5A1	Blocking mAb antibody	CSF-1			[39]
M279	Blocking mAb antibody	CSF1-R			[40]
H27K15	Blocking mAb antibody	CSF-1R		Impairs receptor dimerization	[41]
AFS98	Blocking mAb antibody	CSF-1R			[42]
12-2D6	Blocking mAb antibody	CSF-1R		Blocks either CSF-1 or IL-34 binding [44]	[43]
2-4A5	Blocking mAb antibody	CSF-1R		Blocks CSF-1 but not IL-34 binding [44]	[43]
mAb: Monoclonal Antibody; CSF-1: Colony Stimulating Factor-1; CSF-1R: CSF- 1 Receptor					

Table 1: CSF-1R signaling inhibitors cited in the text.

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