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REVIEW

Classic and new mediators for *in vitro* modelling of human macrophages

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Abstract

Macrophages are key immune cells in the activation and regulation of immune responses. These cells are present in all tissues under homeostatic conditions and in many disease settings. Macrophages can exhibit a wide range of phenotypes depending on local and systemic cues that drive the differentiation and activation process. Macrophage heterogeneity is also defined by their ontogeny. Tissue macrophages can either derive from circulating blood monocytes or are seeded as tissue-resident macrophages during embryonic development. In humans, the study of *in vivo*-generated macrophages is often difficult with laborious and cell-changing isolation procedures. Therefore, translatable, reproducible, and robust in vitro models for human macrophages in health and disease are necessary. Most of the methods for studying monocyte-derived macrophages are based on the use of limited factors to differentiate the monocytes into macrophages. Current knowledge shows that the *in vivo* situation is more complex, and a wide range of molecules in the tissue microenvironment promote and impact on monocyte to macrophage differentiation as well as activation. In this review, macrophage heterogeneity is discussed and the human *in vitro* models that can be applied for research, especially for monocyte-derived macrophages. We also focus on new molecules (IL-34, platelet factor 4, etc.) used to generate macrophages expressing different phenotypes.

KEYWORDS
macrophages, monocytes, Immunotherapy, innate cell mediated immunity, in vitro model

1 | INTRODUCTION

Macrophages are immune cells with heterogeneous phenotypes and complex functions in tissue homeostasis and innate and acquired immunity. These cells belong to the mononuclear phagocyte system (MPS).[1–3] In the original MPS model, macrophages present in the tissues were all thought to be derived from monocytes.[4] In the 2000s this concept started to change when lineage-tracing studies showed that populations of macrophages with different origins were found in tissues. These cells were capable of self-maintenance independently of circulating monocytes.[5–7] Currently, we know that macrophages have different origins: embryonic yolk sac derived, fetal liver derived, and/or bone marrow monocyte derived macrophages (MDMs).[3,8] In terms of function, tissue-resident macrophages act as "controllers" to maintain tissue homeostasis. They perform several functions, for example, removal of dead cells from tissues,[9] sensing changes in oxygen levels, osmolarity, and iron metabolism.[10–12] Besides the homeostatic functions, tissue-resident macrophages drive local and

Abbreviations: BHA, Butylated hidroxyanisole; CaOx, Calcium oxalate; CD200, cluster of differentiation molecule 200; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9; CX_3CL_1, Fractalkine; CX_3CR_1, fractalkine receptor; HIF-1α/HIF-1β, Hypoxia-inducible factor 1α/β; HSC, hematopoietic stem cell; iPSC, induced pluripotent stem cells; iPSDM, iPSC-derived macrophages; IRF4/5, IFN regulatory factor 4/5; *LIPA*, Lysosomal acid lipase; LL-37, Cathelicidin antimicrobial peptides LL-37; MDM, monocyte-derived macrophage; MPS, mononuclear phagocyte system; PF4, Platelet factor 4; *TREM2*, Triggering receptor expressed on myeloid cells 2.

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systemic defensive responses to pathogens.[13–15] MDMs have been implicated in a wide range of diseases, not only those that encompass inflammatory conditions that lead to immune activation, such as atherosclerosis, sepsis, rheumatoid arthritis, and systemic lupus erythematosus, but also those that are accompanied by immune suppression, such as tolerance to bacteria, or cancer.[16–20]

Considering the important role of both tissue-resident macrophages and MDMs in homeostasis and disease it has always been key to develop representative *in vitro* models to study these cells. For these models it is clearly relevant to define the *in vitro* settings, which can mimic both homeostatic and disease-associated situations. The closer *in vitro* models resemble *in vivo* macrophages, the better they will dictate our understanding and translation to study human disease. In general, MDM *in vitro* models have been relatively unrepresentative of the tissue environment that a monocyte faces when entering a tissue. The methods of monocyte to macrophage differentiation *in vitro* have been rather simplistic in terms of the factors used, disregarding many relevant molecules or factors found in diseases that can impact on monocyte to macrophage differentiation. The use of different factors gives an opportunity for further study and improvement of the *in vitro* models. The purpose of this review is to provide an overview of the different methods used to study human macrophages *in vitro*, with a brief discussion of tissue-resident macrophages and a deeper review of MDMs.

1.1 | Origins and *in vitro* models for tissue-resident macrophages

New advances in the field have shown that tissue-resident macrophages have self-renewing capacities in steady state as well as under inflammatory or infectious conditions.[7–22] Most populations are seeded during embryonic development, and emerge in three sequential waves: the primitive, the transient definitive, and the definitive.[23,24]

The primitive wave starts in the blood islands of the yolk sac and produces primitive progenitors of erythroid cells, megakaryocytes, and macrophages. Microglia originate from these cells.[25,26] In the second wave, termed transient definitive, the erythro-myeloid precursors are generated in the yolk sac and migrate to the fetal liver where they expand and differentiate into fetal liver monocytes.[27] These fetal liver monocytes subsequently migrate into tissues to differentiate into tissue-resident macrophages, such as Langerhans cells in the dermis.[28] The third wave, termed definitive, gives rise to immature hematopoietic stem cells (HSCs) in the aorta-gonads-mesonephros region. These immature HSCs colonize the fetal liver, the main hematopoietic organ during embryonic development, and ultimately seed the bone marrow generating mature HSCs that can differentiate into adult monocytes and maintain monocyte populations throughout life.

The contribution of yolk sac macrophages, fetal liver monocytes, or bone marrow monocytes to the development of tissue macrophages varies over time and it's specific for different tissues. For instance, microglia in the brain are only derived from yolk sac macrophages,[29] whereas Langerhans cells (epidermis) are mainly derived from fetal liver monocytes. The same is true for alveolar macrophages in the lungs and Kupffer cells in the liver. In the case of the pancreas and the heart, the ontogeny is a mix of macrophages differentiated from fetal liver monocytes and a minor contribution from bone marrow monocytes. Finally, in the gut and the dermis most macrophages are bone marrow monocyte derived, although recent studies show a population of macrophages in the gut with self-maintaining capacities that present a different transcriptome from gut MDMs.[30] A common feature for most tissues is that during the early stages of development, there is a contribution of yolk sac macrophages that is gradually replaced by macrophages from other origins with increasing age.[31]

Recent advances have allowed the *in vitro* generation of tissue-resident macrophages from different sources applying induced pluripotent stem cells (iPSC) derived from either stromal cells or embryonic stem cells.[32,33] The iPSCs are more commonly used as the use of embryonic stem cells entails ethical issues. The first protocols for generating iPSC-derived macrophages (iPSDMs) appeared in the early 2010s[34,35] and all commonly used protocols share three main phases.

The first phase of differentiation of iPSCs to iPSDMs consists of specification of iPSCs into hemogenic endothelium. The second phase aims to achieve the endothelial to hematopoietic transition to obtain hematopoietic progenitors. Finally, the third and last phase is the induction of differentiation of progenitors into macrophages by the addition of cytokines such as M-CSF, GM-CSF, or IL-34.[36–41]

For certain applications, it is worthwhile to differentiate the iPSDMs into specific tissue-resident macrophage phenotypes. To generate such specified iPSDMs appropriate combinations of cytokines and growth factors are added, for example, for the generation of iPSDMs microglia a cocktail of M-CSF, IL-34, TGFβ, cluster of differentiation molecule 200 (CD200), and fractalkine (CX_3CL_1) can be used.[42] Another approach to obtain tissue-specialized iPSDMs is by coculturing them with parenchymal cells. This has been shown convincingly for microglia, where coculturing iPSDMs with iPSC-derived neurons gave specification into brain-resident macrophages. Also in mice, the *in vitro* generation of microglia-like iPSDMs has been successful by coculture with neurons.[43] These cells acquired similar morphology and gene expression patterns as isolated primary microglia. In humans the in vitro generation of these cells was validated by comparing the microglia-like iPSDM transcriptomic profile with available transcriptomes of primary microglia, which showed a high degree of similarity.[44] The capacity to generate tissue-specific iPSDMs was also tested *in vivo* by transferring cells into brain and lungs of mice. As a result, microglia-iPSDMs and alveolar-iPSDMs developed in these animals.[45] In terms of function, when the response to LPS from iPSDMs and MDMs is compared at the transcriptomic level, the response is largely conserved and only some minor differences are found in antigen presentation and tissue remodeling-related gene expression.[46]

An important advantage of the use of iPSDMs lies in the source to obtain them. Studying the impact of specific mutations is possible using iPSCs obtained from stromal cells from patients carrying the variant, as the genotype will be maintained. For

instance, in neurodegeneration, patients showing mutations in triggering receptor expressed on myeloid cells 2 (*TREM2*) present deficits in phagocytosis and responses to pathogenic signals. Microglia generated *in vitro* from iPSCs from patients carrying this mutation retain this phenotype and provide highly relevant models for insights into disease mechanisms.[44] Similar studies have been conducted with cells from patients with pulmonary alveolar proteinosis where a mutation in the gene *CSF2R* makes the alveolar macrophages unable to respond to GM-CSF signaling leading to an inactivation phenotype and an accumulation of nonphagocytosed proteins. Using iPSCs, alveolar macrophages carrying this mutation were generated *in vitro*, which resembled functional characteristics of the patient, giving a tool to study not only disease but also potential treatments.[47] This approach was also conducted with cells from patients suffering from Gaucher disease,[48] and the cells generated from these patients produced higher levels of TNF, IL-1β, and IL-6 than cells from healthy volunteers.

Another great advantage of using iPSDMs vs. MDMs is that iPSDMs are relatively easy to manipulate genetically. Different groups have used CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) techniques to generate iPSDMs bearing mutations found in patients, allowing studying the consequences of the mutation for cellular function. As an *in vitro* model to investigate reverse cholesterol transport, iPSCs deficient for ATP binding cassette subfamily A member 1 were used to generate macrophages with impaired cholesterol efflux and shown to have a more proinflammatory phenotype.[49] The same was done for the lysosomal acid lipase (*LIPA*) gene to study the role of LIPA in macrophages and lipid metabolism.[50]

Although it is clear that tissue-resident macrophages are important in health and disease, the contribution of MDMs to the tissue macrophage population in homeostasis and disease increases over time and strongly affects the course and outcome of subsequent inflammation, immune activation, and disease development.[51]

1.2 | Origins of monocyte-derived macrophages: monocytes

Before discussing in detail the available approaches for *in vitro* studies of human MDMs, we deem it important to sketch the current views on the origin, heterogeneity, and functionality of human monocytes.

The original definition of the MPS describes monocytes as the precursors of macrophages.[4] Circulating blood monocytes in humans represent about 10% of leukocytes. Pools of monocytes can be found in the spleen and this reservoir can be mobilized quickly in case of injury or acute inflammation. In mice, these reservoirs and their prompt response were studied in ischemic myocardial injury[52] and similar mobilization was seen in humans after acute myocardial infarction, where a fast reduction of monocytes in the spleen and increased amounts in the heart implied swift deployment of this reservoir.[53]

In adults, monocytes are constantly generated in the bone marrow from HSCs via intermediate progenitors, including the granulocyte-monocyte progenitor, the macrophage and dendritic cell progenitor, and finally the common monocyte progenitor, which differentiate into monocytes (Fig. 1A). Although this linear model for monocyte generation is generally accepted, there are other studies that propose a different model for the generation of monocytes from bone-marrow precursor cells.[54–57] These models propose a less linear process, where the progenitor cells are overlapping populations of precommitted.

Circulating monocytes generated in the bone marrow can be separated into three subsets based on differential expression of CD14 and CD16. Approximately 90% of them, termed "classical monocytes," present CD14 but are negative for CD16 ($CD14^+CD16^-$). The "nonclassical monocytes" are $CD14^{low}CD16^+$.[58] Finally, the third subtype termed "intermediate" has been defined as $CD14^+CD16^+$. However, this latter subtype has recently been under debate as a study by Villani et al. shows that, transcriptionally, only classical and nonclassical subtypes can be distinguished, and the intermediate subset highly resembles a population in transition between the other two subtypes.[59] Yet, others, also applying single cell techniques, showed clear transcriptomic differences between the three subtypes,[60] or identified multiple phenotypic distinctions.[61,62]

The different monocyte subsets also show differences in CD11b, with higher expression by classical monocytes compared to the nonclassical. Also, CD11c and CX_3CR_1 (fractalkine receptor) expressions, involved in monocyte survival, differ with both markers showing highest expression by nonclassical monocytes. Another major monocyte subset marker is CCR2, highly expressed on classical monocytes and a receptor for CCL2. This chemokine plays a role in the mobilization of monocytes from the bone marrow. This molecule is also related to the recruitment of monocytes cells to inflammatory sites such as atherosclerotic plaques or infection sites.[63–65]

The monocyte subtypes possess differences in their capacity to infiltrate tissues (Fig. 1B) based on the differential expression of chemokine receptors such as CCR2 or CX_3CR_1. "Classical" monocytes tend to be recruited first and at higher levels in inflammatory conditions whereas "nonclassical" monocytes have a patrolling function, monitoring the luminal side of blood vessels for tissue damage in the form of dying endothelial cells and promoting recruitment of other immune cells in case of damage.[66,67] Further clear differences between the subtypes with respect to phenotype, size, morphology, and transcriptome has been extensively described elsewhere.[56,68]

1.3 | Monocyte-derived macrophages: differentiation and activation

For clarity, we here discriminate two processes that impact on macrophage phenotype: differentiation and activation (Fig. 1B). Differentiation involves the process by which a monocyte transitions into a more mature state of a macrophage or a monocyte-derived dendritic cell induced by cytokines, growth factors, or other stimuli; monocytes can also differentiate into other cell types, such as osteoclasts. Activation, also sometimes referred to as polarization, refers to the phenotype that mature macrophages acquire upon encountering certain factors such as pathogen-related molecules or cytokines, for example, LPS or IFNγ, respectively. This terminology is still evolving greatly, and others distinguish activation and polarization into two separated terms.

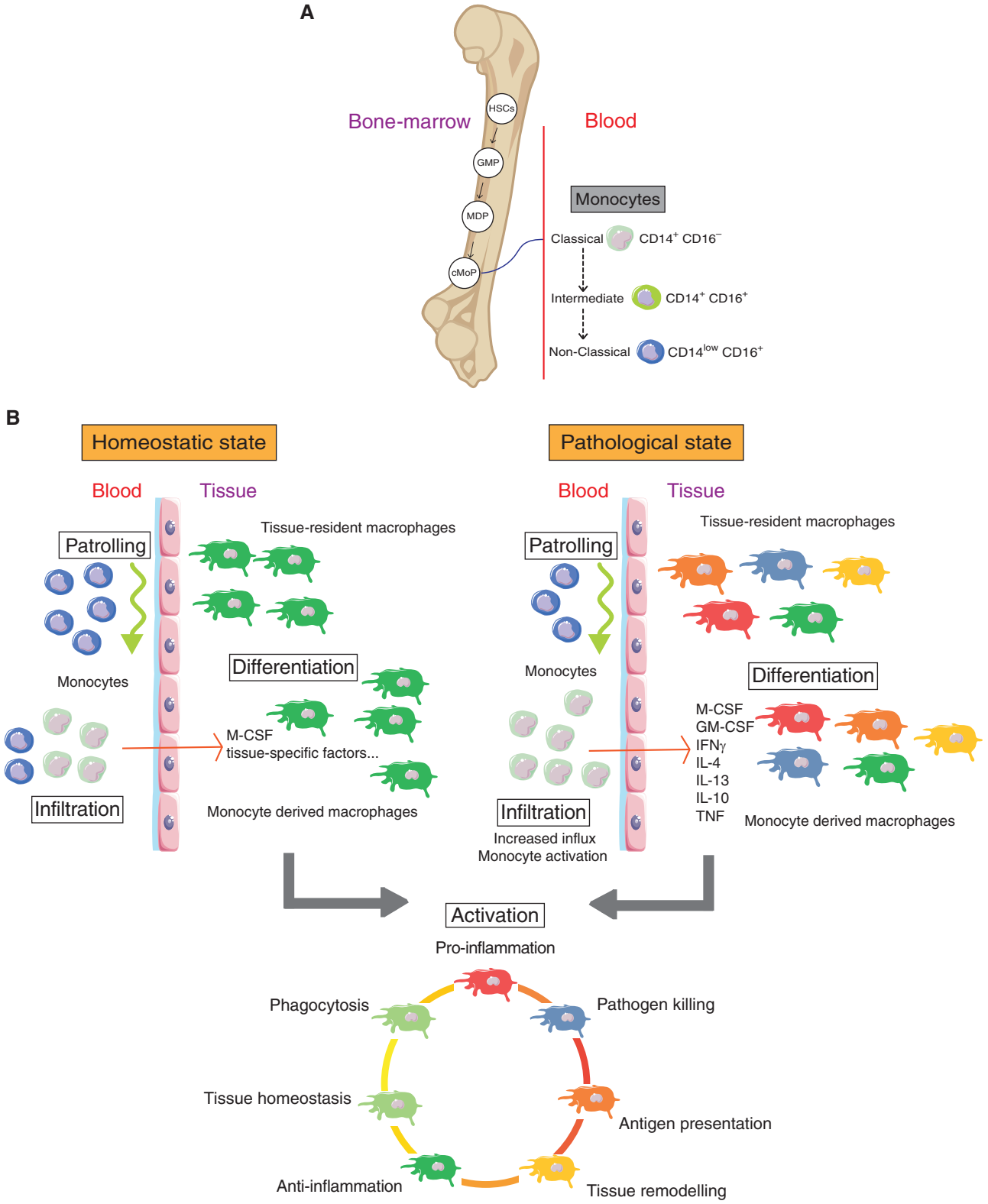

FIGURE 1 **A**: Cell precursors in the bone marrow that give rise to monocytes. Different subtypes of human monocytes found in circulating blood. **B**: Function of monocytes in homeostatic and pathological situations. Under homeostatic situations, classical, and to a lesser extent, non-classical monocytes infiltrate into tissues. Nonclassical monocytes perform patrolling functions on the luminal side of the blood vessel. In pathologic conditions there is an increase in recruitment and infiltration of classical monocytes into tissues. Macrophages, either monocyte-derived macrophage (MDMs) or tissue resident, respond to pathologic stimuli by acquiring an activated phenotype such as proinflammatory, pathogen killing, antigen presenting, anti-inflammatory, or tissue remodeling

The term "polarization" is sometimes used to describe a general phenotype change of macrophages upon certain stimuli, whereas "activation" describes the responsiveness of a cell to certain triggers.[69]

Classically, macrophage activation or polarization was divided into two simplistic subtypes, denominated M1 or M2. The two states represent opposite characteristics and their nomenclature was originally based on Th1 and Th2 cytokines.[70] M1 was referred to as the "proinflammatory" phenotype where the macrophages produced cytokines that enhance responses of the immune system and promote inflammation in tissues. On the other hand the M2 macrophages were considered "anti-inflammatory" cells and have for instance wound-healing capacities.[71] M1 macrophages produce specific chemokines to attract more leukocytes to tissue and to activate other immune cells through co-stimulatory molecules such as CD80. Examples of cytokines produced in the M1 response include IL-6, TNF, IL-12, and IL-1β.[72–74] M2 macrophages are induced by stimuli like IL-4/IL-13, IL-10, or corticosteroids[75] and promote not only wound-healing activities, but also fibrosis. Thus, these cells are important in resolution of the inflammatory state as regulators or dampeners of the immune response.[76,77]

This classical separation of macrophage activation has been expanded and changed by many recent studies (see, e.g., Murray[78] for an overview). Macrophage phenotypes are no longer restricted to two extreme phenotypes but resemble a spectrum (Fig. 1B) from highly proinflammatory to pro-fibrotic, pro-tumoral, anti-inflammatory, and many more. This was particularly well demonstrated by several transcriptome analysis studies where the response of macrophages to a wide range of stimuli led to the induction of a plethora of transcriptional phenotypes.[79,80] This model of a spectrum of responses found *in vitro* was also observed *in vivo* in human after comparison of, for instance, data obtained from alveolar macrophages from bronchoalveolar lavage in smokers, nonsmokers, and chronic obstructive pulmonary disease patients.[81,82] These studies show how specific cues produce unique phenotypes in the macrophages and that not all can be enclosed in simple dichotomy of M1 and M2 activation.

1.4 | M-CSF and GM-CSF: the classical *in vitro* differentiation factors

In vitro differentiation methods of macrophages have also been oversimplified. Historically, macrophages were thought to differentiate mainly from monocytes by the presence of M-CSF or GM-CSF, the latter more under inflammatory conditions.[83] M-CSF is a growth factor readily detected under homeostatic conditions, whereas GM-CSF present in some tissues under homeostatic conditions is not detected systemically unless induced by inflammatory situations.[83] M-CSF is produced by multiple cell types, including macrophages, endothelial cells, and fibroblasts. GM-CSF is produced not only by T cells, mast cells, and natural killer cells but also by macrophages, endothelial cells, and fibroblasts.[84] Endothelial cells produce M-CSF and GM-CSF in response to pro-inflammatory cytokines such as IL-1β, shear stress and also to disease-specific molecules like oxidized LDL in atherosclerosis.[85] Both M-CSF and GM-CSF promote cell survival, monocyte to macrophage differentiation, and enhance monocyte recruitment. The M-CSF and GM-CSF levels produced locally vary depending on the condition; in healthy situations the levels of M-CSF dominate, whereas in pathologic inflammatory conditions, such as rheumatoid arthritis, GM-CSF levels increase.[86–89] An example of a disease where the GM-CSF levels are also increased is multiple sclerosis (MS). In MS patients, CD4+ T cells in the CNS produce GM-CSF, which leads to polarization of macrophages to a more proinflammatory phenotype. These macrophages secrete proinflammatory cytokines such as IL-6, IL-1β, and TNF causing myelin sheath damage in the CNS of the patients. GM-CSF also increases the recruitment of monocytes contributing to disruption of the blood-brain barrier and blood-spinal cord barrier and further demyelination of the neurons in the CNS.[90] In turn, although M-CSF is a key physiologic mediator of macrophage biology, this pathway can become over-active and cause dysregulation as was shown for models of kidney and liver damage[91] and in cancer.[92]

M-CSF and GM-CSF activate cells via distinct signaling pathways. The M-CSF receptor is a homodimer formed by an extracellular domain that contains five immunoglobulin domains, a transmembrane domain and an intracellular domain. This receptor functions via several pathways including PI3K/Akt and MEK-ERK1/2 among others.[93] The GM-CSF receptor is a heterodimeric receptor formed by two subunits: the specific ligand-binding subunit (CSF2Rα) and the common signal-transduction subunit (CSF2Rβ)[84] and activates the JAK2/STAT5 pathway. Transcriptomic analysis of the monocyte differentiation processes by either M-CSF or GM-CSF has shown clear differences between the two cytokines, macrophages differentiated with GM-CSF express higher levels of proinflammatory cytokines genes such as *TNF* or *IL-1β* in response to LPS.[94] Many additional studies have made comparisons between M-CSF- and GM-CSF-induced macrophages that were differentiated *in vitro* from monocytes. In general, differentiation protocols involve culturing the cells between 3 and 7 d in culture medium in the presence of either cytokine. In Table 1 different characteristics of the two populations of cells are summarized.

1.5 | Other mediators used for *in vitro* monocyte to macrophage differentiation

Besides M-CSF and GM-CSF, other cytokines have also been used to differentiate human monocytes into macrophages. The study of alternative differentiation factors is important as monocytes encounter a wide variety of inflammatory mediators that can impact their differentiation while entering the tissue microenvironment. These alternative factors can induce a macrophage subtype different from the well-studied phenotypes observed when differentiated with M-CSF or GM-CSF only.

For instance, IL-34 has similar functions to M-CSF but has a more restrictive expression pattern. This cytokine is also key in the development of osteoclasts attached to bone and of microglia in the CNS, and has been related to rheumatoid arthritis.[105] Monocytes differentiated with IL-34 are like M-CSF macrophages as both molecules bind to the CSF-1 receptor and activate the same pathway for differentiation. Even though these macrophages are similar, there are differences in response after activation as IL-34-differentiated

TABLE 1 Comparison of GM-CSF- vs. M-CSF-differentiated macrophages

Type of study	Results	References
Transcriptomic	GM-CSF-induced macrophages express more genes related to the immune/inflammatory response and higher levels of proinflammatory cytokines after stimulation.	80, 94, 95
Transcription factor activation	GM-CSF activates STAT5. IRF4/5 are up-regulated in GM-CSF and IRF4 is down-regulated in M-CSF-differentiated cells.	94, 96
Cytokine production and inflammatory mediator production	M-CSF differentiated macrophages have lower production of proinflammatory cytokines compared to GM-CSF stimulation. Higher levels of IL-10 in M-CSF differentiated macrophages after stimulation. M-CSF derived macrophages produce higher levels of eicosanoids in response to bacteria.	97–100
Morphology	GM-CSF and M-CSF derived macrophages present different morphology.	94, 95
Surface markers	CD163 is expressed higher in M-CSF differentiated macrophages after dexamethasone stimulation. Differential expression of CD14, lower in GM-CSF differentiated macrophages.	96, 101
Lipid metabolism	Differences in expression of genes related to lipid metabolism, higher apolipoprotein E levels in GM-CSF differentiated macrophages. Differential activation of inflammasome, higher in GM-CSF differentiated macrophages.	102, 103
Translation	GM-CSF derived macrophages have a similar phenotype to alveolar macrophages from patients from pulmonary sarcoidosis and pulmonary neoplasia compared to M-CSF.	104

macrophages show increased phagocytic capacity and higher IL-10 and CCL-17 production after stimulation. These differences might be explained by the capacity of IL-34 to bind to receptors other than CSF-1R.[106] Macrophages differentiated with IL-34 show a clear anti-inflammatory, immunosuppressive phenotype that in tumors is associated with lower levels of infiltration of cytotoxic CD8 T cells.[107] Another example factor impacting on differentiation is platelet factor 4 (PF4) also known as CXCL4, a chemokine secreted during acute vascular injury. PF4-mediated differentiation of monocytes prevents apoptosis in monocytes and induces a macrophage-like morphology with cells presenting pseudopodia as well as increased expression of macrophage maturation markers, but showed lower expression of HLA.[108] Transcriptomic studies of macrophages differentiated with M-CSF compared to CXCL4[109] showed that the CXCL4 differentiated macrophages acquire macrophage-like morphology and express CD45 and CD68, thus confirming bona fide macrophages. In terms of comparison with M-CSF macrophage differentiation, there is a correlation in genes expressed after M-CSF and CXCL4 differentiation but in terms of function, the CXCL4 differentiated macrophages express higher levels of cytokines such as IL-6 and TNF and can thus be seen as more proinflammatory.[110]

CCL2, also known as MCP-1, is a classical chemokine that drives the recruitment of monocytes to tissues. The expression of CCL-2 and its receptor, CCR2, in macrophages varies depending on the cytokine used for the differentiation, seeing higher levels of CCL-2 expression in M-CSF macrophages. The presence of CCL-2 during polarization leads to the presentation of a less proinflammatory phenotype with reduced levels of IL-6 expression.[111] CCL2 is also important in the tumor microenvironment where CCL-2 in combination with IL-6 promotes de survival of CD11b+ cells by increasing the expression of anti-apoptotic proteins (e.g., cFLIP, Bcl-2, and Bcl-X) and blocking the cleavage of the caspase-8. These cells also show increased expression of anti-inflammatory markers such as the mannose receptor CD206.[112] IL-6 alone is also capable to impact on macrophages in the tumor microenvironment by activation of the STAT3 pathway, increasing the expression of CD206, CD163, and the production of IL-10 and TGFβ.[113] These data suggest that both CCL2 and IL-6 drive macrophages toward a cell-phenotype with reduced inflammatory and increased immunosuppressive characteristics, both relevant in tumor growth.

The IL-32 cytokine also presents differentiation capacities. When monocytes are differentiated in the presence of IL-32 there is an increase in the expression of CD14 and a blockage of the effect of GM-CSF+IL-4 in the differentiation of monocytes toward DC,[114] showing a decrease in CD64. The IL-32-generated cells also show phagocytic capacities. IL-32 promotes monocyte to macrophage differentiation by activation of the p38-MAPK and NF-$\kappa\beta$ pathways.[115] The presence of IL-17 in cultured monocytes increased the expression levels of genes for proinflammatory cytokines, chemokines, and pathways related with leukocyte trans-endothelial migration.[116]

A considerable number of molecules are considered to be able to promote monocyte differentiation by the observation of increased levels of macrophage markers in the cells, for example, CD64 and CD80 after using TNF or IFNγ for the differentiation.[117] The phenotype in terms of function of the obtained macrophages generated in the presence of these two factors has not been characterized in detail. However, it is well known that when used for polarization, IFNγ drives a phenotype that is important in the defense against intracellular pathogens. Both IFNγ and TNF stimulation induce a proinflammatory phenotype with increased expression of IL-1β, IL-12, and reduced IL-10.[118,119] Both TNF and IFNγ induce the Th1 phenotype in T cells as a result of the IL-12 produced inducing the Th1 response.[120,121]

Table 2 captures the best-defined examples of molecules with the ability to induce monocyte differentiation, either alone or in combination with M-CSF or GM-CSF and a brief discussion of the phenotypes observed.

It is important also to highlight the role of nonimmune molecules in the monocyte to macrophage differentiation process. Metabolites are widely present and some of them may impact on differentiation. For instance, calcium oxalate (CaOx), a constituent of kidney stones and

TABLE 2 Inflammatory factors used to differentiate human monocytes into macrophages

Factors	Phenotype relative to M-CSF macrophages	References
IL-4, adiponectin	Macrophages with greater anti-inflammatory phenotype.	122
Microparticles (from platelets)	Microparticles stimulate monocytes to express increased CD11b, CD14, and CD68 after 7 d culture. No comparison against M-CSF macrophages was made.	123
M-CSF or GM-CSF + butylated hidroxyanisole (BHA)	BHA affects the differentiation of M-CSF macrophages by modifying their morphology and reducing CD11b and CD163 expression.	124
M-CSF or GM-CSF, with or without serum-containing media	The absence of serum in the media causes a loss of the M-CSF elongated morphology and decreased CD163 expression.	125
Platelet factor 4 (PF4)	PF4 up-regulates expression of macrophage differentiation markers. Similar capacities of preventing monocyte apoptosis when differentiation is performed with M-CSF or GM-CSF.	108
IL-34	Overall similar phenotype to M-CSF derived macrophages. Less phagocytic capacity than M-CSF after alternative activation. Higher IL-10 and CXCL11 production than M-CSF derived macrophages after activation with LPS + IFNγ.	106
Cathelicidin antimicrobial peptides LL-37 (LL-37) with M-CSF	Lower IL-10 and higher IL-12p70 after LPS stimulation in M-CSF + LL-37 differentiated macrophages.	126
Hemoglobin or IL-4	Different phenotype between macrophages differentiated in the presence of hemoglobin or IL-4. Mannose receptor and CD163 are higher in hemoglobin-differentiated macrophages. Hemoglobin prevents foam cell formation. No comparisons to M-CSF derived macrophages were made.	127
IL-32	Increased expression of macrophage markers (CD14) and phagocytic capacities. Differentiation via NF-κB pathway. No comparisons to M-CSF macrophages were made.	115
IFNγ + GM-CSF + IL-4	IFNγ blocks the GM-CSF + IL-4-mediated dendritic cell differentiation by stimulating M-CSF production and inducing macrophage development.	128
IFNγ, IL-4, IL-10, TNF, dexamethasone, M-CSF, and GM-CSF	Differentiation of macrophages with different mediators induces surface markers at different levels depending on the factor used. Macrophages differentiated with IL-10 present similar levels of CD163 and CD16 compared to M-CSF macrophages.	117
CaOx	Similar morphology to GM-CSF macrophages. CD68 and CD86 expression but no CD163 or CD206. Proinflammatory phenotype with higher levels of IL-12 and TNF and lower IL-10 compare to M-CSF.	129
Lactate	Leads the differentiation of monocytes toward alternative activated macrophages instead of DCs. Increased expression of CD14and lower CD1a. Macrophages with tumor promoting and alternative activated immuno-suppressive phenotype. Increase production of VEGF and some proinflammatory characteristics high IL-1β but absence of Th1-inducing response (low IL-12)	130–134
Hypoxia	Lower phagocytic capacities, CD206, and CD40. Higher production levels of VEGF supporting angiogenesis.	135

associated with kidney disease, induces the differentiation of monocytes into proinflammatory macrophages in the kidney. Monocytes differentiated in the presence of CaOx present a macrophage-like morphology similar to GM-CSF derived macrophages, show expression of CD68 and CD86 but not CD163 or CD206. These macrophages also produce higher levels of IL-12, TNF, and lower IL-10[129] compared to M-CSF derived macrophages. Therefore, they seem to have a clear proinflammatory phenotype.

Other nonimmune parameters to consider are the environment where the differentiation takes place. For example, in tumors or inflamed tissues it is common to find hypoxia. During the monocyte differentiation in hypoxia there is an increase in the expression of hypoxia-inducible factor 1 (HIF-1α and HIF-1β), which give them the ability to respond to hypoxia.[136] If the differentiation takes place in hypoxia conditions the survival rate of the cells it is not affected.[137] Macrophages generated under these conditions compared to normal oxygen levels showed lower phagocytic capacities and lower CD206 and CD40 but higher levels of vascular endothelial growth factor (VEGF).[135]

A major metabolite that is also present in tumor microenvironment and whose production is increased in hypoxia is lactate. This metabolite has not been tested as a sole differentiation factor but has great impact on macrophages. One study has tested the effects of lactate on DC differentiation of DCs from monocytes. When DCs are differentiated in the presence of lactate producer cells, the resulting cells presented an alternative activated macrophage phenotype instead of a DC phenotype. The cells expressed higher levels of CD14 and less CD1a. The cells also induced Th2 responses in T cells. Therefore this study shows how the presence of lactate is capable to shift monocyte differentiation away from DCs toward an alternatively immuno-suppressive macrophage type.[130] The impact of tumor cell derived lactate on monocyte to macrophage differentiation may

be crucial in cancer situations. It is well established that lactate drives macrophages toward a tumor-promoting phenotype through induction of VEGF and alternatively activated immune-suppressive characteristics.[131–133] Such changes were recently linked to lactate-mediated histone modifications.[138] Of note, recent data shows that the shift in the phenotype is not that clear-cut anti-inflammatory. Paolini et al showed that in human macrophages lactate drives a phenotype that has some proinflammatory characteristics (e.g., high IL-1β), but lacks Th1-driving capacity (e.g., low IL-12) and has tumor-promoting activity, for instance by producing of growth and proangiogenic factors.[134]

2 | CONCLUSIONS

The macrophage field is evolving rapidly, and the nuance of the M1/M2 paradigm, is a good example of this evolution. Originally in the M1/M2 paradigm only two closed activation states were considered, this idea is changing to understanding the macrophage activation as a spectrum of different activation states. Incorporation of transcriptional studies, and especially those at the single cell level, has revealed the complexity of the MPS and disclosed anatomically specific profiles.[139,140] The diverse cellular ontogeny of macrophages adds an additional layer of functional heterogeneity to these cells. Based on these advances revisions to macrophage nomenclature were proposed based on origin and ontogeny and then on the function, location, and phenotype.[141]

In the same way that the nomenclature is evolving, protocols to generate macrophages have also advanced. Ex vivo studies with human macrophages are very difficult to carry out due to logistical or ethical considerations, so developing representative *in vitro* models is necessary. The use of the different models (MDMs, iPSDMs. or cell lines) will vary based on the scientific question and each approach has its merit.

Other *in vitro* methods to study macrophages, which aim to reproduce the *in vivo* settings of macrophages in the context of organs, are 3D cultures and organoids. These methods are very valuable to study the interaction of macrophages with other cell types in a 3D structure and investigate how the organ structure affects their function, morphology, maturation, migration, among others.[142,143]. The 3D cultures and organoids are widely used in cancer research where these techniques help to understand how cancer cells modify the macrophage phenotype.[144] For instance, when monocytes are added to a coculture of pancreatic tumor cells and fibroblasts the monocytes added differentiate into macrophages, showing an increase in CD68 expression. These cells present an alternative activated phenotype with high levels of CD163 and CD14 and low levels of CD86 and HLA-DR.[145] Another important aspect that can be studied in cancer by 3D/organoids is how macrophages infiltrate in the tumor; for instance, macrophages can use podosomes with proteolytic capacities that break the extracellular matrix to enter into tissues.[146,147] Besides cancer, 3D cultures and organoids could also be used, for example, to study how macrophage play their role in tissue remodeling or wound healing.[148,149] Another application of 3D cultures is related to microglia in the brain. In this case the organoids used are cells derived from the neuroectodermal lineage, and the microglia generated in this 3D cocultures present phagocytosis capacities and similar morphology and transcriptomic response after inflammatory stimulation compared to post-mortem isolated microglia.[150]

The use of iPSDMs has made great progress in generating protocols for the development of tissue-specific macrophages, to date, and protocols used to generate MDMs have been predominantly based on the use of M-CSF and GM-CSF as differentiation factors. However, many additional inflammatory mediators have the ability to stimulate differentiation of monocytes into macrophages alone or in combination with other factors. Indeed, monocytes infiltrating tissues in health or disease will encounter a range of mediators rather than (G)M-CSF in isolation. Furthermore, application of tissue-specific environments in the induction of MDM differentiation remains an underexplored field, in our view. Therefore, work is needed to advance our knowledge on the impact of multiple mediator-induced macrophage phenotypes with emphasis on tissue-specific factors.

Many of these new possible differentiation molecules are cytokines related to alternative activation of the immune system (IL-4, IL-13, IL-10), the impact of these factors on differentiation is not well understood. These cytokines are for instance important in the pathogenesis of allergic airway disease and asthma. On the other hand, IL-10, an anti-inflammatory cytokine, has been linked to chronic fibroproliferative diseases, such as chronic pancreatitis, pulmonary fibrosis, chronic kidney disease, and others.[151,152] Additional cytokines with the potential to induce monocyte differentiation are IL-32, IFNγ, and TNF, and many of these are key drivers of immune-mediated inflammatory diseases such as IFNγ in rheumatoid arthritis.[153] IL-32 in cardiovascular diseases[154] and TNF in inflammatory bowel disease.[155]

Other molecules besides cytokines also showed differentiation-inducing capacities, including adiponectin, butylated hidroxyanisole (BHA), PF4, and hemoglobin. These molecules can be found in tissues under inflammatory conditions, for example, adiponectin in vascular diseases[156] and PF4 in heart failure and lupus nephritis.[157,158] When studying macrophages in these diseases, it would be valuable to include these mediators as part of the microenvironment during monocyte to macrophage differentiation.

Generally, *in vitro* models are restricted in terms of the heterogeneity of the cell populations when compared to those found *in vivo* in disease. However, *in vitro* models continue to provide valuable systems to understand cellular mechanisms and opportunities to modulate these in order to intervene pathogenic processes. However, with the availability of many new technologies it is important to make the next step in *in vitro* modelling of cells and to broaden the way macrophages are generated and activated. Improvement of *in vitro* protocols will provide the much-needed translation to humans and human disease.

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AUTHORSHIP

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DISCLOSURES

P.K.M. is an employee and shareholder at GlaxoSmithKline. The rest of the authors declare no conflicts of interest.

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REFERENCES

1. Gordon S. The macrophage: past, present and future. *Eur J Immunolo*. 2007;37(S1):S9-S17.
2. Jenkins SJ, Hume DA. Homeostasis in the mononuclear phagocyte system. *Trends Immunol*. 2014;35(8):358-367.
3. Hume DA, Irvine KM, Pridans C. The mononuclear phagocyte system: the relationship between monocytes and macrophages. *Trends Immunol*. 2019;40(2):98-112.
4. Van Furth R, Cohan ZA, Hirsch JG, et al. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull World Health Organ*. 1972;46(6):845.
5. Merad M, Manz MG, Karsunky H, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol*. 2002;3(12):1135.
6. Ajami B, Bennett JL, Krieger C, et al. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci*. 2007;10(12):1538-1543.
7. Hashimoto D, Chow A, Noizat C et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;38(4):792-804.
8. Stremmel C, Schuchert R, Wagner F, et al. Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat Commun*. 2018;9(1):75.
9. Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. *Cell*. 2015;160(5):816-827.
10. Gordon S, Martinez-Pomares L. Physiological roles of macrophages. *Pflügers Archiv—Eur J Physiol*. 2017;469(3):365-374.
11. Soares MP, Hamza I. Macrophages and iron metabolism. *Immunity*. 2016;44(3):492-504.
12. Okabe Y, Medzhitov R. Tissue biology perspective on macrophages. *Nat Immunol*. 2015;17:9.
13. Doebel T, Voisin B, Nagao K. Langerhans cells—the macrophage in dendritic cell clothing. *Trends Immunol*. 2017;38:817-828.
14. Deckers J, Hammad H, Hoste E. Langerhans cells: sensing the environment in health and disease. *Frontiers Immunol*. 2018;9:93.
15. West HC, Bennett CL. Redefining the role of langerhans cells as immune regulators within the skin. *Frontiers Immunol*. 2018;8:1941.
16. Chinetti-Gbaguidi G, Colin S, Staels B. Macrophage subsets in atherosclerosis. *Nat Rev Cardiol*. 2014;12:10.
17. Katsiari CG, Liossis S-NC, Sfikakis PP. *The pathophysiologic role of monocytes and macrophages in systemic lupus erythematosus: a reappraisal*. in *Seminars in arthritis and rheumatism*. 2010;39(6):491-503. WB Saunders.
18. Porta C, Rimoldi M, Raes G, et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB. *Proc National Acad Sci*. 2009;106(35):14978-14983.
19. Cohen J. The immunopathogenesis of sepsis. *Nature*. 2002;420:885.
20. Udalova IA, Mantovani A, Feldmann M. Macrophage heterogeneity in the context of rheumatoid arthritis. *Nat Rev Rheumatol*. 2016;12:472.
21. Davies LC, Rosas M, Smith PJ, et al. A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *Eur J Immunol*. 2011;41(8):2155-2164.
22. Schulz C, Perdiguero EG, Chorro L, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. 2012;336(6077):86-90.
23. Zambidis ET, Peault P, Park TS, Bunz F, Civin CI. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood*. 2005;106(3):860-870.
24. Hoeffel G, Ginhoux F. Fetal monocytes and the origins of tissue-resident macrophages. *Cell Immunol*. 2018;330:5-15.
25. Tober J, Koniski A, McGrath KE, et al. The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood*. 2007;109(4):1433-1441.
26. Sheng J, Ruedl C, Karjalainen K. Most tissue-resident macrophages except microglia are derived from fetal hematopoietic stem cells. *Immunity*. 2015;43(2):382-393.
27. Palis J, Yoder MC. Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp Hematol*. 2001;29(8):927-936.
28. Hoeffel G, Wang Y, Greter M, et al. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med*. 2012.209(6):1167-1181.
29. Ginhoux F, Greter M, Leboeuf M, et al. Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science*. 2010;330:841-845.
30. De Schepper S, Verheijden S, Aguilera-Lizarraga J, et al. Self-Maintaining Gut Macrophages Are Essential for Intestinal Homeostasis. *Cell*. 2019;176(3):676.
31. Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. *Immunity*. 2016;44(3):439-449.
32. Karlsson KR, Cowley S, Martinez FO, et al. Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. *Exp Hematol*. 2008;36(9):1167-1175.
33. van Wilgenburg B, Browne C, Vowles J, Cowley SA. Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. *PLoS One*. 2013;8(8):e71098.
34. Choi K-D, Vodyanik M, Slukvin II. Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells. *Nat Protoc*. 2011;6(3):296.
35. Senju S, Haruta M, Matsumura K, et al. Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy. *Gene Ther*. 2011;18(9): 874.
36. Yu P, Pang G, Yu J, Thomson JA. FGF2 sustains NANOG and switches the outcome of BMP4-induced human embryonic stem cell differentiation. *Cell Stem Cell*. 2011;8(3):326-334.
37. Leung A, Ciau-Uitz A, Pinheiro P, et al. Uncoupling VEGFA functions in arteriogenesis and hematopoietic stem cell specification. *Dev Cell*. 2013;24(2):144-158.
38. Sturgeon CM, Ditadi A, Awong G, Kennedy M, Keller G. Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nat Biotechnol*. 2014;32(6):554.

39. Blauwkamp TA, Nigam S, Ardehali R, Weissman IL, Nusse R. Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nat Commun*. 2012;3:1070.
40. Kumano K, Chiba S, Kunisato A, et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity*. 2003;18(5):699-711.
41. Metcalf D. Hematopoietic cytokines. *Blood*. 2008;111(2):485-491.
42. Abud EM, Ramirez RN, Martinez ES, et al. iPSC-derived human microglia-like cells to study neurological diseases. *Neuron*. 2017;94(2):278-293.e9.
43. Takata K, Kozaki T, Lee CZW, et al. Induced-pluripotent-stem-cell-derived primitive macrophages provide a platform for modeling tissue-resident macrophage differentiation and function. *Immunity*. 2017;47(1):183-198.e6.
44. Garcia-Reitboeck P, Phillips A, Piers TM, et al. Human induced pluripotent stem cell-derived microglia-like cells harboring TREM2 missense mutations show specific deficits in phagocytosis. *Cell Rep*. 2018;24(9):2300-2311.
45. Lopez-Yrigoyen M, Fidanza A, Cassetta L, et al. A human iPSC line capable of differentiating into functional macrophages expressing ZsGreen: a tool for the study and in vivo tracking of therapeutic cells. *Phil Trans R Soc B*. 2018;373(1750):20170219.
46. Alasoo K, Martinez FO, Hale C, et al. Transcriptional profiling of macrophages derived from monocytes and iPS cells identifies a conserved response to LPS and novel alternative transcription. *Sci Rep*. 2015;5:12524.
47. Lachmann N, Happle C, Ackermann M, et al. Gene correction of human induced pluripotent stem cells repairs the cellular phenotype in pulmonary alveolar proteinosis. *Am J Respir Criti Care Med*. 2014;189(2):167-182.
48. Panicker LM, Miller D, Awad O, et al. Gaucher iPSC-derived macrophages produce elevated levels of inflammatory mediators and serve as a new platform for therapeutic development. *Stem Cells*. 2014;32(9):2338-2349.
49. Gupta RM, Meissner TB, Cowan CA, et al. Genome-edited human pluripotent stem cell-derived macrophages as a model of reverse cholesterol transport—brief report. *Arterioscler Thromb Vasc Biol*. 2016;36(1):15-18.
50. Zhang H, Shi J, Hachet MA, et al. CRISPR/Cas9-mediated gene editing in human iPSC-derived macrophage reveals lysosomal acid lipase function in human macrophages—brief report. *Arterioscler Thromb Vasc Biol*. 2017;37(11):2156-2160.
51. Dey A, Allen J, Hankey-Giblin PA. Ontogeny and polarization of macrophages in inflammation: blood monocytes versus tissue macrophages. *Front Immunol*. 2015;5:683.
52. Swirski FK, Nahrendorf M, Etzrodt M, et al. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. *Science*. 2009;325(5940):612-616.
53. van der Laan AM, Horst ENT, Delewi R, et al. Monocyte subset accumulation in the human heart following acute myocardial infarction and the role of the spleen as monocyte reservoir. *Eur Heart J*. 2013;35(6):376-385.
54. Iwasaki H, Akashi K. Myeloid lineage commitment from the hematopoietic stem cell. *Immunity*. 2007;26(6):726-740.
55. Hettinger J, Richards DM, Hansson J, et al. Origin of monocytes and macrophages in a committed progenitor. *Nat Immunol*. 2013;14(8):821.
56. Guilliams M, Mildner A, Yona S. Developmental and functional heterogeneity of monocytes. *Immunity*. 2018;49(4):595-613.
57. Zhao Y, Zou W, Du J, Zhao Y. The origins and homeostasis of monocytes and tissue-resident macrophages in physiological situation. *J Cell Physiol*. 2018;233(10):6425-6439.
58. Ziegler-Heitbrock L, Ancuta P, Crowe S, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;116(16):e74-e80.
59. Villani A-C, Satija R, Reynolds G, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. 2017;356(6335):eaah4573.
60. Gren ST, Rasmussen TB, Janciauskiene S, et al. A single-cell gene-expression profile reveals inter-cellular heterogeneity within human monocyte subsets. *PLoS One*. 2015;10(12):e0144351.
61. Ong S-C, Teng K, Newell E, et al. A novel, five-marker alternative to CD16-CD14 gating to identify the three human monocyte subsets. *Front Immunol*. 2019;10:1761.
62. Thomas GD, Hamers AAJ, Nakao C, et al. Human blood monocyte subsets: a new gating strategy defined using cell surface markers identified by mass cytometry. *Arterioscler Thromb Vas Biol*. 2017;37(8):1548-1558.
63. Boyette LB, Macedo C, Hadi K, et al. Phenotype, function, and differentiation potential of human monocyte subsets. *PLoS One*. 2017;12(4):e0176460.
64. Landsman L, Bar-On L, Zernecke A, et al. CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood*. 2009;113(4):963-972.
65. França CN, Izar MCO, Hortêncio MNS, et al. Monocyte subtypes and the CCR2 chemokine receptor in cardiovascular disease. *Clinical Sci*. 2017;131(12):1215-1224.
66. Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007;317(5838):666-670.
67. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*. 2014;14(6):392-404.
68. Olingy CE, Dinh HQ, Hedrick CC. Monocyte heterogeneity and functions in cancer. *J Leukoc Biol*. 2019;106:309-322.
69. van Beek AA, Van den Bossche J, Mastroberardino PG, et al. Metabolic alterations in aging macrophages: ingredients for inflammaging? *Trends Immunol*. 2019;40:113-127.
70. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13(1):453-461.
71. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol*. 2000;164(12):6166-6173.
72. Unanue ER. Antigen-presenting function of the macrophage. *Annu Rev Immunol*. 1984;2(1):395-428.
73. Barros MHM, Hauck F, Dreyer JH, Kempkes B, Niedobitek G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS One*. 2013;8(11):e80908.
74. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*. 2010;11(10):889.
75. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Front Immunol*. 2014;5:514.
76. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549-555.
77. Ramon S, Dalli J, Sanger JM, et al. The protectin PCTR1 is produced by human M2 macrophages and enhances resolution of infectious inflammation. *AM J Pathol*. 2016;186(4):962-973.
78. Murray PJ. Macrophage polarization. *Annu Rev Physiol*. 2017;79:541-566.
79. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime Rep*. 2014;6:13.
80. Xue J, Schmidt SV, Sander J, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*. 2014;40(2):274-288.
81. Shaykhiev R, Krause A, Salit J, et al. Smoking-dependent reprogramming of alveolar macrophage polarization: implication for

pathogenesis of chronic obstructive pulmonary disease. *J Immunol*. 2009;183:2867-2883.
82. Woodruff PG, Koth LL, Hwa Y, et al. A distinctive alveolar macrophage activation state induced by cigarette smoking. *Am J Respir Crit Care Med*, 2005;172(11):1383-1392.
83. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. *Nat Rev Rheumatol*. 2016;12(1):37.
84. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*. 2008;8:533.
85. Montanari E, Stojkovic S, Kaun C, et al. Interleukin-33 stimulates GM-CSF and M-CSF production by human endothelial cells. *Thromb Haemost*. 2016;116(08):317-327.
86. McInnes IB, Buckley CD, Isaacs JD. Cytokines in rheumatoid arthritis—shaping the immunological landscape. *Nat Rev Rheumatol*. 2016;12(1):63.
87. Hume DA, MacDonald KP. Therapeutic applications of macrophage colony-stimulating factor (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. *Blood*. 2011;119:1810-1820.
88. de Groot RP, Coffer PJ, Koenderman L. Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. *Cell Signal*. 1998;10(9):619-628.
89. Fleetwood AJ, Cook AD, Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. *Crit Rev Immunol*. 2005;25(5):405-428.
90. Shiomi A, Usui T, Mimori T. GM-CSF as a therapeutic target in autoimmune diseases. *Inflamm Regen*. 2016;36(1):8.
91. Hamilton JA, Cook AD, Tak PP. Anti-colony-stimulating factor therapies for inflammatory and autoimmune diseases. *Nat Rev Drug Discov*. 2017;16(1):53.
92. Baghdadi M, Endo H, Takano A, et al. High co-expression of IL-34 and M-CSF correlates with tumor progression and poor survival in lung cancers. *Sci Rep*. 2018;8(1):418.
93. Stanley ER, Chitu V. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb Perspect Biol*. 2014;6(6):a021857.
94. Lacey DC, Achuthan A, Fleetwood AJ, et al. Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. *J. Immunol*. 2012;188:5752-5765.
95. Hashimoto S, Suzuki T, Dong HY, Yamazaki N, Matsushima K. Serial analysis of gene expression in human monocytes and macrophages. *Blood*. 1999;94(3):837-844.
96. Lehtonen A, Matikainen S, Miettinen M, Julkunen I. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced STAT5 activation and target-gene expression during human monocyte/macrophage differentiation. *J Leuko Biol*. 2002;71(3):511-519.
97. Xu W, Zhao X, Daha MR, van Kooten C. Reversible differentiation of pro-and anti-inflammatory macrophages. *Mol Immunol*. 2013;53(3):179-186.
98. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and T H 1-T H 17 responses. *Nat Immunol*. 2011;12(3):231.
99. Jaguin M, Houlbert N, Fardel O, Lecureur V. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell Immunol*. 2013;281(1):51-61.
100. Lukic A, Larssen P, Fauland A, et al. GM-CSF- and M-CSF- primed macrophages present similar resolving but distinct inflammatory lipid mediator signatures. *FASEB J*. 2017;31(10):4370-4381.
101. Vogel DY, Glim JE, Stavenuiter AWD, et al. Human macrophage polarization in vitro: maturation and activation methods compared. *Immunobiology*. 2014;219(9):695-703.
102. Waldo SW, Li Y, Buono C, et al. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *Am J Pathol*. 2008;172(4):1112-1126.
103. Budai MM, Tőzsér J, Benkő S. Different dynamics of NLRP3 inflammasome-mediated IL-1β production in GM-CSF- and M-CSF-differentiated human macrophages. *J. Leukoc Biol*. 2017;101(6):1335-1347.
104. Lescoat A, Ballerie A, Augagneur A, et al. Distinct properties of human M-CSF and GM-CSF monocyte-derived macrophages to simulate pathological lung conditions in vitro: application to systemic and inflammatory disorders with pulmonary involvement. *Int J Mol Sci*. 2018;19(3):894.
105. Masteller EL, Wong BR. Targeting IL-34 in chronic inflammation. *Drug Discov Today*. 2014;19(8):1212-1216.
106. Boulakirba S, Pfeifer A, Mhaidly R, et al. IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential. *Sci Rep*. 2018;8(1):256.
107. Baghdadi M, Wada H, Nakanishi S, et al. Chemotherapy-induced IL34 enhances immunosuppression by tumor-associated macrophages and mediates survival of chemoresistant lung cancer cells. *Cancer Res*. 2016;76(20):6030-6042.
108. Scheuerer B, Ernst M, Dürrbaum-Landmann I, et al. The CXC-chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. *Blood*. 2000;95(4):1158-1166.
109. Gleissner CA, Shaked I, Little KM, Ley K, et al. CXC chemokine ligand 4 induces a unique transcriptome in monocyte-derived macrophages. *J. Immunol*. 2010;184(9):4810-4818.
110. Domschke G, Gleissner CA. CXCL4-induced macrophages in human atherosclerosis. *Cytokine*. 2019;122:154141.
111. Sierra-Filardi E, Nieto C, Domínguez-Soto A, et al. CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J. Immunol*. 2014;192(8):3858-3867.
112. Roca H, Varsos ZS, Sud S, et al. CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. *J Biol Chem*. 2009;284(49):34342-34354.
113. Fu X-L, Duan W, Su C-Y, et al. Interleukin 6 induces M2 macrophage differentiation by STAT3 activation that correlates with gastric cancer progression. *Cancer Immunol Immunother*. 2017;66(12):1597-1608.
114. Gschwandtner M, Derler R, Midwood KS. More than just attractive: how CCL2 influences myeloid cell behavior beyond chemotaxis. *Front Immunol*. 2019;10:2759.
115. Netea MG, Lewis EC, Azam T, et al. Interleukin-32 induces the differentiation of monocytes into macrophage-like cells. *Proc Natl Acad Sci*. 2008;105(9):3515-3520.
116. Wang CQ, Suárez-Fariñas M, Nograles KE, et al. IL-17 induces inflammation-associated gene products in blood monocytes, and treatment with ixekizumab reduces their expression in psoriasis patient blood. *J Invest Dermatol*. 2014;134(12):2990.
117. Ambarus CA, Krausz S, van Eijk M, et al. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. *J Immunol Methods*. 2012;375(1-2):196-206.
118. Mantovani A, Sica A, Locati M, Macrophage polarization comes of age. *Immunity*. 2005;23(4):344-346.
119. Donlin LT, Jayatilleke A, Giannopoulou EG, et al. Modulation of TNF-induced macrophage polarization by synovial fibroblasts. *J Immunol*. 2014;193(5):2373-2383.
120. Wilke CM, Wei S, Wang L, et al. Dual biological effects of the cytokines interleukin-10 and interferon-γ. *Cancer Immunol Immunothe*. 2011;60(11):1529.
121. Teng MW, Bowman EP, McElwee JJ, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med*. 2015;21(7):719.

122. Lovren F, Pan Y, Quan A, et al. Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages. *Am J Physiol Heart Circ Physiol*. 2010;299(3):H656-H663.
123. Vasina E, Cauwenberghs S, Feijge MAH, et al. Microparticles from apoptotic platelets promote resident macrophage differentiation. *Cell Death Dis*. 2011;2(9):e211.
124. Zhang Y, Choksi S, Chen K, et al. ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. *Cell Res*. 2013;23(7):898.
125. Rey-Giraud F, Hafner M, Ries CH. In vitro generation of monocyte-derived macrophages under serum-free conditions improves their tumor promoting functions. *PLoS One*. 2012;7(8):e42656.
126. van der Does AM, Beekhuizen H, Ravensbergen B, et al. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *J Immunol*. 2010;185:1442-1449.
127. Finn AV, Nakano M, Polavarapu R, et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. *J Am Coll Cardiol*. 2012;59(2):166-177.
128. Delneste Y, Charbonnier P, Herbault N, et al. Interferon-γ switches monocyte differentiation from dendritic cells to macrophages. *Blood*. 2003;101(1):143-150.
129. Dominguez-Gutierrez PR, Kusmartsev S, Canales BK, et al. Calcium oxalate differentiates human monocytes into inflammatory M1 macrophages. *Front Immunol*. 2018;9:1863.
130. Selleri S, Bifsha P, Civini S, et al. Human mesenchymal stromal cell-secreted lactate induces M2-macrophage differentiation by metabolic reprogramming. *Oncotarget*. 2016;7(21):30193.
131. Colegio OR, Chu N-Q, Szabo AL, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. 2014;513(7519):559-563.
132. Bohn T, Rapp S, Luther N, et al. Tumor immunoevasion via acidosis-dependent induction of regulatory tumor-associated macrophages. *Nat Immunol*. 2018;19(12):1319.
133. Ivashkiv LB. The hypoxia–lactate axis tempers inflammation. *Nat Rev Immunol*. 2020;20(2):85-86.
134. Paolini L, Adam C, Beauvillain C, et al. Lactic acidosis together with GM-CSF and M-CSF induces human macrophages toward an inflammatory protumor phenotype. *Cancer Immunol Res*. 2020;8(3):383-395.
135. Staples KJ, Sotoodehnejadnematalahi F, Pearson H, et al. Monocyte-derived macrophages matured under prolonged hypoxia transcriptionally up-regulate HIF-1α mRNA. *Immunobiology*. 2011;216(7):832-839.
136. Oda T, Hirota K, Nishi K, et al. Activation of hypoxia-inducible factor 1 during macrophage differentiation. *Am J Physiol Cell Physiol*. 2006;291(1):C104-C113.
137. Roiniotis J, Dinh H, Masendycz P, et al. Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. *J Immunol*. 2009;182(12):7974-7981.
138. Zhang D, Tang Z, Huang H, et al. Metabolic regulation of gene expression by histone lactylation. *Nature*. 2019;574(7779):575-580.
139. Günther P, Schultze JL. Mind the map: technology shapes the myeloid cell space. *Front Immunol*. 2019;10:2287.
140. Dutertre C-A, Becht E, Irac SE, et al. Single-cell analysis of human mononuclear phagocytes reveals subset-defining markers and identifies circulating inflammatory dendritic cells. *Immunity*. 2019;51(3):573-589.e8.
141. Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. 2014;41(1):14-20.
142. Linde N, Gutschalk CM, Hoffmann C, Yilmaz D, Mueller MM. Integrating macrophages into organotypic co-cultures: a 3D in vitro model to study tumor-associated macrophages. *PLoS One*. 2012;7(7):e40058.
143. Van Goethem E, Poincloux R, Gauffre F, et al. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. *J Immunol*. 2010;184(2):1049-1061.
144. Rebelo SP, Pinto C, Martins TR, et al. 3D-3-culture: A tool to unveil macrophage plasticity in the tumour microenvironment. *Biomaterials*. 2018;163:185-197.
145. Kuen J, Darowski D, Kluge T, Majety M. Pancreatic cancer cell/fibroblast co-culture induces M2 like macrophages that influence therapeutic response in a 3D model. *PLoS One*. 2017;12(7):e0182039.
146. Wiesner C, Le-Cabec V, El Azzouzi K, et al. Podosomes in space: macrophage migration and matrix degradation in 2D and 3D settings. *Cell Adh Migr*. 2014;8(3):179-191.
147. Van Goethem E, Guiet R, Balor S et al. Macrophage podosomes go 3D. *Eur J Cell Biol*. 2011;90(2-3):224-236.
148. Friedemann M, Kalbitzer L, Franz S, et al. Instructing human macrophage polarization by stiffness and glycosaminoglycan functionalization in 3D collagen networks. *Adv Healthc Mater*. 2017;6(7):1600967.
149. Zhu Y, Sköld CM, Liu X et al. Fibroblasts and monocyte macrophages contract and degrade three-dimensional collagen gels in extended co-culture. *Respir Res*. 2001;2(5):295.
150. Ormel PR, de Sá DV, van Bodegraven EJ, et al. Microglia innately develop within cerebral organoids. *Nat Commun*. 2018;9(1):1-14.
151. Xue J, Sharma V, Hsieh MH, et al. Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis. *Nat Commun*. 2015;6:7158.
152. Sziksz E, Pap D, Lippai R, et al. Fibrosis related inflammatory mediators: role of the IL-10 cytokine family. *Mediators Inflamm*. 2015;2015:764641.
153. Karonitsch T, Beckmann D, Dalwigk K, et al. Targeted inhibition of Janus kinases abates interfon gamma-induced invasive behaviour of fibroblast-like synoviocytes. *Rheumatology*. 2017;57(3):572-577.
154. Damen MS, Oppa CD, Neta MG, et al. Interleukin-32 in chronic inflammatory conditions is associated with a higher risk of cardiovascular diseases. *Atherosclerosis*. 2017;264:83-91.
155. Fischer R, Maier O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. *Oxid Med Cell Longev*. 2015;2015.
156. Ebrahimi-Mamaeghani M, Mohammadi S, Arefhosseini SR, Fallah P, Bazi Z. Adiponectin as a potential biomarker of vascular disease. *Vasc Health Risk Manag*. 2015;11:55.
157. Gokaraju S, Haque A, Vanarsa K, et al. Assessing the disease specificity of urinary PF4 for active lupus nephritis. *Am Assoc Immnol*. 2018;200
158. McMillan R, Bansal V, Skiadopoulos L. et al. Role of platelet activation in the pathogenesis of heart failure in end-stage renal disease patients. *Am Soc Hematology*. 2015;126:4636.

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