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Review

Phenotypic and functional heterogeneity of monocytes in health and cancer in the era of high dimensional technologies

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ABSTRACT

Monocytes have been traditionally classified in three discrete subsets, which can participate in the immune responses as effector cells or as precursors of myeloid-derived cells in circulation and tissues. However, recent advances in single-cell omics have revealed unprecedented phenotypic and functional heterogeneity that goes well beyond the three conventional monocytic subsets and propose a more fluid differentiation model. This novel concept does not only apply to the monocytes in circulation but also at the tissue site. Consequently, the binary model proposed for differentiating monocyte into M1 and M2 macrophages has been recently challenged by a spectrum model that more realistically mirrors the heterogeneous cues in inflammatory conditions. This review describes the latest results on the high dimensional characterization of monocytes and monocyte-derived myeloid cells in steady state and cancer. We discuss how environmental cues and monocyte-intrinsic properties may affect their differentiation toward specific functional and phenotypic subsets, the causes of monocyte expansion and reduction in cancer, their metabolic requirements, and the potential effect on tumor immunity.

1. Monocyte heterogeneity and functions in circulation

Monocytes are the first line of innate immune defenses and play critical functions in fighting infections and eliminating tumor cells. Based on the expression of CD14 (LPS receptor) and CD16 (FcγRIII), circulating monocytes have been conventionally classified in three distinct cell populations: classical (CD14 + CD16-, CM), intermediate (CD14 + CD16+, IM) and non-classical monocytes (CD14dim CD16+, NCM) [1]. Besides the phenotypic differences, these three subsets are also functionally different [2]. Specifically, CM have been shown to be primed for phagocytosis, innate sensing and migration mainly due to their higher expression of several chemokine receptor and increased ability to produce reactive oxygen species compared to the other monocyte subsets. IM express the highest levels of antigen presentation-related molecules and, although their exact role in the immune responses still remains elusive, they have been shown to regulate cytokine secretion and apoptosis. Conversely, NCM have been mostly described as the subset that patrols the luminal side of the endothelium surveying the vasculature [3–5].

Studies investigating the development and kinetics of human monocytes in steady-state and inflammatory conditions have demonstrated a sequential ontogeny scenario, whereby CM progressively convert into NCM (Fig. 1, left side, box view). Specifically, by administering deuterium-labeled glucose in healthy volunteers, Yona and collaborators have shown that, after a lag phase of 38 h, deuterium incorporation was first found in CM, indicating that these are the first monocytes to emerge from the bone marrow (BM). The observed delay in the release of the CM from the BM results from a post-mitotic maturation phase, which is essential to guarantee the rapid replenishment of circulating monocytes in inflammatory conditions. CM progressively differentiate into IM, which show a peak in deuterium uptake at day seven post-administration. Intermediate monocytes finally give rise to NCM [6]. The conversion from CM to NCM does not only occurs in circulation but also in BM and spleen. Notably, a recent study unraveled that in the BM the transition zone vessels that are more abundantly localized in the epiphysis regulate this conversion [7]. Overall, the emerging idea is that the three monocytic subsets do not represent discrete entities but rather a continuum (Fig. 1). More recent studies have further supported this concept by characterizing the phenotype and functions of monocytes with the aid of novel high dimensional technologies coupled with more comprehensive and unbiased analysis pipelines [9,10]. It is now clear that the definition of CM, IM, and NCM is

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rather simplistic. Indeed, already in health conditions the functional and phenotypic heterogeneity of monocytes goes well beyond three discrete populations and is characterized by a range of transition phases that are challenging to capture by performing only a simple phenotypic analysis using conventional flow cytometry [11,12]. The heterogeneity of monocytes can vary in the context of disease, when the emergence of functionally and phenotypically distinct monocyte populations further complicates the discrimination of monocytes from other cells of the mononuclear phagocytic system (MPS).

Among the groups who attempted to characterize the heterogeneity of the cells belonging to the MPS, Hamers and collaborators identified eight monocyte subsets by mass cytometry and validated these subsets by further performing functional and transcriptomic studies [9] (Fig. 2a).

Using two independent algorithms, the authors identified three subsets within the NCM population (subsets 4,5,7), four subsets belonging to the CM (subsets 1,2,6,8), and one single subset (subset 3) within the IM.

Within the CM, cluster 1 was characterized by high CD9 and CD61 expression and was the only subset showing TREM-1 positivity; subsets 2 and 6 showed a similar phenotypic profile and could be distinguished by the higher expression of CD93 and CD11a in cluster 2. Conversely, subset 6 showed the lowest expression of common monocyte markers such as HLA-DR, CD86, CD11a, and CD11c. Finally, cluster 8 was characterized by the expression of IgE, CD14, CD1c, and CD163.

Within the NCM, subsets 4 and 5 showed the highest expression of slan (also known as CD162), an unsialylated O-linked carbohydrate modification of P-selectin glycoprotein. Clusters 4 and 5 showed a similar marker expression and could be distinguished from one another based on the higher expression on clusters 4 of CD61 and CD9, which may be involved in cell adhesion and platelet binding. Conversely, cluster 7, similar to cluster 3 belonging to intermediate monocytes, did not express slan. However, compared to cluster 3, cluster 7 showed reduced expression of known monocyte markers such as CD14, CD36, CCR2, CD64, and CD163.

Analysis of ki67 expression revealed that CM had higher proliferative capacity than NCM. However, all four CM clusters showed a similar ki67 profile.

Analysis of the identified monocytes subsets in patients with coronary artery disease (CAD) revealed that while patients with severe or mild CAD did not show any difference in CM, they differed in the percentage of NCM. Specifically, the Slan- cluster 7 was reduced, and the Slan +cluster 4 and 5 were increased in patients with more severe CAD. Furthermore, functional migration experiments showed that the Slan+ clusters migrated significantly more toward CXCL16, explaining their higher abundance in severe CAD.

Similar to the findings by Hamers and collaborators, a recent study by Vinci found that NCM were more abundant in patients with acute coronary syndrome (ACS) experiencing plaque rupture compared to ACS patients without plaque rupture. In the same study the authors described a novel monocyte subset, $CD14^{int}CD16-$, which was defined as "pre-classical monocytes" (PCM). The frequency of these PCM was higher in patients with ACS compared to patients with chronic coronary syndrome and in patients experiencing plaque rupture compared to patients with intact plaques [13].

Using a similar methodology described by Hamers and collaborators combined with machine learning approaches, Dutertre and colleagues demonstrated that the CD1c + CD163+ CD14+ (cluster 8 of classical monocytes in Hamers' s study) were not monocytes but a phenotypically and functionally distinct subset of CD14-expressing cDC2 that showed overlapping functions with the DC3 described by Villani [14]. These findings have been confirmed in a more recent study combining

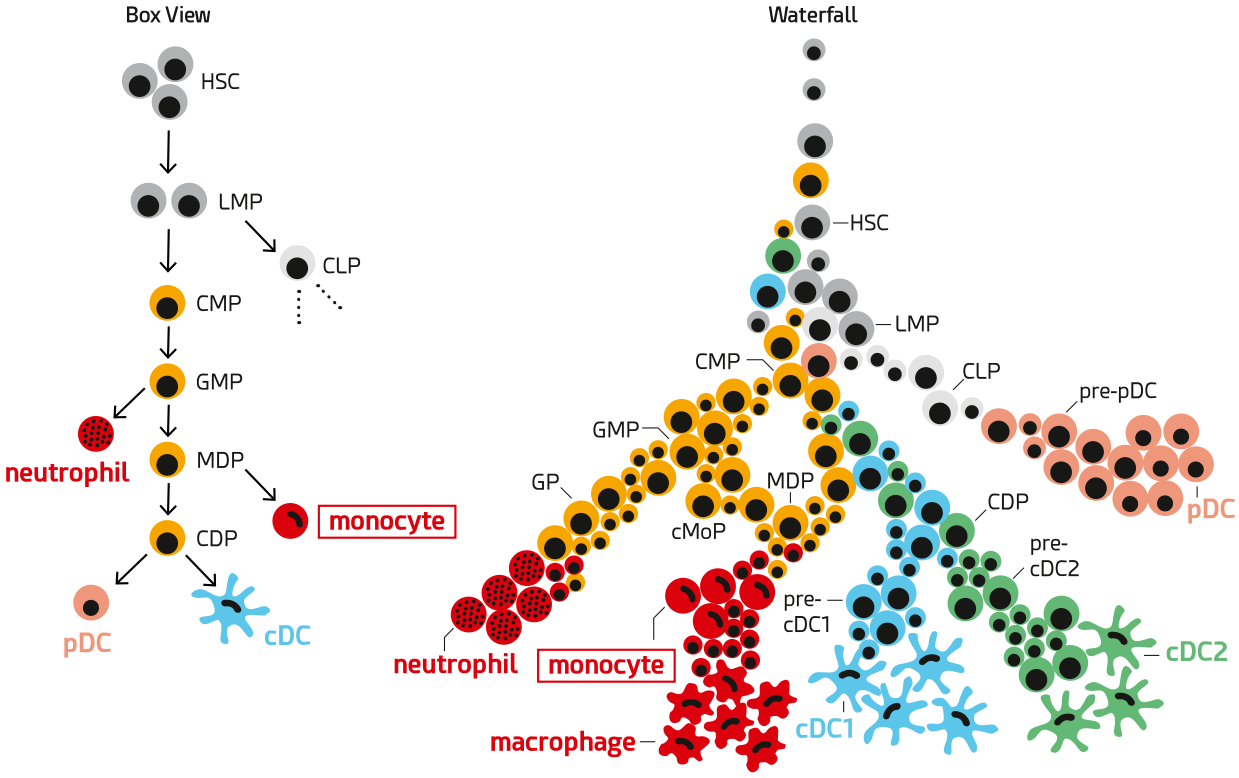

Fig. 1. From a classical (left, box view) to a more recent fluent (right, waterfall) understanding of myelopoiesis using single-cell omics. Historically, hematopoiesis is viewed as an ordered stepwise process with fixed progenitors, such as: the myeloid-cell-committed CMP that would give rise to a GMP with monocyte/neutrophil-potential or the potential to differentiate to a MDP and then CDP – the ultimate progenitor of pDCs and cDCs (left). Various single-cell -omics have since given deep insights into myeloid ontogeny. Therefore, we now know that immune cell development and differentiation is a flowing process involving multiple progenitor stages, with gradually committed progenitors and differentiation (right).

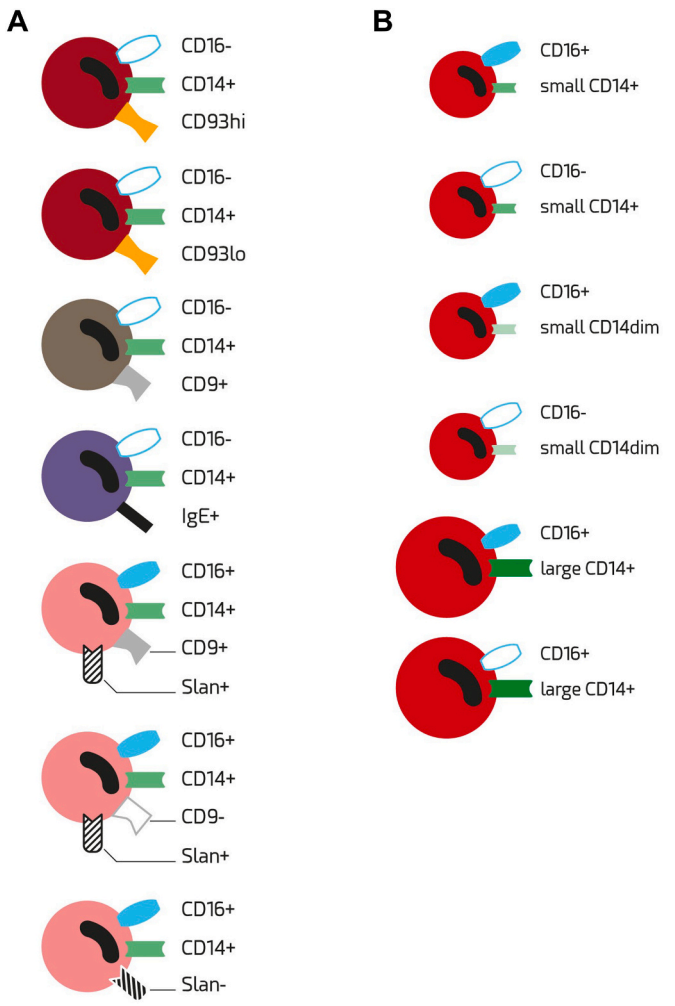

Fig. 2. Monocyte subsets in steady state and disease. (A) Within the CM (top four populations), one cell cluster was characterized by high CD9 and CD61 expression. Another cell cluster has a similar phenotypic profile and could be distinguished by the higher expression of CD93. Conversely, subset three showed the lowest expression of common monocyte markers such as HLA-DR, CD86, CD11a, and CD11c. Finally, the fourth CM subset was characterized by the expression of IgE, CD14, CD1c, and CD163. Within the NCM, two cell clusters showed the highest expression of slan (also known as CD162). CD61 and CD9 could distinguish the two cell clusters. The last cell cluster belongs to intermediate monocytes and did not express slan. However, the other cell clusters showed reduced expression of known monocyte markers such as CD14, CD36, CCR2, CD64, and CD163. Analysis of ki67 expression revealed that CM had a higher proliferative capacity than NCM. (B) Using a multi-omics approach of flow cytometry and imaging flow cytometry our view on conventional CD14+ monocyte subsets are expanded by including their physical size. They consist of small and large monocytes. Based on CD14 and CD16 expression, four subpopulations in the small monocytes and two populations in the large monocytes can separated. Interestingly, the expression of conventional monocytic markers such as chemokine receptors, antigen presentation, and adhesion molecules on the six identified monocyte subsets are similar.

scRNASeq and high dimensional flow cytometry to identify all phenotypic intermediates in the CD14^{high} cDC1^{low} to CD14^{low}cDC1^{high} spectrum. This study found that DC3 share markers with cDC2 (CLEC10A and FCER1A) and monocytes (S100A8, S100A9, CD14, and CD163) but are generated from different progenitors [15]. This finding may explain the results by Lavin and collaborators showing that in lung cancer patients cDC2 and CD14+ monocytes at the tumor site were functionally related as demonstrated by the similar cytokine profile [16]. Interestingly, at the tumor site the DC3 give rise to a population of cDC1+ CD14+ cells, which neither cDC2 or monocytes are able to generate in vitro and in vivo [15].

Using a combination of flow and imaging flow cytometry, another recent study identified 6 monocyte subsets in healthy donors based on size and CD14 and CD16 expression [17]. Specifically, in addition to the conventional monocytes (identified as small by forward and side scatter), which represent the majority of the CD14+ cells, the authors identified an additional monocytic population (defined as large monocytes), which represents approximately 8% of the CD14+ fraction. Based on CD14 and CD16 expression, 4 populations were identified in the small monocyte fraction and 2 populations were identified in the large monocyte fraction (Fig. 2b). Interestingly, the expression of conventional monocytic markers such as chemokine receptors, antigen presentation and adhesion molecules on the six identified monocyte subsets revealed common traits among subsets of donors. Specifically, based on the expression of CD43, CD49d and CD62L the monocytes among all healthy donors could be grouped in 4 phenotypic subsets (a, b, c, d), which appeared to be independent on the age but dependent on the sex of the donors.

While it remains unclear the relation between these monocytes subsets and the ones previously described by Hamers and other groups, based on the results of this study, it is tempting to speculate that there may be additional, yet unidentified factors that can further contribute to monocyte heterogeneity in health and disease.

2. Monocyte heterogeneity and functions at the tumor site

The monocytes' extraordinary phenotypic and functional heterogeneity is further enhanced when considering that besides their role in circulation, monocytes and monocyte-derived cells can also migrate into tissues where they can perform very specialized functions, further increasing their heterogeneity [18]. In this context, it was long thought that the CM are mere precursors of tissue-resident macrophages. However, a large body of literature has now convincingly shown that tissue-resident macrophages are primarily derived from precursors that migrate into tissues from the yolk sac during embryonic development and possess self-renewal capacities [19–21]. Nevertheless, circulating monocytes are still the primary source of resident macrophages in barrier tissues such as the intestine and the skin [22,23].

Interestingly, in inflammatory conditions circulating monocytes can give rise to macrophages and dendritic cells (DC) in tissues, therefore facilitating the quick replacement of tissue-resident cells [24,25].

The cues in the inflamed tissue or sensed in circulation, including cytokines, chemokines and locally secreted factors play an important role in the functional heterogeneity of monocyte-derived myeloid cells. Accordingly, in tissues, monocyte-derived macrophages and DC can exert pro-inflammatory functions and contribute to extracellular matrix degradation, activation and proliferation of T cells or can acquire anti-inflammatory functions and contribute to tissue fibrosis, angiogenesis and inhibition of T cell responses [26–28]. In vitro, pro-inflammatory macrophages (M1), which are associated with resistance against intracellular parasites and tumors have been mainly traditionally generated by the stimulation with IFN-γ, LPS, and TNF-α [29]., Conversely, anti-inflammatory macrophages (M2) can be generated following stimulation with IL-4, IL-13, immune complexes, and specific Toll-like receptors ligands [30,31].

However, the mechanisms and the cues underlying the conversion of monocytes into phenotypically and functionally different subsets of myeloid cells in vivo in inflammatory conditions is still a matter of intense research in the field. As already pointed out for the phenotypic characterization of monocyte subpopulations, the functional distinction between M1 and M2 introduced by Mills in 2000, is insufficient to capture the functional heterogeneity of macrophages and other monocyte-derived myeloid cells in vivo [32].

In transcriptome-based network analysis, Schultze and colleagues challenged the current M1 versus M2 polarization model by proposing a

"spectrum model", which more realistically reflects the in vivo complexity [33]. This study showed that the traditional M1/M2 binary model is only obtained when monocytes are stimulated with classical M1 and M2 polarizing factors alone or in combination with other stimuli. Conversely, the use of other stimuli not linked to M1 and M2 but found at tissue sites in inflammatory conditions such as HDL, fatty acids, PGE2, IFN-β resulted in a spectrum of at least nine distinct macrophage activation programs. This spectrum model has represented a first step toward a deeper understanding of how the integration of multiple signals can locally shape the functional phenotype of monocyte-derived macrophages.

Consistent with this spectrum model, a recent study by Gubin and collaborators found that in the tumor setting the treatment with antibodies against immune checkpoint inhibitors a-PD-1 + a-CTLA-4 resulted in a dynamic remodeling of intra-tumoral monocyte-derived macrophages [34]. scRNASeq and CyTOF analysis identified 5 and 8 subsets of monocytes/macrophages respectively that correlated with tumor progression or regression. Functionally, subset 1 expressing the CM marker Ccr2 and subsets 5 expressing Nos2 were both characterized by the up-regulation of IFN-γ response. Subset 2 expressing Cx3cr1 and Mrc1 showed enhanced oxidative phosphorylation and respiratory electron transport. Subset 3 expressing Mrc1 showed a reduction of the signature described for subset 2. Subset 4 characterized by the exclusive expression of Cd1d1 displayed pathway enrichment in NF-kB signaling, inflammatory responses, and hypoxia. Interestingly, there are striking similarities between these five functional subsets from mouse tumors and the nine subsets described in the spectrum model, suggesting that the dynamic remodeling in tumor following immunotherapy may be relevant in humans.

As recently shown by Laviron et al. an important determinant for tumor associated monocyte/macrophage heterogeneity is the spatial location in the tissue [35]. In a spontaneous model of breast cancer and human biopsies they found that both peripheral monocytes and locally proliferating macrophages contribute to the pool of tumor associated macrophages (TAM) and the phenotype and function of these TAM is largely determined by their localization in the different tumor areas. Specifically, the stromal macrophages (SM) originated mostly from peripheral monocytes, localized in the proximity of adipocytes and at the border of the tumor and showed the highest ability to stimulate CD8 + T cell proliferation and IFN-γ production. Conversely, the ductal macrophages (DM) mostly generated by proliferation of local precursors, localized within the tumor and were either associated to early tumors (CD11b- DM) or evolved toward the malignant DM (DM TAM) that correlated with bad prognosis and, despite being highly phagocytic, were unable to efficiently present antigens to the CD8 + T cells.

Overall, these studies suggest that the extraordinary plasticity of monocytes and monocyte-derived myeloid cells opens opportunities for their potential rewiring by modifying the local cues that are responsible for shaping their functional and phenotypic heterogeneity.

On the other hand, besides the tissue-specific cues, an important question is how the intrinsic properties of the multiple monocyte subsets affect their conversion into functionally distinct subsets in the TME. Since most of the in vitro studies in human have relied on the differentiation of CD14+ cells, which are a heterogeneous mixture mainly composed by classical and intermediate monocytes, it remains unclear whether different monocyte subsets differ in their ability to give rise to macrophages and dendritic cells [36–38].

In one of the few studies investigating the in vitro differentiation potential of different monocytic subsets, Boyette and collaborators showed that in the presence of GM-CSF and IL-4, only CM acquired phenotypical and functional properties of DC including their ability to stimulate T cell proliferation and IFN-γ secretion. In the presence of Flt-3 and IL-3 all monocyte subsets acquired DC and macrophage morphology, but only CM expressed a few markers of plasmacytoid dendritic cells (pDC) without producing IFN-α. Conversely, NCM were the major source of IFN-α but did not express markers of pDC. In the presence of GM-CSF or M-CSF all monocyte subsets were able to give rise to macrophages with the CM-derived macrophages showing the highest phagocytic activity. Therefore, based on this study, while in vitro all monocytes have the ability to differentiate into macrophages, only CM can give rise to dendritic cells and none of the monocyte subsets can differentiate into pDC [39]. Interestingly, the recruitment to the TME is regulated by the secretion of chemokines and since different monocyte subsets express different receptors, there is evidence that they may be recruited to different areas of the tumor and hence undergo distinct differentiation programs. CD62L/CD62L ligands, CX3CR1/CX3CL1, CCR2/CCL2, and VEGFR1/VEGF-A are the main receptor-ligand pairs involved in the recruitment of monocytes to the tumor site [40]. Notably, CD62L ligands are highly expressed in the inflamed endothelium, CCL-2 is highly produced by the epithelial regions of the tumors and VEFG-A is mainly localized in the hypoxic regions of the tumor [41,42]. Since CD62L, CCR2 and VEGFR1 are expressed by CM but not NCM, there are evidences from in vivo studies that these receptors guide the selective localization of CM to the perivascular, epithelial and hypoxic regions of the tumors, respectively. Conversely, CXC3R1 is mostly expressed by NCM but not CM. In steady state NCM patrol the blood vessels [43]. In inflammatory conditions, the production of TNF-α, IL-1 and IFN-γ up-regulates the expression of CX3CL1 on the inflammatory endothelium and NCM are rapidly recruited to the inflammation site where they can play different and often divergent roles in tumor development and progression [44]. For instance, NCM have been shown to up-regulate markers associated with pro-tumorigenic TAM such as arginase 1, Fizz1 and IL-4Rα [45]. Conversely, NCM closely associated to the vasculature have been described to rapidly phagocytose tumor cells thus preventing metastatic spreading [46]. Furthermore, in a model of breast cancer Nr4a1−/− mice that lack NCM showed a higher tumor burden [47].

In addition to their ability to replenish tissue-resident macrophages, new evidence suggests that monocytes can also retain their own phenotypic features once migrated into the tissues and perform specialized functions such as antigen presentation, trafficking to the draining lymph nodes and survey of the tissue environment, further emphasizing the heterogeneous and dynamic nature of monocytes in different settings [48].

3. Causes of monocytopenia and monocytosis in cancer

Adult monocytes originate from the BM after birth and their release into the circulation or retention in the BM is critically controlled by the CCR2 and CXCR4 signaling respectively [49,50]. In steady state, upon entering the circulation, the monocytes progressively differentiate into NCM that patrol the endothelium before undergoing death and elimination [51]. This tightly controlled cycle ensures that the number of monocytes does not deviate from homeostatic conditions.

Nevertheless, in disease conditions including various malignancies abnormal monocyte counts are often described and include both the increase or the reduction in the circulating monocytes caused by multiple mechanisms.

The development of monocytopenia with total monocyte counts lower than 0.5 × 10 [9]/L is frequently described as a joint adverse event of aggressive anti-cancer treatments, especially chemotherapy, which exert toxic effects on the BM [52]. Accordingly, a recent study showed that chemotherapy regimens were the primary cause of SARS-CoV-2 complications in cancer patients due to the induction of monocytopenia. In contrast, immunotherapy, hormone therapy, and targeted therapy did not increase clinical worsening or death risk [53].

Accordingly, treatment with checkpoint blockade or other targeted immunotherapy such as anti-CD64 rarely has been reported to induce transient episodes of non-severe monocytopenia without significant clinical sequelae [54,55].

Monocytopenia can also result from neoplastic disorders such as hairy cell leukemia, acute lymphoblastic leukemia, Hodgkin lymphoma,

corticosteroid or immunoglobulin therapy, and gastric or intestinal resection. Because of the role of monocytes as the first line of defense against pathogens, monocytopenia is often the primary cause of treatment-related mortality in patients due to the higher incidence of lethal infections and sepsis. Hairy cell leukemia patients are particularly susceptible to life-threatening infections due to profound neutropenia and monocytopenia [56]. Therefore, several studies have focused on identifying effective interventions to limit monocytopenia. In this context, a clinical study has reported using M-CSF to limit chemotherapy-induced myelotoxicity and consequent monocytopenia in patients with primary ovarian cancer [57].

Another recent study using preclinical mouse models demonstrated the efficacy of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) as a novel agent in reducing the hematological toxicity 5-Fluorouracil (5-FU). In addition, mice treated with PLAG had a delayed onset and reduced duration of monocytopenia [58].

Interestingly, monocytopenia can occur in cancer patients also independently of the treatment. For instance, a clinical study found that abnormalities in the monocytes characterized newly diagnosed stage IV, untreated melanoma patients. Specifically, by performing flow cytometry analysis, the authors found that melanoma patients had a significantly lower abundance of circulating monocytes, which mainly affected the CM subset with no impact on IM and NCM,neutrophils and T cells [59]. In addition to their reduction, CM from melanoma patients showed decreased functions, as indicated by their modest production of inflammatory cytokines following TLR3 stimulation and the significant down-regulation of HLA-DR. Furthermore, they iupregulated PD-L1 expression, potentially leading to sub-optimal antigen presentation and inhibition of T cell functions[39]. The mechanisms responsible for the reduced frequencies and functions of CM in patients with advanced melanoma are currently unknown. In addition to excluding a direct effect of the treatment, the authors also ruled out the influence of age and gender and hypothesized that the interaction with melanoma cells and/or exposure to tumor-derived factors are the probable mechanisms driving the monocyte defects observed in these patients.

Genetic mutations in the hematopoietic transcription factor GATA2 strongly affect the generation and function of hematopoietic stem and progenitor cells and all subsequent blood lineages and represent another important cause of severe monocytopenia in patients [60]. It is currently unclear why GATA2 deficiency also causes a reduction of lymphocytes such as T cells even in the presence of intact GATA3, which is required to develop the T cell lineage [61]. However, GATA2 deficiencies have been described as a new predisposing factor for familial and sporadic myeloid dysplastic syndrome (MDS) and acute myeloid leukemia (AML). In addition, the clinical evolution in MDS or AML of patients with germline GATA2 mutations is frequently associated with a partial or complete deletion of chromosome 7. This observation supports the hypothesis that GATA2 mutations could act as a preleukemic event, resulting in an overt transformation due to the loss of genes located on chromosome 7 [62].

Similar to monocytopenia, monocytosis is also a common condition in patients with blood-borne or solid cancers and results from the enhanced mobilization of precursors cells from the BM or increased monopoiesis, both described in cancer [63]. According to the World Health Organization's definition, persistent monocytosis is diagnosed when the absolute monocyte count is higher than $>1 \times 10$ [9]/L, with monocytes accounting for >10% of leukocytes for over three months.

As extensively reviewed by Mangaonkar and collaborators, monocytosis can have reactive or clonal causes. Common examples of reactive monocytosis can be observed in cancer patients recovering BM following chemotherapy or in patients undergoing a stressful event such as splenectomy. While in these patients, monocytosis is often transient, several treatments such as radiation, corticosteroid therapy, post-chemotherapy G-CSF treatment, and anti-thymocyte globulin administration in severe aplastic anemia can cause persistent monocytosis [64].

While it is still unclear why monocytosis can result in unfavorable prognosis in cancer, studies in human and mice have contributed to elucidate its underlying mechanisms. During cancer there are often increased levels of factors that control myelopoiesis such as KITLG, G-CSF, GM-CSF, and M-CSF, which can be caused by genetic alterations or by the persistent systemic low-grade inflammation both associated with cancer development and progression [63]. Consequently, there is an expansion of hematopoietic stem cells (HSC) that preferentially give rise to myeloid cell precursors such as common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP) and common dendritic cell progenitors (CDP) [65]. The early commitment of HSC toward the myeloid lineage depends on M-CSF, which promotes their survival and can induce in the HSC the expression of PU.1, the key transcription factor for monocytes and macrophages. Furthermore, the heterodimer PU.1-IRF8 activates KLF4, a transcription factor that specifies the monocyte identity [66]. Notably, cancer does not only reprogram myelopoiesis in the BM but can also support the occurrence of extramedullary myelopoiesis in secondary lymphoid organs such as the spleen. This phenomenon has been described both in animal models and cancer patients and is caused by the recruitment of the myeloid progenitors from the circulation and local proliferation [67,68]. Interestingly, a recent study used mice bearing breast cancer driven by the mammary epithelial expression of the polyoma middle T oncoprotein to dissect the mechanisms underlying monocytosis in a model that progresses from benign tumors to metastatic stages. By assessing BrDU incorporation over time, this study unraveled that there were no changes in the CMP, CDP, common monocyte progenitors (cMoPs) nor in their upstream progenitors and that the higher number of monocytes in circulation was due to increased proliferation of monocytes in the BM. In line with previous studies, there was no further proliferation in the circulating monocytes [69].

The development of acute and chronic neoplasms is the most common example of clonal monocytosis, which can occur at diagnosis or during the disease; notably, persistent monocytosis is used to diagnose chronic myelomonocytic leukemia (CMML) in the absence of BCR-ABL1-driven chronic myeloid leukemia, PDGFRA, *PDGFB*, and *FGFR1* rearrangements or *PCM1-JAK2* fusions [70]. In CMML, monocytosis is often associated with somatic variants in several genes such as *DNMT3, IDH1/2, SRSF2, SF3B3, JAK2,* and *RAS*. The exact molecular mechanisms of monocytosis in CMML and other chronic myeloid neoplasms are still unclear. However, clinical evidence suggests that these gene variants may confer hypersensitivity to granulocyte macrophage-colony stimulating factor (GM-CSF) signaling [71]. In addition, several clinical studies show that the expansion of the CM fraction and the NCM contraction are standard features of CMML [72]. However, considering the heterogeneous nature of the monocytes as revealed by recent high dimensional studies, the use of additional markers other than CD14 and CD16, such as CD36, CCR2, HLA-DR, and CD11c, may help increase the accuracy of CMML diagnosis, especially in the presence of rheumatologic conditions that could affect the phenotype and proportion of monocyte subsets.

On the side of solid cancers, a recent study examining 438 healthy individuals and 219 patients with pancreatic cancer found that in the time frame of 6 months to 1-month from primary pancreatic ductal adenocarcinoma (PDAC) diagnosis, the patients showed a significantly higher abundance of circulating monocytes compared to controls [73]. Although patients with monocytosis showed a worse overall survival than patients without monocytosis, this difference was not statistically significant when accounting for the tumor stage, suggesting that the tumor stage rather than the increase in monocyte counts is the primary driver of the difference in overall survival.

Furthermore, a recent retrospective study found that patients with hepatocellular carcinoma (HCC) show increased circulating monocyte counts compared to healthy individuals. In the same study, the authors determined that a cutoff of 4.01 of monocyte to lymphocyte ratio (MLR) represented a superior indicator of worse overall survival and disease-free survival in HCC patients with curative resection compared to the commonly used parameters such as neutrophil to lymphocyte ratio

(NLR) and platelet to lymphocyte ratio (PLR) [74].

Similar results were obtained in another retrospective study conducted on 351 patients with HCC, where the authors showed that preoperative high monocyte counts correlated with shorter disease-free survival and reduced overall survival [75]. In line with this evidence, a systemic meta-analysis of 3826 patients with colorectal cancer (CRC) revealed that high monocyte counts are associated with worse overall survival [76]. In addition, several other cancers, including T-cell lymphoma, metastatic melanoma, head and neck cancer, and metastatic renal cell carcinoma, are characterized by peripheral monocytosis, which has been associated with poor prognosis and decreased survival [77–80].

Since the blood monocyte counts were mainly obtained from historical clinical evaluation, all the studies mentioned above did not perform any detailed analysis to assess the distribution of the different monocyte subsets in cancer patients relative to healthy individuals and the tumor stage.

4. Functional and phenotypic alterations in circulating and tissue-associated monocytes and monocyte-derived myeloid cells in cancer and implication in anti-cancer treatments

As highlighted in the previous paragraph, clinical studies indicate that the altered frequency of monocytes is a hallmark of several malignancies. Although several studies in humans and preclinical models have shown that higher circulating monocytes correlate with worse survival, it is emerging that monocytes can also represent a favorable prognostic factor in certain conditions. These findings suggest that monocytes and monocyte-derived cells can exert multiple roles in cancer development, progression, and response to treatment, which are highly dependent on the differentiation cues that these cells encounter in circulation and at the tumor site. In addition, both CM and NCM can mediate tumor cell killing via different mechanisms, including phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) [81].

One of the first pieces of evidence supporting the antitumorigenic role of monocytes/macrophages emerged from a study showing that intraperitoneal injection with ascites of tumor-bearing mice into naïve tumor-bearing mice led to influx and activation of peritoneal macrophages, which killed cancer cells [82]. Later studies in patients confirmed that infiltrating macrophages in the primary tumor correlated with reduced metastases and overall improved clinical outcomes in CRC patients [83,84].

Using high-throughput technologies and multi-omic approaches coupled with machine learning methods for single-cell mass and flow cytometry studies (FAUST and CATALYST), we and others have recently shown that in patients with melanoma an enhanced frequency of $CD14^{+}CD16^{-}HLA\text{-}DR^{hi}$ CM is a strong predictor of responsiveness to anti-PD-1 immunotherapy [85,86]. Other studies have also shown that increased HLA-DR positive cells are associated with enhanced CD8+ T cells in circulation as well as in the tumor and with increased IFN-γ signature in the tumor microenvironment [87,88].

Furthermore, a recent study using spectral flow cytometry identified in humans with viral infections and in patients with pancreatic adenocarcinoma (PDAC), HCC, colorectal liver metastases, and melanoma, populations of CM and IM expressing CD169 (Siglec-1) phenotypically distinct from DCs. The authors showed that CD169 expression on these monocytes was driven by IFN-α, which also resulted in up-regulation of HLA-DR and enhanced ability to cross-present antigens to CD8+ T cells [89].

Mechanistically, cancer can affect the monopoiesis in the BM and extramedullary sites mainly via the production of GM-CSF and IL-6. Accordingly, in HCC-bearing mice, HSC in the spleen were reprogrammed to generate circulating myeloid cells with the ability to produce arginase-1, which conferred them an immunosuppressive phenotype. Interestingly, this phenomenon was only observed when HSC from tumor-bearing mice, but not healthy animals were transplanted into the spleen, further confirming the ability of the tumor to skew monopoiesis [68]. Similarly, using scRNASeq, a recent study found that compared to healthy mice, splenic monocytes in mice with breast cancer showed a modified gene signature characterized by the up-regulation of genes involved in inflammation, angiogenesis, and chemotaxis [90]. It is also worth noting that, although GM-CSF can generate immunosuppressive monocytes, the production of GM-CSF by T cells has also been associated with the ability to create inflammatory monocytes with potential anti-tumor effector functions. In line with this observation, GM-CSF has been used as an adjuvant in anti-cancer vaccines [91]. This contrasting data suggest that the effect of GM-CSF on monocyte functions is likely dependent on their developmental stage at the time of exposure.

Clinical studies have found that among the most common features of circulating monocytes in cancer patients is the downregulation of HLA-DR and costimulatory molecules such as CD86, which negatively impacts their antigen presentation functions [92–94]. This phenotype closely mirrors the anergic monocytes first described in the context of sepsis, whereby the continuous exposure to microbial stimuli induces LPS tolerance and an anergic phenotype characterized by the inability to produce inflammatory cytokines and by the down-regulation of HLA-DR molecules [95]. Besides sepsis, HLA-DR low monocytes have been described in several non-malignant states including burn injuries, pancreatitis, chronic liver inflammation, amyotrophic lateral sclerosis and in all these conditions the event triggering these immunosuppressive monocytes is the acute or chronic inflammatory process [96–98]. In contrast with the granulocytic myeloid derived suppressor cells (G-MDSC), which are immature cells generated during the process of emergency myelopoiesis, evidences that monocytic MDSC (M-MDSC) are immature cells or represent immunosuppressive monocytes that leave the BM are still lacking [99]. Independently on their origin, STAT3 and NF-kB are essential for the acquisition of their immunosuppressive functions. Additional immunosuppressive mechanisms in M-MDSC from cancer patients include the production of reactive oxygen and nitrogen species (ROS and RNS), the up-regulation of PD-L1, the reduced secretion of inflammatory cytokines such as IL-1β, IL-6, and TNF-α and the increased production of inhibitory and pro-angiogenic factors such as TGF-β and VEGF-α respectively [100–103].

Interestingly, by performing flow cytometry analysis on three different cohorts of PDAC patients, Trovato and collaborators identified four populations of MDSC, out of which MDSC1 (CD14 + IL-4Rα+) and MDSC4 (CD14 + HLA-DR−/low) were of monocytic origin (M-MDSC) [104]. Notably, functional studies on purified CD14+ monocytes from the same patients unraveled a population of immunosuppressive monocytes and a population of non-immunosuppressive monocytes. The immunosuppressive activity was mainly mediated by the activation of the STAT3/arginase-1 axis. While the differences in the functionality of these two subpopulations were not dependent on the differential abundance of the four sub-classes of MDSC, the authors found that the immunosuppressive monocytes were more frequently found in patients with metastatic disease. In contrast, non-immunosuppressive monocytes were more common in patients with non-metastatic PDAC, suggesting that the tumor-induced reprogramming of monocytes in PDAC is dependent on the tumor stage. Similar to the findings in PDAC patients, Meyer and collaborators showed that the frequency of MDSC tended to be higher in melanoma patients with severe metastatic disease [105].

Conversely, studies comparing the transcriptomic profile of circulating monocytes from 360 CRC patients found that factors secreted by the transformed but not from the healthy colon imprinted in the circulating monocytes a signature that was similar between early tumor stage and disease progression, therefore suggesting that it could be used as a robust biomarker for early diagnosis of CRC [106].

Another clinical study found that the monocytes from breast cancer patients showed an immunosuppressive profile similar to those of patients with sepsis, which was mainly dependent on the expression of HMGB1 and several metalloproteases [101].

All the studies mentioned above found that monocytes can also be

reprogrammed in the absence of emergency myelopoiesis, therefore demonstrating that the tumor-mediated reprogramming does not only occur at the HSC level but also in circulating monocytes. Notably, there is a large body of literature showing that circulating monocytes and not distinct precursors give rise to M-MDSC in circulation [107]. Yet, the molecular mechanisms underlying the generation of these immunosuppressive monocytes in cancer are not entirely clear and do not appear to be different from the induction of M-MDSC in the non-malignant settings. Specifically, similar to sepsis, in several tumor types cytokines such as TGF-β and IL-1β or steroid hormones such as cortisol, prednisolone and dexamethasone can directly or indirectly reduce the expression of HLA-DR on circulating monocytes [108–110].

There are increasing evidence suggesting that the evaluation of M-MDSC could be a valuable prognostic marker of response to cancer treatment. Accordingly, although the immunotherapy with a-CTLA-4 did not affect the overall M-MDSC frequency, melanoma patients responsive to a-CTLA-4 treatment had lower frequencies of these immunosuppressive cells [111]. Furthermore, lower M-MDSC have been shown to correlate with better overall survival and with increased numbers of CD8 + T cells [112]. Interestingly, there is evidence that immunotherapeutic drugs may indirectly fuel the development of M-MDSC as demonstrated in mouse model of neuroblastoma whereby treatment with a-PD1 induced the production of M-CSF by T cells, which in turn supported the development of M-MDSC [113]. These results were confirmed in patients with glioblastoma and provide a rationale for the combination of a-PD1 and a-CSFR1 [114]. Additionally, clinical studies have shown that several therapeutic agents such as tyrosin kinase inhibitors (TKI), TRAIL-R2 antibody, GM-CSF decrease the frequencies of M-MDSC or restore the expression of HLA-DR and co-stimulatory molecules via modification of their proliferation, migration and metabolism [91,115–117].

Because of the extensive studies showing that M-MDSC can negatively affect T cell functions, it is tempting to speculate that M-MDSC may also impair the therapeutic efficacy of CAR T cells. Although direct evidences from cancer patients are still missing, data in animal models indicate that the treatment with CAR T cells may result in the expansion of M-MDSC with similar mechanisms described in glioblastoma patients treated with a-PD-1 [118]. The advantage of using M-MDSC as prognostic marker for anti-cancer treatment is that they may be informative of the tumor site but can be easily studied in the peripheral blood by flow cytometry without the need to sample the tumor tissue. While there seems to be growing consensus that M-MDSC can be defined as CD11b + CD14 + CD33 + HLA-DR−/low, to date the lack of unified approaches to isolate and phenotypically and functionally characterize these cells represents a significant obstacle to their wider use as biomarkers [119,120].

Although steady-state monocytes are not the precursors of macrophages in the majority of the tissues, during inflammatory conditions such as cancer, circulating CM can give rise to tumor-associated myeloid cells, especially macrophages. Accordingly, there is often a positive correlation between the number of circulating monocytes and the macrophages in the tumors [81].

Therefore, the phenotypic and functional modifications described in circulating monocytes can also impact the differentiation potential of monocytes at the tumor site. In line with this hypothesis, a study found that monocytes from breast cancer patients showed reduced expression of *ID2* and *MAFB*, which are essential for the differentiation of DCs and macrophages, respectively [121,122]. In support of these results, DCs differentiated in vitro from the monocytes isolated from patients with breast cancer showed impaired ability to induce T cell proliferation and generated a higher number of regulatory T cells [123].

Studies in preclinical models have revealed that monocytes at the tumor site do not generate a homogenous population but rather a heterogeneous mixture of tissue-associated myeloid cells whose differentiation is dependent on time and location. This can be further complicated because tissue-resident macrophages can also give rise to TAM [81].

There is a large body of literature describing the pro-tumorigenic functions of TAM. However, a growing body of literature supports the antitumorigenic functions of TAM in several cancers [124]. Accordingly, we have described that higher monocytes counts in the circulation of patients with a positive response to immunotherapy were mirrored by a higher infiltration of macrophages in the tumor, which exhibited increased effector functions [85]. In line with our findings, higher CD169+ in the tumor microenvironment positively correlated with increased immune infiltration and overall survival in patients with endometrial cancer [89]. Furthermore, TAM are positively associated with T cell infiltration [125].

Interestingly, using scRNASeq on CD14+ HLA-DR+ cells from metastatic lymph nodes and primary breast cancer tissues, a recently published study described two distinct cell populations: TREM2+ macrophages, which were poorly represented in healthy tissues and increased with tumor progression, and a population of folate receptor 2 (FOLR2) + tissue-resident macrophages that co-expressed LYVE1 and CD206 and were primarily abundant in the healthy mammary glands. Notably, FOLR2+ macrophages localized close to CD8 + T cell clusters in the tumor stroma, and their presence correlated with better prognosis, demonstrating an antitumorigenic role for this newly described macrophage subset [126].

Although DCs are usually generated from distinct precursors, monocytes can also give rise to DCs (moDCs) in inflammatory conditions [127]. Studies in animal models have shown that the depletion of CD11c + cells prevents the development of anti-tumor immune responses, which could be restored by adoptive transfer of monocytes, therefore demonstrating that moDCs exert antitumorigenic functions [128]. Additionally, moDCs contribute to the effectiveness of chemotherapy in a preclinical model of melanoma [129]. While the molecular mechanisms underlying the differentiation of monocytes toward moDC or TAM in cancer remain unclear, a recent study in mouse models and humans has shown that TLR and NOD sensing preferentially skews the monocytes toward TAM or moDC respectively [130].

The generation and functions of TAM have been extensively reviewed elsewhere [81,127]. The studies mentioned above on the pleiotropic functions of monocytes and monocyte-derived myeloid cells highlight the extreme heterogeneity of monocytes and the need to identify how this heterogeneity can be exploited for more effective anti-cancer treatments.

5. Monocytes and macrophage metabolism in cancer

Besides altering their number, phenotype, and effector functions, cancer can change the metabolism of monocytes, therefore directly affecting the cellular processes involved in energy production [131,132].

The process of oxidative phosphorylation (OXPHOS) driven by the mitochondria has been described as the most efficient way for the cells to produce energy in the form of ATP by oxidizing glucose, amino acids, and fatty acids. However, immune and non-immune cells often use aerobic glycolysis, also known as the “Warburg effect,” as a preferential way to produce ATP via an oxygen-independent process, whereby pyruvate is converted into lactate. This process was first described in 1920 by Otto Warburg, who noticed that proliferating cancer cells changed their metabolic profile from OXPHOS to aerobic glycolysis to meet their higher demand for nutrients and ATP [133]. Likewise, in the inflammatory tumor microenvironment, monocytes meet the requirement of biomass accumulation, cytokines secretion, and differentiation to macrophages by increasing their glucose uptake, which is then catabolized through aerobic glycolysis [134]. Despite providing less ATP, this process allows for faster production of energy, which is essential to support highly proliferative or highly activated cells [135]. As such, besides cancer cells, activated, proliferating, and cytokine-producing immune cells such as monocytes, macrophages, DCs, and T cells tend to switch

their core metabolism from OXPHOS to aerobic glycolysis [134,136].

In the inflammatory tumor microenvironment, monocytes are extremely tolerant to anoxia because of their ability to enhance glycolysis even in anaerobic conditions [137]. Accordingly, lactate produced by cancer cells stimulates gluconeogenesis in human monocytes by stabilizing HIF-1α activity [138].

Because of their extraordinary plasticity and adaptability, monocytes can revert their metabolism in glucose-deprived conditions. Monocytes treated with LPS in the absence of glucose can switch back to oxidative phosphorylation, therefore compensating at least partially for the increased energy demand during this activation phase by using fatty acids as a source of carbons [139]. Importantly, fatty acids synthesis, whereby cholesterol is converted into phosphatidylcholine, is the driving force of the differentiation from monocytes to macrophages [140], suggesting that fatty acids synthesis/oxidation plays a crucial role during the adaptation of monocytes to the nutrient deprivation in the tumor environment.

Studies in healthy donors have shown that CM and IM have higher levels of genes involved in glucose metabolism and have a proglycolytic phenotype. Conversely, NCM have higher levels of genes involved in the OXPHOS process, which is also up regulated in anti-inflammatory macrophages at the tumor site [141].

A study by Qorraj and collaborators showed that monocytes from patients with chronic lymphoid leukemia (CLL) have an altered metabolic phenotype characterized by reduced glucose uptake, lower levels of glucose transporters, and down-regulation of molecules involved in glucose metabolism. Accordingly, compared to healthy donors, macrophages differentiated in vitro from CLL monocytes in GM-CSF showed reduced glucose uptake and reduction of lactate, which is a surrogate marker of glycolysis [142]. Interestingly, the authors found that the programmed death-1 (PD-1) was highly expressed in the CLL monocytes and that the interaction with its ligand, PD-L1 expressed in the CLL cells, was responsible for the reduced glycolytic activity of the monocytes. Blockade of PD-L1 restored the glycolytic activity of the CLL monocytes, therefore providing a solid rationale for exploiting a PD-L1/PD-1 checkpoint blockade to reverse the immune-metabolic dysfunctions of the myeloid compartment in CLL patients.

In a study on patients with PDAC, Trovato and collaborators compared the gene expression profile of immunosuppressive and non-immunosuppressive circulating monocytes and found that several were involved in metabolism among the differentially expressed genes. Specifically, immunosuppressive monocytes up-regulated fatty acid and lipoprotein metabolism-related genes, such as *CD36, LYPLA1,* and *CERS5*; energy (ATP) metabolism-associated genes, such as *ATP51C, ATP5G2,* and *SDHB*; genes involved in amino acid metabolism and modification and arginase-1 [104].

Emerging evidence suggest that the metabolic reprogramming of monocytes in cancer profoundly impacts also the immune functions of monocyte-derived myeloid cells at the tumor site by acting either locally or systemically [132].

A study by Ramos and colleagues showed that in patients with breast cancer, the co-culture of monocytes with tumor-derived supernatants induced the differentiation of $CD163^{high}CD86^{low}IL\text{-}10^{high}$ immunosuppressive macrophages via TGF-β, M-CSF VEGF, and IL-10 [122]. Interestingly, the authors also showed that compared to healthy donors, the monocytes from breast cancer patients were refractory to differentiate into M1 macrophages when cultured with GM-CSF/IFN-γ and produced higher levels of IL-10, CCL2, TGF-β1, and TGF-β3.

The transition toward aerobic glycolysis is one of the prerequisites for the differentiation of monocytes into inflammatory and anti-tumorigenic macrophages. In line with this notion, glycolysis supports the secretion of pro-inflammatory cytokines, phagocytosis, and the production of ROS [143]. These latter are an important regulator of anti-tumorigenic macrophage functions as they sustain inflammation by mediating the MAPK-dependent secretion of inflammatory cytokines such as IL-6, IL-1β, and TNF-α.

A proteomic analysis study by Liu and collaborators revealed that the treatment of BM-derived macrophages with tumor extracts from breast cancer patients gave rise to cells with TAM features, which up-regulated vital mediators of the aerobic glycolysis such as hexokinase-2 and the downstream molecules, PFKL and ENO1 [144].

The role of glycolysis in anti-inflammatory macrophages in cancer has not been extensively studied. However, there is evidence that, although the glycolysis can occur in non-inflammatory macrophages, it is not essential for their polarization, which is strictly dependent on OXPHOS [143].

Aerobic glycolysis and OXPHOS have been consistently associated with M1 and M2 polarization, respectively [145]. Yet, this is likely an oversimplification as the tumor microenvironment is spatially and temporally heterogeneous and there is poor evidence that the polarization described for in vitro differentiated macrophages occurs in a similar distinct way in vivo. Notably, in addition to activated immune cells, cancer cells are also highly dependent on glycolysis. Due to their higher proliferative potential, the cancer cells often deprive the tumor of glucose, limiting nutrients for the immune cells and the polarization of antitumorigenic TAM [146]. Furthermore, aerobic glycolysis results in the production of lactate, which blunts immune cell functions [147]. This makes the TME a highly temporally heterogeneous compartment where glycolytic TAM with pro-inflammatory and anti-tumor functions switch to oxidative TAM with pro-tumorigenic functions as the tumor progresses.

In addition to the regulation of monocytes and macrophage's effector functions, metabolic reprogramming in conjunction with epigenetic modifications is essential for establishing "trained immunity", which has been recently described for monocytes and macrophages [148]. *Candida albicans* and Bacillus Calmette-Guerin are the best-documented stimuli known to induce the formation of immune memory in innate cells, which, after a first immune challenge, increases resistance to reinfection with the same or a different stimulus [149]. In trained immunity, which is dependent on the intermediate of the cholesterol synthesis, mevalonate, monocyte, and macrophage metabolism is skewed toward aerobic glycolysis. Although this phenomenon has been described in association with infections, experimental evidence indicates that the stimuli affect the circulating monocytes and HSC in the BM. This suggests that the metabolic rewiring of myeloid precursors may represent an attractive avenue to restore their anti-tumor effector functions and increase the efficacy of the current immunotherapeutic approaches.

Practice points

- Monocytes play important roles in tissue homeostasis
- Monocytes are highly heterogenous in terms of phenotype and functions both in circulation and at the tumor site
- Due to their plasticity, there is high potential for therapeutic strategies aiming at skewing monocytes to specifically support beneficial phenotypes and functions in pathological conditions.

Research agenda

- High dimensional studies using mass cytometry, single cell RNA-Seq (scRNA-Seq) in combination with novel integrative bioinformatic tools to unravel the phenotypic and functional heterogeneity of monocytes in health and disease.
- Studies focusing on understanding the ontogeny and the signals required to support specific subsets of monocytes that are beneficial in defined pathological conditions
- Understanding how the local cues and the intrinsic properties of specific monocyte subsets orchestrate their phenotypic and functional differentiation at the tissue site.
- Studies aimed at deepening the understanding of how modulation of monocyte metabolism can be used to support cancer therapy

Future considerations

The application of novel technologies has drastically changed our understanding of monocyte biology. It is becoming increasingly clear that monocytes comprise very heterogenous and highly plastic cell populations, whose functions and phenotypes can be shaped by the environment as well as by intrinsic properties. While it was previously thought that monocytes were simply the precursors of more specialized tissue-associated myeloid cells, novel studies suggest that monocytes can exert a plethora of functions, which includes but is not limited to antigen presentation and modification of the tissue microenvironment.

We think that future studies should further focus on the challenging task of capturing the multiple shades of monocytes, therefore moving toward a continuum model, which has already been proposed as an alternative to the dichotomic and limited view of pro- and anti-tumorigenic monocytes. To achieve these goals, there will be a need to more extensively implement novel high dimensional technologies including the high dimensional imaging techniques coupled with spatial scRNAseq, which will be essential to unravel the functions, the phenotype and the interactions of monocytes with other immune and non-immune cells in intact tissues.

We believe that the results of these studies will further disrupt our conventional view of monocytes and realistically pave the way for exploiting these cells for novel therapeutic approaches.

Author contributions

SG and CK wrote and finalized the review. All authors approved the submission.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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