MONOCYTE HETEROGENEITY AND FUNCTION

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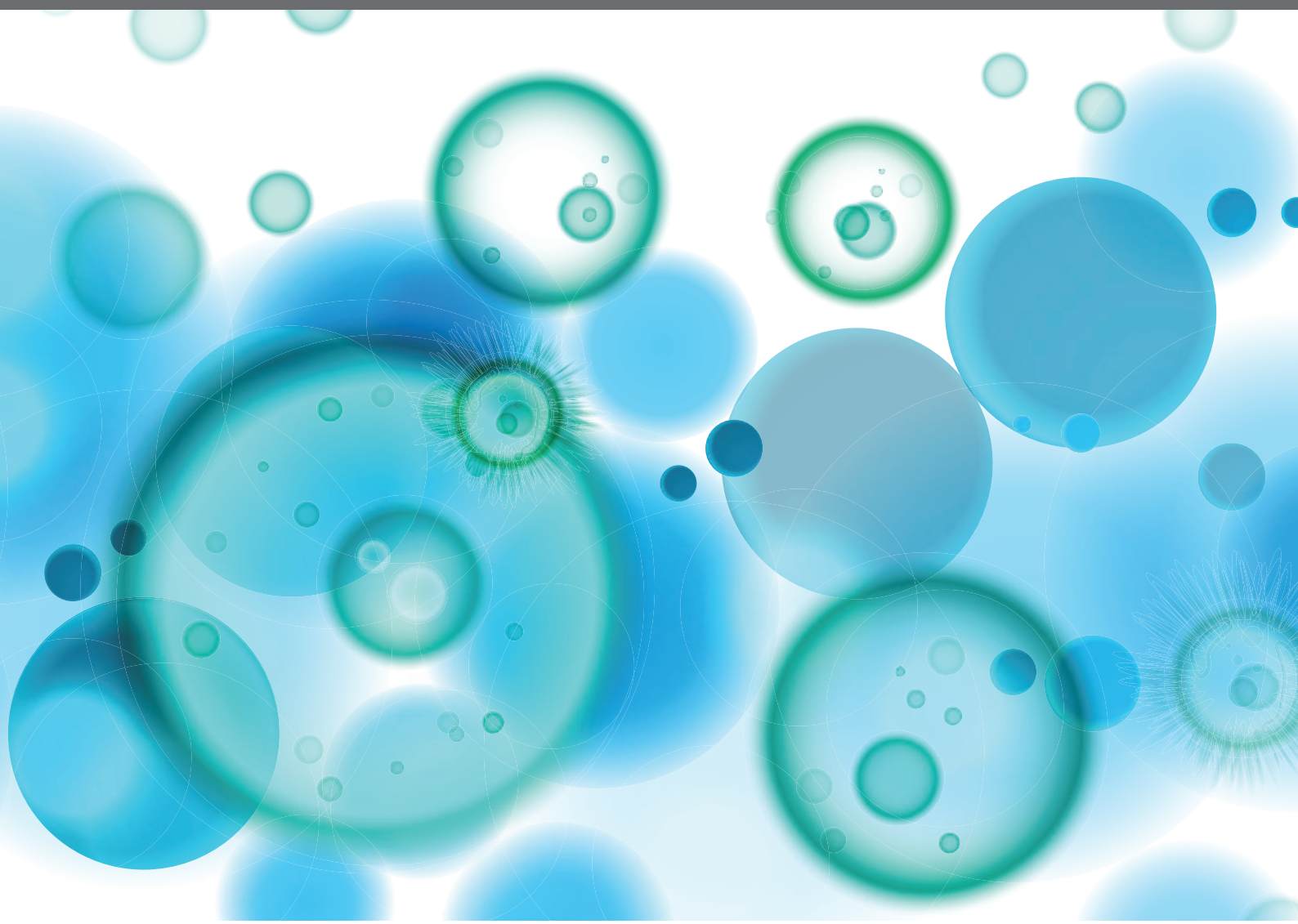

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MONOCYTE HETEROGENEITY AND FUNCTION

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EDITORIAL
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Editorial: Monocyte Heterogeneity and Function

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An Editorial on

Monocyte Heterogeneity and Function

Monocytes originate from bone marrow hematopoietic stem cells and circulate in the bloodstream. Monocyte extravasation and differentiation serve multiple immune functions. The differentiation of monocytes into tissue macrophages at steady state can serve homeostatic functions. Monocytes can also fuel acute inflammatory reactions and anti-microbial immunity by differentiating into inflammatory macrophages. Finally, monocytes also actively contribute to the resolution of inflammation and tissue regeneration.

The subset classification of monocytes is a rapidly emerging field. Recent progress in single-cell genomics and high dimensional approaches in phenotyping have highlighted additional subsets of monocytes. Monocytes might also adopt new dynamic transcriptional states associated with inflammation and reflecting their subset heterogeneity. This brings the research community to face a significant challenge of assigning monocyte heterogeneity to specific functions. The goal of this Research Topic is to gather contributions bringing new insights into:

i. Monocyte subsets and their ontogeny
ii. Monocyte heterogeneity and inflammatory diseases
iii. Monocyte heterogeneity and the regulation of inflammation
iv. Monocyte heterogeneity and cancer

MONOCYTE SUBSETS AND THEIR ONTOGENY

Kapellos et al. provide an updated classification of human monocytes by compiling multiple profiling studies 1. Monocytes are composed of two main subsets: $CD14^+$ and $CD16^+$ monocytes in humans and $Ly6C^{high}$ and $Ly6C^{low}$ monocytes in mice.

While originally referred to as "inflammatory" monocytes, $Ly6C^{high}$ and $CD14^+$ monocytes are now termed "classical" monocytes. Although classical monocytes constitutively enter tissues during homeostasis, upon inflammation, they rapidly extravasate and, depending on the needs of the environment, can differentiate into multiple cell types such as monocyte-derived macrophages.

$CD16^+$ and $Ly6C^{low}$ monocytes are termed as "non-classical" (Kapellos et al.). Non-classical monocytes display an intra-vascular function, interacting dynamically with endothelial cells (1). In humans, the existence of "intermediate" $CD14^+CD16^+$ monocytes complicates the distinction of "classical" vs. "non-classical" (Kapellos et al.). Expression of 6-sulfo LacNac sugar antigen linked to the cell surface protein PSGL-1 is termed as SLAN. SLAN expression defines non-classical $CD16^+$ in humans and provides a practical way to distinguish them from the "intermediate" $CD14^+CD16^+$ monocytes (Hofer et al.).

The developmental pathways by which monocytes arise from hematopoietic stem cells have received a lot of attention. Since the identification and characterization of Granulocyte-Monocyte Progenitors by Akashi et al. in 2000 (2), the ontogenetic pathways of monocytes have been continuously revisited. Geissmann et al. identified progenitors endowed with a mixed potential for monocytes and dendritic cells but devoid of granulocyte potential (3).

In this topic, Wolf et al. review the current line of evidence supporting the existence of multiple differentiation pathways for monocytes (Wolf et al.). GMP-dependent monocytes would co-exist with MDP-derived monocytes. The later encompass monocytes progenitors endowed with a potential for the generation of monocyte-derived DCs (moDCs). Fate mapping studies with $Ms4a3^{cre}/Ms4a3^{CreERT2}$ driver lines identifies the GMP-dependent pathway for the generation of monocytes and assesses precisely its contribution to the tissue resident macrophages (TRMs) pool (4).

The differentiation of monocytes into cellular products endowed with DC-like features has been historically evidenced *in vitro* (5). Since then, multiple lines of evidence suggest the physiological relevance of this process. In this topic, Coillard et al. review the current evidence suggesting that moDCs actually accumulate in patho-physiological conditions with important outcomes for the exacerbation or regulation of inflammation (Coillard and Segura).

Duroux-Richard et al. summarize the current of knowledge on the regulation of monocytes subsets by microRNAs. For instance, mir146a regulate the pool of classical monocytes with a regulatory role in osteoclastogenesis (Duroux-Richard et al.).

Assessing developmental pathways underlying the production of monocyte subsets during homeostasis should not obliterate the fact that monocytes are highly dynamic and regulated by both environmental and genetic factors (Kapellos et al.; Patel and Yona). In this topic, Patel et al. and Kapellos et al. provide a fascinating inventory of factors impacting on monocyte populations thereby emphasizing that "steady state is not a single physiological condition" (Patel and Yona) and providing some insights on how changes in lifestyle impact on monocyte subsets (Kapellos et al.).

Deciphering the aegis of specific signals is often complicated by the fact that they act not only on monocytes but also on numerous other myeloid lineages. The inflammatory factor GM-CSF provides a good example of such pleiotropy. Zhan et al. delineate the complex effects on GM-CSF on myeloid lineages and highlight how signal dosage impacts on signaling outcomes and biological functions. Specifically, low doses of GM-CSF activate preferentially the PI3K/Akt while higher doses of GM-CSF are needed to activate the JAK2/STAT5 pathway (Zhan et al.).

MONOCYTE HETEROGENEITY AND INFLAMMATORY DISEASES

Monocyte subsets represent both circulating precursors and effector populations in the bloodstream or within tissues (Kapellos et al.) (6). For instance, $Ly6C^{low}$ and $CD16^+$ monocytes mainly display an intra-vascular function, interacting dynamically with endothelial cells (Hofer et al.) (1). Kapellos et al. extensively review evidences highlighting the modification of the compartment of monocyte associated to COPD, or atherosclerosis for instance.

Monocyte extravasation supports their function of precursors for macrophages. In particular, Dick et al. provide an integrative picture of the remodeling of cardiac macrophages populations imposed by ischemic injury. Some striking features emerging from multiple scRNAseq studies might apply to various cases of acute injury: i) $TIMD4^+LYVE1^+MHCII^{low}$ TRMs tissue resident macrophages rapidly disappear from the inflamed zone but slowly repopulate the organ by local proliferation; ii) monocyte-derived macrophages acquire multiple transcriptional phenotypes including one resembling tissue resident macrophages (despite the absence of TIMD4 and LYVE1 expression) and other transcriptional phenotypes possibly associated to tissue repair functions; iii) $TIMD4^+LYVE1^+MHCII^{low}$ and $CCR2^+$ TRMs pools present before the onset of acute injury orchestrate monocyte infiltration by controlling the diversity of inflammatory macrophages phenotypes with important consequences for the onset of tissue repair (Dick et al.). Further research is needed to establish a map of developmental trajectories linking monocyte subsets to macrophages recruited after myocardial injury, possibly involving a heterogenous spatial distribution (Dick et al.). Coillard and Segura detail and discuss the various markers and methodological options available, in human immunology settings, to probe the monocytic origin of tissue

phagocytes (e.g., chimerism, labeling, and signature genes). However, the analysis of macrophage dynamics in the injured myocardium underlines the difficulties to move beyond the transcriptional definition of cellular populations to an integrated understanding of their functional contributions to both injury and tissue repair (Dick et al.).

Monocytes and macrophages play a major role in autoimmunity. In this topic, Ma et al. provide a broad panorama of the role of monocytes in multiple autoimmune conditions ranging from Systemic Lupus Erythematosus (SLE) to type 1 diabetes (T1D). Understanding monocyte biology has important therapeutic application. In this context, Hamilton delivers a synthesis of the current knowledge and controversies regarding the action of GM-CSF on monocytes and macrophages. A key feature of GM-CSF signaling lies in the activation of the CCL17 axis in monocyte-derived macrophages by a process involving the JMJD3 histone demethylase. Therapeutic blockade of GM-CSF in rheumatoid arthritis reduce CCL17 levels and reduces osteoarthritic pain (Hamilton).

MONOCYTE HETEROGENEITY AND THE REGULATION OF INFLAMMATION

The role of monocyte subsets in the resolution of inflammation is increasingly considered. The differentiation of classical monocytes into microbicidal macrophages is a central feature of anti-infectious innate immunity (7). However, this axis needs to be tightly regulated since microbicidal function can be associated to tissue damage. Postat and Bousso identify quorum sensing as new mechanism to maintain tissue integrity during the onset of anti-microbial innate immune reactions. In this case, nitric oxide release by differentiated anti-microbial monocytes plays a major role in activating the resolution of inflammation by a mechanism involving the suppression of mitochondrial respiration, thereby limiting cytokine and chemokine secretion (Postat and Bousso). Furthermore, IL-10 is also an important immuno-regulatory factor that can promote the acquisition of an immunoregulatory phenotype in differentiated moDCs associated to CD14 re-expression when combined with TLR stimulation (Krakow et al.). Undoubtedly, that understanding the mechanisms generating monocyte-derived cells endowed with immunosuppressive properties has a strong therapeutic relevance. For instance, Iglesias-Escudero et al. show in this topic that the presence of regulatory monocytes descendants is regulated by rapamycin in kidney transplant recipients.

The resolution of inflammation is a dynamic process relying on the efferocytic capacity of monocytes and their progeny, which support the development of anti-inflammatory responses and subsequently will contribute to the reestablishment of tissue resident macrophage pool. In this regard, Butenko et al. evidence that post-phagocytic "satiated" monocyte-derived macrophages express type I IFN and exhibit a transcriptomic profile limiting their pro-fibrogenic activity.

MONOCYTE HETEROGENEITY AND CANCER

Monocytes play a major role in cancer where CCR2-dependent classical inflammatory monocytes largely contribute to the pool of tumor-associated macrophages (TAMs), often with immunosuppressive and pro-tumoral properties (8). By contrast, non-classical monocytes had been shown to limit the metastatic spread of tumors through the blood circulation (9). Wu et al. discuss the relevance and limitations of the M1/M2 paradigm to understand the diversity of transcriptional states observed in TAMs. Wu et al. propose to re-assess TAM heterogeneity by integrating their ontogeny, activation and localization. Laviron and Boissonnas review the current knowledge on the selective contributions and functional contributions of acutely recruited monocytes in cancer versus pre-existing homeostatic tissue resident macrophage would they be derived from embryonic precursors or adult monocytes.

In summary, the very diverse and multidisciplinary contributions in this Frontiers TOPIC highlight the dynamism of monocyte research in finding new developments related to multiple biological processes and pathologies.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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The Role of Monocytes and Macrophages in Autoimmune Diseases: A Comprehensive Review

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Monocytes (Mo) and macrophages (Mϕ) are key components of the innate immune system and are involved in regulation of the initiation, development, and resolution of many inflammatory disorders. In addition, these cells also play important immunoregulatory and tissue-repairing roles to decrease immune reactions and promote tissue regeneration. Several lines of evidence have suggested a causal link between the presence or activation of these cells and the development of autoimmune diseases. In addition, Mo or Mϕ infiltration in diseased tissues is a hallmark of several autoimmune diseases. However, the detailed contributions of these cells, whether they actually initiate disease or perpetuate disease progression, and whether their phenotype and functional alteration are merely epiphenomena are still unclear in many autoimmune diseases. Additionally, little is known about their heterogeneous populations in different autoimmune diseases. Elucidating the relevance of Mo and Mϕ in autoimmune diseases and the associated mechanisms could lead to the identification of more effective therapeutic strategies in the future.

Keywords: monocyte, macrophage, autoimmune disease, count, function, polarization

INTRODUCTION

Monocytes (Mo) and macrophages (Mϕ) possess broad immuno-modulatory, inflammatory, and tissue-repairing capabilities and actively participate in the development of many autoimmune diseases (1). These cells can secrete a wide range of cytokines and chemokines, which stimulate and recruit additional immune cells to diseased tissue (2). In many autoimmune diseases, the presence of autoantibodies and autoreactive B and T cells indicates that adaptive immune system is critical for pathogenesis, but this cannot fully account for the development of autoimmune diseases, and the innate immune response may play a necessary and irreplaceable role as well (1, 3). In fact, Mo or Mϕ infiltration is generally observed in many autoimmune diseases (4–13). Additionally, a change in the count or frequency of Mo/Mϕ is a hallmark of several autoimmune diseases, i.e., systemic sclerosis (SSc), rheumatoid arthritis (RA), primary biliary cholangitis (PBC), Sjögren's syndrome (SS), and inflammatory bowel disease (IBD) (4, 5, 10, 14–17). However, it should be noted that Mo/Mϕ frequency and count in the peripheral blood or afflicted tissues can be affected by several factors including at least bleeding regimes (for instance time of bleeding) and status of the patients (medical treatment, food intake, age, sex etc.). Thus, Mo/Mϕ frequency and count and their correlation with disease stage are usually controversial in different studies.

Although the regulatory mechanism of Mo and Mϕ in the development of autoimmune diseases has not been fully elucidated, consensus appears to suggest that their abnormal activation plays a key role. Typically, M1-polarized Mϕ are pro-inflammatory and secrete interleukin (IL)-12 and tumor necrosis factor (TNF)-α to contribute to local inflammation, while M2-polarized Mϕ produce IL-4 and IL-10 that mount immunomodulatory, wound repair and tissue remodeling functions [as reviewed by Funes et al. (18)]. However, the M1/M2 dichotomy may oversimplify a more complex activation mechanism. In fact, in certain autoimmune diseases, both M1- and M2-polarized Mϕ are detected simultaneously, and both M1- and M2-stimulating cytokines are present on a large scale (19–22). Additionally, Mϕ even exhibit an intermediate activation status by co-expressing both M1- and M2-specific markers in certain diseases (23, 24). Furthermore, in many cases, Mϕ polarization is a dynamic and reversible event that depends upon the local environment and stage of disease (25).

In the present review, we will discuss our current understanding of the properties of Mo/Mϕ in certain autoimmune diseases, highlighting the phenotypical, functional, and activation properties of these cells in disease pathogenesis and the relevant mechanisms (Summarized in **Tables 1**, **2**). Because there are very limited reports regarding the role of Mo/Mϕ in autoimmune Addison's disease, autoimmune thyroid disease, antiphospholipid syndrome, and myasthenia gravis, these four diseases are not discussed in the present article.

MO AND Mϕ IN AUTOIMMUNE DISEASES

Systemic Lupus Erythematosus (SLE)

Mo percentage and count have been analyzed in SLE patients, but the findings vary among different studies. One group found that although the absolute number of the whole Mo population was similar between SLE patients and healthy controls, the rate and absolute number of $CD14^+CD16^+$ Mo was significantly higher in SLE patients, and steroid therapy could down-regulate the percentage and number of these cells in a dose-dependent manner (26). In contrast, a more recent study based on 205 SLE patients and 74 healthy controls reported decreased absolute Mo counts in SLE patients (27). However, there was no significant difference in the proportions of various Mo subpopulations. In addition, neither the absolute count nor the percentage of various Mo subsets was associated with disease activity (27). It appears that the reduction in Mo count in the latter study is supported by an independent study, which showed that Mo and Mϕ are more fragile and likely to undergo apoptosis (analyzed by flow cytometry with annexin V and propidium iodide) when induced by the sera of SLE patients (199). Although the detailed mechanism remains unclear, it appeared that C5a complement was involved in this process while serum IgG autoantibody was not involved, since Mo apoptosis profile correlated positively with C5a level, and depletion of IgG did not affect such apoptosis. In lupus mice, Mϕ depletion leads to attenuated skin and kidney disease severity, suggesting that these cells do play a critical role in SLE pathogenesis (200).

One of the contributions of Mo/Mϕ to SLE pathogenesis is modulation of the adaptive immune system. The binding of co-stimulatory molecule CD40 to its ligand CD40L is required for the activation of humoral immune responses including B cell activation, plasma cell differentiation, antibody secretion, and isotype-switching (201). In patients with SLE, a significant increase in the frequency of CD40L-expressing peripheral Mo was observed compared with healthy controls (28). Consistent with this finding, although B cells from SLE patients and normal controls showed similar CD40 expression levels, recombinant CD40L significantly stimulated the production of total IgG by SLE B cells but not normal B cells (202). In addition, data from murine studies showed that CD40L overexpression could induce lupus-like autoimmune disease, while CD40L neutralization prevented autoreactive B cell activation and autoantibody production in lupus-prone mice (203, 204). Thus, although direct evidence is still lacking, these data do suggest a potential contribution of Mo to the observed B cell hyperactivity in SLE patients through the CD40/CD40L signaling pathways. Moreover, Mo from SLE patients tend to differentiate into dendritic cells that express higher levels of CD86 when induced by IFN-α in the serum, and this potentiates them with higher abilities to present autoantigens to autoreactive T cells and B cells (29, 30).

Overexpression of adhesion molecules may lead to aberrant Mϕ migration and activation. Mϕ from active SLE patients overexpress intercellular adhesion molecule (ICAM)-1, which is associated with tissue recruitment and inflammatory cytokine production, and this is partially offset by corticosteroid therapy (31, 32). SLE Mϕ also express increased levels of sialic acid–binding Ig-like lectin 1 (Siglec-1, CD169), which could be dramatically reduced by high-dose glucocorticoid treatment (33). However, in view of the broadly anti-inflammatory effect of glucocorticoid (205), it should be noted here that this study could not rule out the possibility that the reduction in Siglec-1 expression level might result from a bystander effect of glucocorticoid treatment. Increased Mϕ Siglec-1 expression may constitute a potent inflammatory signal to promote the activation of autoimmune $CD4^+$ or $CD8^+$ T cells (206). In fact, it was suggested that Siglec-1 expression in Mϕ could serve as a potential biomarker for lupus activity, as the percentage of Siglec-1-expressing Mϕ was shown to positively correlate with SLE Disease Activity Index and autoantibody levels (33).

Defective phagocytosis of Mϕ has also been suggested to contribute to autoimmunity in SLE. The phagocytic capacity of Mϕ is crucial for the clearance of dead cells and debris, which otherwise can be important sources of autoantigens. Accumulating data from *in vitro* studies and murine models illustrate that ineffective clearance of apoptotic cells by Mϕ might be an important trigger of the autoimmune process in SLE. Two decades ago, it was observed that non-inflammatory phagocytosis of apoptotic cells by Mo-derived Mϕ (MDMs) was strikingly impaired in SLE patients (34). In addition, apoptotic cells were found to accumulate in the germinal centers of the lymph nodes in patients with SLE, and *in vitro* uptake of autologous apoptotic cells into Mo-derived Mϕ from SLE patients was significantly impaired (35). Interestingly, the

TABLE 1 | Characteristics of Mo and Mϕ in autoimmune diseases.

Disease	Percentage/count alterations	Functional abnormalities	Polarization profiles
SLE	Similar to healthy controls in Mϕ number (26); Decreased Mϕ count (27). Increased $CD14^+CD16^+$ Mo number (26).	Increased expression levels of CD40 (28), CD86 (29, 30), ICAM-1 (31, 32), Siglec-1 (33); Defective phagocytic ability (34–36).	M1 polarization: Higher levels of IL-1β (37), IFN-γ (19), CXCL10 (38), CCL2 (39), GM-CSF (40). M2 polarization: Higher levels of IL-10 (20, 21).
SSc	Number: Increased $CD68^+$ Mϕ (41); Increased $CD14^+$ Mo (42); Increased $CD16^+$ Mo in diffuse SSc (42). Percentage: Increased $CD14^+$ Mo (43).	More profibrotic (44); Increased expression of Siglec-1 (45).	M2 polarization: Higher levels of IL-4, IL-10, IL-13, TGF-β, and PDGF (46–48). Increased expression of CD163 and CD204 (41, 43).
RA	Increased number and percentage of Mϕ (4, 5).	Increased Mo CD80 (49), CD276 (49), and Siglec-1 expression (50).	M1 polarization: Higher levels of TNF-α, IL-1, IL-6, and IL-12 (51–55). Increased expression of CD50 and CD36 while lower expression of CD163 and CD209 (56). Higher M1/M2 Mo ratio (57).
MS	Increased total mononuclear phagocyte number (11, 12, 58, 59).	Increased expression of CD68, HLA and CD86 (60). Abnormal metabolic changes (more glycolysis) (61).	An intermediate status: Co-expression of CD40 and mannose receptor (24).
T1D	Increased $CD14^+$ Mo number (62). Decreased $CD16^+$ Mo number (62).	Decreased phagocytosis ability (63, 64). Cytolytic to islet β-cells (65).	M1 polarization: Higher levels of C-reactive protein (66), IFN-γ (67), CXCL10 (68), CCL2 (68), IL-6 (66, 69), IL-1β (66, 69), TNF-α (70, 71).
PBC	Increased Kupffer cell number in stage 3 and 4 cases (10, 72). Similar number of Kupffer cells at different stages (73). Increased liver $CD14^+$ Mo number (73). Increased circulating $CD14^{high}CD16^+$ and $CD14^{low}CD16^+$ Mo number (74).	More sensitive to TLR ligation (75). Increased Siglec-1 expression (76). Recognition of AMA-apotope complexes (77).	M1 polarization: Higher levels of IL-1β, IL-6, IL-8, IL-12, and TNF-α (75, 78). Increased endotoxin production of biliary epithelial cells (79). Increased expression of CD40L (72).
SS	Increased $CD14^{high}CD16^+$ and $CD14^{low}CD16^+$ Mo number (15, 80).	Decreased phagocytosis ability (81).	M1 polarization Increased levels of IL-6 (82), IL-12 (83), IFN-γ (84), TNF-α, IL-1β, IL-18, CXCL8, and CXCL10 (80, 85–87). Activation of Mϕ NFκB signaling pathway (88).
Celiac disease	Increased $CD68^+$ Mϕ number (7).	Decreased phagocytosis ability (7, 89). Increased antigen-presenting ability (90, 91).	M1 polarization Higher levels of IFN-γ, IL-1β, TNF-α, and IL-8 (22, 90). Increased expression of CD80, CD86, and CD40 (88). Activation of NFκB signaling pathway (88). M2 polarization: Higher levels of IL-4 and IL-10 (22). Increased expression of arginase 1 and 2 after stimulation (92, 93).
IBD	Increased $CD68^+$ Mϕ number in UC and CD (8, 9, 16). Increased $CD163^+$ Mϕ number in CD (16). Increased circulating $CD14^+CD16^+$ while decreased $CD14^{hi}CD16^-$ Mo in CD (94, 95).	Decreased retinoic acid synthesis ability in CD (8). Abnormally accelerated lysosomal degradation of cytokines in CD (96). Defective GM-CSF receptor expression and function in UC and CD (97).	M1 polarization: Increased production of IL-23 and TNF-α in UC (98, 99). Suppressed IL-10 production in UC (98, 99). Higher expression of CD16/32 in UC (98, 99). M2 polarization: Higher IL-13 level in CD (100). Higher CD163 expression in CD (16). Higher CD163 and CD206 expression in UC (16, 101).

percentage of apoptotic polymorphonuclear neutrophils (PMN) from SLE patients was significantly higher than that from healthy controls, and this percentage correlated positively with SLE Disease Activity Index and serum levels of autoantibodies (207). In addition, the phagocytosis defect may be compounded by the serum milieu of SLE patients because serum from these patients had a strong capacity to accelerate the apoptosis rate of PMN and Mϕ, which might further contribute to the high load of potential autoantigens (199, 207). Based on data from murine models, it was found that Mϕ with low expression of

TABLE 2 | Mechanisms of Mo/Mϕ activities in autoimmune diseases.

Diseases	Triggers for Mo/Mϕ recruitment and activation	Molecular mechanisms of Mo/Mϕ function	Mo/Mϕ-derived mediators in disease progression
SLE	TNF-α: Mo NF-κB inflammatory response (102). Anti-dsDNA antibodies: NLRP3 inflammasome activation in Mϕ (103). Microparticle-associated immune complexes: activation of pro-inflammatory Mo (104). IFN-α: B-lymphocyte stimulator expression in Mo (105). Anti-C1q autoantibodies: induction of a pro-inflammatory phenotype in Mϕ (106). HMGB1: Mϕ inflammatory responses (107).	Decreased PPAR-γ, KLF2 and KLF4 expressions: Defective phagocytosis (108, 109). Decreased PPAR-γ expression: pro-inflammatory functions (110). Increased IRF1 expression: enhanced inflammasome activity (111).	IL-1β, IL-6, TNF-α and IL-10: mediating local and systemic inflammation (112–115).
SSc	CCL2: Mo/Mϕ recruitment (116). Type I IFN: Mϕ activation (45). PDGF-BB: dermal infiltration of Mo/Mϕ (117). CX3CL1: Mo/Mϕ recruitment (118). MIF: concentrating Mϕ at inflammatory loci (119).	TLR/MyD88 signaling and the transcription factor Fos-related antigen 2: TIMP1 production by Mo (120, 121).	PDGF and TGF-β: fibrosis development (44, 117, 122) CCL4, CXCL8, and CXCL10: tissue inflammation and fibrosis (123). CXCL13: fibrosis development (124). Versican and CCL2: Mo recruitment (125). TIMP-1: fibrosis development (121, 126).
RA	CCL2: Mo recruitment (13). Activin A: generation of pro-inflammatory Mϕ (56). Neutrophil microvesicles: preventing inflammatory activation of Mϕ (127). GM-CSF and osteopontin: Mo migration (128). MicroRNA-155: survival of Mo (129, 130).	NFAT5: survival of activated Mϕ (131). Succinate/GPR91 signaling: IL-1β production from Mϕ (132). Liver X receptor pathway: potentiating TLR-driven cytokine production from Mϕ (133).	IL-1, IL-6, IL-12, and TNF-α: mediating local and systemic inflammation (134, 135). IL-1, IL-6, and TNF-α: mediating cartilage degradation (136).
MS	CCL2: M1 macrophage recruitment (137). GM-CSF: migration of Mo across the blood brain barrier (138, 139). IFN-γ and α-B-crystallin: activation of microglia/Mϕ of MS-affected brain tissue (140). Acetylcholine-producing NK cells: kill and inactivate $CCR2^{+}Ly6C^{hi}$ Mo (141).	Decreased SHP1 signaling: enhanced inflammatory activity of Mo (142). KLF2: negatively regulate Mϕ activation (143).	NLPR3 inflammasome: T cell recruitment (144). IL-1β, IL-6, and IL-23: Th17 cell generation (145–147). TNF-α, IL-6, IL-12, IL-1β, Reactive oxygen, and nitrogen species: mediating inflammatory responses (140, 148, 149). IL-6 and BAFF: B cell survival and differentiation (150).
T1D	CCL2: Mϕ recruitment (151). MIF: activating Mϕ and driving Th1 cell response (152–154). GM-CSF: Mo activation (155). Acetoacetate: IL-6 and ROS production from Mo (156) and Mo adhesion to endothelial cells (157). Myeloid-related proteins: adhesion of Mo to fibronectin (158).	Increased expression of long-chain acyl-CoA synthetase 1: enhanced inflammatory activity (159). Increased LFA-1 expression: Adhesion to endothelial cells (157). Persistent activation of STAT5: aberrant inflammatory gene expression (155).	IL-1 and IL-6: Th17 cell generation (69).
PBC	CX3CL1: Mo recruitment (160). MIF-3α, osteopontin and CCL2: MDM recruitment (161–163). TLR ligands: Mϕ activation and production of pro-inflammatory cytokines (75, 78). AMA-apotope complexes: MDM activation (164). TNF-α-induced protein 8-like-2: productions of TNF-α, IL-1β, and IL-8 by Mo (165). Exosomes: expression of co-stimulatory molecules on Mo (166).	TNF-α-induced protein 8-like-2 signaling: inhibiting Mo NF-κB pathways and Mo activation (165).	IL-12: differentiation of Th1 cells (74). NLPR3 inflammasome: inducing IL-1β production and promoting differentiation of Th17 cells (167). IL-1β, IL-6, IL-8, IL-12, and TNF-α: promoting liver inflammation and injury (75, 78)
SS	CXCL9 and CXCL10: migration of $CXCR3^{+}$ Mϕ (168). MIF: local infiltration of Mϕ (119). Extranuclear accumulation of DNA: NLRP3 inflammasome activation (169).	MicroRNAs: targeting the canonical TGF-β signaling pathway as opposed to pro-inflammatory IL-12 and TLR/NF-κB pathways (170). Activated NF-κB pathway: amplifying cytokine production and inflammatory response (88).	CCL22: enhancing autoreactive T cell response and recruitment (171). IL-6, IL-18, type I IFN, and BAFF,: mediating pro-inflammatory immune responses (87, 172, 173)

(Continued)

TABLE 2 | Continued

Diseases	Triggers for Mo/Mϕ recruitment and activation	Molecular mechanisms of Mo/Mϕ function	Mo/Mϕ-derived mediators in disease progression
Celiac disease	Gliadin peptides: Mo production of pro-inflammatory cytokines and chemokines (90, 90, 174) IL-15: supporting Th17 and Th1 responses (175).	TLR/MyD88 signaling pathway: mediating pro-inflammatory cytokine production (176–178). NF-κB activation: Mo production of IL-8 and TNF-α (174). TLR4/MyD88/TRIF/MAPK/NF-κB signaling pathway: production of IL-1β by Mϕ (179) lncRNA: facilitating Mϕ inflammatory gene expression (180). Increased STAT3 signaling: Mo activation and IFN-γ production (181).	Tissue transglutaminase: involved in processes contributing to inflammation (182). IL-1β, IL-23, TNF-α, IL-6, IFN-γ: tissue inflammation (179, 181, 183, 184).
IBD	IL-33: induction of Mϕ with tissue-repairing ability (185). Luminal extracellular vesicles: Mϕ migration (186). Gut microbiota (Clostridium butyricum): induction of IL-10-producing Mϕ (187).	PPAR-γ mutation: generation of pro-inflammatory M1 Mϕ (188, 189). Higher expression of Nuclear paraspeckle assembly transcript 1: mediation of the inflammatory responses through exosome-mediated polarization of Mϕ (190).	IL-1β, IL-6, IL-23, TNF-α and TNF-like protein 1A: generation of Th1 and Th17 cells (191–194). IL-23: promoting Th17 cell differentiation and NK cell activation (195–197). NLRP3 inflammasome: promoting experimental IBD development (without detailed mechanisms) (198).

TLR9 and decreased TLR responsiveness to nucleic acids were largely responsible for the immunologically silent clearance of apoptotic cells (108), which was consistent with the finding that TLR9 was required in SLE pathogenesis (208). In addition, transcription factors Kruppel-like factors 2 (KLF2) and 4 (KLF4) are also important regulators of apoptotic cell clearance in SLE prone mice (108). Intriguingly, it appears that cues from the tissue microenvironment dictated these characteristics of Mϕ, as removal of these cells from specific tissues resulted in their inability to engulf apoptotic cells without generating inflammatory responses (108). A second feature of the impaired phagocytic capacity of SLE Mϕ is the delayed clearance of immune complexes (IC). Elegant work by Michael M. Frank and coworkers examined the half-time of IgG-sensitized, ^{51}Cr-labeled erythrocytes as a measure of Fc receptor function (209). They showed that Fc-specific clearance rates were strikingly prolonged in 13 of 15 patients, and this correlated with elevated levels of IC and with disease activity. Supporting this conclusion, another study by Maria et al. has recently showed that decreased Fc receptor function correlated positively with disease activity and renal involvement (36). In addition, certain Fc receptor polymorphisms appears to determine the clearance of IC *in vivo*, and their heritage is associated with the course of SLE in some ethnic populations (210, 211). Abnormal Mϕ activation has also been observed in SLE patients. Labonte et al. demonstrated that higher activation profiles of Mϕ were associated with more active cases of SLE (212). In addition, Mϕ activation syndrome, a rare but usually very severe or even life-threatening complication has been reported in SLE patients (213, 214).

Accumulating findings suggest the predominance of M1 Mϕ in SLE pathogenesis. Excessive pro-inflammatory M1 Mϕ-related cytokines are produced by Mϕ from SLE patients, including IL-1β (37), interferon (IFN)-γ (19), C-X-C motif chemokine 10 (CXCL10) (38), and C-C motif chemokine ligand 2 (CCL2) (39). In addition, the pro-inflammatory serum milieu of SLE patients also favors M1 polarization, including high levels of IFN-γ, TNF-α, and granulocyte-Mϕ colony-stimulating factor (GM-CSF) (40, 112). M1 dominance may come at the expense of insufficient anti-inflammatory M2 polarization. It was shown that serum transforming growth factor (TGF)-β levels were significantly reduced in lupus patients, and TGF-β levels showed a reverse association with disease activity and organ damage in SLE patients (215). In addition, antibodies against scavenger receptors (an M2 Mϕ marker) or scavenger receptor knockout in lupus prone mice led to a compromised ability of Mϕ to engulf apoptotic cells and resulted in more aggravated SLE symptoms (109).

Considering the pro-inflammatory nature of M1 Mϕ and that M2 Mϕ are anti-inflammatory and are capable of engulfing apoptotic cells during apoptosis, it is reasonable to assume that M1 predominance and M2 insufficiency combine to worsen SLE severity. Indeed, researchers are trying to cure SLE by modulating Mϕ polarization. In a murine model of SLE, adoptive transfer of anti-inflammatory M2a Mϕ induced by IL-4 significantly decreased SLE activity (216). In patients with SLE, pioglitazone treatment enhanced M2 polarization of Mo-derived Mϕ, increasing their anti-inflammatory capacity while suppressing their production of various pro-inflammatory cytokines (217).

Available online at: Intriguingly, large amounts of IL-10, which is a hallmark of M2 Mϕ, are commonly detected in patients with SLE, and serum levels of IL-10 correlate positively with disease activity (20, 21). Contrary to its canonical anti-inflammatory functions, IL-10 in SLE acquires a pro-inflammatory capacity. This is largely dependent on high concentrations of type I IFNs, which confer a pro-inflammatory gain of function upon IL-10 and lead to a positive feedback loop of pro-inflammatory cytokine production (113). Priming of primary human Mϕ with IFN-α resulted in significantly enhanced STAT1 activation in the presence of IL-10, leading to activation of several STAT1-dependent genes such as CXCL9, CXCL10, and IFN regulatory factor 1 (113). In addition, IL-10 can directly stimulate production of platelet-activating factor (a phospholipid mediator of inflammation) of Mo of SLE patients

(218). Indeed, IL-10 antagonist administration ameliorated SLE severity effectively during a 6-month therapy, even though this finding was limited by the small sample size of the study (219).

In addition to IL-10, SLE Mo or Mϕ also produce copious IL-6 and TNF-α. Elevated IL-6 levels are positively associated with disease activity or autoantibody levels (114). The underlying mechanism seems to be the stimulation of B cell hyperactivity by IL-6 (220). Indeed, in an open-label phase I dosage-escalation study, IL-6 receptor inhibition showed a significant decrease in the frequency of circulating plasma cells, reduced autoantibody levels in the serum, and significant disease improvement (221). Another cytokine, TNF-α, is generally reported to be elevated in SLE and positively associated with disease activity (112). However, TNF-α blockade therapy in SLE is controversial. Although this therapy was shown to reduce disease severity, autoantibodies to double-stranded DNA and cardiolipin increased during treatment (222). Furthermore, it seems that TNF-α blockade is safe only for short-term treatment, while long-term therapy would likely provoke severe adverse effects such as lymphoma and Legionella pneumonia (223).

Intriguingly, in lupus prone NZB/W and NZW/BXSB mice, nephritic resident $CD11b^{hi}F4/80^{hi}$ Mϕ exhibit little arginase- or iNOS-producing ability even after stimulation with M1 or M2 Mϕ-inducing cytokines, irrespective of the clinical status of the mice (224). Instead, these kidney residents show a mixed pro- and anti-inflammatory phenotype during lupus nephritis (224). In contrast, Mo-derived Mϕ of the same mice were readily responsive to cytokine stimulation and can be induced to differentiate into the correspondingly M1 or M2 cells (224). In addition to these phenotypic differences, differences, functional analysis showed that nephritic resident Mϕ had higher antigen-presenting function and phagocytosis ability compared with MDMs of the kidney (224).

Several molecules and pathways have been suggested to be associated with controlling polarization and inflammatory profiles of Mϕ. For example, using MDMs of normal subjects and SLE patients, Saeed et al. found that epigenetic modification is partly responsible for the Mϕ polarization profile in SLE (225). Their data showed that sodium valproate, an histone deacetylase inhibitor, can potently induce the alternative activation of Mo-Mϕ *ex vivo* and inhibit the pro-inflammatory profile of these cells when stimulated by apoptotic cells *in vitro* (225). The same group also found that aryl hydrocarbon receptor (AhR)-mediated signaling pathway is responsible for the secretion of anti-inflammatory cytokines and expression of M2 markers from MDMs of SLE patients, as AhR agonist treatment of these cells led to a significant downregulation of M1 markers and expression of pro-inflammatory cytokines, coincided with an upregulation of M2 markers and expression of anti-inflammatory cytokines (226). In addition, decreased peroxisome proliferator-activated receptor gamma (PPAR-γ) expression has also been proposed to be involved in the defective efferocytosis and abnormal pro-inflammatory characteristics of MDMs from SLE patients (217).

SSc

More than two decades ago, Ishikawa et al. stained skin specimens from patients with SSc and found that Mϕ infiltration was generally observed around skin adnexa and vessels as well as between collagen bundles, while no close relationship with disease duration was found (6). Later, another group showed that the percentage of peripheral Mo in SSc is significantly higher than that in healthy controls. Notably, this higher percentage of Mo also correlated with worse prognosis and visceral disease involvement (14). However, in this study, Mo were not detected specifically through CD14 but instead were gated indirectly through $CD3^+CD4^-$, weakening the general application of this conclusion. Using a more specific Mϕ marker, another group showed that the number of $CD68^+$ Mϕ was significantly higher in the skin of patients with localized SSc (41). The same group also found that the percentage of $CD14^+$ circulating Mo was significantly greater in SSc patients than in healthy controls (43). In a more recent study, Lescoat et al. found that SSc patients had an elevated count of total peripheral Mo relative to healthy controls (42). Notably, the $CD16^+$ subpopulation count was more significantly increased in diffuse SSc than in limited SSc. In addition, the absolute count of $CD16^+$ Mo was significantly associated with the severity of skin fibrosis, pulmonary fibrosis, restrictive ventilatory defect, and pulmonary function impairment, suggesting a potential link between this subpopulation of Mo and the pathogenesis of fibrosis in SSc (42, 227). A potential mechanism underlying the increased Mϕ count may involve Mϕ migration inhibitory factor (MIF), which is capable of suppressing the random migration of Mϕ to concentrate them at inflammatory loci. Selvi et al. has reported the detection of high levels of MIF in the basal and suprabasal keratinocytes of SSc-affected skin (119). In addition, elevated concentrations of MIF in the peripheral blood of patients with diffuse cutaneous SSc were identified.

Several lines of evidence have implicated the functional abnormality of SSc Mo/Mϕ. It was reported that SSc Mo were more pro-fibrotic, as they displayed increased differentiation potential toward type-1 collagen- and α-smooth muscle actin (SMA)-expressing cells after stimulation (44). In addition, the production of tissue-inhibitor of metalloproteinase-1 (TIMP-1), an important protein capable of inhibiting extracellular matrix degradation, is significantly increased in SSc Mo mediated by TLR/MyD88 signaling and the transcription factor Fos-related antigen 2 (20, 21, 126). SSc Mo/Mϕ also show abnormally expressed markers that are associated with certain functions. First, increased expression of Siglec-1 in tissue Mϕ and circulating Mo of SSc patients was identified, suggesting a potential role for type 1 IFN-mediated Mo/Mϕ activation in SSc (45). In line with this finding, levels of IFN-α mRNA were significantly increased in vascular and perivascular cells in skin samples of SSc patients (228). However, how Siglec-1 is induced and to what extent it contributes to the pathogenesis of SSc need further verification. In a recent study, Moreno-Moral et al. explored the contribution of MDMs in mediating genetic susceptibility to SSc. By conducting genome-wide genotyping and RNA-sequencing, their work shows that gene expression in Mϕ from SSc patients is altered, especially higher expression of the *GSDMA* and *GRB10* genes (229). However, the relevance of these results at the protein level has yet to be examined in the future.

Mounting evidence suggests the predominant M2 polarization of Mϕ in SSc. The elegant work of Nobuyo et al. showed an evident increase in the number of $CD14^{bright}CD163^{+}CD204^{+}$ Mϕ in the fibrotic areas of the SSc skin (41, 43), suggesting that this cell subset may be potential a regulator of fibrosis in SSc skin. Of note, CD204-deficient mice failed to develop silica-induced fibrosis, suggesting a critical role for this scavenger receptor in fibrosis (230). This finding was underpinned by the works of several other groups, which reported that a soluble form of CD163 (sCD163), released from the Mϕ cell surface, was increased in the sera of SSc patients relative to the general population (231–233). Intriguingly, sCD163 secretion by PBMCs *ex vivo* may serve as a biomarker of SSc progression, as increased production of sCD163 by PBMCs was associated with worse prognosis of SSc (233). In addition, urinary sCD163 concentrations were also higher in SSc patients, but the difference was not statistically significant (232). In line with these findings, several reports have shown elevated serum levels of M2-inducing cytokines, i.e., IL-4, IL-13, and IL-10, in patients with SSc (234–236).

A growing body of data has suggested that M2 Mϕ play crucial roles in the activation of resident fibroblasts and the progression of fibrosis, mainly through the release of TGF-β, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (237, 238). Indeed, high levels of TGF-β and PDGF as well as their contribution to SSc have been reported by several groups (46–48). Data from skin samples of SSc patients and healthy control subjects showed that in SSc, the production of TGF-β by Mϕ was partly induced by Cadherin11, which has been implicated in both pulmonary and skin fibrosis (48). In murine studies, blockade of Cadherin11 led to fewer myofibroblasts and decreased dermal thickness in established fibrosis (48). However, whether this treatment may be therapeutically effective needs further verification.

Intriguingly, a recent study revealed that a considerable number of M2 Mo ($CD204^{+}CD163^{+}CD206^{+}$) co-expressed M1 markers (CD80 and CD86) in the PBMCs of SSc patients, and this subset of cells constituted a significant feature that characterized SSc (23). In addition, down-regulation of the IL-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway was identified in SSc Mo-derived Mϕ (239). These data suggest a more complex activation profile of SSc Mo/Mϕ, consistent with the remarkable plasticity of these cells. Further investigation into the polarization state of Mo/Mϕ in different stages of SSc is needed, and the exact role of these cells should be clarified.

RA

Mϕ infiltration in the synovia is one of the most important hallmarks of RA. There is ample evidence that the frequency and absolute number of Mϕ are markedly increased in the synovial tissues of patients with RA (4, 5). More importantly, this phenomenon could serve as a reliable biomarker for disease activity. Mulherin et al. showed that synovial Mϕ number correlated positively with articular destruction in RA (240). In a study based on 66 patients with RA, it was found that local disease activity in particular was positively associated with the number of synovial Mϕ as well as levels of IL-6 and TNF-α, two major Mϕ-derived cytokines (241). Accordingly, it was suggested that synovial Mϕ count may also reflect the therapeutic efficacy of RA. An early study by Ghada et al. found that the number of synovial $CD68^{+}$ Mϕ was significantly reduced 12 weeks after treatment with sodium aurothiomalate (242). A further study investigated synovial tissue biopsies from 88 patients with RA participating in various clinical trials, and the authors found that the number of synovial Mϕ correlated significantly with disease activity score, and that a decrease in this number was positively correlated with clinical improvement of RA, independent of the therapeutic strategies these patients received (243). In line with these findings, it was important to find that sublining Mϕ did not change in response to placebo or ineffective treatment (243, 244). These findings were corroborated by data from rodent models of arthritis. It was recently shown that experimental arthritis was accompanied by enhanced survival of synovial Mϕ and would be markedly improved in genetically modified mice in which Mϕ were more susceptible to apoptosis (131). In this study, Mϕ survival is induced by increased expression levels of nuclear factor of activated T cells 5, the expression of which is stimulated by the inflammatory tissue microenvironment of the arthritic mice. Importantly, experimental arthritis was significantly alleviated after local Mϕ depletion by knee joint clodronate liposome injection (245). Moreover, inhibition of Mϕ differentiation from Mo also ameliorated synovial inflammation in experimental arthritis (246). These findings suggest that Mϕ play a key role in RA pathogenesis.

A growing number of studies have highlighted the central role of Mϕ activation in RA pathogenesis. To be specific, unrestrained pro-inflammatory M1 polarization with incomplete M2 polarization usually leads to more severe joint pathology, and thus Mϕ polarization modulation usually alters the outcome of experimental arthritis. In a collagen II-induced arthritis mouse model, it was found that cyclophilin A, a potent pro-arthritic protein, aggravated the severity of arthritis through the induction of pro-inflammatory M1 Mϕ polarization and cytokine production in the knee joint (247). On the other hand, efficiently repressed M1 polarization or increased anti-inflammatory M2 polarization suppressed synovial inflammation and held promising potential as a targeted therapy for RA. In collagen II-induced murine arthritis and spontaneous arthritis in Hes1-GFP/TNF-transgenic mice, inhibited M1 polarization and simultaneously enhanced M2 polarization of Mϕ significantly reduced the inflammatory response in the knee joints (248, 249). Likewise, collagen-induced arthritis was efficiently ameliorated by the administration of mesenchymal stem cells, which have potent immunomodulatory capabilities (250–252). In addition, IL-10 was able to suppress the observed effects of pro-inflammatory M1 Mϕ in experimental arthritis, partly due to inhibition of the inflammation-associated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway or pro-inflammatory cytokine secretion from Mϕ (253, 254). Data from murine model of RA showed that synovial tissue-resident Mϕ and MDMs play different roles in experimental RA. Misharin et al. found that $Ly6C^{-}$ Mo are recruited into the synovial tissue and differentiate into pro-inflammatory M1

Mϕ during the effector phase of arthritis, thus driving initiation and progression of joint inflammation. During the resolution phase, these cells are polarized toward an alternatively activated phenotype and contribute to the resolution of arthritis (13). In comparison, synovial tissue-resident Mϕ are anti-inflammatory throughout the course of arthritis and inhibit joint inflammation during the initiation phase (13).

Activated Mϕ are a potent source of various pro-inflammatory cytokines, which are essential mediators of the effects of Mϕ during the development of RA (56, 132, 255). TNF-α is a key cytokine that is produced by synovial Mϕ and is of critical importance in the pathogenesis of RA (51, 249, 256). This cytokine is present in most arthritis biopsies, and its overexpression induces spontaneous inflammatory arthritis, whereas its inhibition suppresses various rodent arthritis models (134, 135). Accordingly, therapeutic targeting of TNF-α signaling has yielded clinical efficacy in patients with established RA, which has also been corroborated by a number of mouse model-based results (257–259). Other Mϕ-derived cytokines such as IL-1, IL-6, and IL-12 are also abundantly present in the arthritic synovium of patients with RA (134, 135). Similarly, they are indispensable for the inflammatory responses in the synovia of patients with RA, and blockade of their signaling pathways improves clinical or experimental arthritis (52–55).

Multiple Sclerosis (MS)

In progressive MS, central nervous system (CNS) inflammation is characterized by widespread activation of mononuclear phagocytes (MPs), which include both Mo-derived Mϕ and resident microglia (58). These MPs are found in both gray and white matter lesions, are close to degenerating areas, and are associated with chronic tissue damage (11, 12). In addition, in normal-appearing white matter, MP infiltration is associated with the formation of microglial nodules that lead to disease pathology (260). It has been suggested that staging of MS lesions can be determined based on the presence of CD68-positive Mϕ and human leukocyte antigens, together with the degree of myelin loss (59). The detrimental role of MP-driven pathology in MS is also supported by evidence from murine models, which has shown that the overall burden of MPs correlates with brain atrophy (261), impaired neuronal function (262), and decreased regenerative responses (263). These findings are underpinned by evidence from clinical trials, as induction of Mϕ apoptosis by IFN-β showed a significant benefit in MS (264). In addition, in murine models, Mϕ depletion showed significantly suppressed CNS damage and clinical signs of experimental autoimmune encephalomyelitis (265, 266).

Using brain autopsy tissue from patients with MS, Tobias et al. found that the main functional changes in Mϕ and microglia are increased expression levels of molecules associated with inflammation, including CD68 (phagocytosis), human leukocyte antigen (HLA) and CD86 (antigen presentation and co-stimulation), and inducible nitric oxide synthase (iNOS) (microglia activation) (60). Another group, George et al. found that Mϕ of MS patients display deficient SHP-1 mRNA and protein expression, leading to heightened activation of STAT1, STAT6, and NF-κB signaling and a corresponding enhanced inflammatory profile (142). In addition, data from experimental autoimmune encephalomyelitis (EAE), an animal model of MS, has shown a critical role for Mϕ in triggering adaptive immune responses. For example, Mϕ NLPR3 inflammasome plays a key role in inducing migration of autoreactive T cells into the CNS in EAE (144). Mϕ also produce several key cytokines (i.e., IL-1β, IL-6, and IL-23) to promote the generation and maintenance of Th17 cells, a key cell subset mediating CNS autoimmunity in EAE (145–147). In addition, TLR7-mediated productions of IL-6 and B cell-activating factor (BAFF) are crucial cytokines for autoreactive B cell survival and differentiation (150). In consistent with these findings, Mϕ depletion or anti-GM-CSF treatment inhibits the induction of myelin antigen-specific Th17 cells and protects mice from clinical symptoms of EAE (146, 267–269).

Ample evidence indicates that inflammatory Mϕ in MS show abnormal metabolic changes. Generally, Mϕ activated by inflammatory stimuli switch their core metabolism from oxidative phosphorylation (OXPHOS) to glycolysis (61). Recent evidence shows that inflammatory Mϕ accumulate succinate, which inhibits the function of prolyl hydroxylase enzymes during this metabolic shift, thereby inducing the transcription and secretion of IL-1β as an additional pro-inflammatory signal (61). In line with this finding, Luca et al. recently showed that inhibition of succinate release from MPs can reprogram their metabolism back to OXPHOS, resulting in an anti-inflammatory phenotype of Mϕ and ameliorated experimental autoimmune encephalomyelitis (270).

Many lines of evidence indicate that Mϕ play divergent roles in the pathogenesis of MS as they exacerbate tissue injury but also show remarkable growth-promoting and neuroprotective effects (271, 272). Obviously, this dual role of Mϕ in MS can be explained by their polarization state. In fact, both M1 and M2 subsets are present in MS lesions. The pro-inflammatory M1 response is rapidly induced and then maintained at sites of CNS injury. In comparison, the immunoregulatory M2 response is comparatively weaker and more transient (271). Thus, when inflammatory signals released by type 1 MPs are suppressed by neural stem cell-derived immunoregulatory factors, significantly ameliorated CNS inflammation can be observed (270). On the contrary, sodium chloride treatment of Mϕ induced an enhanced pro-inflammatory activity of these cells and aggravated CNS autoimmunity in EAE-diseased mice (273). In addition, IL-33 treatment induced significantly ameliorated EAE, accompanied by M2 polarization of Mϕ. Of note, adoptive transfer of IL-33-treated Mϕ attenuated EAE development, suggesting the importance of IL-33-mediated Mϕ polarization in the development of EAE (274). In consistent with this finding, Miron et al. found that immunomodulatory M2 Mϕ were essential for oligodendrocyte differentiation through activin A production (275). Notably, the dichotomy of Mϕ polarization in MS is not accurate, as the majority of Mϕ in active MS lesions show an intermediate activation status, characterized by the co-expression of both M1- and M2-specific markers (24). In addition to their polarization state, the dual role of Mϕ in MS pathogenesis can also be accounted by the origins of CNS Mϕ. In fact, resident microglia and Mo can both give rise to

Mϕ that exhibit distinct expression profiling in the CNS (276). Yamasaki et al. found the distinct functional capacities of these two Mϕ in EAE. They showed that resident macroglia were associated with debris clearance and demonstrated a signature of globally suppressed cellular metabolism during disease initiation, whereas Mo-derived Mϕ were highly phagocytic and inflammatory and actively participated in demyelination demyelination initiation (277).

Type 1 Diabetes (T1D)

There are scant data describing correlations between Mo and Mϕ counts and T1D development. In one study, the absolute count of circulating Mo was significantly increased in patients with T1D, while the number of $CD16^+$ Mo decreased in patients with diabetic complications (62). Unfortunately, this study did not analyze the correlation between Mo number and T1D development. Another study found that decreased Mo counts significantly correlated with insulin resistance in T1D, although this study lacked data on healthy controls and thus could not prove a relationship between Mo number and T1D development (278).

Two independent studies showed that Mϕ from diabetes-prone non-obese diabetic (NOD) mice showed markedly compromised phagocytosis relative to those from normal mice (63, 64). Since Mϕ engulfment of apoptotic cells is an important mechanism of self-antigen clearance, it was thus suggested that deficiencies in apoptotic cell clearance by Mϕ represent a potential factor in predisposition to T1D. In addition, Mϕ from NOD mice were shown to be abnormally activated and exhibited direct cytolytic activity toward islet β-cells (65). Accordingly, *in vivo* depletion of Mϕ by clodronate liposomes abolished diabetes effectively.

In T1D, Mϕ play a key role in triggering the adaptive immune responses. Vomund et al. showed that islet beta cells can transfer some of their secretory granules to resident Mϕ. In autoimmune diabetes, these Mϕ present the transferred antigens to autoreactive $CD4^+$ T cells, resulting in the activation of these cells and initiating the autoimmune diabetic process (279). Mϕ are also involved in the trafficking of autoreactive $CD8^+$ T cells into the islets. Marro and colleagues found that depletion of Mϕ or genetic ablation of *ifnar* on Mϕ aborted lymphocytic choriomeningitis infection-induced T1D (280). Mechanistically, disrupted type-I IFN signaling in Mϕ restricted trafficking of $CD8^+$ T cells into the islets, thus prohibiting the further development of murine T1D (280).

In T1D, the abnormal activation of Mϕ is exemplified by the pro-inflammatory M1 phenotype of these cells, which play a critical role in T1D pathogenesis. The pro-inflammatory serum milieu of T1D patients that favors M1 Mϕ polarization is exemplified by excessive amounts of C-reactive protein (66), IFN-γ (67), CXCL10 (68), and CCL2 (68). This M1 dominance of T1D Mo is reflected in the elevated IL-6- and IL-1β-secreting ability of these cells, regardless of whether they were in a resting state or after lipopolysaccharide stimulation (66, 69). It was suggested that a main function of these two cytokines is to induce the generation of Th17 cells, which is another key cell population in T1D pathogenesis (69). In addition to the aforementioned two cytokines, several lines of evidence have shown elevated levels of Mϕ-derived TNF-α in T1D patients (70, 71). However, the function of TNF-α in T1D pathogenesis seems controversial. Although TNF-α blockade therapy showed clinical efficacy in some cases, others showed disturbance of glycemic control after treatment, and one study even reported induction of T1D during anti-TNF-α therapy in a RA patient (281, 282).

While pro-inflammatory M1 Mϕ promote T1D development, adoptive transfer of immunosuppressive M2 Mϕ reduces the onset of T1D in NOD mice (283). In fact, more than 80% of NOD mice were protected against T1D for at least 3 months after a single transfer of M2 Mϕ, even if the treatment was conducted just prior to clinical onset. Moreover, *in vitro* induced M2 Mϕ can also reduce hyperglycemia, kidney injury, and insulitis in diabetic mice (284).

The pancreas contains both MDMs and resident Mϕ that exert different functional capacities. Bone marrow Mϕ have been found to prevent stem cell mobilization into peripheral blood in diabetic mice (285). In contrast, the islet resident Mϕ exhibit an activation signature with higher expression of various pro-inflammatory cytokines and mount an inflammatory immune response in NOD mice (286). Consistent with these findings, in a study conducted in C57BL/6 mice, islet Mϕ express genes and cell surface markers that categorize them as M1-like and exhibited typically pro-inflammatory characteristics. In contrast, the interacinar Mϕ expressed M2-like transcripts and exhibited anti-inflammatory and tissue-supportive functions (287). Accordingly, depletion of islet resident Mϕ through CSF-1 neutralization resulted in reduced $CD4^+$ T cell infiltration in the pancreatic islets, impaired presentation of insulin epitopes to T cells and reduced severity of autoimmune diabetes (288).

PBC

In 1994, Mathew et al. found that while Kupffer cell counts were not altered significantly in stage 1 and 2 PBC, increased Kupffer cell numbers were clearly identified in periportal and periseptal zones of stage 3 PBC and in the parenchymal areas of stage 3 and stage 4 cases (10). This finding was supported by another independent study (72). In contrast to these findings, the work of Leicester and colleagues showed that the total number of $CD68^+$ Mϕ in the liver remained constant at different stages of fibrosis and did not differ significantly from that of controls (73). This discrepancy may result from distinct disease staging strategies or different hepatic Mϕ immune-staining and quantification methods. In addition to Mϕ, several lines of evidence also showed increased Mo counts in PBC patients. Leicester et al. revealed that while few $CD14^+$ Mo could be observed in control livers, these cells were increased markedly in PBC livers, especially in patients with advanced stage of fibrosis (73). The work of Peng et al. showed that the frequencies of peripheral blood $CD14^{high}CD16^+$ and $CD14^{low}CD16^+$ subpopulations of Mo were elevated in patients with PBC (74). Intriguingly, the frequency of $CD14^{low}CD16^+$ cells was positively associated with disease progress. Consistent with these findings, increased levels of Mo chemotactic proteins were also identified in PBC livers (289). These findings are supported by data from murine models of PBC. In dominant-negative TGF-β receptor type II

transgenic mice, clusters of Mϕ are observed in the parenchyma and portal tracts of the liver (290). In another PBC mouse model, the 2-octynoic acid-conjugated bovine serum albumin immunization-induced autoimmune cholangitis, interestingly, it was found that while MDMs ($CD11b^{hi}F4/80^{int}CX3CR1^{hi}$) were enriched around the portal triads, liver resident Kupffer cells ($CD11b^{int}F4/80^{hi}CX3CR1^{neg}$) were significantly reduced (161). In this study, MDMs play a key role in the development of experimental PBC, as inhibition of their recruitment either by genetic deletion of CCR2 or by pharmacological antagonization of CCR2 resulted in ameliorated autoimmune cholangitis (161).

The dysfunction of Mϕ in PBC is reflected in several findings. In 2005, Mao et al. showed that Mo isolated from the peripheral blood of patients with PBC were more sensitive to toll-like receptor (TLR) ligation and thus produced higher levels of pro-inflammatory cytokines (75). This finding was supported by another independent study, which demonstrated that the expression of TLR4 and its negative regulator RP105 were altered on PBC Mo, making them hyperreactive to LPS and leading to increased production of various pro-inflammatory cytokines (78). In an *in vitro* co-culture model using human peripheral blood Mo and T cells, it was shown that circulating $CD14^{low}CD16^{+}$ Mo could promote Th1 cell proliferation by IL-12 production and direct contact of $CD4^{+}$ T cells (presumably through HLA-DR-, CD80-, and CD86-mediated mechanisms). In line with these findings, circulating $CD14^{low}CD16^{+}$ Mo were positively associated with Th1 cell frequency in PBC patients (74). Other molecules, such as Siglec-1, were also found to be abnormally overexpressed by PBC Mo (76). A great breakthrough in the abnormally altered functions of Mo and Mϕ in PBC may be achieved in studies illustrating their ability to recognize anti-mitochondrial antibody (AMA)-apotope complexes (77, 164). Apoptotic biliary epithelial cell-derived autoantigens might remain immunologically intact and can be recognized by circulating AMAs in apoptotic bodies (164). Of note, these AMA-apotope complexes are capable of activating Mo-derived Mϕ of the liver, thus stimulating the secretion of various pro-inflammatory cytokines from these cells. This effect leads to further biliary epithelial cell apoptosis, thus perpetuating local inflammation and eventually causing bile duct damage (77).

Many lines of evidence indicate a pro-inflammatory M1 polarization of Mϕ in PBC. These Mϕ express high levels of TLR4 and are highly sensitive to endotoxin stimulation, leading to markedly increased secretion of several pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12, and TNF-α (75, 78). Interestingly, endotoxin, which is a strong stimulator of M1 Mϕ activation, is increased in biliary epithelial cells of patients with PBC (79). In addition, levels of CD40L, which interacts with its corresponding receptor CD40 and mediates potent inflammatory signals, are significantly elevated in PBC Mϕ (72). The same study also found that this increase in CD40L expression was mainly stimulated by LPS and IFN-mediated signals.

SS

Increased levels of peripheral mature ($CD14^{low}CD16^{+}$) Mo were described in patients with SS (15), even though their direct aetiopathogenic role remains undefined. Another Mo subset, pro-inflammatory $CD14^{bright}CD16^{+}$ Mo, is also increased in the salivary glands of SS patients, accompanied by overexpression of IL-34, a cytokine that specifically stimulates the growth and differentiation of Mo (80). In addition, the salivary profile of CCL2, a potent Mo chemoattractant, is highly expressed in patients with SS (85). Until now, there has been no direct evidence concerning the association of Mϕ or Mo numbers with human SS disease activity, even though elevated expression of Mϕ-derived molecules (i.e., molecules of the chitinase family) indeed corresponded to more severe SS (291). In addition, a study analyzing saliva proteomics showed that proteins associated with Mϕ differentiation represented one of the biomarker signatures of SS (292). In mouse models, it has been shown that Mϕ are critical mediators of SS pathogenesis and have intimate crosstalks with autoreactive T cells. Using autoimmune regulator-deficient mice as an animal model of SS, Zhou et al. demonstrated that Mϕ infiltration the limbus, corneal stroma, and lacrimal glands were mediated by autoreactive $CD4^{+}$ T cells (293). Importantly, local infiltration of Mϕ correlates with ocular surface damage, and Mϕ depletion by clodronate liposomes led to significant improvements in lacrimal gland pathology (293), indicating the immunopathologic involvement of these cells in SS. In another mouse model of SS wherein NFS/sld mice are thymectomized on day 3 after birth, Ushio and colleagues found that tissue resident Mϕ of the salivary gland mediated $CD4^{+}$ T cell recruitment by effective production of CCL22 (171). Moreover, CCL22 was found to enhance IFN-γ production from T cells in these mice (171). Of note, numerous CCL22-producing Mϕ can be observed in the salivary gland tissue specimens of SS patients (171).

Functional abnormalities of SS Mϕ are exemplified by impaired phagocytosis ability of them. Mϕ isolated from an SS mouse model showed defective phagocytosis of apoptotic cells (294). This finding is in line with previous reports in SS patients, as Mo from these patients showed reduced engulfment of apoptotic epithelial cells and were unable to promote an immunosuppressant cytokine profile (81). In addition, elevated levels of MIF have been shown to be associated with hypergammaglobulinemia in patients with SS (295).

There is a paucity of data on the polarization of Mϕ in patients with SS. Although Baban et al. reported the presence of M1 and M2 Mϕ along with T and B cells in the salivary glands of SS mouse model, the balance of M1 and M2 Mϕ has not been characterized (296). However, accumulating data indicate that pro-inflammatory M1 polarization is the predominant phenotype of SS Mϕ. It has been reported that systemic and local concentrations of IL-6 are significantly increased in SS patients (82). In addition, serum IL-12 levels are associated with more active disease, while an immunosuppressant cytokine, IL-35, is associated with lower disease activity (83). It has also been shown that peripheral IFN-γ levels are increased in patients with SS (84), which is suggested to be stimulated by the synergistic functions of IL-33, IL-12, and IL-23 (297). Additionally, salivary levels of the pro-inflammatory cytokines and chemokines TNF-α, IL-1β, IL-18, CXCL8, and CXCL10 are also significantly higher in SS patients than in non-SS controls (80, 85–87). Notably, levels of pro-inflammatory cytokines or chemokines that are directly secreted by Mo and

Mϕ, i.e., IL-6, IL-18, type I IFN and BAFF, are significantly higher in SS patients (87, 172, 173). In accordance with the increased pro-inflammatory cytokine levels of SS Mo, these cells express reduced levels of NF-κB inhibitor (IκBα), indicating the abnormal activation of the NFκB signaling pathway (88). In addition, Adrienne et al. used freshly isolated peripheral blood Mo and found that SS-associated microRNAs collectively suppressed immunoregulatory TGF-β signaling as opposed to the pro-inflammatory IL-12 and NF-κB signaling pathways (170). Interestingly, in thymectomized NFS/sld mice, an animal model of SS, tissue resident Mϕ of the salivary gland contain two main subsets ($CD11b^{low}F4/80^{+}$ and $CD11b^{high}F4/80^{+}$) (171). These two subsets of Mϕ display different phenotypes and functions. For example, $CD11b^{low}F4/80^{+}$ Mϕ express higher levels of pro-inflammatory M1 markers including MHC-II, CD11c, and CD86, while $CD11b^{high}F4/80^{+}$ Mϕ express higher levels of M2 markers such as CD206 and CD204 (171). In addition, $CD11b^{high}F4/80^{+}$ Mϕ showed significantly higher phagocytic activity compared with $CD11b^{low}F4/80^{+}$ ones (171).

Celiac Disease

Numerous $CD68^{+}$ tissue Mϕ were present in duodenal biopsies from patients with celiac disease (7). Of note, these Mϕ showed strikingly impaired phagocytosis ability, as reduced expression levels of Mϕ-associated scavenger receptors, i.e., CD36, thrombospondin-1 and CD61, were identified in the duodenal mucosae of patients with the active phase of celiac disease, accompanied by the accumulation of apoptotic bodies in these areas (89). However, direct evidence for the phagocytosis ability of Mϕ is lacking. In addition, Mϕ from patients with celiac disease exhibit greater antigen-presenting ability, which is exemplified by the upregulated expression of the co-stimulatory molecules CD80, CD86, and CD40, in concert with higher CD40L expression and a more highly activated state of T cells (90, 91). However, more direct evidence is warranted to support this conclusion.

The cytokine milieu of patients with celiac disease implicates a simultaneous M1- and M2-related profiles. For one thing, significantly higher levels of M1-associated pro-inflammatory cytokines, i.e., IFN-γ, IL-1β, TNF-α, and IL-8 have been identified in celiac disease sera (22). More specifically, gliadin peptides could induce significantly higher levels of IL-8 and TNF-α production by Mo from patients with celiac disease relative to those from healthy donors. This pro-inflammatory cytokine secretion is accompanied by a more pro-inflammatory activation state of Mo expressing higher levels of M1 markers, i.e., CD80, CD86, and CD40, as well as higher activation of the NF-κB signaling (90). In addition, it was shown that gliadin fragments could induce RAW264.7 cells and mouse peritoneal Mϕ to secrete TNF-α and CCL5, and to produce increased levels of nitric oxide in the presence of IFN-γ, which is also associated with the activation of NF-κB signaling (298–300). The interaction of gliadin with Mϕ involved a myeloid differentiation factor 88 (MyD88)-dependent pro-inflammatory cascade, while this was neither TLR2- nor TLR4-dependent (176). Intriguingly, even in patients with celiac disease on a gluten-free diet whose duodenal biopsy specimens are histologically normal, intraepithelial lymphocytes and intestinal epithelial cells exhibit increased expression of TNF-α and MIF (301). This may help explain the rapidity with which the celiac mucosa responds to gliadin challenge.

Additionally, M2-associated immunosuppressive cytokines are also frequently detected in celiac disease. For example, IL-10 concentration is significantly higher in celiac disease sera (22). Importantly, serum levels of IL-10 is significantly correlated with levels of autoantibody titers (22). In addition, IL-10 polymorphisms are correlated with more severe mucosal damage and early-onset of celiac disease (302), even though IL-10 secretion abnormalities are suggested to be more a cause than a consequence of this disease (303). Using Mo from patients with celiac disease or healthy subjects, Amelia et al. found that gluten peptides induced the expression of arginase 1 and arginase 2, both of which are typical markers of M2 Mϕ (92). This finding was supported by data from the same group showing that gliadin stimulation significantly activated the arginase pathway in human Mo as well as in RAW264.7 cells (93).

IBD

In IBD, the intestinal mucosa is characterized by extensive Mϕ infiltration (8, 9). Elevated $CD68^{+}$ Mϕ count in the colonic and ileal mucosae were observed in both Crohn's disease (CD) and ulcerative colitis (UC), while a CD163-positive subset in the colon mucosa was increased only in CD but not UC patients (16). In patients with CD, the mesenteric fat tissue also exhibits considerable Mϕ infiltration (9, 304). Regarding circulating Mo, it was found that Mo with a $CD14^{+}CD16^{+}$ phenotype are increased significantly and are the main contributor to the inflammatory infiltrate in the CD mucosa, while classical Mo ($CD14^{hi}CD16^{-}$) are decreased (94, 95). A dramatic increase in peripheral $CD14^{+}CD16^{+}$ Mo was observed in patients with active CD, particularly in those with colonic involvement and a high Disease Activity Index (95). Intriguingly, a significant correlation between the percentage of $CD14^{+}CD16^{+}$ Mo and clinical activity index has been shown in both CD and UC patients, suggesting the potential involvement of this cell subset in the inflammatory drive of IBD (305). Of note, computational simulations conducted by Wendelsdorf et al. identified that Mϕ and their mechanisms of plasticity are key reasons for mucosal inflammation (188).

The expression level of aldehyde dehydrogenase (ALDH), which is necessary for the synthesis of retinoic acid, is significantly reduced in Mϕ populations of the UC colon, both in active disease and remission (8). Given that retinoic acid has important immunoregulatory properties and is critical for the generation of regulatory T cells (Tregs), local suppressive failure due to a lack of retinoic acid may be involved in driving UC. In line with this finding, Treg numbers in UC patients were lower than that of healthy controls, and Treg number was negatively associated with the clinical activity index of UC (306). In comparison, the percent change in $ALDH^{+}$ Mϕ in CD is controversial, as one study showed that this fraction is similar to that in controls, while another study identified up-regulated ALDH activity in $CD14^{+}$ Mϕ from

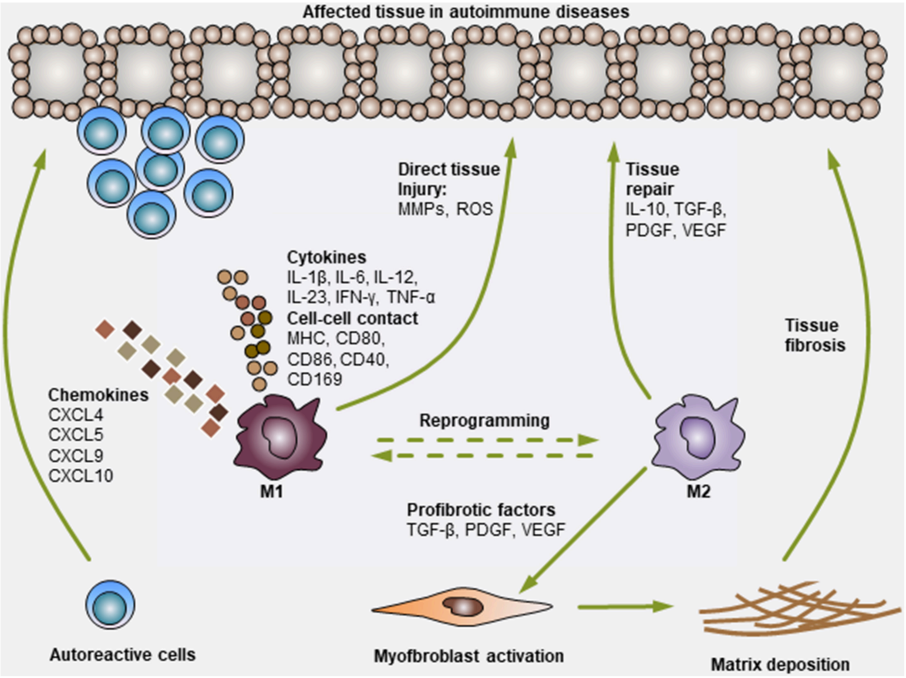

FIGURE 1 | Modulation of autoimmune diseases by Mo and Mϕ. Mo and Mϕ are key players in autoimmune diseases. During the development of autoimmune diseases, pro-inflammatory M1 Mo or Mϕ can secrete various chemokines to recruit additional immune cells (i.e., T cells, B cells, neutrophils, NK cells, and NKT cells) to the affected tissues. Then, Mo or Mϕ can activate these cells via the secretion of various pro-inflammatory cytokines (i.e., IL-1β, IL-6, IL-12, IL-23, IFN-γ, and TNF-α) or through direct cell-cell contact (antigen presentation: MHC, co-stimulation: CD80, CD86 and CD40, and adhesion molecules: CD169). In addition, Mo or Mϕ can also exert direct tissue injury functions by producing matrix metalloproteinases (MMPs) and reactive oxygen species (ROS). Consequently, the activation of Mo or Mϕ and other immune cells synergistically leads to tissue damage. On the other hand, M2 Mo or Mϕ mediate immunosuppressive or tissue-repairing effects during this process, mainly by producing cytokines (i.e., IL-10 and TGF-β) and growth factors (i.e., PDGF and VEGF). M2 Mo or Mϕ can also secrete various pro-fibrotic factors, such as TGF-β, PDGF and VEGF, to activate myofibroblasts in certain tissues, leading to extracellular matrix deposition and fibrosis generation (i.e., cases in PBC and SSc).

CD patients (8, 307). CD Mϕ also showed an abnormally accelerated breakdown of pro-inflammatory cytokines due to faster lysosomal degradation, while cytokine messenger RNA showed normal stability and levels (96). This was shown to lead to impaired neutrophil attraction, causing defective bacterial clearance and thereby boosting the formation of granulomas. However, this case differs strikingly from UC Mϕ, which showed similar or even significantly higher secretion of various cytokines relative to healthy controls in the same study. In addition, there is proof that IBD patients showed defective Mo GM-CSF receptor (CD116) expression and function, which was more prominent in UC than in CD patients, indicating a causal link between the innate immune defect in IBD patients and Mo CD116 expression (97). Intriguingly, CD116 expression in IBD patients was independent of current medications and was not influenced by disease activity.

Several studies have reported the potential interactions between colonic Mϕ and lymphocytes in IBD. Abnormally activated intestinal Mϕ in CD patients produce various cytokines (i.e., IL-1β, IL-6, IL-23, TNF-α, and TNF-like protein 1A) necessary for T cell differentiation, specifically promoting the generation of Th1 and Th17 cells (191–194). A subset of CD14 and CD209 dual positive Mϕ in the lamina propria also possess potent antigen-presenting ability and can strongly evoke the differentiation of Th1 and Th17 cells (194). In addition, these Mϕ can induce the proliferation of naive $CD4^+$ T cells (194). Similarly, in UC patients, IL-23 from $CD68^+$ Mϕ promotes the differentiation of Th17 cells, which are important contributors to the pathogenesis of UC (195–197). In addition, Mϕ-derived IL-23 can strongly promote the activation and cytolytic activities of intestinal NK cells crucially contributing to tissue pathology of UC patients (195). Data from murine model-based studies showed that adoptive transfer of M2a Mϕ to IBD mice increased Th17 and Treg generation, while M1 Mϕ contributed to the disruption of the intestinal epithelial barrier during IBD development (308, 309).

The polarization profile of IBD Mϕ is a complex issue. In CD, Mϕ are more polarized to an M2 profile, which is reflected by several findings. First, CD163 is expressed on a substantial percent of Mϕ in the colonic mucosa as well as in the peripheral blood of CD patients (16). In addition, sCD163 levels are significantly increased in CD patients (310). Upon successful treatment, serum sCD163 levels are dramatically decreased (310). Second, large numbers of Mϕ are found in fibrotic lesions of

TABLE 3 | Pathogenic functions of Mo and Mϕ in autoimmune diseases and the relevant treatment strategies.

Diseases	Pathogenic functions	Relevant strategies of disease treatment
SLE	Enhanced ability to activate autoreactive T and B cells (28, 336, 337). Higher antigen-presenting ability (29, 30). Impaired clearance of apoptotic cells and immune complexes (34, 35).	Adoptive transfer of M2 Mϕ in mouse model (216). Induction of M2 polarization in patients (217). Blockade of TNF-α (222).
SSc	Contributing to skin fibrosis (44). Mo count correlates with disease activity (42). Potentially mediate genetic susceptibility to SSc (229).	Suppression of M2 Mϕ by tocilizumab (338). Blockade of TGF-β (339).
RA	Mediation of local and systemic inflammation (56, 340). Cartilage degradation (136). Synovial Mϕ count correlates with local disease activity (241).	Blockade of TNF-α (257). Blockade of IL-1 (52). Blockade of IL-6 (54).
MS	Higher antigen-presenting ability (60). Positively associated with disease pathology (260, 264). Mediation of myelin damage through iNOS production (60). Mediation of neurotoxicity (271).	IFN-β-induced Mϕ apoptosis (264). Gc protein-derived Mϕ-activating factor treatment (341). Induction of M2 Mϕ (342).
T1D	Impaired clearance of apoptotic cells (63, 64). Mediates death of islet β-cells (65). Production of reactive oxygen species (343).	TNF-α clearance from the circulation (281). Adoptive transfer of M2 Mϕ in mouse models (283, 284). TGF-β-engineered mesenchymal stem cell treatment in mouse model (344).
PBC	Higher ability to produce pro-inflammatory cytokines (75, 78). Promoting Th1 activation (74). Apoptosis induction of biliary epithelial cells (77, 164). Frequency of $CD14^{low}CD16^{+}$ cells correlates with disease progression (74).	Induction of M2 Mϕ by MSC transplantation (345, 346). Blockade of TNF-α (347, 348). Blockade of IL-12/IL-23 (349). Blockade of CCR2/CCL2 signaling (161).
SS	Impaired clearance of apoptotic cells (81). Chitinase levels correlates with SS severity (291). Mediation of local and systemic inflammation (87, 88, 170, 172, 173). MIF concentration correlates with hypergammaglobulinemia (295).	Blockade of TNF-α (ineffective) (350, 351).
Celiac disease	Enhanced ability to activate autoreactive T cells (90, 91).	Parasitic helminth infection (352). TNF-α blockade (353, 354).
IBD	Mediation of local inflammation (94, 355). Percentage of $CD14^{+}CD16^{+}$ Mo correlates with disease activity (305). Boost the formation of granulomas in CD (96).	IL-6 blockade (356). IL-12/IL-23 blockade (357). IFN-γ blockade (358). TNF-α blockade (359). MMP9 blockade (360). Allogeneic mesenchymal stem cell transplantation (361, 362).

CD patients, consistent with the potent tissue-repairing and pro-fibrotic capacity of M2 Mϕ (311, 312). Third, defective bacterial clearance by Mϕ is frequently observed in CD patients, which is presumably due to the impaired pro-inflammatory cytokine secretion of these cells (96). Fourth, IL-13, which is a potent M2 Mϕ inducer, was dramatically upregulated in CD patients (100). In comparison, the Mϕ polarization profile seems much more complex in UC patients. The fact that $CD163^{+}$ Mϕ numbers and serum sCD163 levels are increased in UC patients, coupled with the finding that $CD206^{+}$ Mϕ are enriched in the injured mucosa of these patients, indicates an M2 polarization profile for these Mϕ (16, 101). However, the continuous excessive inflammation in the gut mucosa of UC patients, as well as the significant increase in pro-inflammatory M1 while decrease in M2 Mϕ accompanied by suppressed IL-10 production in mouse models of UC also points to the evident M1 polarization of these Mϕ (98, 99). In various mouse models of IBD, inhibition of the pro-inflammatory activities of M1 Mϕ or induction of tissue-repairing/immunomodulatory M2 Mϕ usually results in attenuated experimental IBD (185, 187, 313, 314).

CONCLUSIONS AND FUTURE PERSPECTIVES

In the present review, we mainly discussed the association of Mo/Mϕ with the development of certain autoimmune diseases. It has been quite well elucidated that Mo/Mϕ are key component of the innate immune system and are involved in both amplifying and suppressing inflammation (2). Mounting evidence suggests that these cells participate in the pathogenesis of autoimmune diseases, mainly through their remarkably pro-inflammatory or fibrogenic properties (1, 2). As discussed above, in different autoimmune diseases, the heterogeneity of Mo/Mϕ subpopulations varies dramatically, and their polarization profile usually plays a key role in disease progression (**Figure 1**). However, in many autoimmune diseases, the phenotypic and functional characteristics of Mo/Mϕ have not been classified unambiguously, as many pro-inflammatory M1-polarized Mo/Mϕ simultaneously express M2-related markers or exhibit immunomodulatory functions (19–22). In addition, in several cases, Mϕ activation is a dynamic and reversible

event in which pro-inflammatory Mϕ can be re-programmed into Mϕ with immunosuppressive or tissue-repairing cells by local microenvironment (13, 25). Thus, future investigation into explaining the seemingly opposing phenotypic and functional programs of Mo/Mϕ and identifying the dynamic changes is clearly needed.

Several possible mechanisms responsible for Mϕ phenotype in autoimmune diseases in general have been suggested by recent findings. For example, genome-wide association studies have identified several candidate genes responsible for the pathogenesis of autoimmune diseases. Among the susceptibility genes, *HLA*, which is closely with the antigen-presenting ability of Mϕ, has been suggested to be involved in the development of SLE (315), SSc (316), RA (317), MS (318), T1D (319–322), SS (323), Celiac disease (324), and IBD (325). In addition, protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), which can be expressed in Mϕ and controls Mϕ activation and polarization, has been identified as a risk gene for RA (317) and IBD (17). Interferon regulatory factor 5 (IRF5), which is mainly expressed by myeloid cells and is a key regulator of Mϕ activation and polarization, has been identified as an important predisposed factor in patients with SLE (326), SS (323), RA (327), PBC (328), and IBD (329, 330). However, functional studies investigating the actual function of these genes in Mϕ should be done to confirm whether they really play a critical role in controlling Mϕ activation in autoimmune diseases.

In recent years, mounting reports have overturned the long-held knowledge that Mϕ in the adult are merely replenished by circulating Mo from bone marrow progenitors (331–333). The new paradigm supports that some Mϕ are embryo-derived and are maintained by self-renewal independent of hematopoietic contribution (332). Intriguingly, this heterogeneity of Mϕ results in distinct phenotypes and, more importantly, totally different biologic functions (334, 335). Thus, it is necessary for future studies to elucidate the roles of tissue-resident Mϕ and bone marrow-derived Mϕ in the initiation, progression and termination of different autoimmune diseases.

Although Mo and Mϕ play a key role in the pathogenesis of certain autoimmune diseases, the development of these diseases is not solely Mo/Mϕ-dependent, and this process involves the interplay of these cells with other immune cells, i.e., autoreactive T and B cells (2). However, most studies fail to explore the interactions of Mo/Mϕ with other immune cells in the local microenvironment. Thus, future work is needed to better determine the synergistic effects and related mechanisms of the interactions between Mo/Mϕ and other immune cells in the development of autoimmune diseases.

To date, although the functions of Mo/Mϕ in several autoimmune diseases have been determined, the clinical translation of this knowledge is still challenging. Certain Mo- or Mϕ-targeted therapies have been developed (see **Table 3**), but whether they are more effective and safer than traditional treatment remains to be verified, and some of them have already proven disappointing (52, 54, 281, 282). However, this does not rule out a potential effective role for Mo/Mϕ as an attractive therapeutic strategy for autoimmune diseases. Thus, further studies are needed to elucidate a more detailed and comprehensive mechanism of Mo/Mϕ regulation in autoimmune diseases; such work, coupled with a wider understanding of the determinant factors of autoimmune diseases (i.e., sex, age, genetics, and environmental factors), which act together but differ between patients, will probably lead to the development of more specific and effective therapies in the future.

AUTHOR CONTRIBUTIONS

W-TM and D-KC designed the structure of this article. W-TM wrote the manuscript. FG and KG revised the manuscript. All authors have reviewed the final version of this article.

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Re-expression of CD14 in Response to a Combined IL-10/TLR Stimulus Defines Monocyte-Derived Cells With an Immunoregulatory Phenotype

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Interleukin 10 is a central regulator of the antigen-presenting function of myeloid cells. It exerts immunomodulatory effects *in vivo* and induces a regulatory phenotype in monocyte-derived cells *in vitro*. We analyzed phenotype and function of monocytic cells *in vitro* in relation to the cytokine milieu and the timing of TLR-based activation. In GM-CSF/IL-4 cultured human monocytic cells, we identified two, mutually exclusive cell populations arising from undifferentiated cells: $CD83^+$ fully activated dendritic cells and $CD14^+$ macrophage like cells. Re-expression of CD14 occurs primarily after a sequential trigger with a TLR signal following IL-10 preincubation. This cell population with re-expressed CD14 greatly differs in phenotype and function from the $CD83^+$ cells. Detailed analysis of individual subpopulations reveals that exogenous IL-10 is critical for inducing the shift toward the CD14+ population, but does not affect individual changes in marker expression or cell function in most cases. Thus, plasticity of CD14 expression, defining a subset of immunoregulatory cells, is highly relevant for the composition of cellular products (such as DC vaccines) as it affects the function of the total product.

Keywords: regulatory dendritic cells, MDSC, monocyte-derived DC, IL-10, macrophages

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INTRODUCTION

Cells of myeloid origin acquire immunostimulatory and immunoregulatory functions depending on the respective milieu. Differentiated type 1 cells, such as type 1 macrophages and dendritic cells, are essential to mount an inflammatory, and antigen-specific response (1). Alternatively activated macrophages, myeloid-derived suppressor cells (MDSCs), and regulatory dendritic cells (DCs) exert multiple immunoinhibitory functions (2–4). In human disease, these cells effectively link innate, and adaptive immunity: e.g., immunosuppressive tumor-associated macrophages can be found in various tumor-entities (5), and MDSCs circulate the blood of cancer patients (4). In contrast, alloreactivity in acute GvHD may be partially based on the dysbalance of the myeloid cell compartment after stem cell transplantation (6, 7).

Monocyte-derived cells, generated *in vitro*, share many of the characteristics of naturally occurring myeloid cell types. Once activated, monocyte-derived dendritic cells are capable of mounting a primary T-cell response, making them central to many tumor vaccination efforts. Alternative culture protocols lead to a regulatory functional profile, providing a cellular tool to address auto- and alloreactivity. As monocytes are readily available, these approaches are being evaluated in clinical trials (8).

Interleukin-10 (IL-10) is a master regulator for generating immunomodulatory cells. Depending on the culture conditions and the timing of IL-10 contact, monocyte-derived cells acquire different phenotypical and functional properties. Nomenclature is ambiguous, making it difficult to draw a general picture. Monocyte-derived macrophages are usually generated by culture with M-CSF and IL-10, whereas GM-CSF and IL-4 is thought to promote a type 1 macrophage/dendritic cell phenotype (9). Within protocols using GM-CSF/IL-4-cultured monocytic cells, the timing of the first contact with IL-10 appears to be crucial: when added directly to $CD14^+$ monocytes, differentiation toward a full dendritic phenotype is thwarted. The cells are described as expressing less costimulatory molecules and less HLA-DR and maintain CD14 (10). Functionally, reduced T-cell stimulatory capacity is documented. Such monocyte-derived cells differentiated with GM-CSF, IL-13, and IL-10, have been simply classified as "macrophages" by Allavena et al. (11). Recently, using a similar approach with GM-CSF, IL-4, and IL-10 (from the start of culture) Heine et al. describe the resulting cells as $CD14^+$HLA-DR^{low} "MDSC-like" cells (12). MDSC have been initially described in the murine system, whereas MDSC in humans still lack definitive classification (4). However, some subsets such as such as LIN^-HLA-$DR^{-/low}$, $CD14^+$HLA-$DR^{-/low}$, and $CD15^+$HLA-$DR^{-/low}$ have been defined (13). $CD14^+$HLA-DR^{low} MDSC have been identified in patients with various cancer types and are associated with a less favorable prognosis (4, 14).

In somewhat parallel investigations, it was noted, that immature dendritic cells, developing under the influence of GM-CSF/IL-4, may be directed toward a regulatory phenotypic and functional profile, once they are in contact with IL-10 (15). As before, it was noted that costimulatory molecules are downregulated, while expression of inhibitory molecules such as ILT4 (16), and PD-L1 increases (17). Again, a robust immunoinhibitory capacity has been noted, as T-cell tolerance is induced. Cells generated with this type of protocol were termed "regulatory dendritic cells," as a fraction, but by far not all, of the cells will express the DC-marker CD83.

In this study, we distinguish between different cell populations arising from standard culture conditions of human GM-CSF/IL-4 cultured monocytic cells in response to IL-10 and an activating trigger. The monocytic cells were primed with IL-10 shortly before triggering them via a TLR. Surprisingly two mutually exclusive populations with distinct phenotypic profiles can be distinguished: $CD14^+$ cells matching in many aspects the phenotypical and functional aspects of MDSC/DC_{reg} and $CD83^+$ cells, displaying markers of type 1 DC. This $CD14^+$ cell population arises from non-differentiated cells, that had already downregulated CD14 as a consequence of GM-CSF/IL-4 culture and then re-express CD14. A fraction of these $CD14^+CD83^-$ cells can routinely be detected following certain TLR-triggers (such as R848 or LPS) even without exogenously added IL-10, but a binary signal from IL-10 and a TLR-trigger is required for maximal differentiation toward this cell type.

Using CD14 as the defining positive marker, we show that rather than a direct effect of IL-10 on individual markers or a specific function, IL-10 shifts a whole cell population toward this altered $CD14^+$ phenotype, while, contrary to the paradigm, many individual markers within this population remain unaffected from the exogenous IL-10.

MATERIALS AND METHODS

Terminology

Classification and terminology of dendritic cell and macrophage subsets remain a matter of intense discussions. Historically, monocyte-derived DCs were described either as "immature," when treated with GM-CSF and IL-4 only, or "mature" when an activating stimulus had been provided (17). Monocyte-derived cells treated with modifying molecules such as IL-10, rapamycin or corticosteroids have been termed "regulatory DCs." A unified nomenclature based on ontogeny has been proposed, which only refers to these cells as "monocyte-derived" (18). For the clearest terms possible, we will refer to the cells evaluated in this work as follows: all cell populations used in this work are human, monocyte-derived cells (moC). Generally, the starting population are cells cultured in GM-CSF/IL-4 containing medium (formerly 'immature DC'), which we refer to as "$_{GM/IL4}$moC." Any further treatment (e.g., with IL-10 or R848) replaces the GM/IL-4 indicator (e.g., $_{IL10/R848}$moC) implying that this treatment was added on top of the GM/IL-4 culture. If various activating conditions are summarized, "act" is put instead of the specific stimulus (e.g., $_{IL10/act}$moC). Once cells are stimulated, we refer to them as "activated" rather than "mature." Morphological distinctions based on CD14 and CD83 expression are added, when these subgroups are evaluated separately (e.g., $_{IL10/R848}moC^{CD14+}$). Functional differences such as a more regulatory or inflammatory profile, are discussed as functions in the paper but are not part of the terminology.

Cell Culture

Peripheral blood mononuclear cells were obtained and cryopreserved from healthy donors, who had been eligible to donate blood in the local blood bank, by washing out leucocyte depletion filter chambers that collect leucocytes as a by-product to platelet collection. Experiments performed with such leucocytes, following pseudonymization of the donor, do not require informed consent according to a decision of our IRB. For the generation of moC, standard procedure was to allow cells to adhere to 6-well plastic dishes for 2 h and subsequently remove the non-adherent fraction by washing. Cells were then cultured in DC Medium (Cellgenix, Freiburg, Germany), supplemented with 1% human serum (Biochrom, Berlin, Germany) and 800 U/ml GM-CSF (Gentaur, Aachen, Germany) and 100 U/ml IL-4 (Peprotech, Hamburg, Germany). Forty eight hours after initiation of the culture, fresh medium was added, including GM-CSF and IL-4. For $_{IL10/R848}$moC, IL-10 (40 ng/ml; Peprotech, Hamburg, Germany) was added at least 1 h before adding the activation stimulus. As activation stimuli, the following reagents were used: R848 (2 μg/ml; Invivogen, San Diego, CA, USA); LPS(*E. coli*) (30 ng/ml; Sigma, St. Louis, Missouri, USA); MPLA-SM (1 μg/ml, Invivogen, France); Poly(I:C) (HMW, 10 μg/ml, Invivogen, France). Additional cytokines used in the

assays were: TNFα (10 ng/ml; Peprotech, Hamburg, Germany), IL-1ß (10 ng/ml, Cellgenix, Freiburg, Germany). Cells were evaluated 16–48 h after activation, depending on the individual question of the assay. Functional grade anti-IL10-antibody and anti-IL10R-antibody was purchased from eBioscience.

Flow Cytometry

Analysis of cell cultures was performed on a FACS Canto II flow cytometer (BD) using 3 lasers. Staining protocols followed standardized procedures at optimized antibody concentrations. The antibodies against the following antigens were used: CD14 (PE; MϕP9; BD Biosciences), CD36 (PerCpCy5.5; eBioNL07; eBioscience), CD80 (PerCpCy5.5; 2D10; Biolegend) CD83 (Brilliant Violet 421TM; HB15e; Biolegend) CD85d (APC; 42D1; eBioscience), CD85k (APC; ZM4.1; eBioscience) CD86 (PerCPCy5.5; IT2.2; Biolegend), CD91 (APC; A2MR-a2; eBioscience), CD163 (FITC; GHI/61; Biolegend), CD206 (FITC; 15-2; Biolegend), CD273 (APC; MIH18; Biolegend) CD274 (FITC; MIH1; BD Biosciences), CD279 (FITC; MIH4; eBioscience), CX3CR1 (PerCpCy5.5; 2A9-1; Biolegend) Viability Dye (eFluor 780; eBioscience).

Endocytosis Assay

Experimental groups were seeded in 96 well-plates using DC medium without serum or cytokines. APC-Dextran (MW: 10,000; 200 µg/ml; Invitrogen) was added at t0. At defined time points (0, 20, 40, 60, 90 min), cells were harvested and immediately washed using cold PBS and placed on ice until FACS analysis.

ELISA

For IL-6 ELISA, supernatant from the differentially activated groups (3×10^6 cells/group) was frozen and later analyzed. ELISAs were performed using kits from ThermoFisher, following the manufacturer's protocol.

T-Cell-Assays

Priming of naïve T-cells was performed following the protocol published previously in detail (19, 20). Briefly, $CD45RO^{-}CD57^{-}CD8^{+}$ T-cells were stimulated at a 10:1 ratio with moC, pulsed with the HLA-A2-restricted, heteroclitic peptide Melan-$A_{(26-35(A27L)}$, immunograde (ELAGIGILTV; jpt, Berlin, Germany). Cells were grown in Cellgenix GMP DC Medium (Cellgenix, Freiburg, Germany). IL-21 (Peprotech, Hamburg, Germany) was added at the start of culture. IL-7 and IL-15 (both Peprotech, Hamburg, Germany) was added on day 3 of culture and refreshed every 2–3 days. Cells were analyzed on day 10 of culture, taking cell counts and performing MHC-multimer-staining (Immudex, Copenhagen, Denmark).

Statistics

Statistical analysis was performed using GraphPad Prism. Error bars always indicate standard deviation. *T*-test or two-way ANOVA was chosen as appropriate and analysis was done taking paired observations into account and correcting for multiple comparisons (Tukey).

RESULTS

CD14 Is Re-expressed on moC Following an IL10/R848 Trigger

For all experiments shown here, human monocyte-derived dendritic cells were generated by selecting the plastic adherent fraction of the cells as described in the methods. The resulting adherent cell population predominantly expresses CD14, which is gradually lost throughout the culture in GM-CSF and IL-4 containing medium. Sometimes retained CD14 expression is reported (8, 10) and is attributed to incomplete differentiation due to culture conditions. Therefore, we initially thought of CD14 as a marker that is gradually lost when monocytes differentiate toward dendritic cells and we expected to see a differentiation stop once IL10 is added to the culture. Indeed, when IL-10 alone was added on day 3 for 24 h, we noticed a higher fraction of $CD14^{+}$ cells, as was already outlined in earlier work (15). However, we also noticed a fraction of the moC expressing even higher levels of CD14, once they had been stimulated with the TLR7/8 agonist R848 and this fraction significantly increased when the cells were pre-incubated with IL-10 followed by R848, an example of which is shown in **Figure 1A**. CD14 expression was mutually exclusive to CD83 expression, as a marker for fully activated DC. To assess whether these differences were truly dependent of the culture conditions, or whether factors inherent to different donors contributed to the results, we repeated this experiment with cell preparations from 19 different donors. Experiments were performed by 3 different researchers. As shown in **Figure 1B**, the range of CD14 expression for each individual donor is high in each of the experimental groups. Specifically $_{GM/IL4}$moC, without any additional manipulation, showed a mean CD14 expression of 4.6% with a standard deviation of 5.5. One explanation may be, as discussed later, that donor-inherent factors (e.g., current *in vivo* cytokine milieu at the time of donation) may influence cell differentiation *in vitro*. Despite this rather large inter-donor variation, the effect of IL-10 on upregulation of CD14, especially when combined with R848 activation, was highly statistically significant ($p < 0.0001$, Two-way ANOVA; **Figure 1B**, right panel). Re-expression of CD14 was dose-dependent, with most robust effects starting in the range of 4–40 ng/ml of IL-10 (**Figure 1C**). These $CD14^{+}$ cells emerge from the $CD14^{-}$ population, as during culture in GM-CSF/IL-4 CD14-expression is rapidly lost (**Figure 1D**, left). Even if residual $CD14^{+}$ cells are depleted, using CD14-microbeads prior to IL-10 exposure (day 3), re-expression of CD14 occurs within 24 h after incubation with IL-10 and R848 (**Figure 1D**, right). Nevertheless, one might argue that 4-day cultured cells are still too undifferentiated and the observed results may be partially affected by incomplete downregulation. We, therefore, prolonged cell culture with GM-CSF and IL-4 for 7 days, and then reevaluated CD14 expression in relation to IL-10 and/or R848. Seven-day-cultured $_{GM/IL4}$moC expressed even less CD14 and adding either IL-10 or R848 alone only resulted in a slight increase in $CD14^{+}$ cells. Combining IL-10 and R848, we observed a similar increase in $CD14^{+}$ cells after a 7-day culture period (**Figure 1E**) to what we had observed in multiple donors in 4-day cultured cells (**Figure 1B**). Likewise, CD83 upregulation

occurred independently of the culture time (4 vs. 7d) but was hindered by IL-10, as has been described in many papers. Of note, excess amounts of GM-CSF or IL-4 (10-fold) had no effect; specifically, it did not counteract the observed upregulation of CD14 (three experiments, data not shown).

As we observed a small percentage of $CD14^+$ cells following activation with R848 only, we suspected that this fraction responded to endogenous IL-10 produced upon TLR-triggering. Experimentally this was confirmed by blocking IL-10 signaling using anti-IL-10- and anti-IL-10R-antibodies. Original plots of one representative experiment, as well as the summary of all 7 experiments are shown in **Figure 1F**. Even with the rather big variation of the $CD14^+$ fraction following R848 activation, the results suggest a significant effect of IL-10 blockade in conditions were no exogenous IL10 was added (left panels). As controls, we also show the experiments with exogenous IL10 added, and then blocked, which was highly statistically significant. We conclude that endogenous IL10, produced during stimulation with R848, contributes to upregulation of CD14 in a fraction of these cells.

CD14 Re-expression Depends on the Activating Signal and the Pre-existing Cytokine Milieu

We next asked whether re-expression of CD14 depends on the stimulus used to activate the cells. Besides R848, triggering through TLR7/8, we also tested LPS(*E. coli*), triggering predominantly via TLR4, monophosphoryl lipid A (MPLA), a less toxic derivative of LPS, used as an adjuvant in vaccines, Poly(I:C), a TLR3 stimulus as well as a maturation cocktail based on IL-1ß, TNFα, and PgE2. The intrinsic capacity of these stimuli, to induce CD14 expression without exogenous IL-10, varied considerably, with LPS inducing a significant fraction of $CD14^+$ cells, whereas cytokine activated cells showing the most significant $CD83^+$ fraction and only a few $CD14^+$ cells. Poly (I:C) alone also did not increase $CD14^+$ cell numbers, but expression of CD83 was poor as well. However, once non-committed cells had been pre-incubated with IL-10, a robust increase in $CD14^+$ cells was observed regardless of the activation stimulus used. Quantitatively R848 and LPS still had the most significant impact on the $CD14^+$ fraction, but re-expression was also observed with Poly (I:C) or cytokines (**Figures 2A**,**B**). Next, we wanted to know, whether non-committed cells could be sensitized for full activation when placing them in a more pro-inflammatory environment early on. $_{GM/IL4}$moC were exposed to titrated amounts of TNFα after 48 h of culture. One group was followed by IL10 incubation 24 h later, whereas the other received no exogenous IL10. Subsequently all cells were stimulated with R848 1 h later. Twenty four hours later, cells were analyzed by FACS. As seen in original plots of one examplary experiment [**Figure 2C** (upper panel)], TNFα greatly reduced CD14 expression in cells activated with R848 only. When exogenous IL-10 was added, preincubation with as little as 0.1 ng/ml TNFα still reduced CD14 expression significantly, whereas the increase in CD83-expression could not be fully restored (**Figure 2C**, bottom panel). When combining data from 5 experiments with different donors, 0.1 ng/ml TNFα was sufficient to significantly inhibit CD14 upregulation (**Figure 2D**).

IL-10 Boosts the $CD14^+$ Subgroup With a Distinct Phenotypic Profile

In all experiments so far, CD14 expression and CD83 expression was mutually exclusive, suggesting that CD14 is a reliable marker for an alternative activation pathway of $_{GM/IL4}$moC. By gating on these two populations, we were able to compare fully activated moC^{CD83+} to the alternatively activated moC^{CD14+}. A third group, which is $CD83^-/CD14^-$ was not taken into account for this analysis. What became evident immediately, is that moC^{CD14+} exhibit many of the phenotypical features formerly attributed to "maturation-resistant DCs," "tolerogenic DCs" or $CD14^+$MDSCs. Just like these cell populations, moC^{CD14+} displayed lower levels of costimulatory molecules such as CD80 and CD86. But the important finding here is, that within each subgroup of cells, IL-10 had little direct effect on CD80, or CD86 expression (**Figure 3**). This seemingly contradicts previously published data, as downregulation of costimulatory molecules is often attributed to IL-10 (2, 17, 21, 22). **Figure 3A** depicts an example of how phenotypes might be analyzed when looking at total cells vs. $CD14^+$ and $CD83^+$ subgroups. To better evaluate IL10 dose dependency on specific markers, **Figure 3B** shows titration curves (mean of 3 different experiment and donors), depicting patterns where IL-10 affects expression of a particular marker in all groups (e.g., CD80), predominantly in one group (e.g., CD163, CD273) or where the effects are only seen on the total (mixed) population (e.g., CD86, ILT4), suggesting a quantitative shift in the population rather than a direct effect on expression.

This was statistically analyzed for different treatment groups (at a fixed IL10 dose) (**Figure 3C**). As has been noted by many groups, the difference between CD86 expression in IL10-treated, activated cells vs. activated cells without IL10 treatment was highly statistically significant when analyzing total cells. However, no difference in the expression level can be observed when looking at CD14 and CD83 subgroups separately. Thus, this difference is explained by the generally lower CD86 expression in moC^{CD14+} and the percentage-wise increase of this cell fraction upon IL-10 preincubation. A very similar pattern was observed for HLA-DR as a marker for MHC class II expression (**Figure 4**). CD273 (PD-L2) was expressed at a higher level in moC^{CD83+} (**Figure 3**), whereas no difference was observed for CD274 (PD-L1) (not shown). ILT4 was expressed at a much higher level on moC^{CD14+}. In that case, analysis of total cells would suggest a direct role of IL10 to induce higher levels of ILT4, as has been described previously (16). However, again, the difference is mainly explained by the striking difference between $moC^{CD14+ILT4+}$ cells vs. the $moC^{CD83+ILT4low}$ cells. Broadening the spectrum of phenotypic markers, we also included markers described to characterize macrophage differentiation. The scavenger receptor CD163 was exclusively expressed on moC^{CD14+}. Exogenous IL-10 enhanced its expression, confirming an IL10-dependent dose-dependency for this receptor (23). CD206(mannose-receptor)

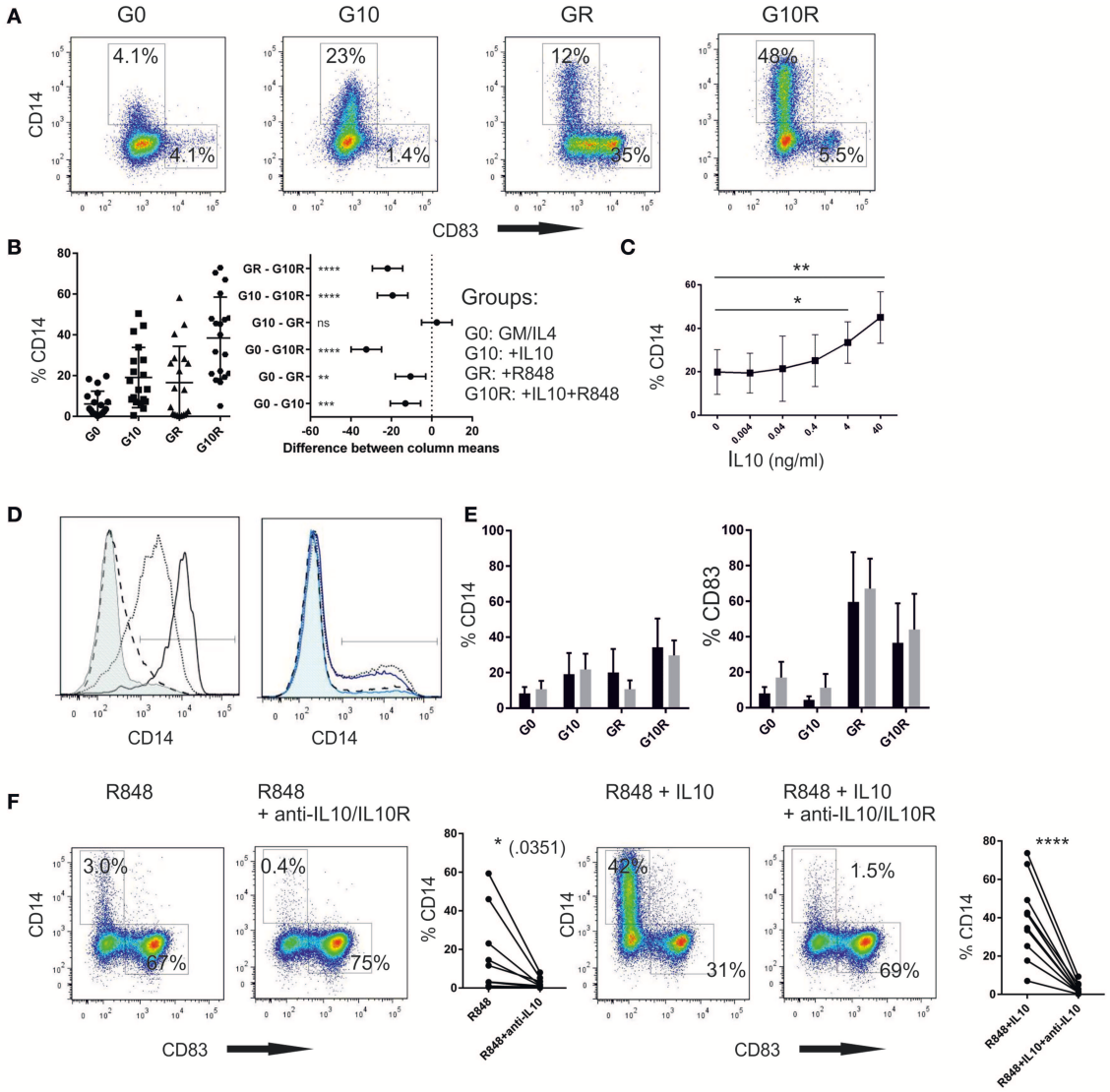

FIGURE 1 | IL-10 in combination with R848 induces re-expression of CD14 in GM-CSF/IL4-cultured monocytic cells **(A)**. Individual plots of cells on d5 of culture after 24 h-incubation R848 (2 μg/ml) without and with IL-10 (40 ng/ml) pre-incubation (1 h), or the combination **(B)**. Summary of 19 different experiments from different healthy donors. (Two-way ANOVA for multiple comparisons; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$) **(C)**. IL-10 dose dependent increase of the percentage of $CD14^{+}$ cells in combination with a fixed dose of R848 (2 μg/ml) **(D)**. Left: Downregulation of CD14 on monocytes during culture in GM-CSF/IL-4 (before experimental treatment): $\%CD14^{+}$: black solid: d1 (94%); dotted: d2 (71%); dashed: d3 (12%); thin solid, tinted: d5 (without activation) (8.6%) (one of 3 experiments). Right: Upregulation of CD14 on day 5 of culture in cells, after treatment on day 4: dotted: IL-10/R848 (33%); solid blue: IL-10/R848 treated, after CD14 depletion on d4 (27%); dashed: R848 only (15%), light blue,tinted: R848(only) after CD14-depletion on d4 (10%) **(E)**. Comparison of $\%CD14^{+}$ cells (left) and $\%CD83^{+}$ cells (right) after the respective treatment following a 4 day (black) culture or a 7 day (gray) culture in GM/IL-4 ($n = 3$) **(F)**. Effect of IL-10 blockade on CD14 re-expression. Functional grade anti-IL10-antibody and anti-IL10R-antibody were added prior to preincubation with IL-10 or prior to R848 addition. CD14 and CD83 expression were measured 16 h later. Examplary plots and a summary from 7 different donors are shown.

remains expressed in $_{IL10/R848}moC^{CD14+}$ as is expression of CD282 (TLR2) (**Figure 4**). CX3CR1, another macrophage-related marker, also was detected exclusively on moC^{CD14+} but was not influenced by IL-10 directly (not shown).

In summary, the phenotypic analysis showed that IL-10 pre-incubation before R848 stimulation gives rise to a macrophage-like, $CD14^{+}$ cell population with increased CD163, CD206, CD282, CX3CR1, and ILT4 expression, and a different level of costimulatory molecules. Contrasting previous interpretations, significant direct effects on marker expression caused by exogenous IL-10, were only seen for CD163, whereas the majority of the effects stems from the shift toward the $CD14^{+}$ population supported by IL-10.

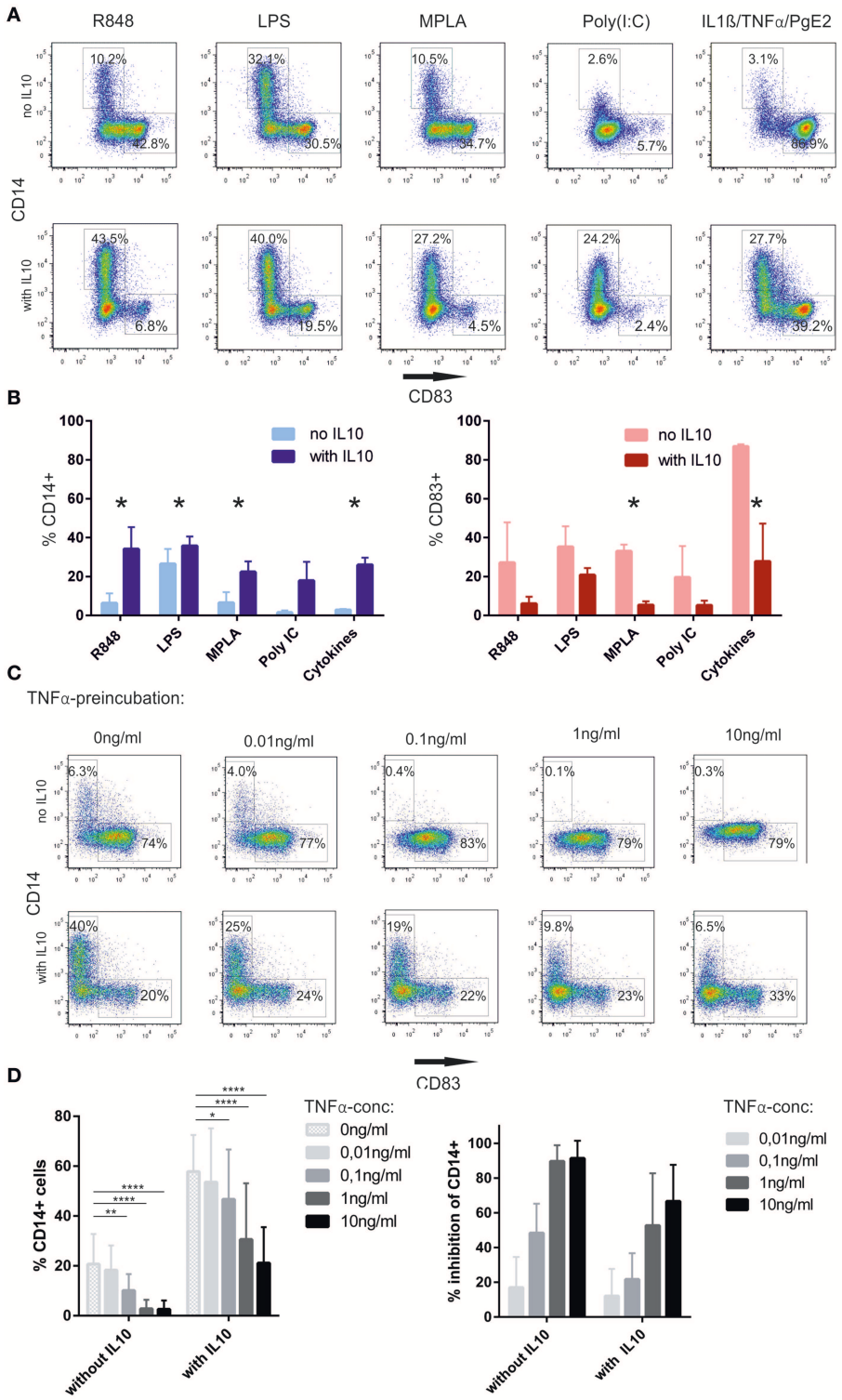

FIGURE 2 | CD14 upregulation depends on IL-10 and the maturation stimulus **(A)**. Monocytes, cultured in GM-CSF/IL-4, were either preincubated with IL-10 or not, and subsequently stimulated for 16–18 h with the indicated substances. Cells were evaluated for CD14 and CD83 expression the following day **(B)**. Summary from $n = 3$ experiments **(C)**. TNFα-preincubation for 24 h prior to adding IL10 hinders CD14 upregulation. Upper row: no IL10 addition, lower row with IL-10 **(D)**. Summary of 5 independent experiments, showing the absolute % of CD14 depending on TNFα-preincubation without or with exogenous IL-10 (left, Two-way-ANOVA for multiple comparisons, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$). The right panel shows the relative inhibition of CD14 expression by TNFα, taking the %CD14 without TNFα of each individual experiment as the reference point (100%). % Inhibition is calculated as: [1-(%CD14(sample)/%CD14(ref.point)] × 100. As these are calculated values from the original data shown in the left panel, no statistical test is shown in this panel.

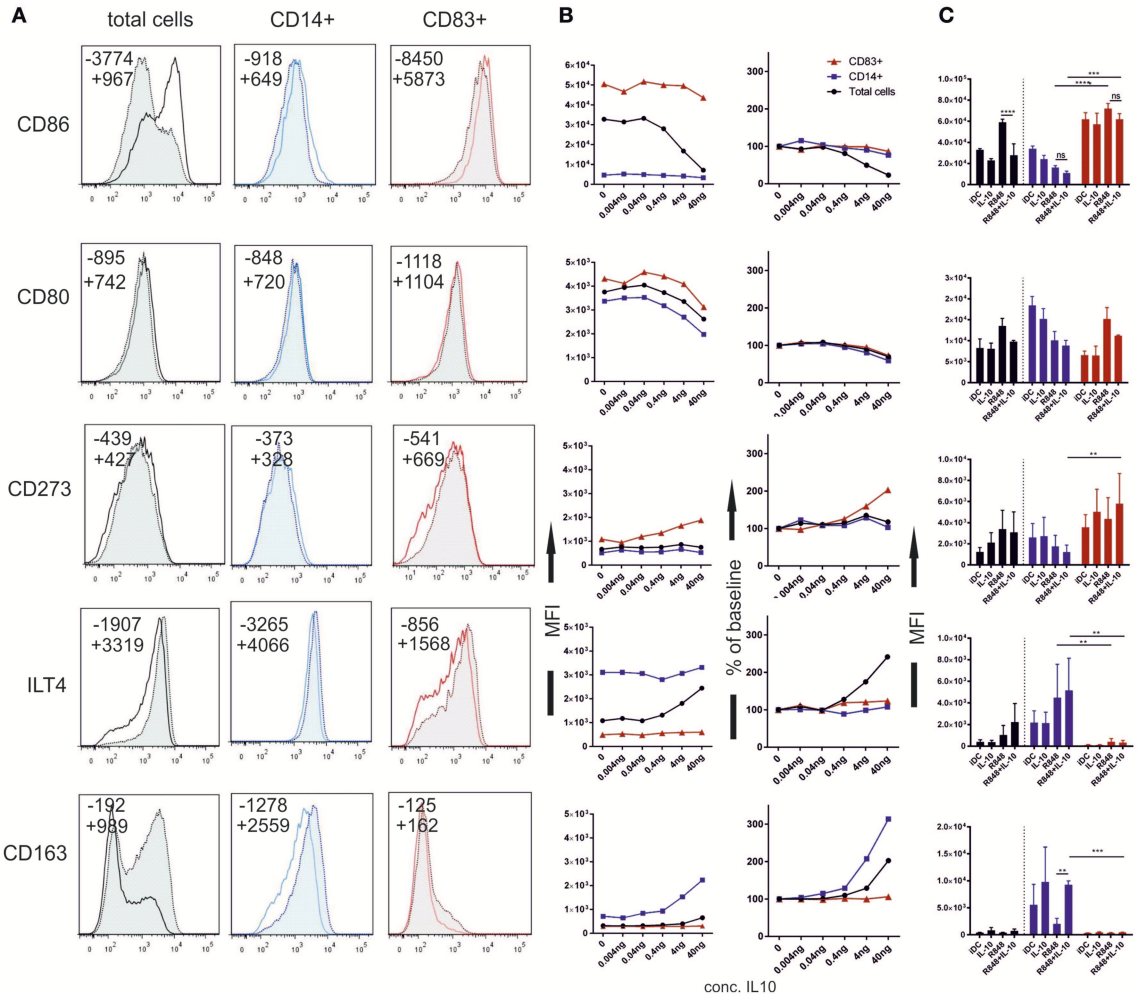

FIGURE 3 | Phenotypic changes of moC depending on IL-10 pre-treatment. moC were pre-treated with IL-10 (40 ng/ml) or not. Cells were then activated using R848 and stained 16 h later. **(A)** Representative histograms for individual markers. Solid lines represent cell populations from the control group without IL-10. Dotted line/tinted filling represent cell populations from the IL-10 treated group. The first panel represents analysis of total cells according to the live scatter gate. The middle panel shows histograms from cells within the $CD14^+$ gate. The right panels show histograms from the $CD83^+$ population. Numbers in each plot indicate the Median fluorescence intensity; −indicates the control group without IL-10, + indicates the IL-10 group. **(B)** Median fluorescence of individual markers (indicated on the left of the figure), in relation to the IL-10 concentration. Black circles indicate analysis of total cells, red triangles indicate $CD83^+$ cells and blue squares indicate $CD14^+$ cells. The left panels show mean absolute values from 3 independent experiments. The right panels show the change from the respective baseline (0 ng/ml) in percent. **(C)** Mean with SD from 5 independent experiments using 40 ng/ml IL-10. Black columns (left) represent total cells, blue columns (middle) represent the $CD14^+$ population, red columns (right) represent CD83+ cells. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Other Non-IL-10 Based Approaches for Regulatory DC Induce a Different Phenotype in moC

These phenotypic changes in response to exogenous IL-10, have been typically described for regulatory DC, but cells are often evaluated as one population, not taking differential CD14 expression into account. Using IL-10 to generate such "regulatory DC" is a crucial concept for the use of such immunomodulatory cells clinically (24). In light of the re-expression of CD14 in cultures treated with IL-10, we wanted to assess alternative protocols to generate regulatory DC. We tested two different protocols: pre-incubation either with rapamycin (25) or corticosteroids (dexamethasone) (26–28), each time followed by R848-activation (**Figure 4**). Rapamycin preceding R848 did not induce any CD14 re-expression, whereas as small $CD14^+$ population was observed following dexamethasone pre-incubation and subsequent activation with R848. Of note, when adding IL-10 on top of either dexamethasone or rapamycin, diverging populations were observed: dexamethasone had additive and similar effects on the phenotype of the $CD14^+$ cells, thus enhancing expression of CD14 itself, but also CD163, CD282, CD206, and ILT4. In contrast rapamycin suppressed CD14 re-expression to some extent and blocked IL-10 mediated CD163 expression. Thus, phenotypical differences in the type of

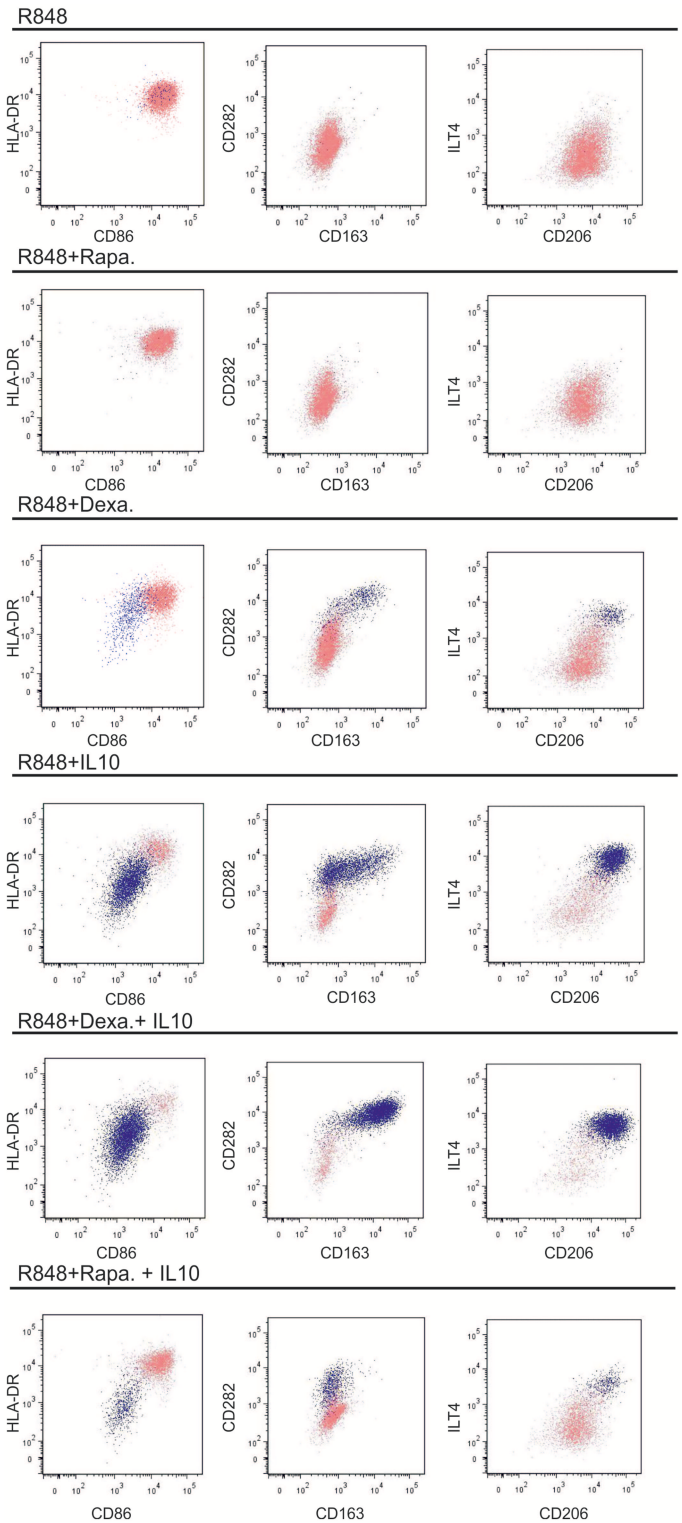

FIGURE 4 | Phenotypic changes in response to other protocols used for generating regulatory DCs. moC were incubated either with IL10 (40 ng/ml), rapamycin (100 ng/ml) or dexamethasone (100 nM) (for 16 h) or left alone. All groups were then activated with R848 (and a second addition of the modulating substance) and stained 24 h later. The light pink population represents $CD83^{+}$ cells, the dark blue population represents $CD14^{+}$ cells. $CD14^{-}CD83^{-}$ non-committed cells were excluded in this analysis. Examplary plots of three experiments are shown.

regulatory cells obtained by the various protocols, are divers, with IL-10 dominating the differentiation toward macrophages.

IL-10 Affects Function by Shaping Regulatory Subgroups Rather Than Affecting Fully Differentiated Cells Individually

Functionally $_{IL10/act}$moC resemble so-called "regulatory dendritic cells." Production of inflammatory cytokines ceases, and cells have been shown to inhibit an allogeneic mixed leucocyte reaction and induce T-cell tolerance (15). As these regulatory characteristics have already been described extensively, we wanted to explore functional characteristics with a focus on CD14 expression.

We first re-evaluated production of IL-12 as the critical inflammatory cytokine to drive TH1-responses. It is known, that after IL-10 pre-incubation, IL-12 production is hindered. IL-10 indeed reduces IL-12 production once cells are stimulated, but this inhibition depends on the stimulus used (**Figure 5A**). Technically, it was not possible, to analyze $_{IL10/R848}$moC^{CD14+} and $_{IL10/R848}$moC^{CD83+} separately, because CD14 upregulation is blocked by the addition of brefeldin A, which is required for the intracellular cytokine staining. However, one likely interpretation is that the shift toward CD14$^+$ cells, which do not majorly contribute to IL-12 production (29), explains reduced pro-inflammatory activity. Similarly, total IL-6 production was reduced in the IL-10-pretreated group as assessed by ELISA (**Figure 5B**). It is well-described that IL-10 treated $_{GM/IL4}$moC start to produce IL-10 endogenously; thus these particular experiments were not repeated.

We next asked how endocytosis, a hallmark of macrophage function, is affected within the different subgroups. Early work by Allavena et al. already showed how the net amount of Dextran-uptake by endocytosis, is increased following IL-10 treatment (11). Subgroup analysis based on CD14 and CD83 expression now allows a refined interpretation: in $_{GM/IL4}$moC cells endocytosis is highest and it is unaffected by exogenous IL-10 (**Figure 5C**, left panel). In comparison, focusing solely on R848-induced CD14$^+$ cells, uptake was lower. Using the MFI of unactivated $_{GM/IL4}$moC after 1 h of Dextran-uptake as the internal reference for the individual experiments, we analyzed how Dextran-uptake varies in R848-activated moC and the influence of IL10: **Figure 5C**, right panel, first shows pooled data of 7 independent experiments, comparing Dextran uptake within the total cell population. In this analysis, IL10 treatment resulted in a highly significant increase in Dextran uptake, when using the 1 h time point as point of comparison. This could be interpreted as a direct effect of IL-10 on the capacity to do endocytosis, as has been mentioned in previous reports (11, 30). However, **Figure 5C** also shows that there is no difference between IL10-treated vs. untreated groups, once CD14+ and CD83+ subgroups are analyzed separately. This means, that there is no dose-dependent effect of exogenous IL-10 on the cells, once the cells have switched to a macrophage-like cell type. This switch is the key event that defines function and phenotype and this step is supported by exogenous IL-10.

Given the heterogenous populations arising from monocytes, we were interested, whether we could detect interactions between these cell populations. Specifically, we asked how $_{IL10/R848}$moC^{CD14+} affect autologous $_{GM/IL4}$moC in the absence of exogenous IL-10. For this experiment, two "effector" populations were generated either by using LPS/IFNγ as a full type 1- stimulus or IL-10 followed by R848 to induce a CD14$^+$ population. Twenty four hour after treatment, these two cell preparations were washed and stained with a membrane dye. Subsequently, cells were added at a 1:1 ratio to autologous $_{GM/IL4}$moC^{dye-} for another 24h. Then R848 was added to the groups to induce differentiation in $_{GM/IL4}$moC^{dye-} followed by FACS analysis 24 h later. Based on the staining with the cell tracker, the "effector" population was separated from the $_{GM/IL4}$moC^{dye-} population. In the exemplary experiment shown in **Figure 5D**, R848 alone induced 13% of CD14$^+$ cells in the presence of $_{LPS/IFN}$moC$^{dye+D14-}$ (**Figure 5D**). In contrast, when $_{IL10/R848}$moC^{dye+} were used as "effectors," the fraction of CD14$^+$ cells within the moC^{dye-} population more than doubled. Similar results were observed when evaluating CD163 expression in the same context. Due to the complexity of this experimental setup, using different preparations of primary cells analyzing sequential events, the overall variation within the three experiments performed is too high, to demonstrate statistical significance. However, the experiment shown in **Figure 5D** is representative of the effects observed. We conclude that even in conditions, where no exogenous IL-10 is present, activated, CD14-polarized cells are capable of affecting unpolarized bystander cells within a culture period of 48 h.

Antigen-Specific T-Cells Are Affected During Priming and Expansion by $_{IL10/R848}$moC^{D14+}

Next, we wanted to assess the role of $_{IL10/R848}$moC^{CD14+} in the context of antigen-specific T-cell activation. There is ample evidence, how regulatory DC affect-cell activation in the context of mixed leucocyte reactions or in response to a CD3/CD28 stimulus (15). Thus, without repeating these assays, it is a safe assumption that cells generated in our hands would have a similar functional profile. We wanted to extend the findings by looking at a more specific and physiological way to activate T-cells. We focused on antigen-specific priming of naïve CD8$^+$ T-cells, using a well-validated experimental system (19, 20). This experimental set-up is calibrated in a way that naïve T-cells specific for the melanosomal peptide antigen Melan-A$_{(26-35(A27L)}$can be efficiently activated, starting from an estimated precursor frequency of 1–10 in 10,000, meaning 20–200 specific T-cells per well-within the starting population, and expanding to a robust, specific cell population of at least 20% at day 10 of culture.

We compared the stimulatory capacity of peptide-pulsed $_{R848}$moC vs. $_{IL10/R848}$moC. As seen for an exemplary experiment in **Figure 6A**, Expansion of MHC-multimer$^+$ T-cells by day 10 was much lower (10.6%) when $_{IL10/R848}$moC were used (which consisted of 20% CD14$^+$ cells). In comparison $_{R848}$moC (with a fraction of 4% CD14+ cells) gave yield to 26.4% of

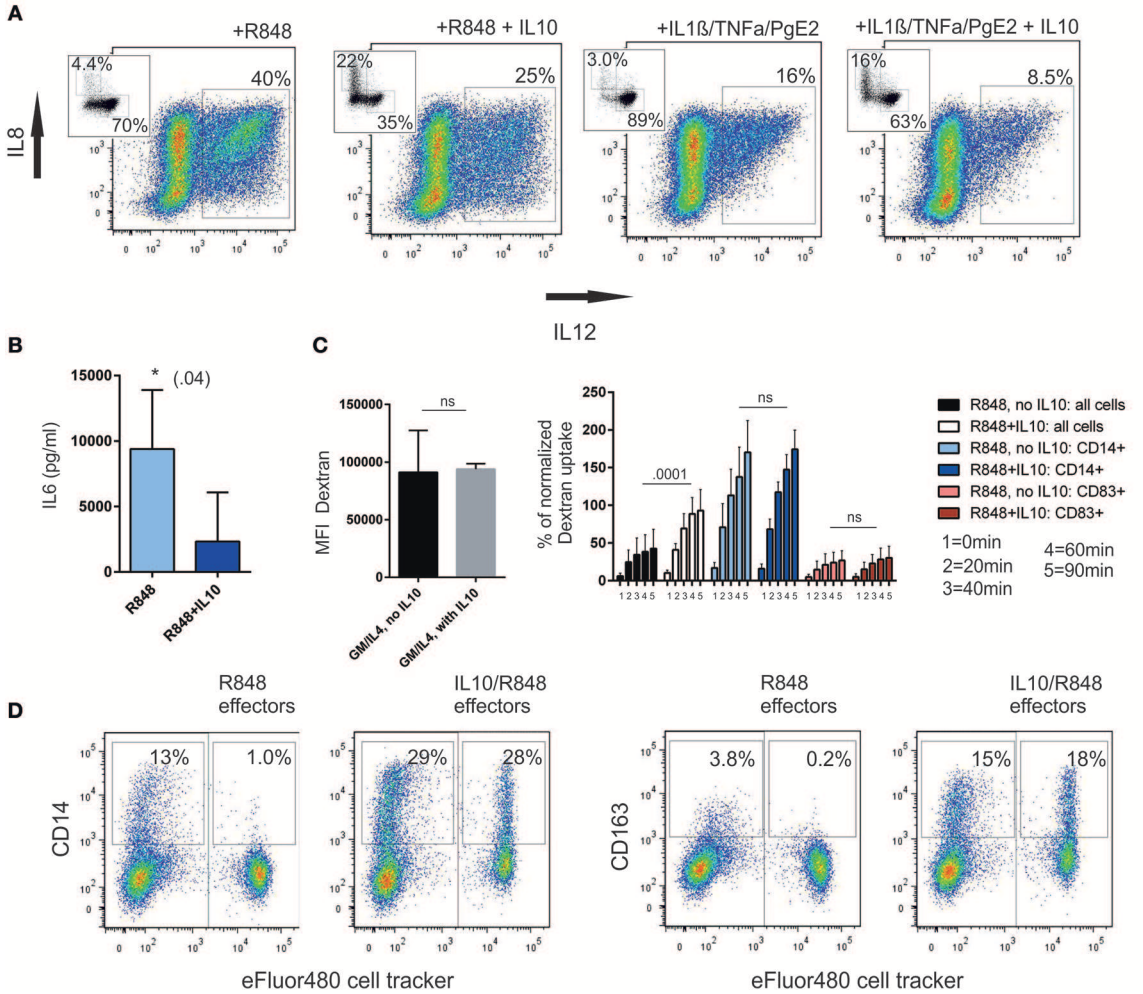

FIGURE 5 | IL-10-mediated effects on the function of moC. **(A)** IL-12 production is reduced. moC were preincubated with IL-10 (or not) and activated either with R848 or a cytokine cocktail. 1 h later, brefeldin A was added for 4 h and cells subsequently stained for intracellular IL-12 and IL-8. Distinction between CD14 and CD83 in the same samples is not possible, as CD14 upregulation is hindered by brefeldin A. CD14(y-axis)- and CD83(x-axis)-staining of a corresponding parallel sample (without brefeldin A) is shown as an inserted dot plot. ($n = 3$) **(B)** IL-6 concentration in the supernatant of differentially treated and activated moC, —pooled data from 5 experiments (*$p < 0.05$) **(C)**. APC-dextran uptake over time in different cell populations. The left panel shows the MFI for Dextran after 1 h of $_{GM/IL4}$moC with or without IL-10 (7 experiments). The right panel shows the analysis of activated cells with or without IL-10. Analysis was either done on total cells, or gated on $CD14^+$ or $CD83^+$ cells, respectively. 1–5 indicates the duration of dextran incubation (1 = 0 min, 2 = 20 min, 3 = 40 min, 4 = 60 min, 5 = 90 min). Each value is normalized to the MFI of $_{GM/IL4}$moC at 1 h within the individual experiment (7 experiments). **(D)** Transmission of the $CD14^+$ phenotype onto non-committed bystander moC. Two 'effector' populations were generated either by using LPS/IFNγ as a full type 1- stimulus or IL-10 followed by R848 to induce a $CD14^+$ population. After 16 h they were stained with cell tracker dye and mixed at a 1:1 ratio with non-committed, autologous $_{GM/IL4}$moC. Twenty four hours later R848 was added to this co-culture. Cells were then analyzed the next day and separated on the basis of the membrane dye. Examplary plot of 1 out of 3 experiments.

antigen-specific T-cells by day 10. Phenotypically, $_{IL10/R848}$moC-expanded T-cells expressed less CD62L in comparison to the $_{R848}$moC primed T-cells. Non-specific bystander T-cells in both groups predominantly retained CD62L expression, indicating that the reduced CD62L expression is due to the specific cell-cell interaction and not due a globally altered microenvironment. The antigen-specific T-cells also proliferated less upon restimulation, which indicates antigen-specific tolerance. The limitations of this assay certainly is the inter-donor variation, as variation in the moC preparation (as documented in **Figure 1**) combines with donor dependent variation due to the low frequency of antigen-specific naïve $CD8^+$ T-cells. However, three separate experiments, (summarized in **Figure 6B**), analyzing between 1 and 4 separate wells, depending on the available cell material, show a comparable pattern with reduced antigen-specific cell numbers, once IL10 was involved as well as reduced CD62L expression. Therefore, similar to the findings with non-specific clonal stimulation or stimulation of memory T-cells shown in earlier work (15), antigen-specific T-cell priming from the naïve T-cell repertoire is quantitatively and qualitatively affected by IL-10 induced moC^{CD14+} as well.

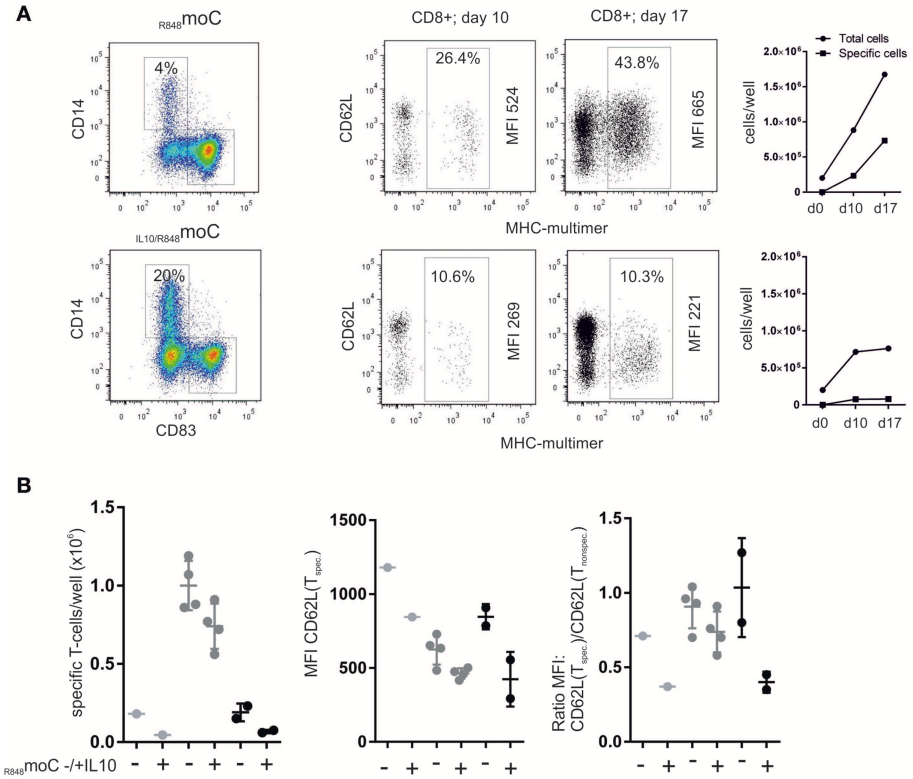

FIGURE 6 | Effects of IL-10-treated moC on antigen-specific priming of naive T-cells. **(A)** moC were differentially treated (left panel) and pulsed with Melan-A peptide. They were then used to prime naive $CD8^+$ T-cells. After 10 days of culture using IL-21, IL-7, and IL-15, MHC-multimer$^+$ cells were determined and phenotypically characterized. T-cells were then restimulated with peptide-pulsed moC and MHC-multimer$^+$ cells were re-evaluated 7 days later. Right panels depict the cell expansion in absolute numbers. **(B)** Summary of three different experiments from different donors. Depending on the cell numbers available (number of APC and number of naïve T-cells), experiments were set up in 1–4 parallel wells. The left panel shows the absolute numbers of antigen-specific (MHC-multimer+) cells per well after 10 days of expansion (based on the precursor frequency the starting cell number in each well varies between 20 and 200 cells). The middle panel summarizes MFI-values for CD62L of the resulting MHC-multimer$^+$ cells. The right panel shows the ratio of the CD62L MFI of specific vs. non-specific CD8+ cells within the same sample.

DISCUSSION

We here provide a new view of how IL-10 affects monocytic cells in culture: instead of assessing its effects on the bulk culture, the identification of mutually exclusive expression of CD14 or CD83 defines heterogeneity within the culture of monocytic cells, which is greatly augmented by exogenous IL-10 in combination with TLR-triggering. Thus, exogenous IL-10 drives a population shift toward macrophages, but it does not—for the most part—affect individual marker expression (such as CD86) of function (such as endocytosis) within the respective subgroup: $_{IL10/act}moC^{CD83+}$ (dendritic cells) do not express less CD86 than their non-IL-10-treated counterparts; $_{IL10/act}moC^{CD14+}$ (macrophages) do not take up more Dextran than non-IL10-treated $_{act}moC^{CD14+}$.

The strong effects seen in the analyses of total cells, which is repeatedly reported in various papers (2, 17, 21, 22), now finds an explanation as a quantitative shift of different cell populations and not a qualitative change of one homogenous dendritic cell population. The shift toward macrophage-like cells alone, and not differences in expression level on differently treated cells, explains for example, why ILT4, an important myeloid-specific receptor to suppress pro-inflammatory responses (31), suddenly seems increased in the total cell population upon IL10-treatment. Thus, at least three populations need to be distinguished, and analyzed separately, based on CD14 and CD83 expression: committed moC^{CD14}, moC^{CD83} and non-committed $moC^{CD14-CD83-}$.

The phenotype- and function-altering effects of IL-10 on immature DC have been known for a long time (15). Early studies by Allavena et al. show, how IL-10 shifted monocytic cells toward macrophages with maintained CD14 expression (11). Of note, in that work, the IL-10 effect was lost, if IL-10 was added at a later time point (e.g., day 3) and no upregulation of CD14 was observed. In a recent paper, $_{GM/IL4}moC$ cultured in the presence of IL-10, from the beginning of culture, were termed "MDSC-like." In this work, upregulation of CD14 has been noted, and interpreted as an indicator for the retention of the monocytic phenotype (12). The protocol to generate prototypic tolerogenic dendritic cells is also based on IL-10, added later at the time of cell activation (15, 24). In an effort for harmonization of the clinical use of tolerogenic dendritic

cells, CD11b+CD14++CD163++CD80+CD86+HLA-DR++ cells have been termed DC-10, arising from monocytes in a process termed "arrested immaturity" (8).

The other critical finding in this work is that CD14, rather than serving as a lineage marker (32), can be re-expressed to indicate alternative cell differentiation toward macrophages. Once moC receive a double stimulus consisting of IL-10 first and TLR-agonist second, CD14 and CD83 serve as mutually exclusive markers to define two cell populations with a different phenotypical and functional profile. Thus, CD14 expression may be less a question of halted differentiation (maintained CD14 expression) (27), rather than a sign of active re-expression as part of the differentiation pathway toward a macrophage-like cell.

Monocyte-derived DC are clinically evaluated as therapeutic DC vaccination for cancer (33). For this purpose, CD83, CD86, and HLA-DR often serves as read-out to assess optimal stimuli (17, 34). Our findings are highly relevant in this context, as the precise definition of a potentially suppressive $CD83^{-}$ subgroup within the DC preparation may help to better understand its effects (or lack thereof). Many studies focus on finding the optimized stimulation cocktail, providing the best Th1-oriented stimulation for these cells. Our pre-incubation experiments with TNFα show that rather than the right combination and dose of the stimulus, the timing, and sequence of activation may be most relevant to counteract intrinsic priming by endogenous IL-10 (**Figure 2**). Once CD14 is fully re-expressed, cells do not convert back to a $CD83^{+}$ inflammatory phenotype.

Likewise in studies on "regulatory DC" the main phenotypical description of such cells is that they express less stimulatory markers (8, 24). Few inhibitory molecules such as ILT4 and CD273 (PD-L2) (17) are known to be expressed at a higher level, but these molecules are not exclusive for a regulatory phenotype. In comparative studies on protocols on the generation of regulatory DC relevant for clinical use, no attention is paid to CD14 expression (24, 35, 36). However, as we now show, CD14, combined with a panel of macrophage markers (**Figure 4**), positively identifies cells with a stable phenotype and regulatory function.

Functionally, we aimed to add to the known characteristics of IL-10-treated cells. Distinction based on CD14 expression reveals that exogenous IL-10 itself does not directly enhance endocytosis as suggested earlier (11, 37), but changes the cellular composition. Moreover, moC^{CD14+} have the capacity to affect non-committed bystander cells, steering them toward the $CD14^{+}$ phenotype once an additional TLR trigger is provided. Although seen in an artificial experimental system with a broad range of variation, this effect may have implications in tumor biology. Once tumor-associated factors dominate the micromilieu and reverse some of the surrounding myelomonocytic cells to tumor-associated macrophages (TAMs), these TAMs may be able to recruit non-committed bystander cells, especially if an additional TLR-trigger is provided, thereby multiplying the tumor-associated effects. Mere activation of the immune infiltrate, e.g., by a TLR-trigger, may cause unintended, suppressive effects, if cells are primed by IL-10, requiring a more orchestrated intervention (38, 39).

Suppression of T-cell responses is a known hallmark of regulatory DCs. We chose to evaluate functional differences in the context of antigen-specific priming of human, naïve CD8+ T-cells (19, 20). Peptide-pulsed $_{IL10/R848}$moC are poor stimulators of a *de novo* T-cell response and the T-cells are tolerant to a second stimulus. Interestingly, despite reduced proliferation, CD62L expression is lower than in fully activated T-cells. This corroborates, on the level of a *de novo* antigen-specific, human immune response, data on the effects of myeloid-derived suppressor cells from murine models (40, 41).

The caveat of these experiments is, that, to some extent, monocyte-derived DCs, are in itself a culture artifact (42). For murine bone marrow cultures, Helft et al. showed, that these cultures are not monomorphic but comprise of conventional DCs and monocyte-derived macrophages (43). Our data extend these findings to human mononuclear cells showing that culture with GM-CSF and IL-4 is not sufficient to definitively tilt monocytes toward DC differentiation. However, understanding how such cell populations, serving as cell therapeutics, may develop and how they might deviate from the projected path is essential to understand their potential clinical impact. For cancer patients significant difficulties have been described to generate fully activated DCs for clinical use and this deficiency has been linked to the presence of regulatory $CD14^{+}HLA\text{-}DR^{lo/neg}$ cells (44). For example, an insufficiently activating vaccine may not be a "null" event, but might even have a negative effect (45). Filipazzi et al. described the occurrence of $CD14^{+}HLA\text{-}DR^{-/low}$ cells with immunosuppressive characteristics following vaccination with GM-CSF and hsp gp96 in melanoma patients (46). Llopiz et al. also observed that IL10-producing "immunosuppressive DC" are induced by therapeutic vaccination with imiquimod-based vaccines, significantly affecting T-cell responses in a murine model (47). Other groups observed elevated levels of IL-10 following vaccination with imiquimod, suggesting that besides the inflammatory activity, a self-regulatory, IL-10-dependent pathway is being triggered. The authors discuss the possibility of using IL-10 blockade clinically, to enhance vaccine effects (48). In this context and in light of our data, it will be interesting to test *in vivo*, whether priming of the vaccination site with TNFα prior to local administration of the TLR-trigger may overcome the described IL-10-dependent pathway.

In summary mutually exclusive, CD14 and CD83-expression in $_{GM/IL4}$moC provides a means to understand functional differences of therapeutically used cell products better. For IL-10, these differences are, for the most part, based on shifts in the magnitude of the respective cell populations rather than a direct regulation on a single molecule level. These findings will help to better design and define cellular products and might help to understand the variable outcome of vaccination in different individuals. Given the prominent role of IL-10 in tumor immunology and the emerging role of $CD14^{+}HLA\text{-}DR^{low}$MDSC in human diseases, these findings may also have a biologically significant counterpart.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files. In very few instances data are briefly mentioned and indicated as "not shown." This data is available on request.

AUTHOR CONTRIBUTIONS

SK, MC, CB, and MW: designed, performed, and analyzed experiments. VW, ME, and PS: analyzed data and provided valuable input throughout the experimental phase. SK and MW: wrote the manuscript. All authors read and approved the manuscript.

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The Ontogeny of Monocyte Subsets

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Classical and non-classical monocytes, and the macrophages and monocyte-derived dendritic cells they produce, play key roles in host defense against pathogens, immune regulation, tissue repair and many other processes throughout the body. Recent studies have revealed previously unappreciated heterogeneity among monocytes that may explain this functional diversity, but our understanding of mechanisms controlling the functional programming of distinct monocyte subsets remains incomplete. Resolving monocyte heterogeneity and understanding how their functional identity is determined holds great promise for therapeutic immune modulation. In this review, we examine how monocyte origins and developmental influences shape the phenotypic and functional characteristics of monocyte subsets during homeostasis and in the context of infection, inflammation, and cancer. We consider how extrinsic signals and transcriptional regulators impact monocyte production and functional programming, as well as the influence of epigenetic and metabolic mechanisms. We also examine the evidence that functionally distinct monocyte subsets are produced via different developmental pathways during homeostasis and that inflammatory stimuli differentially target progenitors during an emergency response. We highlight the need for a more comprehensive understanding of the relationship between monocyte ontogeny and heterogeneity, including multiparametric single-cell profiling and functional analyses. Studies defining mechanisms of monocyte subset production and maintenance of unique monocyte identities have the potential to facilitate the design of therapeutic interventions to target specific monocyte subsets in a variety of disease contexts, including infectious and inflammatory diseases, cancer, and aging.

Keywords: monocyte subsets, monocyte progenitors, monocyte ontogeny, monopoiesis, bone marrow

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INTRODUCTION

Monocytes are innate immune cells of the myeloid lineage that are produced throughout life and play diverse roles all over the body, including in tissue development and homeostasis, host defense, initiation and resolution of inflammation, and tissue repair. They are produced during homeostasis by hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (steady-state monopoiesis), and their production is enhanced during "emergency monopoiesis," which occurs in diverse circumstances including in response to infectious and inflammatory stimuli, in the presence of tumors, and during chronic psychosocial stress (1–5). In addition to quantitative

changes, there are also qualitative changes with the production of functionally distinct monocytes and monocyte-derived cells in response to stress.

Monocytes initially arise in the fetal liver from late yolk sac-derived erythromyeloid progenitors during the transient-definitive wave of hematopoiesis, from around embryonic day 8.5 (E8.5) in mice [reviewed in (6)]. At E10.5, immature hematopoietic stem cells (HSCs), which arise from the aorta-gonad-mesonephros (AGM) region of the embryo, colonize, and establish definitive hematopoiesis in the fetal liver, which serves as the major hematopoietic organ for the developing immune system. HSCs subsequently seed the bone marrow but are not fully functional until several days after birth, so liver (and spleen) HSCs continue to supply monocytes during the peri-natal period until the establishment of nascent adult-like HSCs.

In the post-natal bone marrow, monocytes are produced by HSCs via progenitors with progressively restricted lineage potential that ultimately commit to monocyte production. In both mice and humans, monocytes arise from multilineage common myeloid progenitors (CMPs), which also produce neutrophils, dendritic cells (DCs), erythrocytes and megakaryocytes (7, 8). Monocytes arise via two independent pathways in mice (**Figure 1**) and probably also in humans: granulocyte-monocyte progenitors (GMPs) produce monocytes and neutrophils, and monocyte-DC progenitors (MDPs) yield monocytes as well as conventional and plasmacytoid DCs (cDCs and pDCs) (7, 9–11). Monocyte-committed progenitors—GMP-derived MPs and MDP-derived cMoPs, which are discussed in more detail below—have been isolated in both mice and humans (11–14). The adult spleen also contains a reservoir of monocytes that can be rapidly recruited in response to injury or inflammation (15). Extramedullary monopoiesis has also been reported in the adult mouse spleen under inflammatory conditions, including in the presence of tumors, in models of psychosocial stress, and upon aging (3, 16–18).

Recent studies have revealed the diverse origins of macrophages resident in different tissues [reviewed in (6, 19)]. Microglia arise exclusively from yolk sac progenitors, independent of HSCs and monocytes. Other tissue macrophages are monocyte-derived, but with diverse temporal origins. Langerhans cells, alveolar macrophages, and Kupffer cells, for instance, initially originate from yolk sac progenitors, but are subsequently replaced by fetal liver-derived monocytes. Macrophages in the heart, pancreas, gut and dermis, in contrast, are originally derived from fetal liver monocytes, but significantly or almost entirely replaced by bone marrow-derived monocytes after birth, or later in life, in a tissue-specific manner.

Two major types of monocytes have been extensively characterized in both mice and humans: classical ($Ly6C^{hi}$ $CD43^{-}$ monocytes in mice, which correspond to $CD14^{+}$ $CD16^{-}$ monocytes in humans) and non-classical ($Ly6C^{lo}$ $CD43^{+}$ monocytes in mice, which correspond to $CD14^{lo}$ $CD16^{+}$ monocytes in humans) (20–23). In the steady-state, reserves of classical monocytes are maintained in the bone marrow and other extramedullary sites, such as the spleen, where they are available for immediate deployment to infected or injured tissues and can give rise to macrophages or monocyte-derived dendritic cells (moDCs) with diverse roles in controlling infection, limiting inflammatory damage, and initiating tissue repair. Non-classical monocytes, on the other hand, are recruited to non-inflamed tissues in a CX3CR1-dependent manner, and are characterized by their ability to patrol the resting vasculature, remove cell debris, and repair the endothelium during homeostasis (21–25).

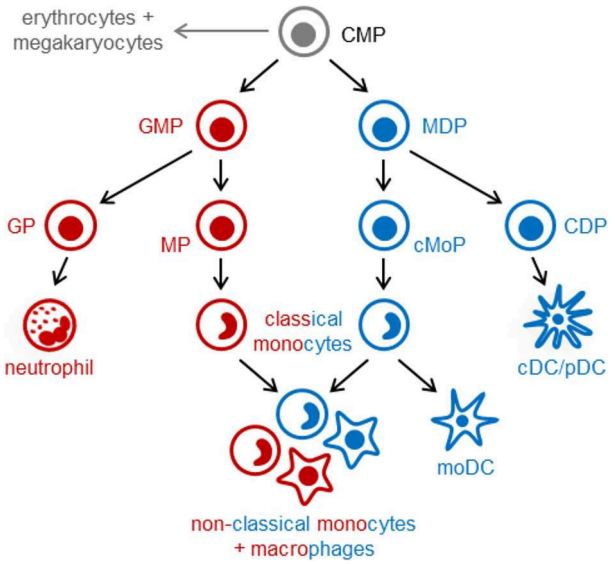

FIGURE 1 | Pathways of myeloid cell differentiation. In the steady-state, distinct mouse monocyte subsets arise independently from common myeloid progenitors (CMPs; LKS^{-} $CD34^{+}$ $Fc\gamma R^{lo}$ $Flt3^{+}$ $CD115^{lo}$ cells) via granulocyte-monocyte progenitors (GMPs; LKS^{-} $CD34^{+}$ $Fc\gamma R^{hi}$ $Ly6C^{-}$) and monocyte-DC progenitors (MDPs; LKS^{-} $CD34^{+}$ $Fc\gamma R^{lo}$ $Flt3^{+}$ $CD115^{hi}$). GMPs also produce neutrophils (via granulocyte progenitors, GPs), and MDPs yield cDCs and pDCs (via common DC progenitors, CDPs). Functionally distinct subsets of classical monocytes ($Ly6C^{hi}$ in mice) are produced by both GMPs and MDPs. Non-classical ($Ly6C^{-}$) monocytes and macrophages, also arise via both pathways and may exhibit functional differences. In contrast, monocyte-derived DCs (moDCs, which are ontogenetically and functionally distinct from cDCs and pDCs) arise exclusively from MDP-derived monocytes, and GMPs produce a neutrophil-like subset of classical monocytes. Monocyte-committed progenitors arising from GMPs (known as MPs) and MDPs (known as cMoPs) are both found in the LKS^{-} $CD34^{+}$ $Fc\gamma R^{hi}$ $Ly6C^{+}$ $CD115^{hi}$ fraction of mouse bone marrow; it is not currently possible to separate them using surface markers, but MPs and cMoPs are revealed as distinct cell clusters by single-cell RNA sequencing.

Non-classical monocytes are less proliferative than classical monocytes, but they remain in the circulation longer (25, 26). Most evidence indicates that they arise from classical monocytes in both mice and humans (23–26). Intermediate monocytes ($Ly6C^{int}$ $CD43^{+}$ monocytes in mice and $CD14^{+}$ $CD16^{+}$ monocytes in humans) have also been characterized (23, 26–30). They possess many of the inflammatory characteristics of classical monocytes, but express similar levels of CX3CR1 to non-classical monocytes, although they do not actively patrol the vasculature.

It is, however, becoming increasingly clear that monocytes are much more heterogeneous than previously appreciated. In this review, we highlight recent insights into the production and

programming of monocytes with distinct functional attributes during homeostasis and in the context of infection, inflammation and cancer. We discuss how monocyte origins influence their function by considering the developmental pathways of monocyte production and reviewing how monocyte function is programmed during differentiation and influenced by signals that instruct or promote monopoiesis.

MONOCYTE HETEROGENEITY

Multiparametric single-cell studies using flow cytometry, mass cytometry and single-cell RNA sequencing have recently revealed further heterogeneity among mouse and human monocytes, and combined with functional studies, have provided insight to support the identification of monocyte subsets. Some subsets are distinct stages of a linear differentiation pathway, whereas others represent functionally distinct monocytes, including new subsets that arise under emergency conditions. In the context of infection and inflammation, for example, elevated monocyte numbers may reflect amplification of steady-state subsets and/or the appearance of new populations with the ability to promote inflammatory responses, initiate tissue healing, or induce fibrosis.

The CXCR4$^+$ subset of Ly6C^{hi} monocytes in mouse bone marrow is a transient population of pre-monocytes that lose CXCR4 expression as they mature, which facilitates their exit from the bone marrow (31). Similarly, TREML4$^-$ Ly6C^{hi} monocytes can produce Zbtb46$^+$ moDCs capable of cross-priming CD8$^+$ T cells, whereas it appears that TREML4$^+$ Ly6C^{hi} monocytes are intermediate monocytes that have lost the potential to produce moDCs, but can still give rise to Ly6C^{lo} monocytes, which are also TREML4$^+$ (32). A recent single-cell RNA sequencing study also revealed heterogeneity among human intermediate monocytes (29), which may at least in part reflect different stages of classical to non-classical conversion.

Surface expression of 6-sulfo LacNAc (slan), a carbohydrate modification of P-selectin glycoprotein 1 (PSGL1), has been reported to distinguish intermediate (slan$^-$), and non-classical (slan$^+$) human monocytes (33), although a recent mass cytometry study revealed a subset of slan$^-$ non-classical monocytes (30). The latter study reported 8 monocyte subsets in peripheral blood from healthy human subjects, including CD61$^+$ and CD9$^+$ subsets of non-classical monocytes (30). The CD9$^+$ subset was also detected in mice and likely reflects platelet binding to these monocytes.

Functionally distinct moDCs—CD103$^+$ DCs produced by Ly6C^{hi} CCR2^{hi} monocytes, and CD11b^{hi} DCs derived from Ly6C^{lo} CCR2^{lo} monocytes—have previously been reported in the steady-state lung (34). Moreover, a recent study described two distinct populations of Ly6C^{hi} monocyte-derived macrophages resident in multiple mouse and human tissues: antigen-presenting Lyve1^{lo} MHCII^{hi} CX3CR1^{hi} macrophages located adjacent to nerve bundles and fibers, and Lyve1^{hi} MHCII^{lo} CX3CR1^{lo} macrophages, which reside near blood vessels and are functionally optimized for tissue repair (35).

Distinct mouse monocyte subsets detectable in the steady-state have also been reported to give rise to inflammatory macrophages and monocyte-derived DCs (36). Expression of MHCII and CD209a (one of the eight mouse homologs of DC-SIGN) defines a subset of Ly6C^{hi} monocytes present in small numbers in the steady-state (∼5% Ly6C^{hi} monocytes in the bone marrow) that are capable of differentiating into moDCs under inflammatory conditions, whereas Ly6C^{hi} monocytes lacking CD209a and MHCII expression (∼90% of Ly6C^{hi} monocytes) produce iNOS$^+$ inflammatory macrophages.

Single-cell RNA sequencing has also revealed a population of steady-state Ly6C^{hi} monocytes (∼15%) with neutrophil-like properties, including strong expression of granule proteins (11). Monocytes with a neutrophil-like gene signature have also been reported in mouse and human lung tumors and peripheral blood (37). In humans, these monocytes were classical (CD14$^+$), whereas the mouse equivalents, which were also present in tumor-free lung tissue, included both classical and non-classical (Ly6C^{hi} and Ly6C^{lo}) monocytes. Moreover, fibrosis-promoting Ceacam1$^+$ Msr1$^+$ non-classical monocytes with granulocytic properties (named segregated-nucleus-containing atypical monocytes, SatMs) are not detected in the steady-state, but appear *de novo* following bleomycin administration (38). It is currently unclear whether or how these neutrophil-like subsets are ontogenetically or functionally related to one another.

Some monocyte subsets appear to contribute to tissue damage, whereas others promote tissue repair. Patients with coronary artery disease, for instance, have elevated numbers of slan$^+$ CXCR6$^+$ non-classical monocytes, which correlate with disease severity (30), and asthma severity in humans has been reported to correlate with elevated numbers of circulating TGF-β1-producing classical monocytes, which differentiate into fibrocytes instead of macrophage-like cells (39). In contrast, MHCII$^+$ Sca-1$^+$ CX3CR1$^-$ Ly6C^{hi} monocytes, which are thought to limit immunopathology via production of prostaglandin E2 (PGE2) and IL-10, arise in the bone marrow following acute gastrointestinal infection with *Toxoplasma gondii* [(40); **Figure 2A**], and immunoregulatory Ym1$^+$ Ly6C^{hi} monocytes have also been observed during the recovery phase of tissue injury (41).

Monocyte subsets that promote anti-tumor immunity, or conversely, support tumor growth have also been reported [reviewed in (5)]. For instance, tumor antigen-presenting CD103$^+$ Ly6C$^+$ monocytes have been reported to be required for efficient cross-presentation of tumor antigens and responsiveness to immunotherapy and immunogenic chemotherapy (42). In contrast, a subset of tumor-infiltrating pro-angiogenic Tie2$^+$ non-classical monocytes has been described in tumors and the circulation of tumor-bearing mice and cancer patients (as well as healthy controls) (43, 44). Immunosuppressive classical monocytes, often termed monocytic myeloid-derived suppressor cells (M-MDSCs), are also prevalent in tumor-bearing mice and humans, as well as in inflammatory contexts such as sepsis and autoimmunity [reviewed in (4)]. They are characterized by their ability to suppress cytotoxic T cell and NK cell activation, promote anti-inflammatory and immunoregulatory responses (IL-10, regulatory T cells etc.), and support tumor progression and metastasis. However, in these contexts, it is unclear whether all classical monocytes exhibit these properties

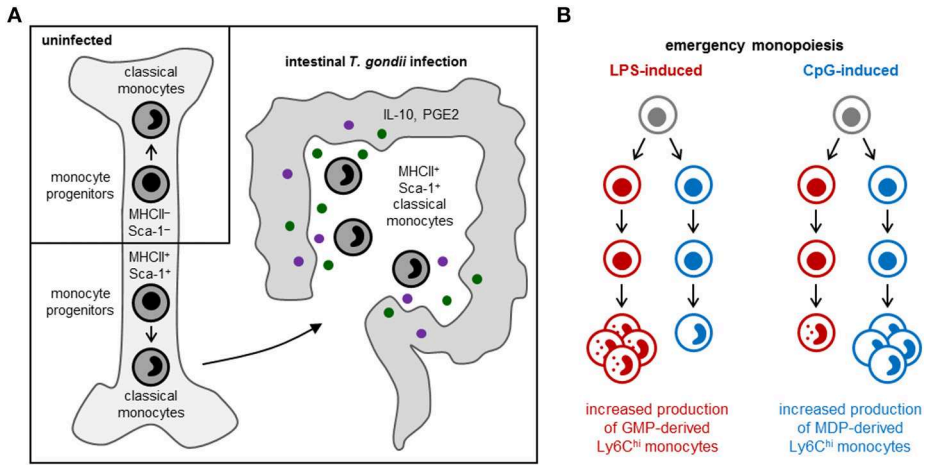

FIGURE 2 | Emergency monopoiesis. Under emergency or stress conditions, functionally distinct monocyte subsets may arise in the bone marrow or spleen, and production of monocyte (and other myeloid cell) subsets may be selectively enhanced. **(A)** In response to intestinal *T. gondii* infection in mice, MHCII^{+} Sca-1^{+} CX3CR1^{-} Ly6C^{hi} monocytes are produced by monocyte-committed progenitors that, unlike their steady-state counterparts, also express MHCII and Sca-1 (40). **(B)** LPS and CpG promote monocyte production by murine GMPs and MDPs, respectively; LPS also stimulates neutrophil production by GMPs, whereas CpG enhances cDC production by MDPs (11).

or just a fraction of them, because T cell suppression is evaluated using bulk populations of monocytic cells. A single-cell RNA sequencing study recently revealed 3 monocyte transcriptional states (both classical and non-classical, including the neutrophil-like monocytes mentioned above), as well as several macrophages and moDC subsets, in human and mouse lung tumors (37), although the functional significance of these subsets remains to be determined.

CONTROL OF MONOCYTE SUBSET PRODUCTION AND FUNCTIONAL PROGRAMMING

Signals from the microenvironment can influence monocyte gene expression and function in a tissue-specific manner, but the presence of multiple distinct subsets of monocytes or monocyte-derived cells in the same tissue indicates that they may have independent origins. We will consider how signals sensed by HSPCs, such as cytokines and microbial components, shape the repertoire of monocytes produced, both in the steady-state and under emergency conditions. This may occur via epigenetic and metabolic programming of the differentiating cells, which is discussed in this section, and/or via selective promotion of specific differentiation pathways that yield distinct monocyte subsets, which is considered in the next section.

Cytokines released by hematopoietic or non-hematopoietic cells in the bone marrow niche, or originating from outside the bone marrow via the circulation, can influence monocyte production and functional programming upon detection by progenitors. For instance, production of regulatory MHCII^{+} Sca-1^{+} CX3CR1^{-} Ly6C^{hi} monocytes in response to *T. gondii* infection is instructed by IFN-γ produced by NK cells in the bone marrow, and elevated expression of MHCII and Sca-1 is already evident at the monocyte progenitor stage [(40); **Figure 2A**]. Sca-1 upregulation by myeloid progenitors is also induced by other inflammatory stimuli, including type I interferons and TNF-α [reviewed in (1)]. HSPCs also express a variety of pattern recognition receptors, allowing them to directly sense microbes [reviewed in (45)]. Exposure of endogenous or purified HSPCs to whole microbes (bacteria, viruses and fungi) or microbial components induces them to produce monocytes and other myeloid cells, but can also program the function of the macrophages they produce. For instance, HSPCs exposed to TLR2 agonists differentiate into macrophages that are less inflammatory (produce lower levels of inflammatory cytokines and reactive oxygen species) than those derived from unexposed HSPCs (46).

Progenitor programming is also thought to underlie observations of innate immune memory. Although cells of the innate immune system do not possess the antigen-specific memory of T and B cells, epigenetic and metabolic changes induced by microbial stimuli can alter their responses to subsequent stimulation. Lipopolysaccharide, for example, can tolerize macrophage cytokine responses and prime microbicidal responses via selective chromatin remodeling (47). Similarly, detection of fungal β-glucans or the Bacillus Calmette-Guérin (BCG) vaccine trains monocytes and macrophages to enhance their responsiveness to secondary stimulation by inducing changes in histone modifications and a metabolic shift toward glycolysis (48, 49). Innate immune memory mechanisms are thought to last weeks, months or possibly even years, and may contribute to protection against subsequent infections. Recent studies have also demonstrated epigenetic and metabolic changes in HSPCs, which may contribute to the persistence of such effects. *In vivo* BCG administration induced myeloid progenitor expansion and sustained production of macrophages epigenetically trained to more effectively kill *Mycobacterium*

tuberculosis (50). β-glucan injection similarly promoted myelopoiesis and induced metabolic alterations in progenitors, consistent with those observed in β-glucan-trained mature monocytes and macrophages (51).

Beyond microbial infection, it is likely that other stimuli also impact the functional programming of monocytes during differentiation, whether or not they concomitantly boost monocyte numbers. For instance, PGE2 induced by UV skin irradiation causes epigenetic and metabolic changes in bone marrow progenitors, and the monocytes, macrophages and DCs they produce have a restricted capacity to migrate in response to chemoattractants and inflammatory mediators (52–55). Immunosuppressive monocytes produced in the context of tumors may also acquire their suppressive properties during differentiation, because monocytic cells isolated from the circulation, spleen and bone marrow of cancer patients and tumor-bearing mice have been reported to inhibit T cell activation and promote tumor growth (4, 5, 56). Tumor-derived factors are therefore thought to instruct the programming of pro-tumor monocytes during differentiation, in addition to their local effects in the tumor itself.

The spleen has been shown to be a key source of monocytes and neutrophils recruited to tumors and may be an important site for monocyte functional programming. In murine lung cancer models, extramedullary myelopoiesis was detected in the spleen (16) and splenectomy reduced tumor progression (16, 56). The underlying mechanisms in these splenectomy models appear to vary with tumor type, but include reduced recruitment of monocytes to the tumors, fewer immunosuppressive monocytes, and more anti-tumor macrophages. Human patients with invasive pancreatic or colon cancer have also been reported to have more splenic myeloid progenitors and monocytes than individuals without invasive cancer (16). Myeloid progenitor recruitment from the bone marrow to the spleen has also been reported in a mouse model of psychosocial stress, in which splenic monopoiesis provides monocytes that traffic to the brain and induce anxiety-like behavior (3).

DISTINCT PATHWAYS OF MONOPOIESIS

Distinct monocyte subsets may also arise via independent differentiation pathways. A study from our lab recently revealed the existence of two independent pathways of monopoiesis in the steady-state, which yield functionally distinct monocyte subsets (11). We demonstrated that neutrophil-like Ly6C^{hi} monocytes arise from GMPs (which also produce neutrophils), whereas MHCII^{+} CD209a^{+} Ly6C^{hi} monocytes capable of producing moDCs are derived from MDPs (which also produce cDCs and pDCs) (**Figure 1**). Other studies applying lineage trajectory analyses of single-cell RNA sequencing datasets similarly predicted the existence of two pathways of monocyte differentiation (57, 58). Macrophages produced via the two monocyte lineages are also probably functionally distinct. Indeed, we observed higher CD86 expression by CD11c^{+} MHCII$^{-/lo}$ macrophages in GM-CSF cultures of MDP-derived monocytes compared to the same cell fraction in GMP-derived monocyte cultures (11). We also demonstrated that LPS and CpG differentially target the GMP and MDP pathways, respectively, to boost monocyte production (11) (**Figure 2B**), although it is currently unclear whether this effect is direct (due to TLR-mediated detection by the progenitors themselves) or indirect.

While the GMP vs. MDP origins of most monocyte subsets are as yet unknown, some emerging studies provide additional evidence of independent pathways for the production of distinct monocyte subsets. Immunoregulatory Ym1^{+} Ly6C^{hi} monocytes, for instance, are reportedly GMP-derived and not MDP-derived (41). New differentiation pathways may also exist under emergency conditions. In support of this possibility, SatMs arose from a subset of FcεRI^{+} GMPs independently of steady-state monocyte-committed progenitors in fibrosis models (38). Monocyte programming that is already evident at the progenitor level, as seen in the context of *T. gondii* infection (40), may specifically affect one pathway or have similar effects in both.

TRANSCRIPTIONAL REGULATORS OF MONOCYTE SUBSET PRODUCTION AND FUNCTIONAL PROGRAMMING

Several transcription factors have been implicated in steady-state and emergency monopoiesis, including some that govern the production of specific monocyte subsets. IRF8 is a key regulator of monocyte differentiation [reviewed in (59)]. It binds with PU.1 to promoters and enhancers to induce the monocyte lineage gene program. IRF8 is dispensable for monocyte lineage specification, but required for the production of steady-state monocytes by monocyte-committed progenitors, as evidenced in IRF8-deficient mice by the accumulation of monocyte-committed progenitors and monoblasts that fail to differentiate into mature monocytes (13). IRF8 induces the expression of monocyte genes both directly and indirectly via induction of the transcription factor Klf4 (60). IRF8 induces the formation of enhancers to direct the expression of monocyte genes (61), and also interacts with the transcription factor c/EBPα to inhibit the granulocyte program (62).

Zeb2 and GATA2 have also been implicated in monocyte differentiation. Zeb2 deletion results in depletion of Ly6C^{hi} monocytes in the bone marrow (63, 64), and GATA2 mutations have been identified in patients with monocyte deficiencies (65, 66). miR146a differentially regulates classical and non-classical monocytes, targeting transcripts for the non-canonical NF-κB family member RelB to restrict classical monocyte expansion during inflammatory challenge without affecting non-classical monocytes (67). We observed high expression of Gfi1, which is important for granulopoiesis, in GMP-derived neutrophil-like Ly6C^{hi} monocytes (11), but its role in the functional programming of these monocytes, including their expression of granule proteins, remains to be determined.

The conversion of Ly6C^{hi} monocytes to Ly6C^{-} monocytes is dependent on the transcription factor c/EBPβ, which is required for the survival of Ly6C^{-} monocytes and maintenance of CD115 expression, at least in part via induction of NR4A1 (Nur77) (24, 28, 68). c/EBPβ also regulates the production of SatM monocytes associated with the development of fibrosis (38), has been implicated in the production of M-MDSCs (69),

and defines the enhancer landscape of moDCs (70). Monocyte differentiation into cross-priming moDCs also requires IRF4, but is BATF3-independent (32). In contrast, p53-drived BATF3 upregulation is reportedly required for differentiation of tumor antigen-presenting CD103$^+$ Ly6C$^+$ monocytes (42).

IMPLICATIONS AND FUTURE OPPORTUNITIES

A growing body of evidence therefore supports the notion that heterogeneity among monocytes in part reflects their origins. A major outstanding question is whether developmental influences have a prolonged impact on the functional programming of monocytes and their derivatives, or whether they are largely overridden by subsequent exposure to other stimuli (cytokines, microbes, tumors etc.) after monocytes leave the bone marrow. This applies in the context of both steady-state and emergency monopoiesis, including in relation to innate immune progenitor memory effects. The role of the splenic microenvironment is also of particular interest under emergency conditions when extramedullary myelopoiesis is often observed.

In terms of ontogeny, it will be important to define which monocyte subsets derive from GMPs vs. MDPs (or via other, as yet undescribed, pathways). For instance, it is unclear whether immunoregulatory monocytes arise via a single pathway or whether their functional programming is independent of their ontogeny. Indeed, it also remains to be determined whether monocytes with pro-tumor properties are ontogenetically distinct from tumor antigen-presenting monocyte subsets. Similarly, Lyve1^{lo} MHCII^{hi} CX3CR1^{hi} and Lyve1^{hi} MHCII^{lo} CX3CR1^{lo} interstitial macrophages appear to arise separately from distinct monocyte subsets (35), but it is unclear at what stage of differentiation their developmental pathways diverge.

Studies of mice and humans have revealed that fetal, neonatal, and young and old adult monocytes have distinct basal transcriptional profiles and exhibit differential responses to cytokines and microbial stimulation (71–73), but single-cell studies are now required to determine to what degree this variation reflects the monocyte subset composition. Microenvironmental differences in the fetal liver and neonatal and adult bone marrow niches likely impact monopoiesis, but monocyte progenitors and differentiation pathways during fetal development and the neonatal period are less well-defined, as are the effects of aging. The impact of the microbiome is another important area of research. Circulating components derived from commensal organisms, as well as short-chain fatty acids they produce, have been demonstrated to impact myelopoiesis (74–77), but further study is required to define their effects on the production and functional programming of specific monocyte subsets.

Multiparametric single-cell profiling (transcriptomic, proteomic and epigenomic), fate mapping and other technical developments have improved our understanding of monocyte subsets, differentiation and ontogeny in recent years, and combinatorial approaches will continue to advance our knowledge in this field as these tools become more widely accessible. Lineage trajectory analyses using omics datasets, along with fate mapping studies, will permit the distinction between monocyte subsets representing transitional states of the same cells vs. cells derived independently via separate pathways. It will also be critical to pair single-cell omics profiling and functional analyses, with careful interpretation where bulk populations of cells are used for functional studies. Precise identification of monocyte subsets will be facilitated by identification of new surface markers and reporter mice that enable tracking of specific subpopulations e.g., suppressive monocytes within a heterogeneous fraction of monocytic cells.

It will of course be particularly important to evaluate the ontogeny of monocyte subsets in humans as well as mice. Single-cell RNA sequencing datasets have already revealed heterogeneity among mouse and human monocytes (11, 28, 29, 36, 37), and allowed comparison between mouse and human subsets, which informs the extrapolation of observations made in studies of murine monocyte ontogeny to humans.

Ultimately, studies defining mechanisms of monocyte subset production and maintenance of unique monocyte identities have the potential to facilitate the design of therapeutic interventions to target specific monocyte subsets in a variety of disease contexts, including infectious and inflammatory diseases, cancer and aging. It remains to be seen whether developmental targeting will be an effective strategy for clinical use.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Ontogeny of Tumor-Associated Macrophages

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Tumor-associated macrophages (TAM) represent the main immune cell population of the tumor microenvironment in most cancer. For decades, TAM have been the focus of intense investigation to understand how they modulate the tumor microenvironment and their implication in therapy failure. One consensus is that TAM are considered to exclusively originate from circulating monocyte precursors released from the bone marrow, fitting the original dogma of tissue-resident macrophage ontogeny. A second consensus proposed that TAM harbor either a classically activated M1 or alternatively activated M2 polarization profile, with almost opposite anti- and pro-tumoral activity respectively. These fundamental pillars are now revised in face of the latest discoveries on macrophage biology. Embryonic-derived macrophages were recently characterized as major contributors to the pool of tissue-resident macrophages in many tissues. Their turnover with macrophages derived from precursors of adult hematopoiesis seems to follow a regulation at the subtissular level. This has shed light on an ever more complex macrophage diversity in the tumor microenvironment than once thought and raise the question of their respective implication in tumor development compared to classical monocyte-derived macrophages. These recent advances highlight that TAM have actually not fully revealed their usefulness and deserve to be reconsidered. Understanding the link between TAM ontogeny and their various functions in tumor growth and interaction with the immune system represents one of the future challenges for cancer therapy.

Keywords: tumor-associated macrophage (TAM), cancer, ontogeny, monocyte-derived cell, cancer therapeutic

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INTRODUCTION

Tumor-associated macrophages (TAM) represent a major component of the tumor microenvironment (TME) that has been extensively studied in the past decades. They play a major role in tumor growth, metastatic dissemination, and therapy failure. Countless reports have described that TAMs can promote angiogenesis, inhibit the anti-tumor immune response, in particular T-cell-mediated cytotoxicity, support tumor growth, and secrete different factors involved in extracellular matrix (ECM) remodeling thus facilitating tumor cell motility and intravasation (1–6). High TAM infiltration is generally correlated with poor outcomes in several types of cancer, such as breast, ovarian, and lung cancer (7–9). However, in some indications TAM can be associated with enhanced anti-tumor immunity (10–12). Although macrophages were originally described as arising exclusively from circulating monocyte precursors (13), it was shown in the recent years that several organs harbor embryonic-derived populations of resident macrophages (ResMac) that maintain and self-renew throughout adulthood (14–16). This

new concept challenges the dogma of TAM origin and questions their relative function. TAM subsets were originally classified as tumoricidal vs. tumor-promoting, often referred as M1/M2 macrophages (17), based on the expression of specific markers. However, the wide diversity of TAM cannot be covered by this nomenclature and many subsets express overlapping markers of the M1/M2 polarization (18–20). Whether TAM heterogeneity originates from their high plasticity or rather from independent specific lineages giving rise to multiple populations is still unclear. Although cellular ontogeny can recapitulate parts of the heterogeneity, it appears that environmental cues are also major determinants in cell education. Macrophage diversity would then be the result not only of ontogeny but also of niche-specific signaling events of tumor immunity (21–24). One can thus wonder whether the origin of TAM dictates their role in tumor development and is associated with various functions. This represent a key issue for anti-cancer therapies as these subsets might be differentially targeted regarding their role in tumor development.

MACROPHAGE ORIGIN AND TURNOVER

Although the precise origin of ResMac is still under debate [For the different models proposed, see review (14)], fate-mapping models highlighted a differential origin of tissue macrophages deriving either from an embryonic precursor (yolk sac, fetal liver) or a monocyte precursor from adult hematopoiesis origin. These precursors seed the tissues in different waves during development and adulthood giving rise to different ResMac. The dynamics of these waves vary between organs, age, and macrophage subsets. In some organs, such as the brain, the lung and the liver, some embryonic-derived ResMac (named here EmD-ResMac) maintain by self-renewal in adults whereas in the gut, the skin, the heart, and the pancreas most subsets are progressively replaced through the differentiation of monocyte precursors from adult hematopoiesis into monocyte-derived ResMac (named here MoD-ResMac) with different turnover rates. The ability of newly recruited macrophages to self-maintain in the tissue and become a ResMac *per se* is proposed to be tightly regulated by space availability and competition for growth factors in the niche (23).

This turnover appears to be variable among subsets in a given organ and could be induced by exposure to homeostatic environmental cues (e.g., mechanical, metabolic) specific of distinct subtissular regions. In the gut, long-lived macrophages with precise subtissular localization are key regulators of physiological functions (25). In the lungs, alveolar macrophages (AM) originate almost exclusively from yolk-sac derived macrophages and self-maintain throughout adulthood (26) whereas lung interstitial macrophages follow a more complex regulation, unveiling further heterogeneity in this subset (27, 28). While some of these interstitial macrophages have an embryonic origin (27), others differentiate from distinct monocyte precursors according to the subtissular niche they colonize, thus becoming the dominant population during adulthood (22). As most studies rest on relative proportion of the different subsets, whether EmD-ResMac are replaced or dominated by MoD-ResMac needs to be confirmed. Along tissue seeding, circulating monocytes undergo significant gene modifications to become truly ResMac sharing strong similarities with their counterpart of embryonic origin. This differentiation is dictated by lineage determining factors but mostly instructed by the local environment (29–31) as even mature macrophages adoptively transferred can be reprogrammed by the tissue to a certain extent (32, 33). Little information is available regarding the functional identity of MoD-ResMac and EmD-ResMac (34), but evidence show that macrophages derived from classical monocytes (named here MoD-Mac) infiltrating the tissue in an inflammatory context harbor distinct transcriptomic profiles, display shorter life span [reviewed in Guilliams et al. (35)] and can be functionally distinct (36).

RECONSIDERING TAM ORIGIN

The characterization of macrophage ontogeny in tissue at steady state has rapidly raised the question of their presence in neoplastic tissues and their differential role in tumor development.

Until recently, TAM were considered to originate exclusively from monocyte precursors undergoing differentiation upon tissue infiltration but the distinction of TAM from different origins led us to reconsider this dogma (37–39). In most cancer models, blocking the CCL2/CCR2 axis leads to a strong decrease in TAM abundance. Because CCR2 is a major receptor involved in monocyte trafficking, it has contributed to the idea that TAM originate from bone marrow-derived $CCR2^{+}$ monocyte precursors (40–42). In an inducible lung carcinoma model, splenectomy resulted in a strong reduction in TAM. These spleen-derived TAM were shown to be also CCR2-dependent, suggesting that CCR2-deficiency does not necessarily account for a direct bone marrow provenance of TAM progenitors (43). However, deletion of *Ccr2* did not result in full depletion of macrophages suggesting that a CCR2-independent TAM accumulation or compensatory mechanisms might exist. CCR2-deficiency did not impact the relative proportion of TAM in the spontaneous PyMT-MMTV mammary carcinoma, but the use of $Ccr2^{DTR}$ system led to an almost complete elimination of TAM suggesting their monocytic origin (44). However, CCR2 expression by ResTAM could not be excluded, and would also sensitize them to the toxin.

Recent studies have confirmed that TAM of different origins accumulate within the TME in mouse cancer models. Using parabiotic mice and bone marrow transfer, it was shown that the pool of TAM was composed of both newly recruited MoD-Mac and ResMac in a model of pancreatic ductal adenocarcinoma. Fate mapping models strongly support that a significant fraction of these ResTAM have embryonic origin and actively proliferate along with tumor growth (38). Although no difference in tumor weight was observed in $Ccr2^{-/-}$ mice, ResTAM depletion using anti-CSF1R antibody and clodronate was associated with a strong reduction of tumor burden suggesting a dominant role of this population in tumor growth (38).

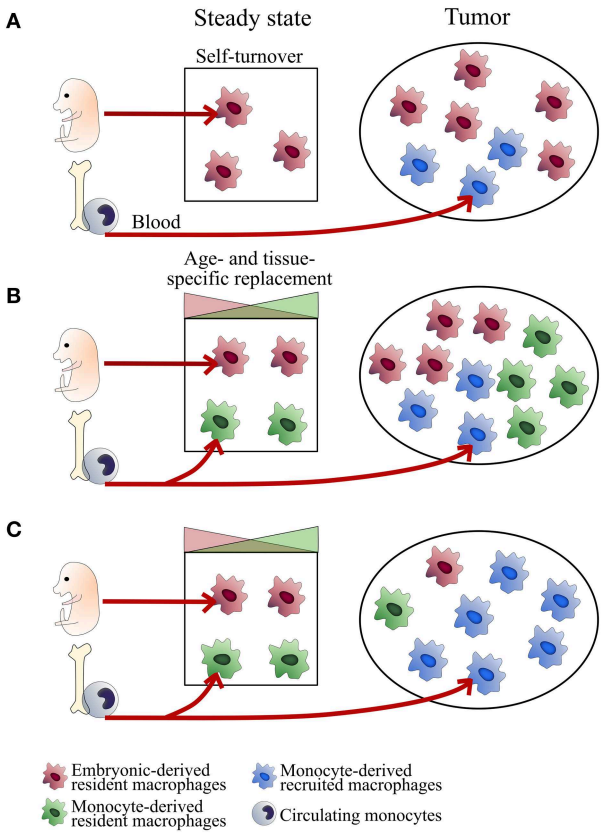

FIGURE 1 | Tissue-dependent heterogeneity of TAM origin. Schemes represent different scenarios of TAM ontogeny. TAM composition may depend on the regulation of macrophage self-maintenance and turnover. This regulation is tissue- and subset-specific. In some tissues, embryonic-derived resident macrophages self-maintain over time (typically microglial cells in the brain, alveolar macrophages in the lungs; Scenario **A**). Other subsets are progressively replaced by monocyte-derived macrophages with turnover rates depending on the subtissular niches (typically, rapid turnover for certain macrophages of the gut or the dermis and slow turnover for interstitial macrophages of the lung, heart and pancreas; Scenario **B**). At tumor onset, classical monocytes are recruited to the tumor (mainly in a CCR2-dependent manner) and differentiate into inflammatory TAM (MoD-TAM). Depending on tumor localization and the inflammatory state, ResMac proliferate (scenarios **A** and **B**) or not (scenario **C**) and contribute more or less to the pool of TAM (ResTAM), exerting distinct functions in tumor development. Scenarios **(A,B)** are expected in brain and lung tumors respectively. The use of inflammatory ectopic tumor models may bias toward scenario **(C)**.

The expansion of resident interstitial macrophages with the development of multifocal lung tumors was also observed by Loyher et al. (37). Fate mapping experiments unveiled that at least a fraction of these TAM had an embryonic origin and greatly expand with tumor development. Interestingly AM, the typical embryonic-derived macrophages in the lung, did not expand and the relative proportion of ResTAM and recruited MoD-TAM was dependent on the anatomical niche of tumor development (37).

In the brain, conflicting results have been published regarding TAM origin (45). Microglial cells were shown to be the major contributor in several studies whereas others supported an accumulation of MoD-TAM (46–48). As several models used irradiation to test whether classical monocytes were able to replenish the brain, the disruption of the blood brain barrier may have artificially increased the accumulation of MoD-TAM (34, 49). Major contribution of this last population was demonstrated in primary and metastatic brain tumors (39). Different transcriptional profiles as well as different epigenetic landscapes were observed between microglia and MoD-TAM, associated with different activation patterns. The comparison with macrophages from healthy brain tissue revealed that some features shared by both TAM populations were not dependent on ontogeny but were "taught" by the TME. Additionally, CD49d was identified as a potent marker to discriminate microglia- vs. MoD-TAM in both murine models and human brain tumors (39).

Based on this work, single cell RNA sequencing was performed on macrophages from glioma or non-malignant human tissues (50). From 237 lineage-specific murine TAM genes, they compared the homologous genes in human samples and identified two TAM subsets that correlated with microglial enriched or bone marrow-derived TAM enriched genes. These two profiles were thus hypothesized to reflect the differential ontogeny of TAM in human brain tumors (50).

ONTOGENY AS A NEW FEATURE OF TAM DIVERSITY

So far, few works support that TAM can be composed of newly recruited MoD-TAM mostly in a CCR2-dependent manner, but also ResTAM of embryonic origin (EmD-ResTAM) or arising from adult hematopoiesis (MoD-ResTAM) that locally proliferate and accumulate with tumor expansion. Whether this assumption can be generalized to other models deserve further investigation and the transposition to human tumors is even more hypothetical due to the lack of knowledge in macrophage ontogeny. Combining fate mapping models with RNA sequencing from mice to identify specific signature based on homologous human genes might be a valuable approach to track macrophage ontogeny in humans.

According to the model proposed for macrophages niches at steady state (23), the relative proportion of the different TAM subsets may vary with age, organs, subtissular niches, and the inflammatory state of tumor development (**Figure 1**). Understanding the relative importance of ResMac vs. MoD-Mac in the pool of TAM is limited by the lack of clear markers to discriminate them both in mice and human. Moreover, the use of experimental ectopic tumor models inducing local inflammation could bias the composition of the TAM compartment (scenario C in **Figure 1**). Ontogeny may represent a source of heterogeneity, hence an alternative classification in TAM diversity in addition to the common M1/M2 nomenclature.

THE M1/M2 NOMENCLATURE MODEL IN TAM ORIGIN

The common characterization of TAM subsets relies on the M1/M2 polarization model induced by different *in vitro*

stimuli (18). This model rapidly finds limitation in complex environments (*in vivo*) in which M1 and M2 stimuli can be present and generate very dynamic microanatomical niches. Tumors should be considered as an evolving tissue in which space availability and growth factors expression are changing over time (51, 52) and where inflammatory signals are generated by the loss of tissue integrity and immune cell infiltration (53). It is thus not surprising to find a wide range of activation profiles in the TME (18–20, 45). No typical M1/M2-associated marker defined one or the other TAM subset in lung unveiling heterogeneity among each subset (37). No direct link between TAM origin and the commonly described pro- or anti-tumor profile could be achieved in this study. One could expect that macrophage ontogeny and their anatomic localization define specific niches dictating their polarization toward a specific phenotype and function.

TAM FUNCTION ACCORDING TO THEIR ORIGIN

Despite recent works discriminating resident TAM vs. recruited TAM, their relative function in the TME has been barely addressed. The absence of phenotypic markers defining TAM according to their origin limits the possibility for functional studies. As previously mentioned, *Ccr2* deletion has been very useful to generate a TME with a largely reduced infiltration of recruited MoD-TAM while ResTAM seemed to be less affected. The variable extent of macrophage deletion observed between the different models may be related to the relative proportion of resident and recruited TAM. In most cases, the impaired macrophage accumulation in the TME was associated with a better control of the tumor and reduced metastatic dissemination (54–57) suggesting a major role for MoD-TAM in these processes. For instance, no difference in lung tumor burden was observed in CCR2-deficient mice compared to WT although nodules were smaller and more disperse suggesting that both MoD-TAM and ResTAM contributed to tumor growth but the presence of the former was associated with increased tumor cell spreading (37). Accordingly, CCL2 secretion by breast tumor cells activated Wnt-1 production by mammary intra-epithelial macrophages inducing an epithelial/mesenchymal transition-like signaling on cancer cells and driving early cancer dissemination (58).

In $Ccr2^{-/-}$ mice engrafted with colorectal cancer, the reduction in TAM was associated with reduced tumor burden along with altered ECM composition (59). Genomic and proteomic analyses revealed upregulation of collagen synthesis and deposition in monocytes differentiating into TAM. CCR2-dependent TAM were shown to have a primary role in shaping the TME, thus promoting tumor expansion. On the other hand, Madsen and colleagues showed that $CCR2^{+}$ MoD-TAM were responsible for collagen degradation in the TME in various tumor models. Transcriptomic analysis of these cells revealed a catabolic signature related to ECM degradation in this subset (60). These paradoxical observations suggest that different CCR2-dependent TAM subsets might be implicated in deposition and degradation of collagen in the TME. However, Res-TAM from pancreatic ductal adenocarcinoma were also shown to exhibit a pro-fibrotic profile, with increased expression of genes involved in ECM deposition and remodeling, which is a hallmark of this cancer. On the other hand, MoD-TAM were more efficient antigen-presenting cells (38).

Finally, in brain tumor, microglial-cells were enriched in pro-inflammatory genes as well as factors involved in ECM remodeling while MoD-TAM exhibited an immunosuppressive signature associated with immune suppression (39). In human glioma samples, MoD-TAM infiltration correlates with tumor grade. These TAM also exhibit an immunosuppressive profile with increased immunosuppressive cytokine expression. As observed by Chen et al. in a mouse model (61), these cells localize in necrotic regions and perivascular areas while microglia-derived TAM were found at the edge of the tumor (50).

Altogether, most studies rely on transcriptomic analysis and highlight functional profiles of resident vs. recruited TAM that cannot be fully associated with their origin across the different models. In addition, very little information is available regarding suppression of the adaptive response which is a key feature of TAM biology. Functional differences might be linked with the differential cues from the TME that polarize the macrophages in a niche-specific manner in addition to their ontogeny-specific features. Live imaging studies represent a complementary approach to compare functional difference between TAM subsets as reported in the lungs (37) and recently in the brain (62). Further studies using fluorescent strains and lineage-tracing models (63) will be necessary to better address the functional features of TAM subsets to better understand their role in tumor development as well as resistance to anti-cancer therapies and unveil key target for immunotherapy.

RESPONSE OF TAM SUBSETS TO ANTI-CANCER THERAPIES

Apart from their direct impact on tumor cells anti-cancer therapies display many immune-mediated effects. In addition to conventional treatments, many immunotherapies to boost the anti-tumor immune response are under investigation. TAM are usually considered as a factor of resistance to many therapies (64–66) but paradoxical roles in their efficacy are reported. Whether these contrasting roles are related to their ontogeny is unknown. Therefore, elucidating how TAM subsets are impacted by anti-cancer treatments is crucial especially in the context of combined therapies. So far, very few studies have addressed the selective targeting of TAM from different origins.

Following myeloablative chemotherapy using cyclophosphamide, we showed that both resident and recruited TAM were depleted by the alkylating agent in lung tumor (37). Recruited TAM rapidly recovered through a transient and massive wave of bone marrow-derived monocytes and TAM, while ResTAM recovery was much more limited. This wave contributed to tumor cell destruction and phagocytosis suggesting that in certain cases TAM are potent effector of the anti-tumor response. Specific targeting of TAM displaying

protumor function without affecting tumoricidal activity is thus required in these conditions.

For instance, anti-CSF1R is quite efficient to deplete TAM in both human and mouse tumors (67) but its clinical efficacy is limited and leads to compensatory mechanisms (68). Mouse models suggest that anti-CSF1R treatment depletes efficiently certain subsets of ResMac but its effect on monocytes showed conflicting results that could be explained by variable dependency on CSF1R across different tumor microenvironment (45, 69, 70). In a lung tumor model, anti-CSF1R treatment blocked monocyte accumulation and differentiation into MHC-II^{lo} TAM, indicating a role for this axis in monocyte recruitment beyond CCL2/CCR2 (69). However, the impact on tumor growth was not reported. Another study in the lung showed strong depletion of TAM following anti-CSF1R administration although monocytes were not affected (70). No effect on tumor growth was observed, suggesting either that ResTAM are irrelevant to tumor growth or that some macrophage subsets involved in anti-tumor response could also be depleted. These studies were performed with different anti-CSF1R clones, which might have different pharmacological action.

PD1/PDL1 represents another promising approach to target macrophages as PD1 expression by macrophages increases along tumor growth (71). Anti-PD1 therapy was shown to induce a macrophage-dependent anti-tumor efficacy in a subcutaneous injected colon cancer cell line (71). Using bone marrow transplant of RFP^{+} cells it was shown that PD1^{+} TAM were mainly of medullar origin, although the use of fully reconstituted irradiated chimera may impact the compartment of resident MoD-TAM.

Restoring antigen presentation in the TME is essential to induce an effective T-cell anti-tumor response (72). The SIRPα/CD47 signaling axis is a "don't eat me" signal that is being hijacked by tumor cells to abrogate phagocytosis by TAM, thus impairing antigen processing. CD47 has been shown to be overexpressed in several cancer indications. Immunotherapy targeting CD47 has shown promising results in various tumors, including brain tumors (11, 73, 74). CD47 blockade was tested in glioblastoma pre-clinical models and showed a differential response of ResTAM vs. MoD-TAM. Both subsets showed enhanced phagocytosis upon treatment, but microglia-derived TAM displayed less inflammatory response. This was associated with prolonged mouse survival. The anti-CD47 effect on microglia was maintained in CCR2-deficient mice although the survival did not reach the same value as in WT mice (75). These results indicate that microglia-derived TAM might be the main subset involved in antigen presentation to T-cell in glioblastoma.

The development of immunotherapies targeting the myeloid compartment is challenging as targeting TAM is a double-edged sword process and the selective depletion of pro-tumoral macrophages without affecting the anti-tumor function would be idealistic.

CONCLUDING REMARKS

The characterization of TAM ontogeny is still in its infancy. The lack of specific markers to discriminate and selectively target them for functional studies represents a technical limitation. Fate-mapping models and fluorescent reporters have revealed a differential contribution of tissue-resident and inflammatory macrophages in the pool of TAM in several tumor models, but no specific functional profile could be attributed to their origin across different cancer indication so far. Indeed, the contribution of TAM subsets follows complex spatio-temporal dynamics as macrophage niches evolves throughout life with specific regulation at the subtissular level depending on the organ and the age. Better characterization of how these subsets are differentially affected by anti-cancer therapy is of major importance to be able to selectively target them and thus promote the anti-tumor immune response.

AUTHOR CONTRIBUTIONS

ML and AB wrote the manuscript.

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MINI REVIEW
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In vivo Differentiation of Human Monocytes

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Circulating monocytes can infiltrate mucosal or inflamed tissues where they differentiate into either macrophages or dendritic cells. This paradigm is supported by numerous studies conducted in mice and in different *in vitro* settings for human cells. Determining whether it holds true *in vivo* in humans is essential for the successful design of monocyte-targeting therapies. Despite limitations inherent to working with human samples, there is accumulating evidence of the existence of *in vivo*-generated monocyte-derived cells in humans. Here, we review recent studies showing the recruitment of human monocytes into tissues and their differentiation into macrophages or dendritic cells, in normal or pathological settings. We examine the methods available in human studies to demonstrate the monocytic origin of infiltrating cells. Finally, we review the functions of human monocyte-derived cells and how they might contribute to pathogeny.

Keywords: human, monocyte, macrophage, dendritic cell (DC), inflammation

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INTRODUCTION

Numerous studies in mice have shown that monocytes circulate in the blood and are recruited to mucosal tissues or inflammation sites, where they can differentiate into monocyte-derived macrophages (mo-Mac) or monocyte-derived dendritic cells (mo-DC) (1, 2). In models of inflammatory disorders, monocyte-derived cells have been shown to exert a deleterious role, in particular by fueling the inflammation and inducing tissue damage. Blocking their recruitment to inflamed tissues, using nanoparticules that induce apoptosis (3) or that contain si-RNA against CCR2 (4), reduces inflammation and improves the pathogeny in mouse models of colitis, peritonitis, and atherosclerosis. Monocytes have therefore emerged in the past few years as an attractive therapeutic target.

However, findings from mouse models do not always translate to humans due to genetic, physiological, and environmental differences. In particular, whether mice represent an appropriate model for analyzing inflammatory responses and chronic inflammatory diseases has been controversial (5–7). Despite inherent limitations, observations in humans are therefore essential to complement mouse studies to fully understand monocyte biology and the contribution of monocyte-derived cells to inflammatory disorders.

MONOCYTE LIFE CYCLE

Circulating monocytes are classified into three subsets based on the expression of the surface markers CD14 and CD16: "classical" CD14^{high}CD16-monocytes (around 85% of monocytes), "intermediate" CD14+CD16+ monocytes (5–10%) and "non-classical" CD14-CD16^{high} (5–10%) monocytes. The life cycle and relationship of these subsets has been the subject of recent *in vivo* studies.

Several lines of investigation point to a linear differentiation relationship between monocyte subsets. Using *in vivo* labeling with a short pulse of 6,6-2H2-glucose in healthy volunteers, two studies have reported the sequential enrichment in the blood of labeled CD14^{high} monocytes, then CD14+CD16+ monocytes and finally CD16^{high} monocytes (8, 9). Similarly, following autologous stem cell transplantation, CD14^{high} monocytes reappeared first in the blood after 7 days, followed by CD14+CD16+ monocytes and then CD16^{high} monocytes after 10 days (10). Moreover, after *in vivo* endotoxin challenge in healthy volunteers, monocytes disappeared from the circulation within 2 h with CD14^{high} monocytes recovering after 4 h, then CD14+CD16+ monocytes and CD16^{high} monocytes after 24 h (9, 11). Mathematical modeling indicated that CD14^{high} monocytes have a short lifespan in the blood of 1–2 days, before differentiating into CD14+CD16+ monocytes or disappearing from the circulation (8, 9).

Consistent with this model, single-cell RNA-seq (scRNA-seq) analysis of blood monocytes showed that CD14+CD16+ monocytes are a heterogeneous population with mixed transcriptional profiles (12). In an independent scRNA-seq analysis, purified CD14^{high}CD16- monocytes were shown to contain two subsets: one with a typical transcriptional profile of CD14^{high} monocytes and one with a profile closer to CD16^{high} monocytes, suggesting that part of the CD14^{high} monocytes are already en route to differentiate before up-regulation of CD16 (13).

Collectively, these observations support the notion that CD14^{high} monocytes represent the precursor population of both CD16^{high} monocytes in blood and monocyte-derived cells in tissues.

MONOCYTE RECRUITMENT INTO TISSUES

In mice, circulating monocytes leave the bloodstream to infiltrate mucosal tissues or inflamed sites, or to reside in the spleen. What is the evidence that the same scheme applies to humans?

Several studies have shown monocyte recruitment in the context of acute inflammation. In dialysis-induced bacterial peritonitis, CD14+ monocytes number was increased in the peritoneum 1 day after infection (14). In the acute inflammation model of skin blister, a high proportion of CD14+ cells was observed 24 h after blister formation, suggesting monocyte recruitment (15). Similarly, CD14+ cell number increased in the nasal mucosa 12 h after allergen challenge in a model of allergic rhinitis (16) and in the bronchoalveolar lavage 8 h following LPS inhalation (17). Furthermore, S100A8/9+ cells accumulated in the bronchial mucosa of patients who died from asthma attack as compared to non-atopic controls (16). Monocytes also infiltrate the heart following acute myocardial infarction, as shown by the increase in CD14+CD16- and CD14+CD16+ cells as compared to heart tissue from donors who died of other causes (18). This strong influx of monocytes correlated with a decrease in the proportion of CD14+ cells in the bone marrow and spleen, suggesting that the spleen could be a monocyte reservoir in humans. This is consistent with the presence of *bona fide* monocytes in human spleen (19).

Monocytes also infiltrate tissues in chronic inflammation. In inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC), an increased infiltration of monocytes was described in the colon. Following injection of radiolabelled monocytes, radioactive CD14+ cells were detected in the intestine of patients with intestinal inflammation (20) and in joints of patients suffering from rheumatoid arthritis (RA) (21). Moreover, CD14^{high}CD11c^{high} monocytes were increased in the inflamed mucosa of CD patients as compared to control samples (22–24). During multiple sclerosis (MS), infiltrating monocytes were detected in MS lesions (25). CD14+ CCR2+ CD16- MerTK- cells were identified in the synovial fluid from gouty arthritis patients, suggesting recruitment of monocytes (26). In cancer, monocytes were detected in lung tumors (27) and breast tumors (28) using scRNA-seq analysis.

Monocyte recruitment in steady-state tissues has also been evidenced in a few studies. Extravascular monocytes have been observed in lung from organ donors (29). Monocyte-derived cells have been described in non-diseased intestine, liver and skin (see below).

To conclude, there is ample evidence that, similarly to mice, human monocytes are recruited at steady state in tissues and mucosa to replenish the niche, and in acute and chronic inflammation. In these different contexts, monocytes will further differentiate.

DEMONSTRATING THE MONOCYTIC ORIGIN OF CELLS ISOLATED FROM HUMAN TISSUES

In mice, tracking monocyte fate from blood to tissues can be accomplished by adoptive transfer or genetic lineage tracing methods. These techniques are obviously not directly transposable to humans. When working with human samples, it is necessary to use alternative approaches to demonstrate the monocytic origin of tissue myeloid cells.

Phenotyping is the most widely used technique. It relies on the postulate that phenotypic markers expressed by blood monocytes will persist in tissues after their differentiation, allowing the identification of monocyte-derived cells. CD14, CCR2, and CX3CR1 are some of the most commonly used markers (**Table 1**). However, these molecules are not exclusive of monocyte-derived cells. CD14 is also expressed by tissue macrophages derived from embryonic precursors (40). CX3CR1 is highly expressed by microglia (1) and pre-DC (12). Finally, a population of pre-DC-derived CCR2+ DC has been described in mouse intestine (41).

Recently, S100A8/9 (calprotectin, an intracellular protein) has been proposed as a reliable marker for monocytes and monocyte-derived cells (34). S100A8/9 was detected both at the mRNA and protein levels in circulating monocytes (35, 42, 43). In the intestine, S100A8/9 expression in DC inversely correlated with Flt3 expression and S100A8/9 was highly expressed in short-lived myeloid cells, but not in long-lived macrophages (34).

TABLE 1 | Surface markers commonly used to distinguish DC, macrophages, and monocytes.

Surface markers	cDC1	cDC2	Resident macrophages	mo-Mac	mo-DC	CD14 + monocytes
CCR2	−	−	−	+	+	+
CD11b	−	Tissue dependent	+	+	+	+
CD11c	+	+	+	+	+	+
CD14	−	−	Tissue dependent	+	+	++
CD141	++	+	low	low	+	−
CD16	−	−	Tissue dependent	+	−	−
CD163	−	−	Tissue dependent	+	−	−
CD172a (Sirpa)	−	+	+	+	+	+
CD1a	−	Tissue dependent	−	−	+	−
CD1b	−	+	−	−	+	−
CD1c	−	+	−	−	+	−
CD206	−	Tissue dependent	Tissue dependent	+	++	−
CD209	−	−	+	+	+	−
CD226	+	−	−	−	+	−
CD64	−	Tissue dependent	+	+	+	low
CD88	?	?	?	+	+	−
Clec9A	+	−	−	−	−	−
CX3CR1	−	Tissue dependent	Tissue dependent	+	+	+
FceRI	−	+	−	−	+	−
HLA-DR	+	+	+	+	+	+
MerTK	−	−	+	+	−	−
S100A8/A9	−	−	−	+	+	++
Main references	(30–32)	(30–33)	(30, 33–35)	(13, 14, 35–37)	(13, 14, 37–39)	(31)

Collectively, this evidence suggests that S100A8/9 can be used as a marker for monocyte-derived cells.

Phenotyping is an easy way to characterize cellular identity. However, most markers are not specific to monocytes and monocyte-derived cells, and are tissue-dependent. Analyzing a combination of markers can increase the robustness of this approach.

Chimerism in the context of transplantation is another method to demonstrate a monocytic origin. This approach is based on the fact that following transplantation, cells from the recipient will repopulate the transplanted tissue while long-lived cells remain of donor origin, resulting in cellular chimerism. Resident macrophages derived from embryonic precursors are self-maintaining (1). Studying the replacement of tissue resident macrophages by monocyte-derived cells can be performed by analyzing markers of donor-recipient mismatch. For example, cells derived from the recipient's monocytes were evidenced in transplanted heart using *in situ* hybridization for Y chromosome (36). Cells derived from the donor or the recipient have also been distinguished by HLA-mismatch in the intestine (34, 35).

This method is an elegant way to analyse monocyte recruitment and differentiation in human tissues. However, it is not broadly available due to restricted access to transplanted tissue samples.

Another procedure to analyse monocyte fate is labeling, *ex vivo* or *in vivo*. For example, autologous monocytes were radiolabelled *ex vivo* and re-injected to patients with IBD (20) or RA (21). Monocytes can also be labeled *in vivo* through injection of ultra small particle iron oxide (USPIO). Labeled cells were observed in brain lesions of MS patients (25). This method allows a direct tracking of monocytes. However, it remains unconventional as it requires very specific procedures and ethics agreements.

Specific gene expression signatures can also be used to infer developmental origin. This approach is based on the idea that ontogeny will leave a transcriptomic imprint in monocyte-derived cells. One method is to perform comparative transcriptomic analysis, including blood monocytes as a reference. As an example, intestinal CD103-SIRPa+ cells (44) or CD14+ cells (34) clustered with blood monocytes, suggesting that these populations are related. Another method is to use transcriptional signatures specific of mo-Mac and mo-DC for enrichment analysis. scRNA-seq data of macrophages found in human fibrotic lung was annotated with signatures from bulk RNA-seq data from mouse macrophages and DC (45). This analysis revealed that pro-fibrotic macrophages may originate

from monocytes. Similarly, scRNA-seq data from human ascite myeloid cells was analyzed using gene signatures generated from bulk RNA-seq of tissue-derived and *in vitro*-generated mo-DC and mo-Mac (46).

This approach has the advantage of being unbiased and requires no prior knowledge of putative "marker genes." However, it is essential to use robust transcriptomic signatures as a reference.

The monocytic origin of tissue macrophages and DC can be demonstrated using various techniques. As none of these methods can lead to definitive conclusions, it is necessary to combine them to provide strong evidence that the cells of interest derive from monocytes.

IN VIVO DIFFERENTIATION INTO MO-MAC AND MO-DC

Human monocytes can be differentiated *in vitro* into mo-Mac or mo-DC in various culture conditions. However, do monocytes have the capacity to differentiate into both macrophages and DC *in vivo* in humans?

How to Distinguish mo-Mac From mo-DC?

Distinguishing DC from macrophages by phenotyping is challenging as they share a lot of markers. MerTK, CD68, CD163, and the transcription factor MAFB are considered robust markers of macrophages, while DC express CD1a, CD1b, FcεRI, and CD226 (**Table 1**). Other techniques can help confirming cell identity. Analyzing cell morphology is a robust method for this (14, 36, 37, 47). Macrophages are large cells containing many phagocytic vesicles. By contrast, DC are smaller and display dendrites on their surface. Finally, transcriptomic approaches can also be used to distinguish mo-Mac from mo-DC, for instance by performing enrichment analysis for transcriptional signatures specific of DC and macrophages (34, 44, 46, 48–50).

Identification of mo-Mac and mo-DC in Human Tissues

Many studies describe the presence of mo-Mac in different tissues at steady state. In the small intestine, two subsets of macrophages were replaced 3 weeks following transplantation by recipient cells (35), demonstrating that they derive from monocytes. Monocytes also participate in the replenishment of skin macrophages (33). Following allogeneic hematopoietic stem cell transplantation, CD14+ cells reappeared after 8 days in blood and after 12 days in normal skin. This sequential detection of CD14+ cells suggested that blood monocytes give rise to skin CD14+ macrophages (33). Monocyte differentiation into mo-Mac also occurs in tissues with lower self-renewal capacities. For example, mo-Mac were identified in the heart (36), lung (43), and liver (51).

Furthermore, mo-Mac have been described in different inflammatory settings. The increased presence of macrophages has been observed in the colon of IBD patients (52, 53). CCR2 expression on their surface suggested their monocytic origin. In the cantharidin-induced skin blister model, following monocyte recruitment, HLADR+CD14+CD16+ cells increase their expression of CD163 suggesting that they differentiate into mo-Mac (15). In dialysis-induced peritonitis, CCR2+ mo-Mac are increased in the peritoneal cavity as compared to normal dialysis (14). Finally, mo-Mac are detected in tumors. In glioma patients, mo-Mac were identified in the brain by CX3CR1 expression and transcriptomic analysis (49). In melanoma, scRNAseq analysis evidenced one population expressing both macrophage and monocyte genes suggesting the presence of mo-Mac (48).

Similar findings apply to mo-DC. At steady state, mo-DC are mainly described in the intestine. Intestinal SIRPa+CD103- DCs (44) and SIRPa+CD103-CD14+ DCs (34) are transcriptionally related to blood monocytes. Moreover, S100A8/9 expression suggested that this population derives from monocytes (34). The presence mo-DC expressing CCR2 and S100A8/9 has also been suggested in non-diseased lung (54). In CD, CD14+CD163-MerTK- cells from inflamed gut exhibited a typical DC morphology and scRNA-seq showed signatures of monocyte lineage, suggesting that these cells are mo-DC (47). CCR2+ DC were also evidenced in the peritoneum at steady-state (12).

There is also evidence of monocyte differentiation into mo-DC in an inflammatory context. In atopic dermatitis and psoriasis, early studies have suggested monocyte differentiation into mo-DC (55–57). An increased proportion of "inflammatory" DC was found in atopic dermatitis and psoriasis patients in comparison to healthy skin (55). Their phenotype (CD1a+ FceRI+ CD206+) is reminiscent of that of mo-DC identified in subsequent studies. Similarly, DC displaying a phenotype consistent with mo-DC were observed in pleural effusions of tuberculosis patients (58) and evidenced in colorectal and breast tumors (38, 50). In lung cancer, phenotypic analysis as well as transcriptomic signatures in scRNAseq data suggested the presence of mo-DC (27, 38, 59). Using gene signatures, peritoneal ascite DC from ovarian cancer patients were also identified as mo-DC (46).

Collectively, these observations relying on phenotypic, morphological and transcriptomic analysis support the notion that human monocytes can differentiate *in vivo* into both macrophages and DC.

FUNCTIONAL PROPERTIES OF MO-MAC AND MO-DC

Classical DC and tissue resident macrophages play major roles in the initiation and resolution of immune responses. Do the DC and macrophages derived from monocytes display the same functions as their classical counterparts, or have specific functional properties?

Secretion of Soluble Mediators

A major property of myeloid cells is the secretion of soluble mediators. Mo-DC and mo-Mac have been reported as strong

producers of pro-inflammatory cytokines such as TNFα and IL1β. Mo-Mac from healthy intestine or from the colon of CD and UC patients secreted higher levels of TNFα as compared to their tissue resident counterparts, with or without *ex vivo* restimulation (35, 52, 60). Mo-DC from the inflamed colon of CD patients produced high levels of IL1β (36, 50). Peritoneal ascites mo-DC and mo-Mac also secrete high levels of TNFα and IL1β (37). Furthermore, heart mo-Mac are potent producers of IL1β in contrast to CCR2- tissue resident macrophages (36).

IL23 secretion is more specific to mo-DC. Mo-DC from the inflamed intestine of CD patients or from peritoneal ascites of cancer patients secreted significant levels of IL23 with (37) or without *ex vivo* restimulation (47). Similar results were obtained with mo-DC from pleural effusions of tuberculosis patients restimulated *ex vivo* with *Mycobacterium tuberculosis* (58). Although IL23 seems to be mainly produced by mo-DC, it has also been reported for mo-Mac from CD patients (52). By contrast, IL12 is produced by mo-DC but not mo-Mac (46).

Finally, a few studies reported the production of the anti-inflammatory cytokine IL-10 by mo-Mac. In the context of CD, mo-Mac from inflamed colon secreted high levels of IL-10 with (52) or without restimulation (60). *Il10* mRNA levels were upregulated in mo-Mac from CD patients after 24 h of culture without any stimulatory signal (36). Finally, mo-Mac from glioma were enriched for *Il10* expression (49).

Taken together, these findings indicate that mo-DC and mo-Mac are strong producers of pro-inflammatory cytokines, which are essential for the recruitment of immune cells at an injured site and for the initiation of immune responses. However, in chronic inflammation, cytokine secretion by mo-DC and mo-Mac is exacerbated and contributes to the pathogenesis.

Fibrosis

Macrophages are key actors in wound healing and tissue repair by secreting growth factors for fibroblasts. The dysregulation of this mechanism can lead to fibrosis (61). There is evidence that mo-Mac participate in fibrosis. CX3CR1+ mo-Mac expressing pro-fibrotic Platelet Derived Growth Factor AA (PDGFAA) accumulated in fibrotic regions of lungs in comparison with non-fibrotic regions (45). Mo-Mac from cardiomyopathic heart expressed genes coding for growth factors and extracellular matrix components known to be involved in fibrosis (36). Moreover, these mo-Mac accumulated in scar or fibrotic tissues regions.

CD8 T Cell Responses

Few studies have investigated the role of monocyte-derived cells in CD8 T cell responses. Both mo-DC and mo-Mac isolated from peritoneal ascites were able to cross-present antigens using a non-conventional intracellular pathway (46), consistent with another study using peritoneal mo-Mac and mo-DC from peritoneal dialysis (14). Of note, the ability to cross-present was specific of mo-Mac as compared to lymphoid organ macrophages (62). However, only mo-DC could provide costimulatory signals for the differentiation of effector cytotoxic CD8 T cells (46).

CD4 T Cell Responses

One of the major roles of classical DC is to orient CD4 T cell polarization. Several studies have shown that mo-DC, but not mo-Mac, have the same property. Mo-DC isolated from synovial fluid of RA patients were better activators of CD4 T cell proliferation than mo-Mac from the same environment and induced Th17 polarization (37, 63). Th17 polarization was also induced by mo-DC from pleural effusions of tuberculosis patients (58) and from the inflamed colon of CD patients (47). Of note, mo-DC from synovial fluid of RA patients and from pleural effusions of tuberculosis patients were able to induce the proliferation of autologous CD4 T cells, showing that they can present antigens that were captured *in vivo* (58, 63). This Th17 polarization was associated with high secretion of IL-23 which is known to promote Th17 cells (37, 47, 58). In other studies, mo-DC from healthy small intestine or inflamed mucosa of CD patients preferentially induced Th1 polarization over Th17 (44, 52). This IFNγ production contributed to the pathogenesis of CD (52).

Finally, mo-DC from synovial fluid of RA patients and from peritoneal ascites induced CXCL13 secretion by CD4 T cells, suggesting Tfh polarization (64).

Collectively, these observations underline the capacity of mo-DC to polarize naïve CD4 T cells. In particular, Th17 polarization could contribute to the maintenance of chronic inflammation in Th17-driven pathologies such as RA and IBD.

In conclusion, mo-DC and mo-Mac share with their classical counterparts some of their hallmark functions (T cell stimulation for DC and tissue repair for macrophages). Ontogeny seems to influence mostly cytokine secretion, with mo-DC and mo-Mac being stronger producers of pro-inflammatory mediators than classical DC or resident macrophages from the same tissues. More studies using side-by-side comparisons will be needed to confirm this mixed functional profile.

CONCLUSION

Despite methodological limitations inherent to human samples, numerous studies support the notion that human monocytes can differentiate *in vivo* into DC or macrophages. This process occurs at steady state to replenish the niche, but can also play a major role in the initiation and maintenance of chronic inflammatory diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases

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Human monocytes are divided in three major populations; classical ($CD14^{+}CD16^{-}$), non-classical ($CD14^{dim}CD16^{+}$), and intermediate ($CD14^{+}CD16^{+}$). Each of these subsets is distinguished from each other by the expression of distinct surface markers and by their functions in homeostasis and disease. In this review, we discuss the most up-to-date phenotypic classification of human monocytes that has been greatly aided by the application of novel single-cell transcriptomic and mass cytometry technologies. Furthermore, we shed light on the role of these plastic immune cells in already recognized and emerging human chronic diseases, such as obesity, atherosclerosis, chronic obstructive pulmonary disease, lung fibrosis, lung cancer, and Alzheimer's disease. Our aim is to provide an insight into the contribution of human monocytes to the progression of these diseases and highlight their candidacy as potential therapeutic cell targets.

Keywords: human monocytes, atherosclerosis, diet, respiratory diseases, neurodegeneration

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INTRODUCTION

Human monocytes were originally defined by their distinctive morphology at the beginning of the previous century by Paul Ehrlich and Ilya Metchnikoff [reviewed in (1)]. The invention of flow cytometry in the 1970s enabled the design of a monocyte-specific antibody panel based on the surface protein levels of the pattern recognition receptor CD14 and the Fc gamma III receptor CD16 (2).

Two populations were identified; the classical ($CD14^{++}CD16^{+}$) and the non-classical ($CD14^{dim}CD16^{+}$) (2). Subsequently, an intermediate for CD14 and CD16 ($CD14^{+}CD16^{+}$ $HLA\text{-}DR^{+}CD86^{+}CD11c^{+}$) monocyte population with a distinct transcriptomic profile (*LYZ*, *S100A8*, *CD14*, *S100A10*, *HLA-DRA*, *CD74*, *IFI30*, *HLA-DPB1*, *CPV*) was discovered (3–5). At this time, it was also suggested that this population can be separated from non-classical monocytes by the expression of 6-sulfo LacNAc (SLAN) (6). These "intermediate" monocytes displayed comparable ROS production and phagocytosis potential, lower adhesion to surfaces, but demonstrated higher class II molecule expression and IL-12 production than classical monocytes (3, 4). In mice, two monocyte subsets were identified in the bloodstream by flow cytometry and intravital microscopy; a short-lived $Gr\text{-}1^{+}CCR2^{+}CX_3CR1^{lo}$ which migrates to tissues during inflammation and a $Gr\text{-}1^{-}CCR2^{-}CX_3CR1^{hi}$ one, which carries out CX_3CR1-dependent patroling of the vasculature during homeostasis (7–9).

Investigation in the developmental trajectories of the three described monocyte subsets with deuterium labeling in humans suggested that intermediate and non-classical monocytes emerge sequentially from the pool of classical monocytes (10). In fact, mathematical modeling of

monocyte differentiation demonstrated a linear trajectory from classical monocytes to non-classical monocytes, although it is very likely that not all of them follow the same path or that the intermediate to non-classical monocyte step occurs outside the bloodstream (11). An elegant study provided another line of evidence to support this concept. In particular, endotoxin challenge led to a rapid loss of all monocyte subsets. However, their re-appearance from the bone marrow or marginated pools followed different kinetic patterns; classical monocyte numbers were restored first, with intermediate and non-classical monocytes following. Of note, the first two subsets followed a peak in CCL2, CCL3, and CCL4 blood levels, in contrast to the latter which were sensitive to CX_3CL1 (12). In mice, monocyte development clearly occurs in the bone marrow where granulocyte-monocyte (GMP) and monocyte-DC (MDC) progenitor pools produce functional monocytes (13). Furthermore, during infections, monocyte progenitor reprogramming happens already in the bone marrow (14).

With the development of multi-dimensional single-cell techniques, assessment at the single-cell transcriptome level unexpectedly suggested 4 monocyte subsets in healthy volunteers; classical, non-classical, and 2 monocyte subsets, one expressing genes involved in cell cycle, differentiation and trafficking and the other being associated with a NK cell-like signature (15). By generating new single-cell transcriptomics data we now have evidence that the latter monocyte subset was due to misclassification of a particular subset of NK cells, indicating that the current model with 3 major subsets is still valid (16).

Classical monocytes were found to be primed for phagocytosis, innate sensing/immune responses and migration, intermediate monocytes were the only subset expressing *CCR5* and were well-suited for antigen presentation, cytokine secretion, apoptosis regulation, and differentiation and non-classical monocytes are involved in complement and Fc gamma-mediated phagocytosis and adhesion (17, 18). However, it was also concluded that the current monocyte subsets are not homogeneous populations, but instead can be clustered in smaller, transcriptionally distinct subsets (17).

Using a mass cytometry approach, Thomas and colleagues showed that traditional gating on CD14 and CD16 frequently led to contaminations of intermediate and non-classical monocytes; instead, the addition of markers, such as CD36, CCR2, HLA-DR, and CD11c enabled more precise separation of human monocytes (19). Another mass cytometry protocol increased the resolution of the non-classical monocyte phenotype and distinguished $CD14^{dim}CD16^{+}SLAN^{-}$ from $CD14^{dim}CD16^{+}SLAN^{+}$ non-classical monocytes. All non-classical monocytes in this study exhibited less CD36, CD64, CCR2, CD11b, and CD33, but more CD45, CD11c, and HLA-DR expression than classical and intermediate monocytes, becoming consistent in terms of useful surface marker selection for reliable monocyte subset isolation (20). Lastly, another study counted 8 monocyte clusters in healthy individuals using a broad range of lineage, adhesion, antigen presentation, migration, activation, cell death, and survival markers. Classical monocyte subsets differed on the levels of IgE, CD61/CD9, and CD93/CD11a, whilst non-classical monocyte subsets were further divided by the expression of CD9 and SLAN which linked them to increased efferocytosis and migration to CCL16 in comparison with $SLAN^{-}$ non-classical monocytes (21). It will be interesting to learn in larger cohorts of healthy and diseased individuals whether such cellular subsets are of functional relevance *in vivo*.

Monocyte subsets have been shown to exhibit distinct functional properties which partly rely on differential methylation status of immune-related genes (22). For example, classical monocytes migrate to CCL2 and CCL3 gradients and are more efficient than intermediate monocytes in producing ROS and constraining fungi (23–25). In fact, $CD14^{+}$ human monocytes express higher levels of chemokine receptors, such as CCR1, CCR2, CCR5, CXCR1, and CXCR2 which highlights their potential to migrate to cues stemming from injured or inflamed tissues (18, 24), but are also characterized by their ability to secrete pro-inflammatory molecules, such as IL-6, IL-8, CCL2, CCL3, and CCL5 (18, 26). Based on evidence originating from murine studies [reviewed in (1, 27)], but also recent human observations (28), it is now widely accepted that classical monocytes have the ability to differentiate into monocyte-derived macrophages (moMϕs) and DCs (moDCs) (29) and play an integral part in shaping inflammation and its resolution in tissues.

Intermediate monocytes express the highest levels of antigen presentation-related molecules (18, 30) and were also shown to secrete TNF-α, IL-1β, IL-6, and CCL3 upon TLR stimulation (18, 26, 31), while Szaflarska and colleagues described an anti-tumoral phenotype for these cells (32). With regard to chemokine receptors, they express more CCR5 than classical monocytes and this likely accounts for their high susceptibility to HIV-1 infection (5, 24, 33). $CD14^{+}CD16^{+}$ monocyte numbers are expanded in the blood of patients with systemic infections, implying that they must play an important role in the rapid defense against pathogens (34, 35). However, their exact role in immunity remains elusive as another report found that they are the main producers of IL-10 upon TLR stimulation (36). Whether these cells can produce pro- and anti-inflammatory mediators simultaneously or whether there are different kinetics of expression for these factors requires further exploration.

On the other hand, a comparison of $CD16^{+}$ and $CD16^{-}$ monocytes revealed that despite the remarkable similarities which suggest a common developmental origin, $CD16^{+}$ cells possess a more mature phenotype -as assessed by transcriptome profiling- and associate with gene ontology terms, such as cell-to-cell adhesion, cell trafficking, proliferation, and differentiation (37). In addition, they express higher levels of CX_3CR1 which

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid beta; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; IPF, idiopathic pulmonary fibrosis; moDC, monocyte-derived dendritic cell; moMΦ, monocyte-derived macrophage; NCD, non-communicable disease; oxLDL, oxidized low density lipoprotein; SLAN, 6-sulfo LacNAc; T2D, type 2 diabetes; IM, interstitial macrophage.

explains the fact that they migrate and adhere more than $CD16^-$ monocytes to fractalkine-secreting endothelium (5, 25).

Non-classical human monocytes express a distinct transcriptomic and metabolic profile (respiratory chain metabolism) in comparison to classical monocytes which utilize carbohydrate metabolism as their energy source (38). Similar to $CD14^+CD16^+$ monocytes, they present antigen processing capabilities, but are distinguished from classical monocytes by their association with wound healing processes (38). Furthermore, they have antagonizing functions to classical monocytes and promote neutrophil adhesion at the endothelial interface via the secretion of TNF-α (39) and do not reach the classical monocyte production levels of pro-inflammatory cytokines (40). Finally, a role for the $SLAN^+$ subset of non-classical monocytes in TNF overproduction in viraemic HIV-infected patients was proposed, suggesting that they might be considered as a major actor in the immune hyperactivation of the disease (41). While SLAN seems to delineate a subset of non-classical monocytes, there is no evidence for transcriptional differences between $SLAN^+$ and $SLAN^-$ cells (16) which requires further work to understand the reasons for the discrepancy between homogeneity at the transcriptional, but heterogeneity at the protein level.

In recent years the concept of trained immunity has been introduced (42). Monocytes exposed to ß-glucan or BCG react toward a related secondary stimulus with a faster onset and a more pronounced inflammatory response (43–45). Surprisingly, it is not entirely clear, whether all monocyte subsets can exert such a response or whether only a subset of monocytes is capable to be programmed in such a way. Furthermore, it is unclear whether there are changes in the training response when monocytes transition from classical via intermediate to non-classical monocytes.

Taken together, human monocyte subsets display remarkable heterogeneity in their surface marker expression and function; classical monocytes exhibit a more pro-inflammatory phenotype via their ability to secrete soluble mediators and to differentiate into monocyte-derived DCs to bridge innate and adaptive immune responses, intermediate monocytes are specialized in antigen presentation and play an important role in HIV infections, while non-classical monocytes are

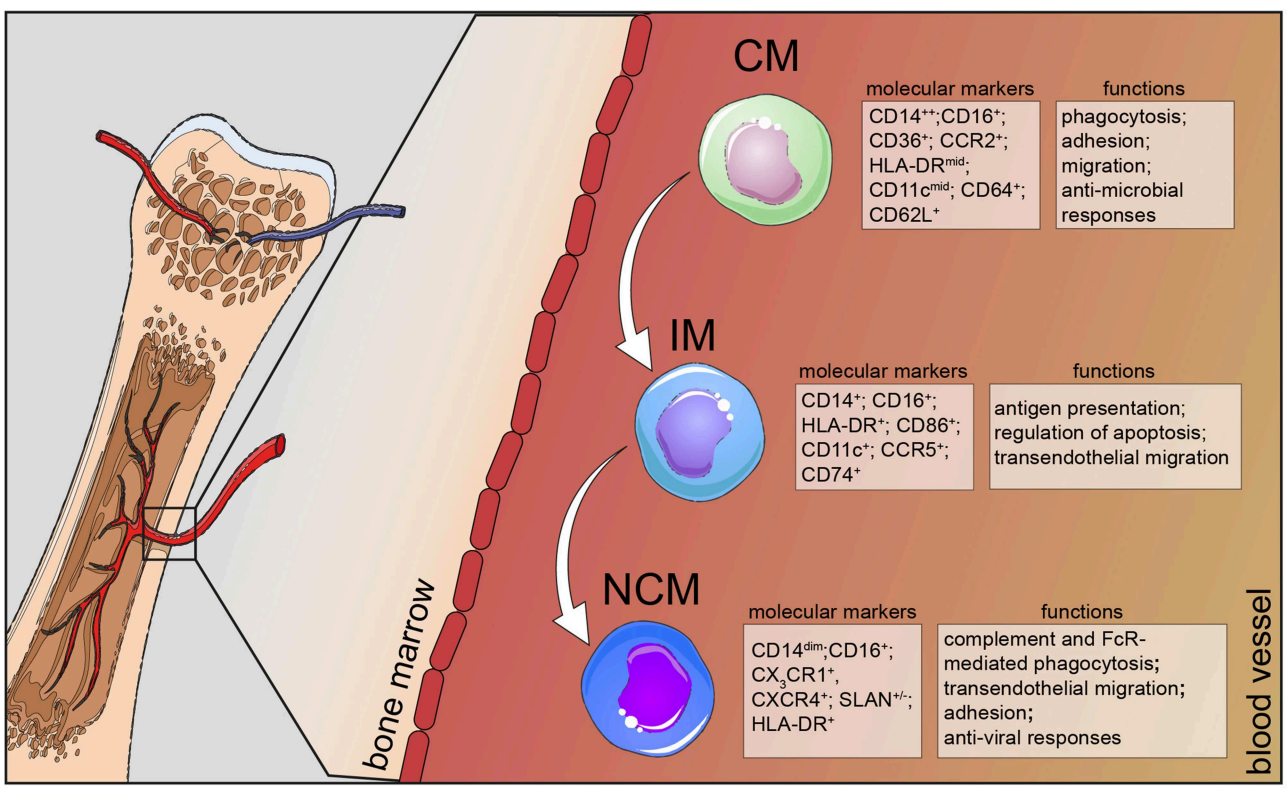

FIGURE 1 | Human monocyte subsets in health. Human monocytes mature in the bone marrow and are subsequently released into the circulation as $CD14^+$ classical monocytes. Progressively, classical monocytes ($CD14^+CD16^-$) give rise to non-classical monocytes ($CD14^{dim}CD16^+$) through an intermediate step of $CD14^+CD16^+$ monocytes. Classical monocytes in humans can be distinguished from the other two subsets by additional markers, such as CD36, CCR2, and CD64 and take part in the host's anti-microbial responses, such as adhesion to the endothelium, migration, and phagocytosis. Intermediate monocytes are characterized by their high expression of CCR5 and HLA-DR molecules and are involved in antigen processing and presentation and transendothelial migration. Non-classical monocytes divide into a $SLAN^+$ and a $SLAN^-$ population, express high levels of CX_3CR1 and specialize in complement and FcR-mediated phagocytosis, transendothelial migration and anti-viral responses. CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes.

responsible for the anti-viral responses of this lineage (**Figure 1**). In this review, we summarize the most recent findings on monocyte behavior in human chronic diseases and put extra emphasis on phenotypic changes that occur and correlate with disease severity or progression. We decided to focus on chronic inflammatory diseases, such as atherosclerosis, diet-induced syndromes, respiratory diseases, and neurodegenerative conditions as case studies for the heterogeneity and plasticity that these cells exhibit in humans (**Figure 2**).

MAJOR LIFESTYLE CHANGES AND THEIR EFFECTS ON HUMAN MONOCYTES

Non-communicable diseases (NCDs) are among the main causes of death in western countries. The close correlation between dietary habits and morbidity and mortality of chronic NCDs has been already extensively characterized (46, 47). In the last 20 years the shift to a more sedentary lifestyle and a Western type high-caloric diet has led to a continuously growing percentage of obese and overweight people (48). According to the World Health Organization, more than half the European population is overweight, of which 30% is obese (49), with this percentage also dramatically increasing in South America and Asia (50). Additionally, obesity is clearly associated with an increased risk of several co-morbidities, such as type 2 diabetes (T2D), cardiovascular disease (CVD), atherosclerosis, kidney and liver failure, sterile inflammation (51, 52), and certain types of cancer (53).

Dietary habits have been shown to drastically affect the number and composition of the three populations of circulating monocytes. Obesity has been shown to induce monocytosis of the intermediate and non-classical subsets (54, 55), while transcriptomic analysis of monocytes in obese donors demonstrated increased expression of *TLR4* and *TLR8* and secretion of pro-inflammatory cytokines, such as IL-1β and TNF in response to LPS or ssRNA stimulation (54).

Moreover, all circulating monocytes in obese donors express more CX_3CR1, implying an increased chemotactic potential toward CX_3CL1-secreting adipocytes (54). In accordance with this observation, obesity has been characterized by an increased amount of monocyte-derived adipose tissue macrophages in both mouse and human (53, 56). Caloric restriction has beneficial effects in many chronic metabolic disorders like T2D, non-alcoholic liver disease and CVD (57–59) and short-term fasting is sufficient to reduce the numbers of all monocyte populations in healthy human subjects (60).

Nevertheless, in depth characterization of mechanistic changes occurring due to different dietary habits is still lacking. Modern high-dimensional technologies (e.g., multi-color flow cytometry, mass cytometry, single-cell RNA-seq) will contribute to understanding primary and secondary effects of diet on the monocyte compartment, possibly dissecting the influence of single macronutrients.

HUMAN MONOCYTE DIFFERENTIATION IN THE GUT IS INFLUENCED BY DIETARY COMPONENTS

Metabolites play a major role in the differentiation of monocytes and affect their functionalities, as exemplified by the short chain fatty acid β-hydroxybutyrate, which upon its release from the liver under prolonged fasting, has been shown to suppress the NLRP3 inflammasome-induced IL-1β and IL-18 production by human monocytes (61). Similarly, Goudot and colleagues found that *in vitro* activation of human monocytes with 6-formylin-dolo(3,2-b)carbazole (FICZ), an endogenous ligand for the environmental sensor aryl hydrocarbon receptor (62), biases monocyte differentiation into moDCs via a BLIMP-1-dependent mechanism (63). Finally, bacterial butyrate imprints a host protection program via epigenetic remodeling during monocyte to macrophage differentiation in the lamina propria (64). In more detail, in the absence of tissue-damaging inflammation, butyrate induces macrophages to upregulate antimicrobial proteins, such as calprotectin.

The mechanisms by which metabolites alter monocyte functions have many aspects in common with the concept of innate immune cell memory where initial priming with a stimulus leads to sustained epigenetic reprogramming that culminates in a phenotypic change upon subsequent challenge (42, 65). Emerging evidence on diet-associated triggers shows that they can induce cellular reprogramming in humans. For instance, *in vitro* exposure of human monocytes to oxidized low density lipoprotein (oxLDL) reprograms the cells to enhance the expression of pro-inflammatory cytokines and chemokines (66). Furthermore, single nucleotide polymorphisms at the gene regions of the inflammasome adaptor ASC and the IL-1 receptor antagonist were identified to have an effect on the training response of human monocytes to oxLDL which proposes the involvement of the inflammasome in this process (67). Taken together, diet-related triggers may induce differential levels of training in human individuals, thus adding another layer of heterogeneity to human monocyte immune responses.

MONOCYTES AND MONOCYTE-DERIVED CELLS IN ATHEROSCLEROSIS

Atherosclerosis is triggered -at least in part- by the elevated levels of oxLDL and LDL which accumulate in the intima of arterial walls (68, 69). A vicious cycle of infiltrated immune cells which store lipid species in the intima and recruit more leukocytes leads to the formation of atherosclerotic plaques, mostly situated in branching points of the vessels. The consequences of ruptured plaques and subsequent clogging of arteries include myocardial infarction and stroke which are the leading causes of death worldwide (70).

Monocytes play a key role in the early formation and maturation of plaques. They are attracted to the arteries by chemokines, such as CCL2 secreted by activated endothelial cells (71–77) and take up lipids within the subendothelial space to differentiate into foam cells (76, 78). Additionally, they can

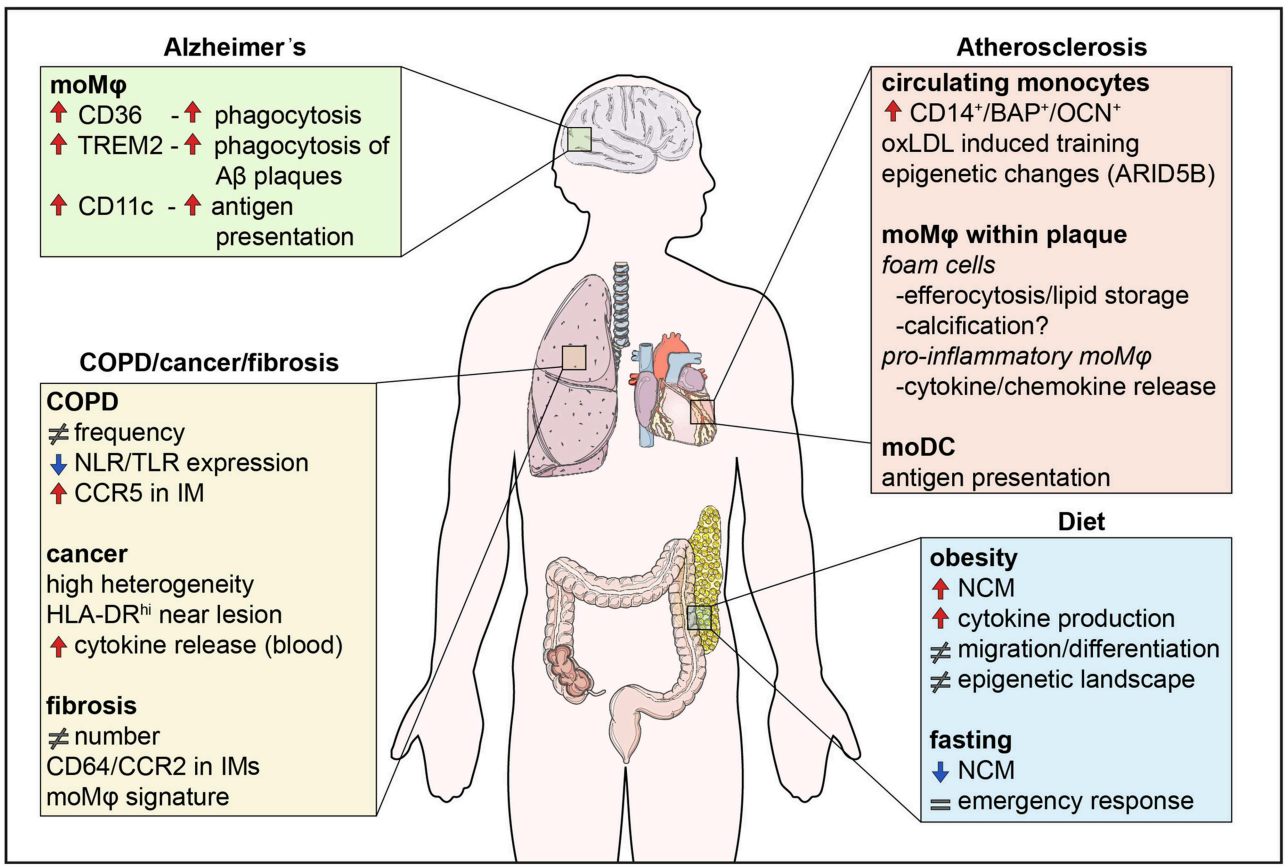

FIGURE 2 | Monocyte functions in disease. Monocytes are involved in human diseases both by their direct functional effects, but also indirectly through their differentiation into macrophages. Diet influences the numbers of non-classical monocytes, monocyte migration, and cytokine production, effects which are counteracted by fasting. In addition, the epigenetic landscape is altered by metabolites in a process called innate immune memory. In atherosclerosis, monocytes differentiate into foam cells which secrete pro-inflammatory cytokines and chemokines, store lipids and are possibly involved in calcification. Differentiation of monocytes to DCs also contributes to antigen presentation. In the lung, changes in monocyte numbers are the most common observation in disease. Monocytes display high heterogeneity and their functions may be impaired like in COPD, whereas monocyte location seems to be crucial in lung cancer, with monocytes close to tumors being immunocompromised. Finally, monocytes infiltrate the brain in neurodegenerative diseases, such as Alzheimer's disease. CD36 and TREM2 are upregulated and enhance phagocytosis of Aβ plaques in monocyte-derived macrophages. NCM, non-classical monocyte; IM, interstitial macrophage.

phagocytose precipitated cholesterol crystals (79) and oxidized lipid species (66, 80, 81) that activate the inflammasome, leading to a highly inflammatory form of cell death called pyroptosis and the induction of innate immune responses (79).

Research on the functional differences of human monocytes in atherosclerosis provided mechanistic insight into their role in the disease. Isolation of monocytes from individuals with symptomatic coronary atherosclerosis and elevated levels of the CVD risk factor lipoprotein(a) displayed a long-lasting pro-inflammatory phenotype (80, 82, 83). These functional differences are accompanied by changes in the monocyte epigenetic landscape. For example, the expression of pro-inflammatory genes such, as *TNF*, *IL6*, *CCL2,* and *CD36* in oxLDL-trained monocytes are regulated by trimethylation of H3K4 residues at the promoter regions (66). Similarly, a large study in control and CVD patients showed that the expression of the transcription coactivator ARID5B positively correlates with CVD. It acts by removing repressive H3K9me2 histone marks from its target genes which are related to inflammatory/immune responses, chemotaxis, extravasation, and phagocytosis (84).

To date, epidemiological studies investigating the correlation between circulating monocytes and the occurrence of cardiovascular events or atherosclerosis severity using flow cytometry have yielded contrasting results due to technical and experimental design reasons (74, 85–96). Briefly, Hamers and colleagues first showed that $SLAN^{+}CXCR6^{+}$ non-classical monocytes are more frequent in patients with atherosclerosis. This subset presents with a higher capacity to migrate toward the chemokine CXCL16 secreted by macrophages in plaques and is probably involved in the clearance of apoptotic cells from the necrotic core (21). On the other hand, a longitudinal study on a larger cohort revealed a correlation of classical monocytes with reverse cardiac events and a negative association of intermediate monocyte numbers with plaque thickness (96). The correlation

of elevated monocyte counts and higher risk for cardiac events has been confirmed in other reports, as well (90). However, classical monocyte counts could not be associated with plaque stability or increased chance of cardiac events after carotid endarterectomy in patients with already existing atherosclerotic plaques (94). Additionally, other studies demonstrated that elevated intermediate monocyte counts play a pivotal role in the growth and stability of already existing atherosclerotic plaques or cardiac attacks (86–88, 97, 98). Elevated CCL2 levels in the early phases of the development of atherosclerotic plaques may lead to increased classical monocyte counts and could thus be considered a predictive marker, while later on, the presence of necrotic cores may rather recruit non-classical monocytes to control vascular homeostasis and the clearance of debris (21, 99, 100).

Computational deconvolution of whole transcriptome data from 126 human carotid plaques using known signatures for leukocytes revealed that macrophages represent about 50% of cells in human atherosclerotic plaques (101). The origin of these cells in human atherosclerotic plaques is not fully resolved. Lin and colleagues argued for the monocyte origin of all described macrophage subtypes in murine atherosclerotic plaques by lineage tracing of bone marrow-derived myeloid cells (102). Interestingly however, only a proportion of the foam cells exhibits a monocyte origin (103) which is compatible with the concept of smooth muscle cell transdifferentiation into foam cells and macrophage-like cells (104–107).

The role and heterogeneity of monocyte-derived cells in the atherosclerotic plaques needs to be further investigated as they could serve as therapeutic targets. As a matter of fact, single-cell RNA-seq analysis of atherosclerotic plaques provided an insight into the heterogeneity of macrophages present in transgenic mouse models. Three main subsets of macrophages have been identified; the resident-like macrophages, which probably overlap with aortic resident macrophages present in steady state, a set of pro-inflammatory macrophages and a subtype of macrophages with a high expression of *Trem2* and genes associated with lipid-metabolic pathways and cholesterol efflux (101, 108). As shown by Kim and colleagues, the *Trem2*^{hi} cells probably reflect the lipid-laden foam cells (103). The most diverse macrophage spectrum in atherosclerotic plaques was so far presented in mice (102), with some of the conserved markers also being validated in human atherosclerotic plaques (101, 108). Furthermore, all single-cell RNA-seq studies of the murine plaque environment defined subsets of DCs. Whereas Kim and colleagues subdivided DC subsets into DC1 and DC2 (103), Cochain et al. found only one DC subset which they hypothesized to be monocyte-derived (108). Indeed, monocyte lineage tracing also included a subset of DC-like cells which was termed as CD74^{hi}MHC^{hi} macrophages (102). These results indicate that at least part of the DCs found in atherosclerotic plaques may be of monocyte origin. Interestingly, the moDCs of the latter study differentially express *Ahr* which has been associated with monocyte to DC differentiation and they are more present during plaque progression rather than regression (63, 108).

Monocyte-derived cells may also contribute to the calcification of the cap, another major feature of the atherosclerotic plaque, despite the earlier consensus that it is mainly mediated by the transdifferentiation of smooth muscle cells to osteoclast-like cells (109). Single-cell RNA-seq of murine plaques revealed a macrophage subset expressing osteoclast genes like osteopontin and human plaques express their protein products (108). Notably, there is a unique osteogenic monocyte subtype in humans defined by the expression of CD14^{+}, bone alkaline phosphatase and osteocalcin which is linked to the degree of calcification and the burden of necrotic cores (110). These cells may also differentiate into calcium-depositing macrophages upon transmigration.

In summary, the composition and ontogeny of monocyte-derived cells in the atherosclerotic plaques has been well-described in mice. Monocytes are recruited to the intima of arteries upon lipid deposition and differentiate into a spectrum of pro-inflammatory macrophages, lipid-laden foam cells, and DCs. Experimental limitations still hamper the translation of these findings to humans. In addition, the contribution of different monocyte subsets to disease progression suffers from low temporal and functional resolution in epidemiological studies. A focus on high-dimensional phenotyping of plaque-associated macrophages, monocytes and their progenitors in humans will allow a deeper understanding of disease development and will hopefully lead to novel therapeutic targets.

MONOCYTE PHENOTYPE AND FUNCTIONS IN HUMAN RESPIRATORY DISEASES

At steady state, the myeloid compartment of the human lung consists of the CD163$^{+/++}$CD206^{+}CD64^{+}CD14^{lo} alveolar macrophage population, CD169^{-} interstitial macrophages, CD14^{+} tissue monocytes and two populations of (CD1a$^{+/-}$) monocyte-derived cells (111–113). Monocytes express typical blood monocyte markers, such as CD14, CD11b, CCR2, and CD16, but at extravascular sites they possess higher levels of CD141, CD11c, HLA-DR, and CCR7, indicating a tissue-imprinted phenotypic change that is reminiscent of DCs (111, 114). Indeed, location is key for monocyte functions, as exemplified by the enrichment of intermediate monocytes in distal airways and the weaker production of pro-inflammatory mediators than in the peripheral blood (114). Similarly, accumulation of CD141^{+}CD14^{+} pulmonary mononuclear phagocytes at the T cell zones of draining lung lymph nodes likely facilitates antigen presentation and T cell-mediated immunity (111).

Changes in monocyte counts have been observed in muco-obstructive lung diseases and fibrotic disorders (115, 116). For example, total numbers of monocytes and the non-classical subset change in the blood of patients with chronic obstructive pulmonary disease (COPD) (117), while classical monocyte counts can be a prognostic marker of mortality in patients with idiopathic pulmonary disease (IPF) (118). However, other monocyte subsets may contribute to disease progression, as shown for intermediate monocytes expressing CD64 or CCR2 (119). Finally, monocytes do not only affect

disease outcome by their direct functions, but also through their differentiation to macrophages. This was shown in IPF patients where alveolar macrophages expressed a gene signature similar to that of monocyte-derived macrophages of bleomycin-treated animals (120).

Studies using bulk transcriptomics suggested that monocytes express a shared gene signature with alveolar macrophages which is overexpressed in COPD compared to healthy individuals and correlates with lung function (121). However, these studies are hampered by the fact that immune cells are treated as homogeneous populations and thus a direct link between peripheral blood monocyte subsets with distinct phenotypes and alveolar macrophage populations in the bronchi of patients with COPD is missing. To resolve this issue, it will be necessary to employ single-cell technologies, such as single-cell RNA-seq and study the differentiation trajectories of peripheral blood monocytes and lung-derived myeloid populations.

Moreover, the frequency of $CD206^+$ non-classical monocytes is reduced, while that of $CD163^+CD206^+CCR5^+$ is increased (117). Congruent with this, intermediate monocytes from patients with COPD also overexpressed CCR5 as a result of high systemic IL-6 and sIL-6R levels. However, their migration capability to CCL5 or CXCR3 chemokines in comparison with non-smokers was reported to be either impaired (122) or not affected at all (123). Lastly, miRNAs could account for monocyte functional dysregulation. Dang and colleagues showed that increased miR-24-3p expression in blood T cells and monocytes from patients with COPD was associated with decreased levels of genes involved in the TLR and NLR pathways which remains to be experimentally validated (124).

Lung cancer is one of the most prevalent cancers worldwide (125) and non-small cell lung cancer (NSCLC) is the most common subtype. Although T cells have been extensively studied in the past, the importance of monocytes in the disease is starting to emerge as recent evidence links their levels to a greater risk of recurrence (126) and worsened post-operative disease-free and overall survival rate (127, 128). The power of single-cell transcriptomics to deconvolute the immune cell structure of NSCLC was evaluated both in mouse and humans (129). Lung tissue-derived myeloid cells in NSCLC patients were divided into 14 transcriptional states; three populations carried signatures of monocytes, 9 of macrophages, one of monocytes/DCs and one subset was characterized by cell cycle ontology terms. In alignment with human monocyte subset expression signatures, the lung monocyte populations identified in this study were defined as classical (*CD14*, *FCN1*), non-classical (*CDKN1C*, *LILRB2*, *ITGAL*), and neutrophil-like (*S100A8*, *S100A9*, *CSF3R*). Nevertheless, although these monocyte populations matched to the three major peripheral blood monocyte populations from the same study, on average, considerable transcriptional differences were seen, such as those related to expressed chemokines and chemokine receptors (129).

Similar to the findings in COPD, tumor infiltrating $CD14^+$ cells in patients with early lung cancer express a mixture of FcγRs (CD64, CD32), cytokine receptors (CD115) and scavenger receptors (CD163, CD206). Further phenotyping revealed that monocyte/TAM localization is driven by microenvironmental cues and thus HLA-DR^{hi} TAMs are found at the tumor lesion, whereas HLA-$DR^{lo/-}$ monocytes reside at distant sites (130). Of note, tumor monocytes displayed a compromised ability to stimulate T cells in direct contrast to TAMs. These results are in line with a previous report on stage I lung adenocarcinoma which found that $CD14^+$ and $CD16^+$ monocyte numbers are decreased at the tumor site, express less HLA-DR than macrophages and secreted less IL-8 and IL-1β at the tumor site compared to monocytes at the rest of the tissue (131). With single-cell-omics technologies now entering this field, we anticipate further knowledge about spatial and temporal changes of monocytes in blood, lung parenchyma, and bronchoalveolar lavage in these major lung diseases.

HUMAN MONOCYTES IN NEURODEGENERATIVE DISEASES: CASE STUDY IN ALZHEIMER'S DISEASE

Neurodegenerative diseases are disorders that disturb the proper functioning of neurons in the central nervous system (CNS). They may affect the structure or the survival of the neurons, which are unable to regenerate after the damage, thus leading to cognitive or motor dysfunction. The immune system was only recently found to play an important role in neuronal injury that occurs in an inflammatory milieu through a complex interplay between resident (microglia) and infiltrating myeloid cells (monocytes) (132, 133). The major neurodegenerative diseases include Alzheimer's disease (AD) which affects over 150 million people worldwide (134, 135), Parkinson's disease (136), Huntington's disease (137), and amyotrophic lateral sclerosis (138).

AD is characterized by the accumulation of insoluble amyloid beta (Aβ) in the extracellular matrix which forms plaques and of hyperphosphorylated tau protein in the cytoplasm which forms neurofibrillary tangles (139). Studies have shown that these protein aggregates are strongly associated with neuroinflammation, synaptic loss and impaired neuronal function which ultimately lead to cognitive decline (135, 140, 141). The progressive deposition and aggregation of Aβ peptides in the brain are the result of an imbalance between their production and clearance, a process in which brain-resident microglia and brain-infiltrating peripheral monocytes (moMϕs) are involved (134, 142).

Because of the technical and ethical limitations of human CNS studies, most of the work on the molecular mechanisms of neurodegenerative diseases has been conducted in murine disease models. The infiltration of monocytes in the brain through the blood-brain barrier was shown in murine models to be mediated via the CCL2-CCR2 axis with microglia and recruited monocytes located in the close proximity of deposited Aβ plaques (143), although some controversial studies based on irradiation experiments exist (144). In the context of monocyte infiltration to the brain, monocytes differentiate into moMϕs which upregulate the expression of surface proteins, such as CD11c, TREM2, and CD36 (145). In this study, Martin and colleagues sorted microglia as ($CD45^{mid}CD11b^+$) and moMϕs as

($CD45^{hi}CD11b^{+}$), a common strategy also used in other studies (146–148). The role of monocytes in AD is multi-faceted. In a larger consortium led by the Neher group, we recently provided evidence that a systemic immune response to LPS stimulation can lead to localized immune training in the brain (149). This, alongside the knowledge that AD is often accompanied by a systemic inflammatory response (150), poses the question: how does the interaction behind this bidirectional relationship work? Whether inflammation is a result of AD pathology or not, an earlier causal factor or both of these at once is yet to be answered.

A human study using blood and brain tissue from healthy and old subjects found that increased expression of the myeloid cell surface receptor CD33 is linked to the AD risk allele rs3865444C (151). This is noteworthy as it expands previous work in murine models whereby increases in CD33 expression lead to higher uptake of Aβ42 peptides and lower deposition of Aβ plaques (152). Building on this, another study confirmed the relationship between CD33 and risk allele rs3865444C, further suggesting that it can result in higher surface expression of TREM2, another biomarker of AD pathology in the cortex (153).

Recently, there has been a renewed interest in the link between TREM2 expression and AD pathology, in particular where the late onset forms are concerned (154). In the presence of functional TREM2, CD68-positive microglial activity initially promotes the clearance of Aβ aggregates by triggering microglia clustering around the plaques. However, due to the concomitant overexpression of ApoE levels in the vicinity of the plaques, Aβ deposition is progressively enhanced. On the other hand, TREM2 loss-of-function mutant mice reported higher levels of Aβ seeding, suggesting TREM2 involvement is a double-edged sword.

The characterization of AD in the brain has recently been advanced by emerging single-cell technologies which allow an in-depth look at changes in aging transcriptomes. One study assessed age-related microglia changes by examining gene expression profiles of purified parietal cortex microglia leading to the identification of human-specific signatures. The study suggests that with increasing age, microglia downregulate actin cytoskeleton-related genes (*TLN1*, *PFN1*, *EVL*, *ARPC1A*, *ARPC1B*, *CORO1A*, *CAP1*, *CTNNA2*, *VASP*) and cell surface receptors (*P2RY12*, *IL6R*, *TLR10*) (155). More recently, a dataset consisting of 80,660 single nuclei transcriptomes from AD patients' human prefrontal cortexes at different stages of the disease indicated the existence of heterogeneity in six identified cell types. Four microglial subpopulations were identified and *CD81*, *SPP1*, *APOC1*, *PTPRG,* and *APOE* were highly upregulated in AD samples. In addition, these subpopulation profiles uncover new AD-associated genes, including the complement component *C1QB* and *CD14*, which have not been reported before. Interestingly, transcriptional changes in response to the earlier disease stages were more cell type-specific in comparison with more ubiquitous late stage variations where the genes being upregulated represented a more general stress response (156).

In none of these single-cell sequencing studies on AD presented above have researchers been able to identify bone marrow-derived monocytes. The reason for this could be monocyte exclusion in sorting panels, such as in Galatro et al. (155) or the utilization of known marker genes in cell type classification as in Mathys et al. (156). In contrast, single-cell studies on pre-clinical models of other neuroinflammatory diseases, such as multiple sclerosis, the numbers of microglia and circulating monocytes in the brain have been shown to be increased in comparison with homeostasis (157). Consequently, the way monocytes are involved in neurodegenerative diseases depends on both the condition itself and the severity stage.

CLOSING REMARKS

Human monocytes are still widely studied in context of peripheral blood and the advent of novel single-cell technologies, including sequencing-based methods have fueled new interest in these cells. While higher heterogeneity has been suggested, we still propose classical, intermediate, and non-classical monocytes as the three major subsets within the monocyte cell space. We would suggest further heterogeneity being explained by functional states of these important immune cells. However, this requires a community effort with guidelines on how to define such newly defined cell states in the monocyte compartment. This will also be important in view of the increasing interest in tissue-associated monocytes and their ability to differentiate into moMϕs or moDCs. Of particular interest are current and future studies on spatio-temporal behaviors of monocyte-derived cells within diseased tissues and organs. We are convinced that the new single-cell technologies can help to decipher the role of these important cells during the major chronic, but also acute inflammatory diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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GM-CSF-Dependent Inflammatory Pathways

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Pre-clinical models and clinical trials demonstrate that targeting the action of the cytokine, granulocyte macrophage-colony stimulating factor (GM-CSF), can be efficacious in inflammation/autoimmunity reinforcing the importance of understanding how GM-CSF functions; a significant GM-CSF-responding cell in this context is likely to be the monocyte. This article summarizes critically the literature on the downstream cellular pathways regulating GM-CSF interaction with monocytes (and macrophages), highlighting some contentious issues, and conclusions surrounding this biology. It also suggests future directions which could be undertaken so as to more fully understand this aspect of GM-CSF biology. Given the focus of this collection of articles on monocytes, the following discussion in general will be limited to this population or to its more mature progeny, the macrophage, even though GM-CSF biology is broader than this.

Keywords: cell survival, polarization, inflammation, pain, IRF4, CCL17

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INTRODUCTION

The glycoprotein, granulocyte macrophage-colony stimulating factor (GM-CSF) or CSF2, was originally defined as a hemopoietic growth factor based upon its ability to form colonies *in vivo* of granulocytes, and macrophages from bone marrow precursor cells (1). However, subsequently, it has been viewed more as a cytokine acting via a specific receptor, expressed mainly on myeloid cell populations, such as monocytes/macrophages, neutrophils and eosinophils, to enhance their survival and/or to activate/differentiate them (2–5). While not having a significant effect on steady state myelopoiesis, in the lung GM-CSF signaling normally maintains surfactant homeostasis and its disruption causes pulmonary alveolar proteinosis (PAP) most likely due to compromised alveolar macrophage development (6, 7). This GM-CSF-driven development of lung alveolar macrophages is of fetal monocyte origin (8). Recently it has been proposed that GM-CSF is required for cholesterol clearance in alveolar macrophages with reduced cholesterol clearance being the primary macrophage defect driving PAP pathogenesis (9). There is evidence that GM-CSF also controls non-lymphoid tissue dendritic cell (DC) homeostasis (10).

Seeing that this Review resides within a collection of articles on monocytes its content will generally be focussed on this population and its tissue counterpart, the macrophage, even though GM-CSF biology is broader involving other responding cell types such as neutrophils and eosinophils.

GM-CSF AND AUTOIMMUNE/INFLAMMATORY DISEASE

Based mainly in data using GM-CSF gene deficient mice or neutralizing monoclonal antibody (mAb) in models of autoimmunity and chronic inflammation, it is apparent

that GM-CSF can be a key driver of tissue inflammation and its associated pain. Examples include arthritis, EAE, cardiovascular disease, and lung disease. The data summarizing these findings have been reviewed recently (11–14) although some of this data more pertinent to the main topic of this Review will be mentioned. It should also be noted that systemically administered GM-CSF can have beneficial effects in inflammatory disease (for example, colitis) and host defense (for example, as an adjuvant) although caution should be exercised in assessing the significance of such administration for the role of endogenous GM-CSF in inflamed tissue (5, 14).

Given the potentially wide range of GM-CSF biology involving multiple cellular sources and responding myeloid cell types (5), human conditions that involve both acquired and/or innate immunity could fall within the realm of GM-CSF influence. As a result of some of the basic biology from pre-clinical models and GM-CSF expression in the corresponding human condition, a number of clinical trials using neutralizing mAbs to target GM-CSF or its receptor in autoimmune/inflammatory diseases have been performed and are continuing. There have been beneficial effects on disease severity in rheumatoid arthritis and asthma trials but, for reasons yet to be elucidated, not in plaque psoriasis—the data from these trials have been reviewed recently (11, 13–15).

GM-CSF RECEPTOR AND SIGNALING

The GM-CSF receptor (GM-CSFR) is a type I cytokine receptor comprising in a multimeric complex a binding (α) subunit and a signaling (β) subunit, the latter shared with interleukin 3 (IL-3) and interleukin-5 (IL-5) receptors. These pathways have been linked to key residues in the intracellular regions of GM-CSFR using mainly receptor mutants expressed in cell lines (16–18). Key downstream signaling pathways from GM-CSFR are often those involving JAK2/STAT5 and ERK (16, 17, 19–21) with ERK activity linked to GM-CSF enhancement of human monocyte survival *in vivo* (21). The GM-CSF-driven development of lung alveolar macrophages is dependent on the transcription factors, PU.1 (22) and PPARγ (23). The debated contribution of other transcription factors, namely interferon regulatory factor (IRF) 4 and IRF5, to GM-CSF-driven monocyte/macrophage polarization (24–26), is discussed below.

The various cellular responses (survival, proliferation, activation and/or differentiation) appear to be explained by dose-dependent and sequential activation by GM-CSF of specific signaling pathways downstream of the activated receptor (16, 27). For example, physiological picomolar concentrations of GM-CSF are able to promote Ser585 phosphorylation in the cytoplasmic domain of the GM-CSFR β subunit to regulate cell survival via phosphoinositide 3-kinase activity and in the absence of other biological responses which occur at higher GM-CSF concentrations (18, 28). A time- and dose-dependent licensing process in mouse and human monocytes by GM-CSF *in vivo* has been described that disables their inflammatory functions and promotes their conversion into suppressor cells (29): this two-step licensing requires activation of the AKT/mTOR/mTORC1 signaling cascade by GM-CSF followed by signaling through the IFN-γR/IRF-1 pathway. Consistent with these dose-dependent signaling responses, dose dependent effects of a neutralizing anti-GM-CSF mAb on monocyte-derived activation/polarization vs. cell number levels were found in an inflammation model—indications were that higher local GM-CSF concentrations were needed for the activation/polarization response (30). Monocytes/macrophages generated *in vivo* from mouse bone marrow precursors with different concentrations of GM-CSF differed in function with possible implications for GM-CSF-dependent pathology (31)—cells generated with a high concentration of GM-CSF were more potent in generating cytokines and chemokines. The links between the various signaling pathways listed and their dependence on GM-CSF concentration in monocytes/macrophages requires further analysis to assess their contribution to the various cellular responses mentioned above. Additional signal transduction findings, particularly linked with the role of GM-CSF in inflammation, are described below.

CELLULAR SOURCES OF GM-CSF AND "NETWORKS"

Both hemopoietic [e.g., T and B lymphocytes (12, 32–35) and innate lymphoid cells such as ILC3] (36–38) and non-hemopoietic cell types (e.g., fibroblast, endothelial, and epithelial populations) can produce GM-CSF although usually requiring an activating stimulus (5, 12, 14, 32, 37, 39–43). In accord with this requirement, in the steady state GM-CSF circulates at low levels and tends to be expressed basally in non-sterile tissues such as skin, lung and gut (44, 45). Even though in inflammation GM-CSF can serve as a communication conduit between tissue-invading lymphocytes and myeloid cells, there is some controversy as to which factors can induce GM-CSF production in T helper (Th) cells (12).

To help understand the chronicity of certain inflammatory/autoimmune responses, a "CSF network" hypothesis was originally proposed in which there is an interdependent co-regulation of proinflammatory cytokines, such as IL-1 and TNF, with GM-CSF as part of a positive feedback "loop" involving communication between monocytes/macrophages and neighboring cell populations, such as fibroblasts, endothelial cells etc. (3–5, 46); this concept has been expanded to include cytokines, such as IL-23 and IL-6, as components of an autocrine/paracrine "network" involving macrophages, DCs and Th cells (45, 47, 48). Recently, positive feedback "loops" have also been put forward involving GM-CSF in inflammatory-dilated cardiomyopathy and breast cancer metastasis (49, 50).

GM-CSF AND MONOCYTE/MACROPHAGE FUNCTION

Macrophage Polarization

Based only on increased expression of pro-inflammatory cytokines, GM-CSF-treated monocytes/macrophages have been

termed "M1-like" (51). However, such cells have also been considered to have characteristics of both M1 and M2 cells, for example, as regards their cytokine expression (39, 52), and GM-CSF-activated mouse monocytes have been reported to alleviate experimental colitis (52). Partly on account of the modest overlap with classical M1 polarization and their dual M1/M2 characteristics, it has been recommended that the M1/M2 polarization terminology not be applied to GM-CSF-treated monocytes/macrophages (14, 25, 26, 53). Even though increased mRNA expression for TNF, IL-1β, and IL-6 is readily observed in GM-CSF-treated (primed) monocytes/macrophages *in vivo*, significant cytokine secretion usually requires another stimulus, such as lipopolysaccharide (26, 54, 55).

Endogenous mediators can contribute to the phenotypes of GM-CSF-treated monocytes/macrophages (25). As an example, GM-CSF-mediated macrophage polarization of human monocytes *in vivo* has been reported to be modulated by endogenous activin A (25, 56); it also has been proposed that the GM-CSF-induced PPARγ expression in human macrophages is primarily regulated in this way (57). Endogenous TGF-β has also been invoked to have a similar role in the development and homeostasis of mouse alveolar macrophages (58). Since most, if not all, mediators involved in the host inflammatory response to injury and/or infection are endeavoring to be beneficial by restoring homeostasis, it is important to explore such a role for GM-CSF in its action on monocytes/macrophages.

Monocytes, Macrophages, and DCs

It is debated as to whether GM-CSF can give rise to monocyte-derived DCs (MoDCs) *in vivo* or not (10, 14, 30, 59–61) even though GM-CSF, often in combination with IL-4, is widely used *in vivo* to generate mouse and human DC populations from bone marrow precursors and blood monocytes, respectively (20, 62–64). Two major types of GM-CSF-dependent phagocytes, termed macrophages and inflammatory DCs, have been claimed to have arisen *in vivo* from mouse $CD209^-$ and $CD209^+$ monocyte subsets (65)—their relationships to the *in vivo* generated populations (see below) also need further analysis. Mouse $CD103^+$ DCs (also called cDC1) from different lymphoid and non-lymphoid tissues have distinct functional activities and there has been disagreement about the contribution of GM-CSF to their development *in vivo* (10, 14, 66, 67) with perhaps varying levels of GM-CSF helping to explain the discrepancies between different studies (68). Obviously, more work needs to be done to understand the role of GM-CSF in cDC development in the steady state and during inflammation. It has been proposed that the effector functions of GM-CSF-expanded myeloid cells *in vivo* are guided by their tissue microenvironment (69).

Mouse populations generated by GM-CSF from bone marrow precursors are heterogeneous with cells having both DC and macrophage features being observed—such features include surface markers, morphology, motility, antigen presentation, T cell activation, cytokine production, and gene expression profiles (51, 70–73); in fact their nomenclature is debated as to whether they should be termed DCs or macrophages (25, 73–75). As an advance on the use and interpretation of the data from such cultures, cell sorting strategies have isolated populations from them with macrophage and DC properties (73). Again the GM-CSF concentrations employed likely contribute to the phenotypes of the resulting populations (31). The *in vivo* relationships of the *in vivo* generated populations from mouse bone marrow and human monocyte cultures are not fully defined.

Inflammation/Autoimmunity

In chronic inflammation and autoimmunity myeloid populations, for example, monocyte/macrophages and neutrophils, the cell populations which are potentially responsive to GM-CSF, are likely candidates to be regulating tissue damage and inflammation, being capable of releasing mediators, such as cytokines, chemokines, proteases and reactive oxygen species, as part of this response (5, 12, 26, 76, 77) (**Figure 1**). Of likely relevance to its function in inflammation/autoimmunity, GM-CSF upregulates class II MHC (21, 78, 79) and CD1 expression (80, 81) in human monocytes. However, it is worth noting that it cannot be assumed that monocytes/macrophages are the only myeloid cell types via which GM-CSF functions to regulate autoimmunity/inflammation (5). Amongst members of the macrophage lineage, GM-CSF initiates cardiac disease in resident mouse tissue macrophages (40) while in contrast only $CCR2^+$ $Ly6C^+$ monocytes require GM-CSF to lead to a pathogenic signature for EAE progression characterized by the induction of genes linked to inflammasome function, phagocytosis and chemotaxis, i.e., they become pathogenic DCs (76). Interestingly, it was reported that intrinsic GM-CSFR signaling by mouse monocytes and their precursors is not a prerequisite for the differentiation of monocytes into inflammatory monocyte-derived DCs *in vivo* during acute injuries (10). Nevertheless, moDCs do become more abundant in mice in which levels of GM-CSF are increased indicating again that GM-CSF can still be a critical factor influencing moDC differentiation, particularly under conditions where GM-CSF levels are elevated (61). GM-CSF-responsive $CCR2^+$ moDCs and not $Csf2rb^{-/-}$ moDCs are critical for Th17 induction and EAE progression (60). In addition to being able to preferentially control putative moDC numbers in antigen-induced mouse peritonitis, GM-CSF could also regulate macrophage numbers in the inflamed peritoneal cavity (30, 82, 83). Whether this regulation of monocyte-derived populations was due to effects of GM-CSF on cell trafficking in or out of a lesion and/or cell survival is unknown (30, 83) although effects on the latter parameter in other inflammatory/autoimmune models have been discounted (40, 60). Interestingly, in this context it has been suggested that GM-CSF controls mouse DC survival in non-lymphoid tissues as the mechanism for their homeostasis (10). There is also evidence that during an inflammatory response GM-CSF may act systemically to promote hemopoietic cell mobilization and development (40, 84–87).

GM-CSF vs. M-CSF (CSF-1)

The gene expression profiles of human monocytes differentiated for 7 days in GM-CSF or M-CSF (CSF-1) differ substantially (25) and display distinct bioenergetic profiles (88). Since monocytes/macrophages are in general likely to be exposed

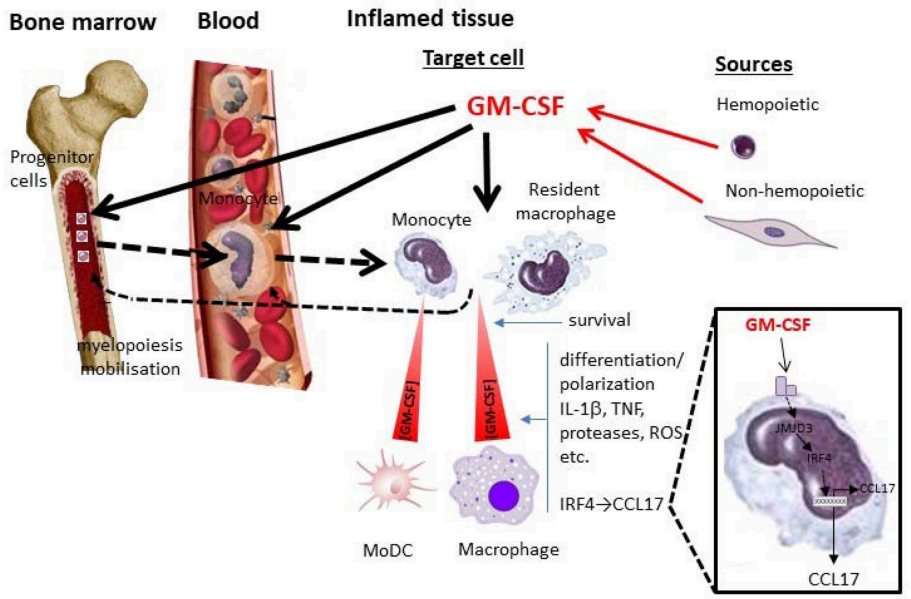

FIGURE 1 | GM-CSF and monocytes/macrophages in inflammation. Depicted are some potential local and systemic actions of GM-CSF on monocyte/macrophage populations during an inflammatory reaction. Whether particular actions operate are currently debated and are likely to depend on the nature of the inflammatory reaction and the levels of GM-CSF attained from hemopoietic (e.g., lymphocyte) and non-hemopoietic (e.g., fibroblast) cell populations. Locally GM-CSF can act in a concentration—dependent manner on target cells (resident macrophages and/or blood-derived monocytes) to promote their survival and/or polarization/differentiation; the latter cell target can give rise to MoDCs. Their polarization/differentiation can be characterized by the production of proinflammatory mediators such as cytokines (e.g., IL-1β, TNF), proteases, reactive oxygen species (ROS), etc. One interesting pathway (zoomed), which seems to be important for GM-CSF-dependent inflammation and associated pain, leads to CCL17 production via JMJD3 and IRF4. GM-CSF can also act systemically in the blood and/or bone marrow, either directly or indirectly (⇢) via its cellular targets in the tissue, leading to migration/mobilization of monocytes or their precursors and/or monocyte development from these precursors (myelopoiesis) (→). MoDC, monocyte-derived DC.

to CSF-1 in the steady state, it has been proposed that pro-inflammatory stimuli, such as GM-CSF and interferon γ, lead to a cellular state of "CSF-1 resistance" or compromised CSF-1 signaling (5). CSF-1 could also be another endogenous mediator contributing to the phenotype of GM-CSF-treated human monocytes (89). Human monocytes differentiated in CSF-1 are widely used as a model for steady state tissue macrophages. In contrast to this widely used practice of employing CSF-1 as the differentiation stimulus, human monocytes treated *in vivo* with GM-CSF for 3 days have been used as a starting population of "macrophages" to analyse the transcriptional regulator networks upon cellular activation by a diverse range of stimuli (75, 90), stressing the need for researchers in the macrophage field to be conscious of the terminology used in any particular article.

GM-CSF and Interferon Regulatory Factors (IRFs)

Based on a number of reports (91–94), the hemopoietic-specific transcription factor, IRF4 (95), appears to be a key signaling molecule regulating the adoption of DC-like properties in GM-CSF-treated precursors such as monocytes. Ly6C^{hi} Trem4^{neg} mouse monocytes can differentiate into Zbtb46^{+} MoDCs in response to GM-CSF and IL-4 in an IRF4 dependent manner (96). Also, GM-CSF-IRF4 signaling upregulates MHC Class II expression in mouse macropahges (97). However, IRF5 rather than IRF4, has been reported to be important for GM-CSF-mediated macrophage polarization (24) although there is disagreement with this conclusion in that IRF4 is considered to be more important based on the divergent data for the relative enhanced expression of the two IRFs by GM-CSF in human monocytes (25, 26). There is no obvious reason for this divergence although subtle differences in culture conditions could perhaps play a role. In support of the importance of IRF4, there is recent evidence that IRF4, most likely acting in monocytes/macrophages, is important in controlling how GM-CSF promotes arthritis and associated pain, as well as inflammatory pain *per se* (26, 98). There is evidence in turn that enhanced JMJD3 histone demethylase activity is required for GM-CSF-induced IRF4 transcription to occur in monocytes/macrophages as well as for GM-CSF-induced inflammatory pain (26) (see below).

GM-CSF/CCL17 Axis

We recently found that the chemokine, CCL17, is the most highly up-regulated gene in GM-CSF-treated human monocytes and, unlike TNF and IL-1β, is secreted at high levels by GM-CSF-treated monocytes and mouse macrophages (26). It was also found surprisingly that CCL17 mediated GM-CSF-driven inflammatory pain as well as GM-CSF-driven and GM-CSF-dependent arthritic pain and disease. These pro-inflammatory

actions of GM-CSF via CCL17 in turn required IRF4 and JMJD3 activity (26) (**Figure 1**). This proposed pro-inflammatory effect of IRF4 in macrophages was also surprising as IRF4 is usually considered to have an anti-inflammatory role in such cells since it down-regulates their production of pro-inflammatory cytokines such as TNF and IL-1β (99–101). Thus, GM-CSF joins the list of cytokines, such as IL-4 and TSLP, which can up-regulate CCL17 expression in monocytes/macrophages. This new GM-CSF → CCL17 pathway appears to be active in rheumatoid arthritis patients since circulating CCL17 levels are dramatically reduced upon anti-GM-CSF receptor monoclonal antibody therapy (102).

More recent studies have indicated that the GM-CSF → CCL17 pathway can be linked with TNF activity (103) as well as regulating experimental osteoarthritic pain and optimal disease (98)—the latter model data have led to a clinical trial being initiated in osteoarthritis using a CCL17 antagonist (NCT03485365 ClinicalTrials.gov). Interestingly, CCL17 may not necessarily be functioning as a chemokine in its regulation of inflammatory pain and arthritic pain/disease (98, 103).

CONCLUSIONS

It would appear from the above that GM-CSF-dependent inflammatory pathways in monocytes (and macrophages) are likely to be critical for the purported role of GM-CSF in inflammation, autoimmunity and host defense. In addition to attempting to summarize the relevant literature on this topic I have tried to highlight some of the contentious issues which are currently being debated. Such issues, which I have endeavored to represent diagrammatically (**Figure 1**), are: (i) when, how and at what concentrations GM-CSF controls cell number and/or activation/differentiation (polarization) *in vivo*, (ii) whether GM-CSF controls MoDC development *in vivo*, (iii) the nature of GM-CSF-induced cell polarization, (iv) whether IRF4- or IRF5- dependent pathways are more important for GM-CSF-dependent biology, (v) when and how endogenous GM-CSF can act systemically in addition to locally in tissues, and (vi) how relevant are the effects of systemically administered GM-CSF to the actions of endogenous GM-CSF.

In order to understand better the role of GM-CSF-dependent pathways, future studies in some of the following areas are likely to be informative: (i) additional clinical trials targeting GM-CSF action and that of other putative downstream mediators, such as CCL17, (ii) human monocyte/macrophage studies, (iii) cellular metabolic responses to GM-CSF, and (iv) the significance of GM-CSF→ IRF4 signaling.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Quorum Sensing by Monocyte-Derived Populations

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Quorum sensing is a type of cellular communication that was first described in bacteria, consisting of gene expression regulation in response to changes in cell-population density. Bacteria synthesize and secrete diffusive molecules called autoinducers, which concentration varies accordingly with cell density and can be detected by the producing cells themselves. Once autoinducer concentration reaches a critical threshold, all bacteria within the autoinducer-rich environment react by modifying their genetic expression and adopt a coordinated behavior (e.g., biofilm formation, virulence factor expression, or swarming motility). Recent advances highlight the possibility that such type of communication is not restricted to bacteria, but can exist among other cell types, including immune cells and more specifically monocyte-derived cells (1). For such cells, quorum sensing mechanisms may not only regulate their population size and synchronize their behavior at homeostasis but also alter their activity and function in unexpected ways during immune reactions. Although the nature of immune autoinducers and cellular mechanisms remains to be fully characterized, quorum sensing mechanisms in the immune system challenge our traditional conception of immune cell interactions and likely represent an important mode of communication at homeostasis or during an immune response. In this mini-review, we briefly present the prototypic features of quorum sensing in bacteria and discuss the existing evidence for quorum sensing within the immune system. Mainly, we review quorum sensing mechanisms among monocyte-derived cells, such as the regulation of inflammation by the density of monocyte-derived cells that produce nitric oxide and discuss the relevance of such models in the context of immune-related pathologies.

Keywords: monocyte, monocyte-derived cell, quorum sensing (QS), macrophage, nitric oxide, metabolism

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QUORUM SENSING: FROM THE BACTERIAL WORLD AND BEYOND

Living cell-based systems, such as multicellular organisms or bacterial biofilms, are very complex collections of biological components (cells) continually interacting with each other. They are organized as coordinated functional communities, which integrity relies on an efficient organization, and therefore, communication. Cells communicate with each other by various mechanisms either dependent on cell contact (e.g., juxtacrine signaling, membrane nanotubes) or dependent on diffusive material (e.g., diffusive signaling molecules, exosomes). Different modes of contact-independent signaling, including autocrine, paracrine, and endocrine signaling, were extensively characterized in the past using mammalian systems (2, 3). Autocrine signaling occurs

when the same cell simultaneously produces and responds to a signaling molecule that stays confined to the cell vicinity, with the help of high-affinity receptors. By contrast, paracrine signaling happens when the responding cell is located at a relatively short distance from the cell producing the signal, and often of a different cell type. Lastly, endocrine signaling is defined when the signaling and the target cells are located at distant sites, the signaling molecule traveling by the bloodstream.

Over 50 years ago, the most well-known bacterial mode of communication named quorum sensing was discovered in the luminous marine bacterial species *Vibrio fischeri* (4). Quorum sensing is a process of chemical communication that bacteria use to orchestrate group behaviors [(5–8); **Figure 1**]. In classic quorum sensing, each bacterium of the same type produces a membrane-diffusive signaling molecule called autoinducer, that can modify bacterial gene expression. The production of autoinducer by individual cells is too low to trigger any significant biological effect. When a threshold of cell density is reached (e.g., by continuous bacterial growth), the autoinducer reaches a sufficient concentration in the local environment to alter gene expression in all cells present in the area. The coordinated alteration of gene expression results in the emergence of group behaviors such as biofilm formation (9), virulence factor expression (10), or swarming motility (11). Therefore, quorum sensing mechanisms in bacteria initiate specific responses only when a sufficient cell density is reached, and rely on a diffusive molecule that acts as a surrogate for cell density (autoinducer). Quorum sensing mechanisms provide the possibility for spatiotemporal regulation of collections of cells, and for the emergence of specific behavior that would be unproductive when undertaken by a single isolated bacterium.

It is only recently that several immune regulatory processes similar to quorum sensing mechanisms have been revealed in the mammalian immune system. Notably, such processes were shown to be triggered locally only when a sufficient number of cells were reached and lead to population-level effects. Also, these mechanisms involve diffusive signaling molecules such as cytokines and chemokines, that resemble autoinducers in that they might be secreted in a reasonably low amount by single cells. Additionally, quorum sensing regulation may occur by mechanisms not only modifying gene expression, but also altering cellular metabolism.

In this mini-review, we will discuss the existence and biological relevance of quorum sensing mechanisms by immune cells at homeostasis and during inflammation, with a particular focus on monocyte-derived cells. We propose that quorum sensing mechanisms are integral to the immune system and that malfunction of such regulatory pathways may lead to uncontrolled monocyte-derived cell accumulation and activation, leading to excessive immune responses and the development of immunopathologies. We will not address the impact of bacterial quorum sensing molecules on monocyte activity, but rather extend the theory originally described in bacteria to the monocyte-derived cells.

MECHANISM OF QUORUM SENSING IN MONOCYTE-DERIVED CELLS

Monocyte-Derived Cell Homeostasis

In mammalian species, the concept of homeostasis establishes that most tissues and organs are made of different cell types whose numbers remain constant at steady-state. Accordingly, monocyte-derived cells such as monocyte-derived macrophages can maintain their cellular density in different organs under physiological conditions (12, 13). Such maintenance is achieved in the different tissues by compensating cell death either by a continuous input of circulating monocytes or by a constant self-renewal of tissue-resident macrophages as for Langerhans cells in the murine epidermis (12, 14–16). However, most of the mechanisms governing monocyte-derived cell homeostasis are still poorly understood. Recently, Antonioli et al. suggested that macrophage homeostasis is controlled under physiological conditions by a quorum sensing mechanism (1, 17). They propose a central role for Colony-Stimulating Factor 1 (CSF1) as the autoinducer for macrophages, as it controls their survival and proliferation at steady-state (18–20). Macrophage density would be controlled by two factors: CSF1 production by stromal cells and its consumption by the entire population. Accordingly, a two-cell circuit-based model between Platelet-Derived Growth Factor (PDGF)-producing macrophages and CSF1-producing fibroblasts was shown to have sufficient stability and robustness to perturbation to allow macrophage/stromal cell homeostasis (21). Further studies will provide new insight regarding the relevance of this model *in vivo* and its potential role not only at steady-state but also during ongoing immune responses. While this particular mechanism somewhat differs from classic microbial quorum sensing in that the autoinducer is produced by stromal cells (which are not the responding cells), it still aims at controlling the macrophage pool size and may be best defined as an indirect quorum sensing mechanism. Finally, the idea that cell density could also affect macrophage behavior at homeostasis has been less investigated. In one study using a model of cultured human monocytes, it was established that the secretion of chondroitin sulfate proteoglycan (structural component of human tissues) by these cells was dependent on their cell density (22).

Quorum Sensing During Immune Reactions

Other mechanisms controlling the size and activity of monocyte-derived cell populations were recently described during immune reactions. For instance, human macrophages infected by the intracellular pathogen *Mycobacterium tuberculosis* restrict bacterial growth more efficiently when cultivated at high density (23). The authors proposed that such finding is compatible with the possibility that high-density cultures release factors that can affect cell behavior [in that case, bacterial behavior]. More recently, TNF-α and IL-10 have been identified as the primary soluble mediators positively and negatively regulating macrophage function, capable of mediating cytokine production in groups vs. in single cells (1). Additionally, it was proposed that the regeneration of hair follicles in response to patterned hair plucking is regulated by a quorum sensing mechanism

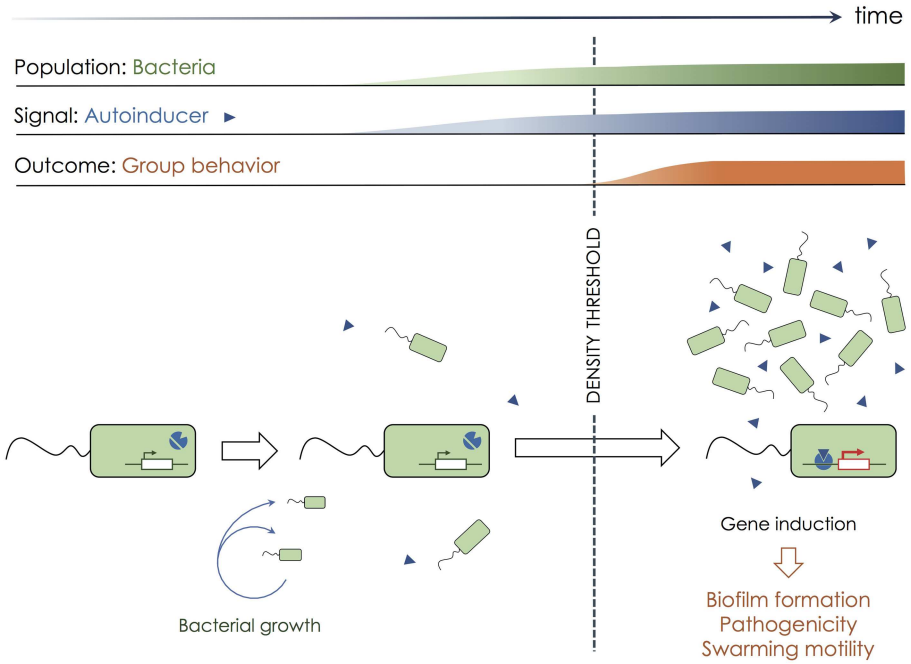

FIGURE 1 | Quorum sensing in bacteria. To communicate and synchronize their behavior, bacteria (green rectangles) use quorum sensing. Each bacterium produces a low quantity of a membrane-diffusive molecule called autoinducer (blue triangles), which biological activity is absent at low concentration. Bacterial growth over time increases cell density together with the concentration of the autoinducer in the extracellular environment. Once a sufficient number of bacteria have accumulated, hence reaching a sufficient density, the autoinducer concentration is high enough to initiate biological alterations. In bacteria, the autoinducer often triggers a switch in genetic expression, after binding to transcriptional regulators. Such a genetic switch leads to the emergence of group behaviors such as biofilm formation, increased pathogenicity, or swarming motility.

involving macrophages (24). The authors uncovered a two-step mechanism where CCL2 released from damaged hairs leads to the recruitment of TNF-α-producing macrophages which accumulate and signal to both plucked and unplucked follicles to stimulate their regeneration.

More recently, a quorum sensing mechanism more similar to what described in bacteria was comprehensively described *in vivo* during the immune response against *Leishmania major* [(25); **Figure 2**]. Local infection with this intracellular parasite in murine skin triggers a massive recruitment of immune cells at the site of infection, including circulating monocytes. Recruited monocytes differentiate into mononuclear phagocytes that not only represent the main population of infected cells but also actively participate in controlling the local inflammatory reaction. Such an immune response can be detrimental to the host by inducing irreversible tissue damage if not adequately regulated and terminated on time (26, 27). Controlling mononuclear phagocyte recruitment, activity, and clearance is therefore essential to resolve inflammation concomitantly to parasite elimination. During cutaneous leishmaniasis, recruited mononuclear phagocytes secrete nitric oxide (NO) that acts to adjust and limit the overall inflammation intensity. NO suppresses mononuclear phagocyte accumulation, as well as cytokine and chemokine production by blocking cellular respiration and decreasing the ATP:ADP ratio. Importantly, such mechanism only exists when a sufficient number of mononuclear phagocytes have accumulated at the site of infection (more than 5,000 cells per mm^3) to produce a high quantity of NO in a collective manner (**Figure 2**). NO acts thereafter by diffusing and targeting all mononuclear phagocytes irrespectively of their intrinsic iNOS expression. In this mechanism, the mitochondria, and most probably the cytochrome c oxidase, represents the target of NO (28, 29). Therefore, mononuclear phagocytes not only produce NO but are also regulated in number and activity by this diffusive molecule, establishing a quorum sensing mechanism for the control of the inflammatory reaction, with NO acting as the autoinducer. Furthermore, it is very interesting to note that such mechanism relies on the modification of cellular metabolism. That shows, in contrast to the current paradigm in bacteria, that quorum sensing mechanisms do not necessarily rely on genetic alteration. Targeting cellular metabolism compared to genetic expression could have several advantages, including the possibility to alter cell activity within a very short period of time and in a rapidly reversible manner (25). In addition to dampening oxidative phosphorylation, NO was also shown to accelerate myeloid cell death (30, 31), a phenomenon that could participate in inflammation resolution (32). We propose that this NO-based quorum sensing mechanism is integral to the biology of inflammatory mononuclear phagocytes and could certainly operate in other models of infections or during cancer development. We additionally propose that malfunction of quorum sensing mechanisms may lead to uncontrolled

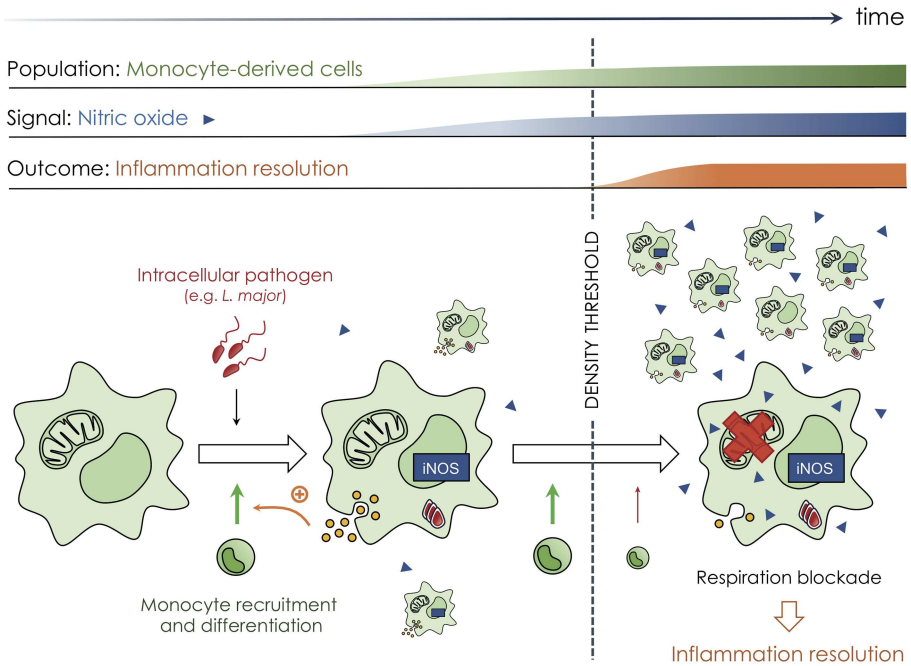

FIGURE 2 | Quorum sensing among mononuclear phagocytes at the site of infection by intracellular pathogens. Mononuclear phagocytes are endowed with a quorum sensing mechanism during the immune reaction against *Leishmania major* parasites. Local skin infection with this intracellular pathogen elicits inflammation and the recruitment of innate immune cells from the blood, including monocytes (small round green cells) that differentiate into mononuclear phagocytes (large rough green cells) at the site of the immune reaction. Such cells sustain monocyte infiltration and differentiation by secreting cytokine and chemokine (yellow circles) but also produce nitric oxide (blue triangles, NO) that diffuses within the microenvironment and helps fight the infection. Such mechanism increases mononuclear phagocyte number at the site of infection during the early phases of the response, allowing for local control of the pathogen load. Once a sufficient number of mononuclear phagocytes have accumulated, NO starts to repress cellular respiration (red cross on the mitochondria), dampening the cellular ATP:ADP ratio and ultimately limiting cytokine and chemokine secretion that is needed for immune cell recruitment. The mechanism relies on NO that diffuses and acts on every mononuclear phagocyte, independently of their iNOS expression, and only exists when a sufficient number of cells have accumulated. Therefore, NO acts as an autoinducer for mononuclear phagocytes, limiting their recruitment and the development of an immunopathology but only when a sufficient number of cells have accumulated to control the infection efficiently.

mononuclear phagocyte accumulation and activation, leading to non-resolving inflammation and therefore immunopathology development (26).

Furthermore, other quorum sensing mechanisms are about to be elucidated during immune reactions. Indeed, using a droplet-based microfluidic approach, Wimmers et al. showed that during human pDCs activation, the fraction of IFN-α-expressing cells at early time points of activation is dependent on their initial cell density. Using microtiter plates, they measured that more than 10^3 pDCs per well should be stimulated together to have a significant percentage of IFN-α-producing cells following activation (33). That suggests that the magnitude of dendritic cell activity significantly increases above a threshold number of cells and that these cells are probably endowed with quorum sensing capability. Similarly, it was recently showed using mathematical modeling that macrophage activation is most probably bimodal, with a proportion of highly activated cells increasing with cell density. Because only a fraction of the population becomes activated, the authors preferably describe such phenomenon as quorum licensing rather than quorum sensing (34). Such studies and early work on human macrophages establish the potential relevance of quorum sensing mechanisms for the control of immune reaction in humans.

Benefits Over and in Conjunction With Autocrine and Paracrine Signaling

Immune cell communication by quorum sensing mechanisms provides many benefits over autocrine or paracrine signaling. Quorum sensing mechanisms can significantly differ from paracrine signaling, a distinction that is not systematically present in the literature. To distinguish the two communication modes, further studies will have to investigate whether the diffusive molecule has any biological effect when the producing cell is present at low density. Indeed, the absence of biological effect of the signaling molecule (autoinducer) when its producer is at too low density is a cornerstone of quorum sensing. In other words, if a diffusive molecule biologically acts when the producer cell is at a negligible density, the communication occurs through paracrine signaling.

A first advantage of quorum sensing is the regulation of the cell number *per se* in tissues during inflammation (35). For instance, a high T cell density is needed for their terminal differentiation into cytotoxic T cells (36). By contrast, an excessive number of cells involved in an immune response can trigger immunopathology (25). Quorum sensing mechanisms may therefore locally adjust the cell density to promote or stop the immune reaction. Next, compared to autocrine signaling,

the use of a diffusive mediator (the autoinducer) that acts simultaneously on numerous cells offers a way to reduce the level of heterogeneity between cells, and therefore coordinate their behavior in their complex microenvironment. For instance, DC populations can synchronize their behavior at late time points of activation provided that a small fraction of them secrete type I interferon rapidly after stimulation (37). Also, compared to paracrine signaling, quorum sensing mechanisms have the potential to temporally and locally adjust cell density and inflammation intensity without the need for external cues or regulatory cells. Paracrine signaling would not allow such self-adjustment to exist because it implies at least two cell types: the producing and the target cells. In a way, quorum sensing mechanisms can be considered as a form of paracrine signaling that depends on the cell population density but in which cells produce both a signaling molecule and its receptor, as in autocrine signaling (38).

Nonetheless, these different types of communication do not seem mutually exclusive. For instance, during the immune reaction against the parasite *L. major* in murine skin, a two-wave immune cell regulation occurs (39). First, a wave of IFN-γ secreted by activated $CD4^+$ T cells spreads away from the site of antigen presentation and induces iNOS expression in numerous infected and bystander monocyte-derived cells, by a mechanism resembling paracrine signaling (40). Next, the subsequent collective production of NO allows for both parasite control (41) and regulation of the inflammation intensity at the tissue level by a metabolism-based quorum sensing mechanism (25). Thus, both paracrine signaling and quorum sensing can act in concert to spread a signal originating from a few discrete sites of cell activation to the level of the entire organ, while keeping the inflammatory reaction locally under tight control. Another example of coupling also exists for type I IFN during pDCs activation. In such an event, IFN-α stimulates its own production and alter cellular metabolism via an autocrine amplification loop (33, 42) but also regulates the fraction of IFN-α-producing cell most probably by a quorum sensing mechanism. Indeed, the fraction of activated pDCs is dependent on their cell density and relies on the diffusion of IFN-α that binds most probably IFNAR1 to mediates its biological effects (33, 43). Hence, combining quorum sensing with either autocrine or paracrine signaling appears to be essential to fine-tune immune cell activity.

QUORUM SENSING BY IMMUNE CELLS: PERSPECTIVES

Quorum Sensing by Other Immune Cell Types

While we focused on monocyte-derived cells, quorum sensing mechanisms have been described as well in other immune cells such as T and B cells.

In T cells, interleukin-2 (IL-2) was shown to be a significant autoinducer involved in a quorum sensing regulatory loop stabilizing T cell population density and phenotype (44–48). With the help of mathematical models and *in vitro* experiments, it was shown that a sufficient density of T_{EFF} cells is critical to reach a minimum threshold of IL-2 above which the phosphorylation of the signal transducer and activator of transcription (STAT) 5 is sustained to allow T cell proliferation (45). It was demonstrated using the same strategy that an excess of IL-2 above a maximal threshold leads to cell death instead of proliferation, participating in the regulation of T cell density to reach homeostasis (46). As well, it was recently evidenced that T cell density can directly modulate T cell differentiation toward T_{EFF} or T_{CM} by a quorum sensing mechanism relying on IL-2 and IL-6 as autoinducers (47). Additionally, during an immune challenge, T cells were shown to establish a negative feedback loop by capturing their cognate pMHC complexes from antigen-presenting cells and presenting them to antigen-experienced $CD4^+$ T cells, thereby inhibiting their recruitment into the ongoing response (49). Finally, it has been proposed that T cell activation require a quorum of lymphocytes to happens (50).

Additionally, B cells were also shown to be endowed with a quorum sensing regulatory mechanism (51). After challenge, activated B cells secrete soluble immunoglobulin G (IgG) which concentration rises in the serum and is detected by the inhibitory receptor FcγRIIB, present on all B cells. At a sufficient concentration of IgG, the binding on this receptor is enough to trigger intracellular inhibitory pathways and prevent further IgM-secreting B cell activation. As a result, the number of IgM-secreting B cells is kept under control (51). So far, only IgG molecules were shown to act as autoinducer for B cells.

Outstanding Questions and Perspectives

We envision that quorum sensing is integral to the regulation of immune responses. In this respect, future studies are critically needed to extend our understanding of this mode of communication. Several outstanding questions remain open: How autoinducer concentrations evolve in tissues during immune reactions? How can the physical tissue architecture and organ structure impact autoinducer diffusion, distribution, and access to the cells? How accurately can a cell population sense its own density based on an autoinducer concentration? Are there other unpredicted advantages of quorum sensing in immune cells? Do regulatory mechanisms for quorum sensing, such as quorum sensing quenching (52), can be triggered by invading pathogens or by the immune cells themselves? Can we target quorum sensing mechanisms in the context of immunotherapies (53). Further studies in both bacterial and mammalian systems will help answer these questions (54–57).

Given the complexity of the immune system and the vast number of quorum sensing mechanisms described in bacteria, we anticipate systems immunology to be essential in unraveling new quorum sensing mechanisms in immune cells (58). Mathematical modeling and computational simulations should help identify new quorum sensing mechanisms in immune cells, as it has been done for T cells (46, 59, 60) and more recently in macrophages (34). Furthermore, the development of new biological tools to measure autoinducer concentration and diffusion in complex tissue microenvironment are needed. For instance, reliable tools to map IL-2 or nitric oxide gradient *in vivo* would be of great help to better decipher quorum sensing mechanisms and characterize the effect of tissue architecture of its efficacy.

CONCLUDING REMARKS

While quorum sensing is the norm in the bacteria world, it is only recently that similar mechanisms were shown to exist in the immune system, including in monocyte-derived cells. Quorum sensing mechanisms provide a way to regulate immune cell activity concurrently in a spatial and temporal manner complementary to what autocrine or paracrine signaling can achieve. They also favor the emergence of group behaviors and synchronized responses, two features decreasing the sensitivity of the system to external perturbations and therefore increasing its robustness. Alteration of immune quorum sensing mechanisms by impaired access or dysregulated response to the autoinducer may certainly trigger the emergence of immunopathology, as demonstrated in the context of the infection by the parasite *L. major*. Future studies are needed to extend the concept to other cell types and models to provide a better understanding of how this unique mode of communication integrates within the complexity of immune reactions.

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REVIEW
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Using High-Dimensional Approaches to Probe Monocytes and Macrophages in Cardiovascular Disease

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High dimensional approaches that characterize single cells at unprecedented depth have helped uncover unappreciated heterogeneity, a better understanding of myeloid cell origins, developmental relationships and functions. These advancements are particularly important in cardiovascular disease, which remains the leading cause of death worldwide. Gradual, monocyte-dependent inflammatory processes, such as the development of atherosclerotic plaque within arterial vessels, contrasts with the robust acute response within the myocardium that occurs when a vessel is occluded. Monocytes and macrophages differentially contribute to tissue injury, repair and regeneration in these contexts, yet many questions remain about which myeloid cell types are involved in a coordinated, organ-level sterile inflammatory response. Single cell RNA sequencing, combined with functional analyses have demonstrated that at least three populations of resident cardiac macrophages exist, and after tissue injury, there is significant diversification of the tissue macrophage pool driven by recruited monocytes. While these studies have provided important insights, they raise many new questions and avenues for future exploration. For example, how do transcriptionally defined sub-populations of cardiac macrophages relate to each other? Are they different activation states along a pre-defined trajectory of macrophage differentiation or do local microenvironments drive newly recruited monocytes into distinct functions? The answers to these questions will require integration of high-dimensional approaches into biologically relevant *in vivo* experimental systems to ensure the predicted heterogeneity possess a functional outcome.

Keywords: macrophages, monocytes, cardiovascular, scRNA-seq, myocardial infarction, atherosclerosis

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BACKGROUND

Mononuclear phagocytes are central mediators of cardiovascular (CV) disease, the leading cause of death worldwide (1). In broad terms, CV disease can be classified into two forms; ischemic and non-ischemic. Ischemic injury initiates within coronary arteries, with gradual accumulation of LDL cholesterol in the artery wall over decades, leading to a smoldering, monocyte-dependent chronic inflammatory response that drives atherosclerotic plaque expansion. Acute plaque rupture leads to diminished blood flow to a segment of the myocardium (myocardial infarction), resulting

in cell death with or without reperfusion injury—both processes that also trigger substantial monocyte recruitment. Non-ischemic cardiovascular injury represents a heterogeneous group of etiologies that include hemodynamic strain (hypertension), inflammatory myocarditis (infectious or autoimmune), cardiotoxicity (such as from chemotherapy), as well as a variety of other factors, all of which also trigger monocyte recruitment (2). After injury, cardiac contractile function can be impaired, promoting the development of heart failure. Importantly, ischemic and non-ischemic etiologies both trigger recruitment of monocytes from circulation and activate resident macrophages that live within the tissue—which together, coordinate the inflammatory and reparative response to injury.

The traditional view for decades has been that a monocyte produced in the bone marrow enters tissue and becomes a tissue macrophage in health and disease (3). This concept, while initially important—overlooked substantial heterogeneity within both monocyte production, monocyte fate after entry into tissue—and separately, the heterogeneity within resident tissue macrophages. Recent technical advancement in genetic fate mapping, multi-dimensional (single-cell mass cytometry [CyTOF], and novel flow cytometric markers; ∼40 markers) and high-dimensional approaches (i.e., single cell RNA sequencing [scRNA-seq]; 1,000–5,000 transcripts), represents a key inflection point in our ability to probe the mononuclear phagocyte system. Subsequent computational analyses can not only help functionally separate closely related cell types in an unbiased fashion but can infer developmental relationships between cells. In this review, we will define our current understanding of monocyte and macrophage heterogeneity in CV disease (heart and vasculature), where limitations exist, and possible opportunities for future investigation in the context of using high-dimensional approaches.

CIRCULATING MONOCYTE HETEROGENEITY

During development monocytes are produced in the fetal liver (through erythroid myeloid progenitors that migrate from the yolk sac) and subsequently from definitive hematopoietic stem cells (HSCs) (4). After birth, definitive HSCs in the bone marrow become the major source of monopoiesis. Blood monocytes, derived from common myeloid progenitor cells, are first produced as Ly6C^{hi} monocytes (CD14^{+}CD16^{-} in humans), which are referred to as classical/inflammatory monocytes due to their ability to extravasate into tissues, where they execute a variety of effector functions following injury. In addition, Ly6C^{hi} monocytes may differentiate into macrophages or dendritic cells depending on the local tissue environment, or they persist as a monocyte subset and exit tissue, as demonstrated in the lung (5). In patients, increased numbers of intermediate CD14^{+}CD16^{+} monocytes have been correlated to increased risk of CV disease, impaired recovery after myocardial infarction, microvascular dysfunction and worse clinical outcomes (6–9).

Examination of chromatin accessibility within the genome of Ly6C^{hi} monocytes has led to the prediction that differentiation from classical to Ly6C^{lo} non-classical monocytes (through an intermediate stage) is the default pathway (10). Non-classical Ly6C^{lo} monocytes (CD14^{lo}CD16^{+} in humans) play an important role in patrolling the vasculature and maintaining vessel wall integrity (11). With the use of scRNA-seq, several groups have attempted to uncover further heterogeneity that exists within the blood monocyte pool at steady-state. These studies suggest a heterogeneous population of intermediate monocytes (murine Ly6C^{int} monocytes and human CD14^{+}CD16^{+} monocytes) (10, 12). Given the heterogeneous nature of intermediate monocytes, variation between individual human donors and different single cell technologies, it is not surprising that some studies failed to demonstrate a defined intermediate population (13). Whether increased intermediate monocytes are a marker of systemic processes driving increased cardiac pathology, or whether CD14^{+}CD16^{+} intermediate monocytes are themselves infiltrating the myocardium and promoting pathology, has yet to be determined. Advances in profiling circulating monocytes using CyTOF have yielded enticing clues about potential novel monocyte subsets that may arise in patients with coronary artery disease, including increased CXCR6 and Slan (6-sulfo-LacNac) expression on non-classical monocytes correlating with increasing severity of atherosclerosis (14) (**Figure 1**). While a detailed and unbiased single cell approach focused on peripheral monocytes (and other circulating cells) has yet to be undertaken in CV disease, we have compelling evidence in animal studies that in the setting of inflammation, novel monocyte subsets are liberated from the bone marrow, which may have important functional implications.

MONOCYTE DIVERSITY TRIGGERED DURING INFLAMMATION, AGING, AND IMPLICATIONS FOR CV DISEASE

During cardiac injury, the monocytic demand is beyond that available in circulation and in bone marrow or splenic reservoirs. This HSC drive leads to the increased production and mobilization of myeloid cells, a process termed "emergency hematopoiesis" (15). The bone marrow senses increased stress at distant sites through soluble factors, such as GM-CSF and IL-1β (16, 17). In the setting of myocardial infarction, a subset of CCR2^{+}CD150^{+}CD48^{-} hematopoietic progenitors with enhanced proliferative capacity are mobilized in the bone marrow through an *Mtg16*-dependent process (18). Deletion of *Mtg16* decreased monocyte production and led to impaired infarct healing. Additionally, myocardial infarction and risk factors for myocardial infarction (sleep deprivation) induces a state of stress—which itself can trigger increased bone marrow HSC activity and promote development of atherosclerotic lesions (19). The chronic months-long process of atherosclerosis progression in mice (decades in humans) vs. the very acute inflammatory and hemodynamic fluctuations of a myocardial infarct trigger very different hematopoietic responses—and while little is known about the types of monocytes produced in both settings, it is tempting to speculate.

For example, scRNA-seq revealed a "neutrophil-like" Ly6C^{hi} monocyte subset that was mobilized in response to LPS injection and contained increased expression of granule enzyme myeloperoxidase protein indicating an enhanced direct pathogen killing function (20). Additionally, SatM monocytes (segregated-nucleus-containing atypical monocytes) were found to be responsible for fibrosis, but not inflammation, in the setting of pulmonary fibrosis (21). A Ym1^{+}Ly6C^{hi} monocyte population has been recently shown to be liberated from the bone marrow to the colon during the resolution phase of colitis (22). Thus, early production of monocytes skewed toward inflammation or fibrosis may be balanced by later production of monocytes that promote tissue repair.

The accumulation of somatic mutations in hematopoietic stem and progenitor cells as we age can lead to the clonal expansion of a particular hematopoietic founder cell, termed clonal hematopoiesis, due to its competitive advantage over others. The role of clonal hematopoiesis in cardiovascular disease has recently emerged, contributing to the aberrant accumulation of inflammatory monocyte-derived macrophages in atherosclerosis, hypertension, and ischemic injury (23, 24). Similarly, increased proliferation and expansion of hematopoietic stem and progenitor cells in the *Apoe*$^{-/-}$ mouse fed a high fat diet led to the development of atherosclerotic lesions (25). This suggests that excessive myelopoiesis is not only a consequence of the inflammatory injury response but when not properly regulated, can promote/exacerbate disease progression. Although conflicting results have been gleaned from clinical trials using anti-inflammatory drugs in ischemic and non-ischemic heart disease (26), a recent clinical trial demonstrated a beneficial role for the IL-1β inhibitor Canakinumab, resulting in decreased cardiovascular events in patients with atherosclerosis (27). This effect has been attributed to its potential ability to blunt excessive hematopoiesis and monocyte production; however, macrophages within advanced atherosclerotic plaque expand numerically through local proliferation rather than continual monocyte recruitment (28), thereby suggesting alternative mechanisms may also be involved.

MACROPHAGE HETEROGENEITY IN STEADY STATE

Several groups, using a combination of genetic fate mapping and single cell transcriptomics have defined three populations of tissue macrophages within the myocardium that are distinct in origin, monocyte-dependence, and function (29–36) (**Figure 1**). TIMD4^{+}LYVE1^{+}MHC-II^{lo}CX3CR1^{lo} macrophages (termed TIMD4^{+}LYVE1^{+} macrophages) represent an embryonically-derived subset that renews almost entirely through *in situ* proliferation without significant blood monocyte input in adult animals, downregulating CX3CR1 as animals age (29, 32). A portion of TIMD4^{+}LYVE1^{+} macrophages upregulate MHC-II and lose expression of TIMD4 and LYVE1 (termed MHC-II^{+} macrophages); which renew *in situ*, but also receive measurable, albeit minimal monocytes in adult animals. Lastly, a numerically smaller population of CCR2^{+}MHC-II^{hi} macrophages exists, which is continuously replaced by monocyte-derived cells. Sex-mismatched heart transplant recipients confirm the peripheral blood origin of CCR2^{+} cardiac macrophages in humans (33).

Both CCR2^{+} and MHC-II^{+} macrophages can process and present antigen to T-cells, however their definitive role during homeostasis is unclear. Analogous populations have been reported to be associated with nerve bundles (34) and it is possible that they suppress inflammation at these sites. Resident macrophages are found in the atrioventricular node of the myocardium, and when depleted, conduction abnormalities can be detected–which suggests they may also reinforce efficient electrical conduction (37). LYVE1^{+} macrophages are found closely associated with the vasculature, promote endothelial cell activation, patterning of coronary vasculature and are efficient in the uptake of apoptotic cell material (29–31, 34). Depletion of resident macrophages in steady state induced development of cardiac fibrosis (34), which together suggest multiple important homeostatic functions.

A fourth population of tissue cardiac macrophages has now been identified through scRNA-seq in the uninjured myocardium, increasing in number after injury (32, 38, 39). This population is characterized by a strong interferon stimulated gene signature (termed ISG MFs). Whether ISG macrophages represent a unique tissue macrophage subset or are part of a spectrum of activation states is unclear. Moreover, their role in homeostasis is also unknown, which highlights the need to develop tools to isolate and study this novel population. Importantly, in the setting of myocardial infarction, blockade of the type I interferon response enhanced infarct recovery suggesting a critical role for this pathway (and possibly this subset) in adverse LV remodeling (38).

A variety of approaches have been used to study monocytes and macrophages within blood vessels, with a focus on the aorta as a surrogate of coronary vasculature. Both embryonic-derived macrophages, and neonatal macrophages, contribute to aortic macrophage composition. ScRNA-seq has been used to examine macrophage heterogeneity within the aorta, with a focus on the atherosclerotic environment (see below). A resident macrophage signature was seen within a single cluster in naïve mice that expressed *Lyve1*, with gene expression similarities to *Lyve1*/*Timd4* expressing cardiac macrophages, however additional heterogeneity within the total macrophage population was not assessed (40, 41). This population of arterial LYVE1^{+} macrophages resides in the arterial adventitia and is maintained locally via self-renewal though interaction with the vasculature smooth muscle cells (42).

DIVERSIFICATION OF CARDIAC MACROPHAGE POPULATIONS IN ISCHEMIC INJURY

In the setting of ischemic injury, the injured myocardium recruits Ly6C^{hi} monocytes in large numbers—an observation established by numerous groups. The parallel fates of resident macrophages and recruited monocytes at single cell resolution

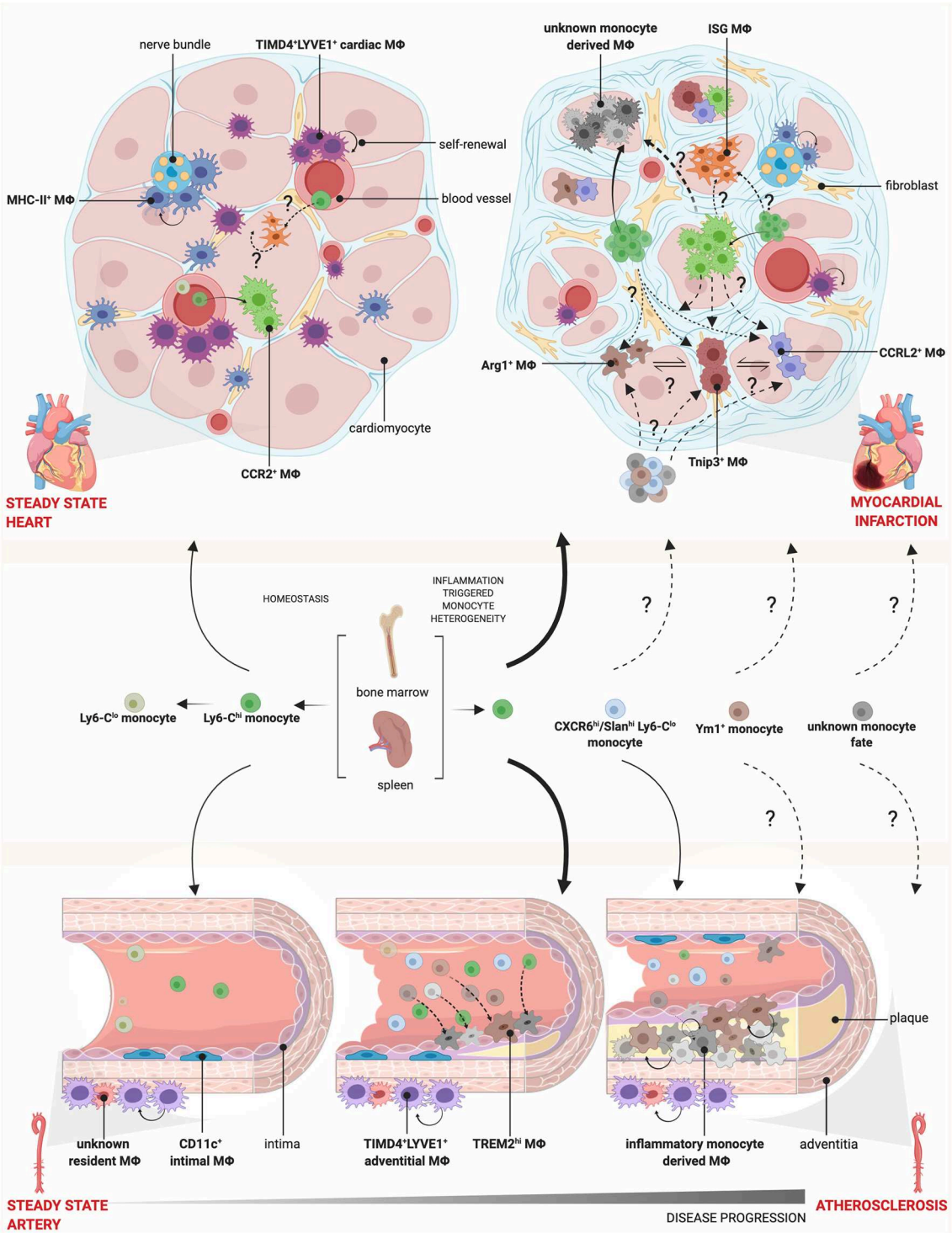

FIGURE 1 | Monocyte and macrophage heterogeneity in steady state and cardiovascular disease. During homeostasis, Ly6C^{hi} monocytes circulate through blood vessels and infiltrate tissue, where they give rise to CCR2^{+} MΦs, while Ly6C^{lo} monocytes patrol the vasculature. Cardiac MΦs are further composed of monocyte-independent self-renewing TIMD4^{+}LYVE1^{+} and MHC-II^{+} resident MΦs, which localize preferentially near blood vessels and nerve bundles, respectively. During myocardial infarction, there is increased monopoiesis and release of Ly6C^{hi} monocytes from the spleen and bone marrow, which are recruited to the injured heart and give rise to diverse MΦ subsets. Whether these MΦ subsets are a spectrum of activation states or arise via pre-defined monocyte fates, such as Ym1^{+} or CXCR6^{hi}/Slan^{hi} Ly6C^{lo} monocytes as identified in other disease models, is not known. Conversely, there is a loss of TIMD4^{+}LYVE1^{+} and MHC-II^{+} resident MΦs. In the vessels, the intima is lined with CD11c^{+} MΦs and the adventitia contain TIMD4^{+}LYVE1^{+} MΦs and other undefined resident MΦ populations. In atherosclerosis, TREM2^{hi} MΦs and inflammatory monocyte-derived MΦs accumulate in the intima, expand via self-renewal and participate in plaque growth. How this fate is defined and the contribution of CXCR6^{hi}/Slan^{hi} Ly6C^{lo} monocytes, found in the circulation of patients correlating with disease severity, is unknown. Mϕ, macrophage.

is less clear. Genetic fate mapping and scRNA-seq reveal that resident TIMD4^{+}LYVE1^{+} and MHC-II^{hi} macrophages are lost within the ischemic zone, presumably due to cell death. Recruited monocytes appear to have two principle paths after tissue entry. The first, observed both in acute, and sub-acutely after infarct involves a unique trajectory relative to the resident macrophage population, characterized by multiple unique transcriptional states (32, 39). The transition from Ly6C^{hi} monocytes to early macrophages tightly correlates with upregulation of hypoxia-inducible genes (*Hif1a*, *Vegfa*, etc), upregulation of mature macrophage genes (*Mertk*) and downregulation of monocyte genes (*Ly6c2*). ScRNA-seq has been performed at day 3, 4, 7, and 11 post-infarct by different groups, demonstrating unique transcriptional identities that become more clear over time (32, 36, 39). Importantly, key findings from different studies support the loss of the resident cardiac macrophage subpopulations, and in parallel–recruitment of monocytes that begin to specify as early as day 3-4, and subsequently their differentiation into a variety of transcriptionally unique populations, including those with more reparative properties.

Secondly, a subset of recruited monocyte-derived macrophages developed overlapping transcriptional identities that were nearly identical to resident macrophages (32). Interestingly, these recruited macrophages did not upregulate a handful of lineage-specifying genes such as *Lyve1* and *Timd4*, which proved useful as cell surface markers to reliably track the original resident macrophage populations without the need for genetic fate mapping. Despite the near transcriptional identity between these recruited macrophage populations, depletion of only resident macrophages resulted in decreased cardiac function and adverse remodeling, suggesting either a functionally (or temporally) non-redundant role. While precise functioning of resident macrophages in this context is unclear, it may be due to their ability to modulate the fate of recruited monocytes. Depletion of tissue resident macrophages increased the number of two recruited monocyte-derived macrophage fates (termed ARG1^{+} and CCRL2^{+} macrophages) (33). This sheds new light on the diversity of monocyte-derived macrophages within the injured heart and highlights tissue resident macrophages as important orchestrators of monocyte fate specification.

Not all resident macrophage populations behave similarly post-ischemic injury. For example, the depletion of CCR2^{+} cardiac macrophages prior to ischemic injury (43) reduced the number of pathologic ISG macrophages (IFIT3^{+}) and increased the number of ITGB7^{+} macrophages. We do not yet know what the function is of ITGB7^{+} cardiac macrophages, however their gene expression profile suggests they could be reparative. It is also unclear whether individual resident tissue macrophage subpopulations (TIMD4^{+} vs. CCR2^{+}) directly influence recruited monocytes, or have the capacity to recruit monocytes that have been shown to have a direct reparative role in other models [Ym1^{+}Ly6C^{hi} monocytes (22)]. It is equally likely that the depletion of individual resident macrophage populations changes the microenvironment rather than acting directly on recruited monocytes, and thus the altered microenvironment directs monocyte fate decisions after monocytes enter tissue.

DIVERSIFICATION OF AORTIC MACROPHAGE POPULATIONS IN ATHEROSCLEROSIS

Although much is known about the role of monocytes in the formation of atherosclerosis [as reviewed in (44)], two recent reports have now shown via scRNA-seq the heterogeneity that exists within the immune cell compartment of atherosclerotic lesions in two independent mouse models fed a high fat diet (*Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$) (40, 41). Consistent with both studies, is the identification of monocytes within atherosclerotic aortas (*Ly6c2*, *Ccr2*). Beyond the resident macrophage population seen in control and atherosclerotic mice (*Lyve1, Pf4*), inflammatory macrophages and TREM2^{hi} macrophages were also demonstrated, the latter being enriched in pathways linked to lipid metabolism and calcification (41) (**Figure 1**). Although macrophage populations were consistent between healthy and diseased aortas, the number of macrophages were increased, as well as the expression of a number of genes implicated in lipid and cholesterol metabolism and oxidative stress. In one study, the authors evaluated earlier (11 weeks) vs. more advanced atherosclerosis (20 weeks) with little difference in macrophage heterogeneity. This is consistent with the observation that lesional macrophages accumulate through local proliferation (28). These initial experiments proved to be informative, however it was not possible to differentiate macrophages isolated from within the artery wall itself (intimal) vs. those that accumulate outside the wall in the surrounding adventitia. Given the very different microenvironments in these two regions, it will be important for future studies to separately investigate each region. Moreover, atherosclerosis tends to develop regionally in the aorta (near the aortic root, lesser curvature of the aortic arch), thus understanding the differences between regions at the single cell level could provide clues to the regional nature of atherosclerosis initiation and progression.

OUTSTANDING QUESTIONS AND CONCLUSIONS

One key outstanding question which remains is how to interpret heterogeneity revealed by single cell data and move forward with functional studies. Retrospectively identifying population clusters bioinformatically is only the first step. Building a differentiation map of infiltrating monocytes and prospectively sorting populations based on robust combinations of surface markers will be an important approach to characterize individual populations. As technology and computational approaches improve, it will be important to integrate single cell mapping with tissue localization. For example, it is possible to perform single cell transcriptomics using methods that preserve tissue localization [MERFSIH, SlideSeq (45, 46)], whereby monocyte fate can be mapped from the blood vessel lumen to varied anatomical niches found within ischemic or atherosclerotic tissue by tracking individual or groups of RNAs. Linking single cell transcriptomic advancements to single cell epigenetics

and proteomics will further enhance resolution (47, 48). The analysis of the comprehensive landscape of cells within tissues has led to the generation of whole mouse and human cell atlas projects which take a relatively broad approach to cell characterization, but highlights the strength of this technology to be able to compare cells across tissues and species (49, 50). These multi-disciplinary approaches require collaboration, given the wide breadth of skills required to integrate different technologies. The use of single cell technologies to assess immune cells come with limitations, such as differential extraction of individual subsets and reduced read depth compared to bulk techniques, which are caveats that must be acknowledged. In addition, most of the initial studies utilized single replicates, thus the reproducibility of individual data sets is still a major question in the field. The identification of new subsets of monocytes and monocyte-derived cells within tissues at steady state and inflammation already highlights the profound role single cell technologies have had revealing previously unknown heterogeneity. Future insights into their function could allow for better therapeutic targets that aim to hinder chronic inflammation while promoting tissue repair and regeneration.

AUTHOR CONTRIBUTIONS

SD and SE wrote the manuscript. RZ created the figure with BioRender.com.

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frontiers in Immunology

REVIEW
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6-Sulfo LacNAc (Slan) as a Marker for Non-classical Monocytes

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Monocytes are subdivided into three subsets, which have different phenotypic and functional characteristics and different roles in inflammation and malignancy. When in man CD14 and CD16 monoclonal antibodies are used to define these subsets, then the distinction of non-classical CD14low and intermediate CD14high monocytes requires setting a gate in what is a gradually changing level of CD14 expression. In the search for an additional marker to better dissect the two subsets we have explored the marker 6-sulfo LacNAc (slan). Slan is a carbohydrate residue originally described to be expressed on the cell surface of a type of dendritic cell in human blood. We elaborate herein that the features of slan+ cells are congruent with the features of CD16+ non-classical monocytes and that slan is a candidate marker for definition of non-classical monocytes. The use of this marker may help in studying the role of non-classical monocytes in health and in diagnosis and monitoring of disease.

Keywords: monocyte subsets, slan, man, monkey, inflammation, cancer, CMML, lymphoma

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INTRODUCTION

The identification of monocytes in human blood has become much easier with advent of flow cytometry and the use of monoclonal antibodies to cell surface molecules. Antibodies to CD14 have been widely used for monocyte identification and with additional staining for CD16 at least three subsets (classical, intermediate, non-classical) can be defined (1). The CD14++CD16- classical monocytes can be clearly separated from the CD14++ CD16+ intermediate monocytes based on an isotype control for CD16 (2). However, the dissection of intermediate and non-classical monocytes is difficult and different approaches based on the level of CD14 expression have been used to set a cut-off between the two (2). Since differential roles in disease of these two CD16+ monocyte subsets have been documented, an unequivocal strategy is required for their dissection and here the use of the slan-marker has been suggested (3).

The slan-marker was first targeted with a monoclonal antibody termed M-DC8. This antibody was generated by immunizing Balb/c mice with human blood mononuclear cells depleted of T and B cells and monocytes (4). The resultant IgM antibody selectively stained about 1% of the mononuclear cells with light scatter properties between lymphocytes and monocytes. Phenotypic analysis of the M-DC8+ cells revealed that they had low CD33 and high CD16 expression levels.

Later on, the molecule recognized by the antibody was shown to be 6-sulfo LacNAc (slan), a sugar structure, which is linked to the cell surface protein PSGL-1 (P-selectin glycoprotein ligand), and the cells were dubbed slan dendritic cells (slanDCs) (5). There was early evidence suggesting that the blood leukocytes, which express the M-DC8 marker, belong to the monocyte lineage based on its similarity to the CD16+ monocytes including the low level expression of CD14 and absence of CCR2 (6, 7).

As illustrated in a CD14 CD16 dot plot, the slan+ cells (green) localize to the gate of non-classical monocytes (**Figure 1**) and here they account for the majority of CD14+CD16++ monocytes. There are a few events within that gate, which are slan-negative (pink color).

In phenotypic analyses similar patterns of cell surface markers were noted for CD16+ monocytes and "slanDCs." Also, similar results were reported for functional analyses such as cytokine production and antigen presentation. The same applies to many clinical studies and to response to anti-inflammatory therapies.

With the advent of transcriptome analysis, unsupervised hierarchical clustering approaches have then demonstrated that the blood slan+ cells cluster with monocytes and not with dendritic cells (8, 9). These findings have provided additional strong evidence for the monocyte nature of the slan+ leukocytes.

In the following, we will summarize the arguments to show that in human blood, slan+ cells are a subset of the CD16+ monocytes. Further, we will argue that slan is an appropriate marker for non-classical monocytes.

CELL SURFACE PHENOTYPE OF CD16+ MONOCYTES AND SLAN+ CELLS

As mentioned, the slan-residue is a sugar structure attached to PSGL1. PSGL1 is expressed by all leukocytes (10) including CD16- and CD16+ monocytes (11) but the slan residue is only found on a subset of CD16+ monocytes. It was shown that CHST2 can link the residue to the PSGL1 protein molecule (5). There are additional transferases including CHST15, which similar to CHST2 shows increased mRNA expression in CD16+ monocytes (12) and B3GALT2, which is increased in slan+ compared to slan- CD16+ monocytes (13). These findings need confirmation and the role of these transferases in generating the slan-residue needs to be determined.

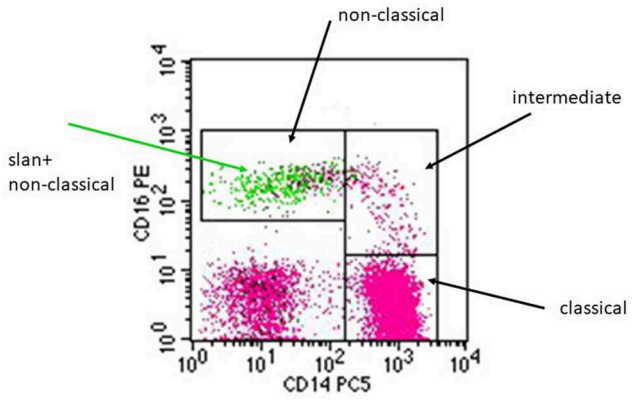

FIGURE 1 | Illustration of the pattern of slan+ positive cells in a CD14 CD16 dot plot. A whole blood sample from a healthy donor was stained for CD14, CD16, DR, and for slan using the FITC-conjugated slan IgM antibody (# 130-117-371, Miltenyi Biotec). Black arrows indicate the monocyte subsets defined via CD14 CD16 staining. The green arrow points at the green dots that represent the slan+ monocytes, which localize within the non-classical monocyte gate.

CD16+ monocytes have been characterized for cell surface markers in a host of flow cytometry analyses. Compared to CD14++ monocytes, higher levels of expression for HLA-DR and lower levels for CD11b, CD14, CD33, and CD64 have been noted for these cells (14, 15). Also, CD11a, c, and d were higher on the CD16+ cells, while CD62L was essentially absent. With respect to chemokine receptors, the CD16+monocytes were found to be CCR2 negative (11, 16) while CX3CR1 was found increased (17) and this went along with an absent and an increased response to the respective chemokine. Finally, CD115 the receptor for macrophage colony stimulating factor (M-CSF-R) was found increased in CD16+ monocytes (18).

Looking at slan+ cells in blood, high levels of HLA-DR, CD11c, CD16, and CX3CR1 and low levels of CD11b, CD14, CD33, and CD64 were noted, while CD62L and CCR2 were absent (4, 7, 19). High levels of CD115 on blood slan+ cells were only reported recently (20, 21). Slan+ cells were shown to express receptors for C3a and C5a (5, 9, 22), while for CD16+ monocytes only expression of C3aR mRNA was noted [see Table S5 in (23)].

Finally, C-type lectin receptors CD368 (Dectin-3) and CLEC5A were found essentially absent both in CD16+ monocytes and in slan+ cells, while classical monocytes showed a strong expression of these markers (24). Looking at these data, it is evident that the pattern of cell surface markers for the CD16+ monocytes and the slan+ cells is very similar. The congruent expression of these various functionally relevant receptors suggests similar functional properties of these cells.

FUNCTION OF CD16+ MONOCYTES AND SLAN+ CELLS

Cytokine Production

In response to LPS (lipopolysaccharide) the CD16+ monocytes were shown to be potent producers of cytokines like TNF (tumor necrosis factor) (25), while the production of the anti-inflammatory cytokine IL-10 was decreased compared to classical monocytes (26). This pattern of high TNF and low IL-10 production in response to LPS was confirmed by others (18, 27, 28). Also, higher TNF production by CD16+ monocytes was seen after stimulation with TLR7/8 ligands, with *Aspergillus fumigatus* conidia and toxoplasma tachyzoites (27, 29, 30). Also for blood slan+ cells, high levels of IL-1, IL-6, IL-12, and TNF protein were reported after stimulation by toll-like receptor ligands (31–34). In addition, TNF levels were shown to be even higher in slan+ cells of HIV-infected individuals (35).

With respect to IL-10, slan+ cells were shown to express lower levels compared to slan- cells (31) and also compared to classical monocytes (21). This latter study, in fact, provided a side-by-side comparison of slan+ cells and CD16+ non-classical monocytes with respect to cytokine production and it confirmed the higher levels of TNF and IL-12 and the lower levels for IL-10 for both CD16+ non-classical monocytes and slan+ cells as compared to classical monocytes. Hence, the two cells share a characteristic cytokine production pattern with high TNF and IL-12 and low IL-10 expression and this includes a stronger

responsiveness to the IFN-gamma-mediated priming compared to classical monocytes (21).

Since TNF and IL-12 play a dominant role in most inflammatory diseases, the concepts regarding the pathophysiological role of slan+ non-classical monocytes revolve around their ability to produce these cytokines. Because of this ability, the slan+ cells may be major players in infection and inflammation. Experiments, which selectively target these cells in disease models, are required to support this concept.

Cell-Cell Interactions

CD16+ monocytes in their original description were noted to express high levels of HLA-DR, i.e., the major MHC class II molecule in man (14). Consistent with the role of HLA-DR in presentation of peptide antigens to T cells, the CD16+ cells show potent induction of IFN-gamma in T cells in response to influenza Type A-antigen and purified protein derivative (36).

For the slan+ cells, antigen presentation studies using keyhole limpet hemocyanin and tetanus toxoid showed efficient induction of T proliferation (5). Here, the response generated by slan+ presenting cells was comparable to the response induced by CD11c+ dendritic cells and this was taken to support the conclusion that the slan+ cells belong to the dendritic cell lineage.

The induction of TH17 cells was shown to be supported both by CD16-positive monocytes and by slan+ cells. When CD4+ T cells were incubated in the presence of LPS with monocyte subsets then CD16+ intermediate monocytes were most potently supporting the generation of IL-17-producing T cells (28). In another study, using superantigen for T cell activation, the CD16+ non-classical monocytes were the strongest inducer of TH17 cells (37). Looking at slan+ cells, these cells were shown to be more potent than CD1c+ dendritic cells in inducing IL-17 in CD4+ CD45RA+ T cells after 7 days of co-culture (19).

In antibody dependent cellular cytotoxicity (ADCC), an effector cell can kill another cell via a bridging antibody that binds to the Fc-receptor on the effector cell and the cell surface antigen of a target cell. Monocytes are equipped with both high and low affinity Fc-receptors for IgG and the CD16+ blood monocytes were shown to efficiently kill B cell lymphoma cells via a CD20 monoclonal antibody (38). CD20-mediated ADCC of lymphoma cells was demonstrated for slan+ cells taken from healthy donors or patients with diffuse large B-cell lymphoma (39).

Furthermore, CD16+ monocytes showed ADCC against cells of the SKBR3 breast cancer cell line mediated via a monoclonal against HER2 (human epidermal growth factor receptor 2) (38). Strong ADCC activity against the same breast cancer cell line with the same anti-HER2 monoclonal antibody had been reported earlier when studying slan+ cells (40).

In the context of malignant melanoma, CD16+ non-classical monocytes were shown to be crucial to immune check-point blockade in that they mediated the killing of regulatory T cells via an antibody against CTLA-4 (cytotoxic T lymphocyte–associated antigen 4) (41). In this study, only patients with high numbers of CD16+ non-classical monocytes showed a decrease in tumor burden in response to therapy. This type of activity has not been reported from the perspective of slan+ cells, as yet.

Both CD16+ non-classical monocytes and slan+ cells have been noted to express the CD16 and CD32 Fc-receptors for IgG but none or little of the high affinity CD64 IgG Fc-receptor. In the context of ADCC, cooperation of CD16 and CD32 has been noted, but there was no role for CD64 (38). For slan+ cells such a cooperation of CD16 and CD32 had been reported earlier (40).

Slan+ cells have been shown to interact with neutrophils leading to an increased production of IL-12 by slan+ cells incubated with LPS plus IFNg (42). Also, neutrophils will reduce the cell death of slan+ cells, which occurs in *in-vitro* co-culture in the presence of LPS (43). Both induction of IL-12 and protection from cells death requires cell-cell contact. In this context, the CD16+ monocytes also have been noted to be susceptible to cell death in culture (44), but a protective effect of neutrophils or an induction of IL-12 has not been reported for CD16+ non-classical monocytes.

Conversely, slan+ cells can activate NK cells via IL-12 (42, 45). Such an activity has not been shown for CD16+ monocytes but it would not come unexpected since these cells are major producers of IL12 (46) and IL-12 is a major NK cell activator (47). Also, the activation of NK cells via transmembrane TNF expressed by slan+ cells (48) has not been shown for CD16+ monocytes yet but given the superior TNF production by CD16+ monocytes it is conceivable that these cells would be able to activate NK cells via this route.

One crucial issue in monocyte biology is the interaction of these cells with vascular endothelium. In *in-vitro* experiments human non-classical and also classical monocytes were reported to show a crawling ("patrolling") behavior (49). No such data are available for slan+ cells. Transmigration across endothelium was shown for CD16+ monocytes and, interestingly slan+ monocytes were mentioned to do the same (50). While PSGL-1 is involved in leukocyte-endothelium-interaction (51), there is no report on the function of the slan residue on PSGL-1, albeit an interaction of slan with lectins and a role in monocyte-endothelial-interaction are likely.

Taken together the interactions with T cells reported for slan+ blood cells under the label "dendritic cells" have also been published for CD16+ monocytes. Also with respect to ADCC similar findings have been reported from the perspective of CD16+ monocytes and the slan+ cells. However, when it comes to interaction with NK cells and neutrophils then the "slan DC" studies provide novel insights for the CD16+ non-classical monocytes.

Transcriptome of CD16+ Monocytes and Slan+ Cells

A comparative transcriptome study looked at CD1c+ dendritic cells, at classical, intermediate and non-classical monocytes and at slan-positive CD16+ and slan-negative CD16+ monocytes. Here, unsupervised hierarchical clustering clearly demonstrated that the slan+ cells cluster with monocytes and not the CD1c+ DCs (8).

Another study used hybridization to a human transcriptome array using cells isolated via magnetic cell sorting and flow cytometry cell sorting. Here the slan+ cells clustered away from both the CD1c+ and CD141+ DCs (9).

These transcriptome data consolidate the conclusion that slan+ cells in blood belong to the monocyte lineage. Therefore, at this stage the features previously described under the M-DC8+/slan+ dendritic cell concept, can now be ascribed to the slan+ non-classical monocytes. Therefore, in this paper the term "slan+ non-classical monocyte" will be used from here on.

Flow Cytometry Approach to Slan+ Monocytes

Monocytes currently are subdivided into three subsets, i.e., classical, intermediate, and non-classical monocytes (1) and in man they are defined using markers CD14, CD16, and DR. Separating non-classical and intermediate monocytes has been difficult within this setting, since different cut-off levels for CD14 have been used. To resolve this, the slan marker has been proposed as an additional marker for a positive definition of non-classical monocytes (18). In fact, molecular and clinical studies have demonstrated the feasibility of this approach (8). A typical staining of whole blood for slan+ non-classical monocytes is shown in **Figure 2A**. Here, we use a CD14 CD16 DR staining to determine the CD16 monocytes and then the slan+ CD16+ cells are defined. In the example shown there are 30.0 slan+ CD16+ cells /μL. In average of $n = 5$ the absolute number of these cells is 37.6 ± 11.4 cells/μL for the mouse IgM antibody.

Consistent with the carbohydrate nature of the slan structure the antibodies generated in the mouse are of the IgM class (4). More recently, a recombinant human IgG1 antibody has been generated at Miltenyi Biotec. This reagent, compared to isotype control, gives a similar staining pattern in flow cytometry (see **Figure 2B**).

Together, these data illustrate a straightforward strategy for determination of slan+ non-classical monocytes, a strategy that might be useful when it comes to monitoring of non-classical monocytes in disease and during therapy.

Recently it has been suggested that there might be subsets of slan+ non-classical monocytes with one subset characterized by an increase in expression of genes like CD41 and CD61 (13). Since these genes encode typical platelet receptors, the nature of this increase still needs to be resolved.

Also, we have to be open to the possibility that there may by some slan-negative cells with features of non-classical monocytes.

There have been reports that described CD16+ dendritic cells, which were identified among lineage-negative DR+ cells (23, 52, 53). While CD14-positive monocytes were excluded in the definition of these cells, the very low CD14-positive monocytes remained within the lineage-negative population. Comparative studies by Calzetti et al. have then demonstrated that cells dubbed CD16+ DCs do, in fact, belong to the CD16+ slan+ non-classical monocytes (21).

Clinical Studies Involving Slan+ Non-classical Monocytes

While there is a host of studies on monocyte subsets in inflammation and cancer, we will herein only highlight selected studies relevant to slan+ cells. When it comes to increases and decreases of the number of slan+ cells in patients, then changes with gender and age in healthy donor control values need to be considered. Here, slan+ monocytes were shown to be significantly higher in infants aged 6–12 months and in the elderly at age 60–70y (54).

Chronic Myelomonocytic Leukemia

The definition of monocyte subsets has emerged as a diagnostic tool for chronic myelomonocytic leukemia (CMML). The WHO classification lists CMML among the myelodysplastic/myeloproliferative neoplasms and requires for diagnosis a persistent blood monocyte count >1,000/μL and > 10% of all blood leukocytes (55). Since monocytosis is not unique to CMML and since cases may present with subthreshold monocyte counts, novel diagnostic approaches were explored. The original finding by Vuckovic et al. (56) noted that "The CD14lowCD16+ monocyte subpopulation was not found in CMML patients." Selimoglu-Buet et al. (57) then studied the diagnostic potential of this lack of non-classical monocytes by looking at the complementary increase of the classical monocytes and defining >94% of classical monocytes as a criterion for CMML. The usefulness of this additional test for diagnosis of CMML was subsequently confirmed (58). Furthermore, myelo-dysplastic syndrome (MDS) patients with subthreshold monocytosis, but increased classical monocytes were labeled "CMML-like" MDS and it was shown that several of these patients developed overt CMML within 1 year (59). Instead of looking at the increase of classical monocytes Hudson et al. (60) focused on the decrease of non-classical monocytes and reported a higher diagnostic specificity. Along these lines, Tarfi et al. (61) have then reported that slan-defined non-classical monocytes also gave a high diagnostic specificity. Hence, the slan marker may become the preferred tool in diagnosis of CMML based on the characteristic depletion of this subset.

Currently, a multicenter prospective ELN study is ongoing to validate the use of monocyte subsets in CMML diagnosis. To this end, the European Hematology Association and the European LeukemiaNet recommends the determination of monocyte subsets in flow cytometry to separate CMML from reactive monocytosis (62).

Cardiovascular Disease

Slan-defined monocyte subsets may be informative in atherosclerosis. Along these lines, Hamers et al. noted in a small study an increase of slan+ non-classical monocytes in patients with severe as compared to mild coronary artery disease (13). Also, an increase of slan+ cells had been noted in patients with peripheral artery disease (63). Given the many studies on the role of intermediate monocytes in cardiovascular disease, including their prognostic value (64), there also is potential for intermediate monocytes defined as CD16+ slan-negative monocytes in this context.

Inflammatory Disease

In systemic lupus erythematosus (SLE) immune complexes are of central pathogenic importance and such complexes can recruit leukocytes and thereby initiate damage. For lupus nephritis with pronounced sub-epithelial immune complex

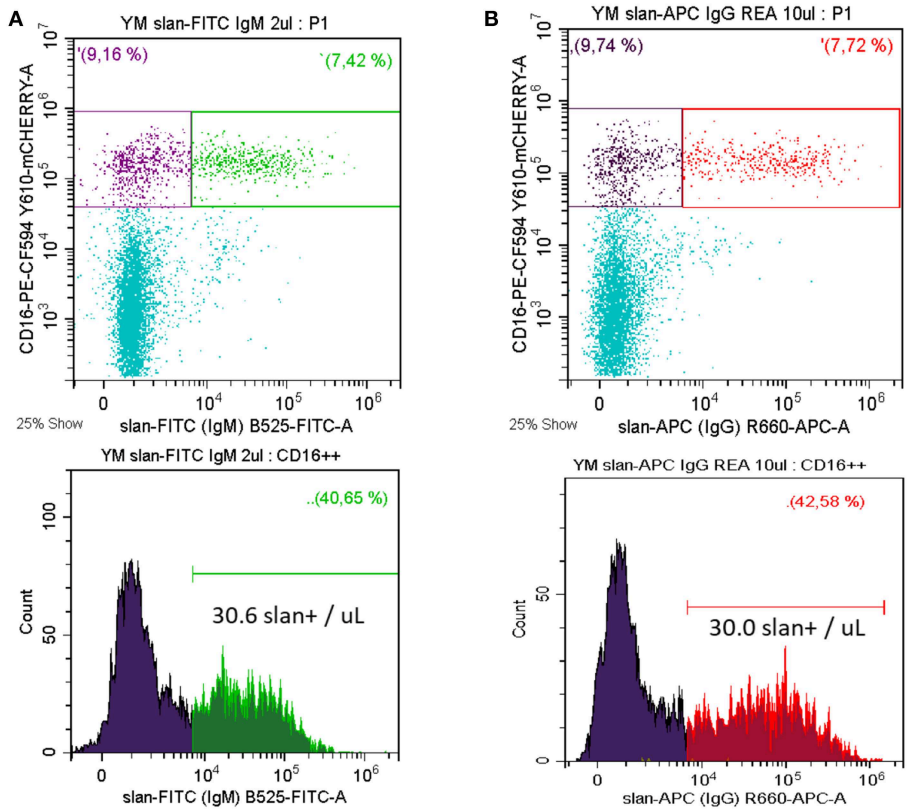

FIGURE 2 | Whole blood staining for slan+ non-classical monocytes. Whole blood samples were stained for CD14, CD16, DR, and slan. Shown is the slan vs. CD16 staining of all CD14+ monocytes in the upper panels. The respective single parameter slan+ histogram for all CD16+ monocytes is given in the lower panel **(A)** FITC-conjugated slan IgM antibody (# 130-117-371, Miltenyi Biotec) **(B)** APC-conjugated slan human IgG antibody (# 130-117-919, Miltenyi Biotec, kindly provided by Miltenyi Biotec). In average of 5 donors the absolute number of FITC-mu-IgM slan+ cells was 37.6 ± 11.4 cells/μL and of APC-hu-IgG slan+ cells it was 39.6 ± 16.8 cells/μL. Venous blood samples were obtained from healthy volunteers after informed consent and with the approval of the Ethics Committee of the LMU Medical Faculty, Munich.

deposits (class III and IV according to the International Society of Nephrology/Renal Pathology Society classification) an increased number of CD16+ cells had already been documented (65). Consistent with these findings in a recent study on lupus nephritis an increase in the frequency of slan+ monocytes in class III and IV glomeruli was shown (66). These slan data are obviously much more informative compared to staining for CD16+ cells because they strongly suggest the presence of non-classical monocytes while the demonstration of CD16+ cells in tissue sections is less specific since this receptor is also present on neutrophils and NK cells.

Cancer

An increased number of blood slan+ non-classical monocytes, associated with a decreased frequency of pDCs, has been found in patients with colorectal carcinoma (CRC) (22) and in diffuse large B cell lymphoma (DLBCL) (39).

In lymph nodes in proximity to metastatic carcinoma cells (where they are well-positioned for tumor cell destruction) slan+ cells can be readily detected (22). However, slan+ cells are not present within the primary sites nor within the metastases tissue in solid cancer.

In contrast to solid tumors, the slan+ cells can be found within lymphoma tissue and here they can display either dendrites that extend into the tissue or they have a more rounded macrophage-like morphology (39). The latter type of cell may be involved in antibody-dependent cellular phagocytosis (ADCP) of tumor cells. In addition, slan+ non-classical monocytes can efficiently destroy B lymphoma cells via anti-CD20 in ADCC (39).

Taken together there are several reports on slan+ non-classical monocytes in disease settings. Given the extensive literature on CD16+ monocytes in inflammation and cancer revisiting these areas with the use of the slan marker may generate novel insight into the monocyte subsets involved.

DRUGS TARGETING SLAN+ NON-CLASSICAL MONOCYTES

Glucocorticoid therapy was shown to selectively reduce the number of CD16+ monocytes, while classical monocytes increase and this was shown both in multiple sclerosis patients and in healthy volunteers (67, 68).

The depletion of CD16+ monocytes is likely to be mediated by induction of apoptosis and was shown to act via the nuclear steroid receptor (68). In a more recent article, the effect of high dose GCs on slan+ cells was studied in multiple sclerosis and here a depletion of these cells in blood was described (69).

Interferon-beta (IFN-beta) therapy in multiple sclerosis patients had been shown to decrease CD16 monocytes with low level expression of CD14 after 4 weeks of therapy (70). Later, such treatment was demonstrated to reduce blood slan+ cells in multiple sclerosis patients (69). These findings were substantiated in a study on hepatitis C patients, which showed an almost complete disappearance of slan+ cells and of CD14low CD16++ monocytes on day 30 of IFN-alpha therapy (71).

An anti M-CSF antibody in a rheumatoid arthritis pilot study showed depletion of both CD16+ non-classical and intermediate monocytes (72). Similarly, in Diffuse Type Tenosynovial Giant Cell Tumor (=Pigmented Villonodular Synovitis) a selective reduction of non-classical monocytes was noted after treatment with the humanized anti-M-CSF receptor antibody emactuzumab, a treatment that reduces the tumor–promoting macrophages within the tumor tissue (73). As detailed before, slan+ are rare among the tumor infiltrating M-CSF-R+ macrophages (74). Therefore, it is unlikely that these slan+ cells in tissue are an important therapeutic target in cancer. Still, the determination of slan+ non-classical monocytes in blood may be a useful tool for monitoring of anti-M-CSFR therapy in cancer.

G-CSF treatment can increase the number of slan+ monocytes (75, 76) and this is in line with earlier studies that suggested an increase of CD16 on total blood monocytes after G-CSF (77).

Lenalidomide is a thalidomide derivative used in therapy of multiple myeloma (78). It binds to the E3 ubiquitin ligase complex and directs its substrate specificity to IKFZ transcription factors leading to their proteasomal degradation (79) and this leads to cell death of myeloma cells. Lenalidomide treatment also leads to depletion of B cells and of non-classical monocytes, which correlates with the intracellular depletion of IKFZ1 protein (80). Earlier work suggest that ubiquitin is relevant to slan+ non-classical monocytes since transcriptome analysis of these cells has revealed a ubiquitin-signature in that altogether 50 UBC-linked genes were selectively up- or down-regulated in these cells (8). It remains to be determined whether any of these differential genes is involved in the lenalidomide depletion of slan+ non-classical monocytes

Laquinimod is a quinolone-3-carboxamide, which is being evaluated as a therapy for multiple sclerosis (81). In a phase I dose escalation study a reduction within 2 weeks of the slan+ cell frequency by 80% was noted (82), while in a separate study no change was seen for numbers of T cells, B cells, NK cells and CD14+ monocytes (83). The mechanism of action remains unclear, but an involvement of the aryl hydrocarbon receptor and of NF-kB has been proposed (82).

Taken together, most studies on drug effects have reported on monocyte subsets defined via CD14 and CD16 but only some have looked at slan+ cells. The slan-marker offers an unequivocal alternative for drug monitoring of non-classical monocytes in blood under various clinical settings.

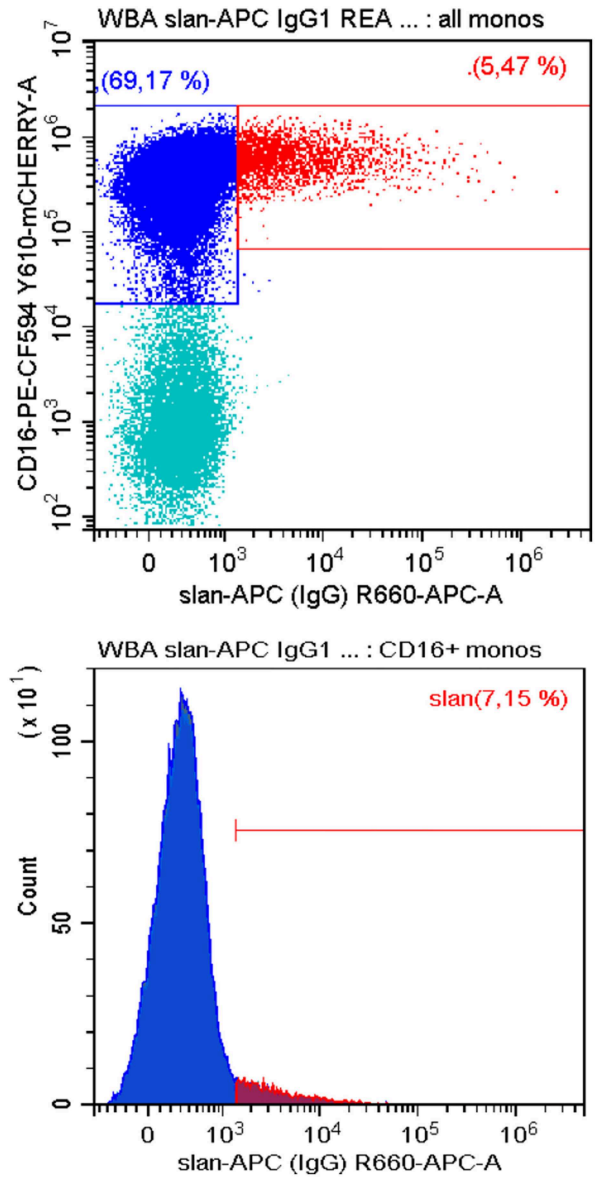

FIGURE 3 | Staining for slan+ non-classical monocytes in common marmosets. Peripheral blood mononuclear cells from −140°C-stored samples of common marmosets (Callithrix jacchus) (Deutsches Primatenzentrum, Goettingen) were thawed and stained for CD14, CD16, CD56, DR, and slan (APC-conjugated slan human IgG antibody REA 1050, # 130-117-919, Miltenyi Biotec). Shown is the slan vs. CD16 staining of all CD14+ monocytes in the upper panel. The respective single parameter slan+ histogram for all CD16+ monocytes is given in the lower panels. CD56+NK cells were excluded. One of three samples is shown. In average of 3 samples the slan+ monocytes account for 3.4 ± 1.8% of all monocytes. Blood sampling was approved by the German Primate Center Ethics Committee and the Lower Saxony State Office for Consumer Protection and Food Safety in accordance with the European Union guidelines on the welfare of non-human primates used in Research and the European Union (EU directive 2010/63/EU).

Slan+ Cells in Tissue

Another intriguing novel aspect is the detection of slan+ cells in various tissues. In normal tissue, these cells are

sparse with a few scattered slan+ cells for instance in the dermis. However, in inflammatory disease such as psoriasis and atopic dermatitis these cells can increase substantially (19, 34, 84).

In tonsils, there is also a low number of these cells but at levels similar to CD141+ dendritic cells (20). Here, the cells localize preferably to the T cell areas (31). Phenotypically the tonsil slan+ cells show lower CD16 and CX3CR1 and higher CD14 compared to blood slan+ non-classical monocytes (20). Of note, the tonsils studied were from patients undergoing tonsillectomy for recurrent infection such that here information is only available on inflamed tissue. Therefore, it is unclear as to whether these differences are due to location or to inflammation or to both.

Also in lymph nodes, slan+ cells are rare but as discussed above they increase with metastasis of carcinomas to the draining lymph nodes (22).

The expression of the slan-marker in skin, tonsils, lymph nodes, and tumor metastasis as presented above would be consistent with the concept that these cells are the progeny of the blood slan+ non-classical monocytes, which have migrated into these tissues. On the other hand, it is possible that the slan-residue is induced via sulfo-transferases in an unrelated type of leukocyte residing in the tissue. However, experiments using tumor-cell conditioned media revealed that the slan marker is very stable and apparently not inducible in other leukocyte populations (22).

In any case, to resolve the relationship of slan+ monocytes in blood and slan+ cells in tissue, a comparative characterization including transcriptomics of slan+ cells in blood and tissue is required.

Slan+ Cells in Other Species

To date all studies on slan+ monocytes have been conducted with human samples. We have tested whether the slan-antibodies can also be used to identify homologous cells in old-world and new world monkeys. Here, monoclonal antibodies targeting human cells surface molecules have been successfully used to define monocyte subsets based on CD14 CD16 markers (85). As shown in **Figure 3** in blood mononuclear cells from common marmosets slan+ cells can be readily detected using the recombinant monoclonal antibody. Here a large proportion of monocytes is CD16 positive such that the percentage of slan+ cells among all CD16+ monocytes is low at 7.2%. However, the percentage of the slan+ cells among all monocytes is at 5.5% and with that comparable to man (see **Figure 2B** upper panel).

These data demonstrate that the new world marmoset monkeys have the potential to serve as a model in the study of slan+ monocytes.

It remains to be determined whether slan or a similar sugar structure exists on PSGL in other mammalian animals including mice.

CONCLUDING REMARKS AND PERSPECTIVE

This review summarizes the evidence, which shows that the slan+ cells in human blood are part of the CD16+ monocytes and their phenotypic and functional properties are identical to non-classical monocytes. It remains to be determined whether slan covers all non-classical monocytes and whether there is heterogeneity among the slan+ cells. Single cell sequencing may be able to address these questions.

In any event, the slan-marker has potential for monitoring of non-classical monocytes in various disease states and the many studies on CD16+ monocytes in inflammation and cancer should be revisited using slan.

Future work should look into selective targeting of these cells in order to demonstrate a crucial role of slan+ non-classical monocytes and their cytokine production in disease. Then there are many open questions regarding the interaction of slan+ monocytes with the endothelium. Finally, it will be important to determine whether there is a structure homologous to slan on mouse non-classical monocytes such that these cells can be studied in experimental animals other than non-human primates.

AUTHOR CONTRIBUTIONS

TH and CS-H performed experiments. TH, AL, CS-H, MC, and LZ-H wrote the paper.

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MicroRNAs: Fine Tuners of Monocyte Heterogeneity

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Small non-coding microRNAs (miRNAs) have been found to play critical roles in many biological processes by controlling gene expression at the post-transcriptional level. They appear to fine-tune the immune response by targeting key regulatory molecules, and their abnormal expression is associated with immune-mediated inflammatory disorders. Monocytes actively contribute to tissue homeostasis by triggering acute inflammatory reactions as well as the resolution of inflammation and tissue regeneration, in case of injury or pathogen invasion. Their contribution to tissue homeostasis can have many aspects because they are able to differentiate into different cell types including macrophages, dendritic cells, and osteoclasts, which fulfill functions as different as bone remodeling and immune response. Monocytes consist of different subsets with subset-specific expression of miRNAs linked to distinct biological processes dedicated to specific roles. Therefore, understanding the role of miRNAs in the context of monocyte heterogeneity may provide clues as to which subset gives rise to which cell type in tissues. In addition, because monocytes are involved in the pathogenesis of chronic inflammation, associated with loss of tissue homeostasis and function, identifying subset-specific miRNAs might help in developing therapeutic strategies that target one subset while sparing the others. Here, we give an overview of the state-of-the-art research regarding miRNAs that are differentially expressed between monocyte subsets and how they influence monocyte functional heterogeneity in health and disease, with descriptions of specific miRNAs. We also revisit the existing miRNome data to propose a canonical signature for each subset.

Keywords: microRNA, monocytes, Ly6C^{high}, Ly6C^{low}, CD14^{+}, CD16^{+}

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INTRODUCTION

MicroRNAs (miRNAs) are a class of short non-coding RNAs (18–22 nt), conserved from worms to mammals that play a regulatory role in gene expression at the posttranscriptional level (1). Since their discovery, many studies have shown that they are involved in biological processes. Quantitative and qualitative assessments of miRNA expression in various disease conditions have revealed considerable changes in their expression profiles.

The biogenesis of miRNAs occurs in the nucleus. MiRNA-encoding genes are transcribed to a primary miRNA and processed by Drosha, a class 2 RNase III enzyme, into a precursor miRNA (pre-miRNA), which is exported to the cytoplasm by exportin-5. In the cytoplasm, mature forms of miRNAs are produced after several steps involving Dicer, a RNase III type protein, and RISC, a RNA-induced silencing complex (2). MiRNA genes can be located in the context of non-coding

transcription units or in the introns of protein-coding genes (3, 4). Almost half of miRNA genes are clustered and can be independently or simultaneously transcribed into single polycistronic transcripts (5, 6).

Currently, more than 2,800 and 2,100 miRNAs have been identified in human and mouse, respectively (miRBase vs22). Both *in vitro* and bioinformatic analyses have determined that more than 500 genes could be targeted by a single miRNA (7, 8). MiRNAs bind mRNA targets by their "seed" sequence interacting with the 3′untranslated region (UTR), and more rarely with the coding region (CDS) or 5′UTR, of the targeted mRNA (9). According to the degree of complementarity, miRNAs lead to mRNA cleavage and degradation or to the inhibition of translation, thus interfering with the downstream protein output (10). MiRNA family members can be highly conserved among vertebrates, in particular in the seed region, which corresponds to nucleotides 2 to 7/8 and is the main determinant of target specificity (11). Thus, miRNAs with similar seed sequence can target similar sets of genes and similar biological pathways.

Extensive work has been performed to identify miRNA-specific signatures in immune cells and to understand how a specific miRNA gene controls the development and function of a specific immune cell population. However, few studies have addressed the role of miRNAs in terms of subset heterogeneity of one specific immune cell type. Here we review reports of miRNAs in monocyte subsets and performed an *in silico* analysis that also includes new data to revisit the current knowledge of monocyte subset functions.

miRNA-BASED SIGNATURES SPECIFIC TO MONOCYTE SUBSETS

Monocytes are composed of two main subsets in both mouse and human (12) that are committed to different functions (13–15): in mice, the "classical" inflammatory Ly6C^{high} and the "non-classical" patrolling Ly6C^{low} monocyte subsets. Their human counterparts are CD14^{+}CD16^{-} and CD14^{dim}CD16^{++}, respectively (12, 13). Ly6C^{high} monocytes secrete inflammatory mediators in response to bacteria and can differentiate into macrophages, inflammatory dendritic cells (DCs), and osteoclasts (OCs) (16–18). Ly6C^{low} monocytes survey endothelial cells and surrounding tissues to detect damage and viral threat and are involved in tissue repair (13). Although mouse and human studies have underscored the relevance of studying monocyte subsets in disease by showing differential accumulation of both subsets, factors that regulate monocyte subset fate and functional heterogeneity under pathophysiological conditions remain poorly explored.

MiRNAs play pivotal roles in regulating monocyte development and functions, including differentiation, tissue recruitment, activation, initiation, and resolution of inflammation (19); however, very little is known about their involvement when considering the heterogeneity of monocytes. An attempt to close this gap in knowledge has been addressed by miRNome analyses of monocyte subsets. Nevertheless, few miRNome analyses of monocyte subsets have been performed with human or mouse samples. To our knowledge and from free-access databases, we identified only three studies (20–22); two focused on miRNAs differently expressed between classical and non-classical monocytes, in humans and mice, without considering "intermediate" monocytes (20, 21). After showing differences in DNA methylation in the three human monocyte subsets—classical, non-classical, and intermediate (23)—Zawada et al. studied miRNA profiling for human "intermediate" monocytes (22).

Thus, in the current review, we combined all existing data with our own unpublished miRNome data for both classical and non-classical monocyte subsets isolated from human and mouse blood to provide novel insights into monocyte subset-specific miRNA signatures.

Analysis of the miRNome of Classical and Non-classical Monocyte Subsets

Briefly, we collected miRNome datasets (GSE52986 and GSE32370) from the GEO bank (http://www.ncbi.nlm.nih.gov/geo/). For each GEO dataset, we compared miRNA expression profiles for classical and non-classical monocyte subsets (i.e., CD14^{++} CD16^{-} and CD14^{+} CD16^{++} for human blood samples, Ly6C^{high} and Ly6C^{low} for mouse blood samples) to obtain a list of miRNAs differentially expressed between the subsets in both species. The technical platforms used in these two studies were Illumina Human v2 and Mouse v1 MicroRNA expression beadchips, respectively. Also, we performed large-scale miRNA screening using a TaqMan low-density array to identify miRNAs differentially expressed between classical and non-classical monocyte subsets in human and mouse. With false discovery rate-adjusted $P \leq 0.05$, we found 25 miRNAs differentially expressed between classical and non-classical monocytes. We then used a Venn diagram to visualize common miRNAs between all four datasets (http://www.irp.nia.nih.gov/bioinformatics/vennplex.html). Only miR-146a was commonly downregulated in classical monocytes, for all available human and mouse datasets, independent of the technological platform used (**Figure 1A**). At the intersection of human datasets, we identified nine miRNAs (miR-132, miR-106a, miR-19b, miR-18b, miR-20b, miR-146a, miR-342-3p, miR17, and miR18a): miR-132, miR146a, and miR-342-3p showed lower expression in classical than non-classical monocytes. At the intersection of mouse datasets, we identified 4 miRNAs (miR-146a, miR-130b, miR-150, and miR-148a); miR-148a and miR-130b were upregulated in Ly6C^{high} versus Ly6C^{low} monocytes. Only a very small number of miRNAs was specific to mouse (miR-150) or human (miR-18b, miR-19b, miR-106a, and miR-132) subsets.

Genomic Organization of miRNAs Specific to Monocyte Subsets

Among the 25 miRNAs identified, 16 showed sequence homology between human and mouse (**Table 1**) and almost 70% were organized in clusters in both species; examples are miR-17/92, miR-106a/363 and miR-106b/25 (**Figure 1B**). Only two miRNAs were not organized in clusters in either species: miR-342/151b and miR-150/5121 in human and mouse, respectively.

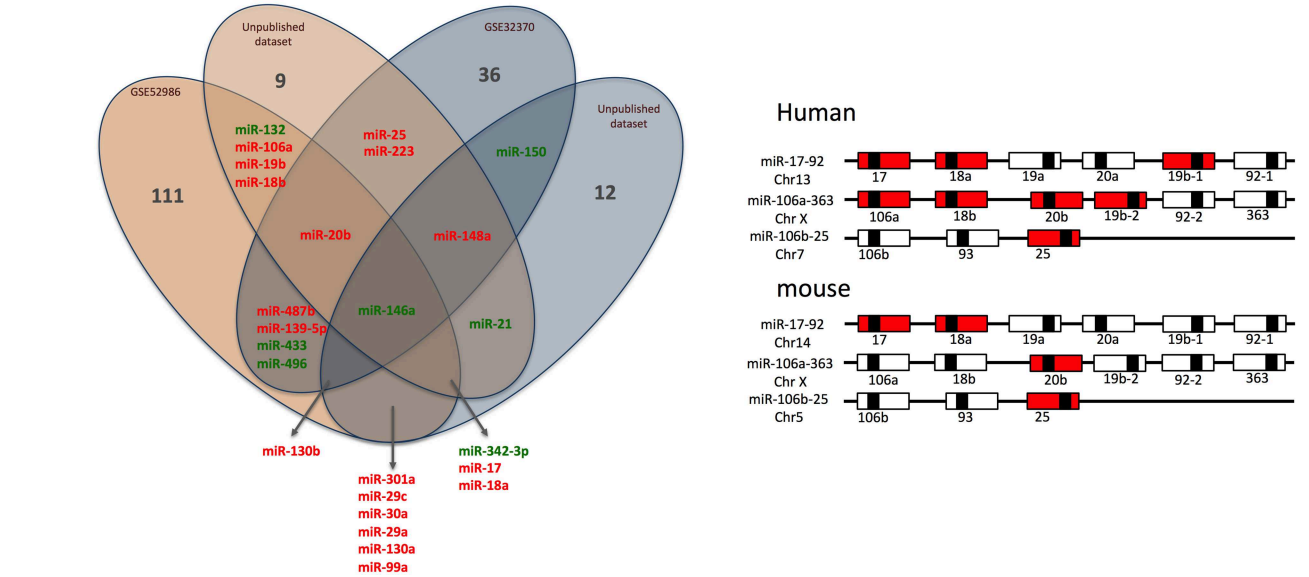

FIGURE 1 | Human and mouse miRNome profiles identifying monocyte subset-specific miRNAs. **(A)** Four datasets were analyzed and clustered to obtain a Venn diagram showing miRNAs differentially regulated between classical and non-classical monocytes and common to human and mouse. Red and green represent miRNAs up- and down-regulated, respectively, in classical vs. non-classical monocytes. **(B)** Schematic representation of members of the miR-17/92 family of miRNA gene clusters in human and mouse. MiRNAs upregulated in classical monocytes vs. non-classical monocytes are in red.

Of note, miR-17, miR-18a/b, miR-19a/b, miR-20b, miR-25, and miR106a are members of the three paralog clusters: miR-17/92, miR-106a/323, and miR-106b/25. These clusters contain miRNAs that are very comparable, regulate similar sets of genes, and have overlapping functions (24). Their genomic organization is highly conserved, which suggests important functions and coordinated regulations. Overall, 7 and 4 miRNAs in human and mouse, respectively, were overexpressed in classical monocytes (**Figure 1B**).

The miR-17/92 cluster is a well-described cluster that plays a role in immune responses (25). In the lymphocyte lineage, this cluster is expressed in B and T precursor cells, and its expression diminishes upon differentiation (26). In the monocytic lineage, monocyte hematopoiesis is affected by loss of the miR-17/92 cluster in humans but is unaffected in mouse (27). Human $CD34^{+}$ hematopoietic progenitor cells differentiate *in vitro* into monocytes upon exposure to macrophage-colony stimulating factor; this differentiation leads to decreased expression of the miR-17/92 cluster, which is inversely correlated with upregulation of the transcription factor acute myeloid leukemia 1, a validated human target of miR-17. In addition, overexpression of the miR-17/92 cluster delays terminal differentiation of monocytes, and its inhibition accelerates differentiation (28). Differences between human and mouse data may be caused by species-specific differences and/or the fact that experiments were performed *in vitro* or *in vivo*. Using genetic mouse models, deletion of the miR-17/92 cluster and its paralog miR-106b/25 led to severe developmental defects, so these two miRNA clusters may act synergistically on cell survival to control embryonic development (27). Moreover, miR-17, miR-20a, and miR-106a, which belong to the two cluster paralogs miR-17/92 and miR-106a/363, regulate macrophage infiltration, phagocytosis, and proinflammatory cytokine secretion via targeting signal-regulatory protein alpha expression, both *in vitro* and *in vivo* (29).

Despite rare reports describing these 25 miRNAs in the context of monocyte subsets (see below), many more exist on their role in monocyte differentiation or inflammation processes. For example, some miRNAs are involved in macrophage polarization; one is miR-148a-3p, which promotes macrophage 1 (M1) polarization and inhibits M2 polarization upon Notch activation (30). Others are involved in osteoclastogenesis [e.g., the miR-29 family regulates osteoclast commitment and migration (31)]. MiR-223 is upregulated during granulopoiesis and fine-tunes the differentiation of myeloid precursors into granulocytes or monocytes and negatively controls the activity of NLRP3 inflammasome in these cell types (32). Also, miR-433 negatively regulates the hematopoietic cell proliferation by directly targeting interferon-induced guanylate-binding protein 2 (33), and miR-130a regulates the expression of macrophage pro-fibrogenic genes in chronic inflammation (34).

Putative Function of miRNAs Specific to Human Monocyte Subsets

With human miRNome data from classical and non-classical monocytes (**Figure 1A**), we quantified the expression of the nine miRNAs commonly deregulated between both subsets by using RT-qPCR and new samples. We confirmed the overexpression of miR-132, miR-146a, and miR-342-3p in human non-classical

TABLE 1 | List of miRNAs common in human and mouse miRNome datasets.

Name	Species	Cluster (miRNA)	Chromosome position	miRNA mature seq	miRNA* mature seq
miR-106a	*hsa*	Yes (miR-18b, miR-20b, miR-19b-2, miR-92a-2, miR-363)	chrX: 134170198-134170278	AAAAGUGCUUACAGUGCA GGUAG	CUGCAAUGUAAGCACUUC UUAC
	mmu	Yes (miR-18b, miR-20b, miR-19b-2, miR-92a-2, miR-363)	chrX: 52742503-52742567	CAAAGUGCUAACAGUGCA GGUAG	ACUGCAGUGCCAGCACUU CUUAC
miR-130a	*hsa*	No	chr17: 59151136-59151221	GCUCUGACUUUAUUG CACUACUCAGUGCAAUAGU AUUGUCAAAGC	
	mmu	No	chr11: 87113004-87113089	CAGUGCAAUAGUAUUGUC AAAGC	GCUCUGACUUUAUUGCAC UACU
miR-130b	*hsa*	yes (miR-301b)	chr22: 21653304-21653385	CAGUGCAAUGAUGAAAGG GCAU	ACUCUUUCCCUGUUGCAC UAC
	mmu	yes (miR-301b)	chr16: 17124061-17124142	CAGUGCAAUGAUGAAAGG GCAU	ACUCUUUCCCUGUUGCAC UACU
miR-132	*hsa*	Yes (miR-212)	chr17: 2050271-2050380	UAACAGUCUACAGCCAUG GUCG	ACCGUGGCUUUCGAUUGU UACU
	mmu	Yes (miR-212)	chr11: 75173388-75173478	UAACAGUCUACAGCCAUG GUCG	AACCGUGGCUUUCGAUUG UUAC
miR-139-5p	*hsa*	No	chr11: 72615063-72615130	UCUACAGUGCACGUGUCU CCAGU	
	mmu	No	chr7: 101475376-101475443	UCUACAGUGCACGUGUCU CCAG	
miR-146a	*hsa*	No	chr5: 160485352-160485450	UGAGAACUGAAUUCCAUG GGUU	CCUCUGAAAUUCAGUUCU UCAG
	mmu	No	chr11: 43374397-43374461	UGAGAACUGAAUUCCAUG GGUU	CCUGUGAAAUUCAGUUCU UCAG
miR-148a	*hsa*	No	chr7: 25949919-25949986	UCAGUGCACUACAGAACU UUGU	AAAGUUCUGAGACACUCC GACU
	mmu	No	chr6: 51269812-51269910	UCAGUGCACUACAGAACU UUGU	AAAGUUCUGAGACACUCC GACU
miR-150	*hsa*	No	chr19: 49500785-49500868	UCUCCCAACCCUUGUACC AGUG	CUGGUACAGGCCUGGGGG ACAG
	mmu	yes (miR-5121)	chr7: 45121757-45121821	UCUCCCAACCCUUGUACC AGUG	CUGGUACAGGCCUGGGGG AUAG
miR-17	*hsa*	Yes (miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a-1)	chr13: 91350605-91350688	CAAAGUGCUUACAGUGCA GGUAG	ACUGCAGUGAAGGCACUU GUAG
	mmu	Yes (miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a-1)	chr14: 115043671-115043754	CAAAGUGCUUACAGUGCA GGUAG	ACUGCAGUGAGGGCACUU GUAG
miR-18a	*hsa*	Yes (miR-17, miR-19a, miR-20a, miR-19b-1, miR-92a-1)	chr13: 91350751-91350821	UAAGGUGCAUCUAGUGCA GAUAG	ACUGCCCUAAGUGCUCCU UCUGG
	mmu	Yes (miR-17, miR-19a, miR-20a, miR-19b-1, miR-92a-1)	chr14: 115043851-115043946	UAAGGUGCAUCUAGUGCA GAUAG	ACUGCCCUAAGUGCUCCU UCUG
miR-18b	*hsa*	Yes (miR-106a, miR-20b, miR-19b-2, miR-92a-2, miR-363)	chrX: 134170041-134170111	UAAGGUGCAUCUAGUGCA GUUAG	UGCCCUAAAUGCCCCUUC UGGC
	mmu	Yes (miR-106a, miR-20b, miR-19b-2, miR-92a-2, miR-363)	chrX: 52742331-52742413	UAAGGUGCAUCUAGUGCU GUUAG	UACUGCCCUAAAUGCCC CUUCU
miR-19b-1	*hsa*	Yes (miR-17, miR-19a, miR-18a, miR-20a, miR-92a-1)	chr13: 91351192-91351278	UGUGCAAAUCCAUGCAA AACUGA	AGUUUUGCAGGUUUGCAU CCAGC
	mmu	Yes (miR-17, miR-19a, miR-18a, miR-20a, miR-92a-1)	chr14: 115044305-115044391	UGUGCAAAUCCAUGCAA AACUGA	AGUUUUGCAGGUUUGC AUCCAGC
miR-19b-2	*hsa*	Yes (miR-106a, miR-18b, miR-20b, miR-92a-2, miR-363)	chrX: 134169671-134169766	UGUGCAAAUCCAUGCAAAA CUGA	AGUUUUGCAGGUUUGCAU UUCA

(Continued)

TABLE 1 | Continued

Name	Species	Cluster (miRNA)	Chromosome position	miRNA mature seq	miRNA* mature seq
	mmu	Yes (miR-106a, miR-18b, miR-20b, miR-92a-2, miR-363)	chrX: 52741983-52742066	UGUGCAAAUCCAUGCAA AACUGA	AGUUUUGCAGAUUUG CAGUUCAGC
miR-20b	*hsa*	Yes (miR-106a, miR-18b, miR-19b-2, miR-92a-2, miR-363)	chrX: 134169809-134169877	CAAAGUGCUCAUAGUGCA GGUAG	ACUGUAGUAUGGGCACUU CCAG
	mmu	Yes (miR-106a, miR-18b, miR-19b-2, miR-92a-2, miR-363)	chrX: 52742113-52742192	CAAAGUGCUCAUAGUGCA GGUAG	ACUGCAGUGUGAGCACU UCUAG
miR-21	*hsa*	No	chr17: 59841266-59841337	UAGCUUAUCAGACUGAUG UUGA	CAACACCAGUCGAUGG GCUGU
	mmu	No	chr11: 86584067-86584158	UAGCUUAUCAGACUGA UGUUGA	CAACAGCAGUCGAUGGGC UGUC
miR-223	*hsa*	No	chrX: 66018870-66018979	UGUCAGUUUGUCAAAUAC CCCA	CGUGUAUUUGACAAGC UGAGUU
	mmu	No	chrX: 96242817-96242926	UGUCAGUUUGUCAAAUA CCCCA	CGUGUAUUUGACAAGCU GAGUUG
miR-25	*hsa*	Yes (miR-106b, miR-93)	chr7: 100093560-100093643	CAUUGCACUUGUCUCGGU CUGA	AGGCGGAGACUUGGG CAAUUG
	mmu	Yes (miR-106b, miR-93)	chr5: 138165321-138165404	CAUUGCACUUGUCUCGGU CUGA	AGGCGGAGACUUGGG CAAUUGC
miR-29a	*hsa*	Yes (miR-29b-1)	chr7: 130876747-130876810	UAGCACCAUCUGAAAUC GGUUA	ACUGAUUUCUUUUGGUG UUCAG
	mmu	Yes (miR-29b-1)	chr6: 31062660-3106274	UAGCACCAUCUGAAAUC GGUUA	ACUGAUUUCUUUUGGUG UUCAG
miR-29c	*hsa*	Yes (miR-29b-2)	chr1: 207801852-207801939	UAGCACCAUUUGAAAUCG GUUA	UGACCGAUUUCUCCU GGUGUUC
	mmu	Yes (miR-29b-2)	chr1: 195037547-195037634	UAGCACCAUUUGAAAUCG GUUA	UGACCGAUUUCUCCU GGUGUUC
miR-30a	*hsa*	No	chr6: 71403551-71403621	UGUAAACAUCCUCGACUG GAAG	CUUUCAGUCGGAUGUUU GCAGC
	mmu	No	chr1: 23272269-23272339	UGUAAACAUCCUCGAC UGGAAG	CUUUCAGUCGGAUGUUU GCAGC
miR-342-3p	*hsa*	Yes (miR-151b)	chr14: 100109655-100109753	UCUCACACAGAAAUCGCA CCCGU	
	mmu	No	chr12: 108658620-108658718	UCUCACACAGAAAUCGCAC CCGU	
miR-433	*hsa*	Yes (miR-337, miR-665, miR-431, miR-127, miR-432, miR-136)	chr14: 100881886-100881978	UACGGUGAGCCUGUCAU UAUUC AUCAUGAUGGGC UCCUCGGUGU	
	mmu	Yes (miR-337, miR-3544, miR-665, miR-3070-1, miR3070-2, miR-431, miR-127, miR-434, miR-432, miR-3071, miR-136)	chr12: 109591715-109591838	AUCAUGAUGGGCUCCUCG GUGU	UACGGUGAGCCUGUCAUU AUUC
miR-487b	*hsa*	Yes (miR-376c, miR-376a-2, miR-654, miR-376b, miR-300, miR-1185-1, miR-1185-2, miR-381, miR-539, miR-889, miR-544a, miR-655, miR-487a, miR-382, miR-134, miR-668, miR-485, miR-323b)	chr14: 101046455-101046538	GUGGUUAUCCCUGUCCU GUUCGAAUCGUACAGG GUCAUCCACUU	
	mmu	Yes (miR-495, miR-667, miR-376c, miR-654, miR-376b, miR-376a, miR-300, miR-381, miR-539, miR-889, miR-544, miR-382, miR-134, miR-668, miR-485, miR-453)	chr12: 109727333-109727414	AAUCGUACAGGGUCAUCCA CUU	UGGUUAUCCCUGUCCU CUUCG

(Continued)

TABLE 1 | Continued

Name	Species	Cluster (miRNA)	Chromosome position	miRNA mature seq	miRNA* mature seq
miR-496	*hsa*	Yes (miR-487a, miR-382, miR-134, miR-668, miR-485, miR-323b, miR-154, miR-377, miR-541, miR-409, miR-412, miR-369, miR-410, miR-656)	chr14: 101060573-101060674	UGAGUAUUACAUGGCCAA UCUC	
	mmu	Yes (miR-544, miR-382, miR-134, miR-668, miR-485, miR-453, miR-154, miR-377, miR-541, miR-409, miR-412, miR-369, miR410, miR-3072)	chr12: 109739119-109739197	UGAGUAUUACAUGGCCAAU CUC	AGGUUGCCCAUGGUGU GUUCA
miR-99a	*hsa*	Yes (miR-let-7c)	chr21: 16539089-16539169	AACCCGUAGAUCCGA UCUUGUG	CAAGCUCGCUUCUAUGG GUCUG
	mmu	Yes (miR-let-7c)	chr16: 77598936-77599000	AACCCGUAGAUCCGAUC UUGUG	CAAGCUCGUUUCUAUGG GUCU

hsa, human; mmu, mouse; chr, chromosome. Red highlights nucleotide differences between humans and mice.

vs. classical monocytes and the overexpression of miR-17, miR-18a, miR-18b, miR-19b, miR-20b, and miR-106a in classical vs. non-classical monocytes (**Figure 2**).

Zawada et al. hypothesized that intermediate monocytes have a distinct miRNA profile as compared with classical and non-classical monocytes and identified 38 miRNAs differentially expressed in intermediate monocytes vs. both classical and non-classical monocytes (22). **Figure 3** gives a schematic representation of the miRNA expression profile patterns for the three human monocyte subsets in the Zawada et al. study. Of note, miRNAs in panel 1 with gradually increasing expression from classical to intermediate and non-classical monocyte subsets included the three miRNAs that we found upregulated in our comparative study (miR-132, miR-146a, and miR-342-3p; **Figure 2A**). Panels 2 and 3, showing decreasing expression from classical to non-classical monocytes, displaying (panel 2) or not (panel 3) differences between intermediate and non-classical monocytes, contained the six miRNAs that we found downregulated in our comparative study (**Figure 2B**). Zawada et al. identified a fourth panel, including miR-150, with downregulated miRNAs in intermediate monocytes as compared with both classical and non-classical monocytes ($p < 10^{-10}$, and > 10-fold difference in expression). Our Venn diagram analysis identified miR-150 as the only miRNA with differential expression between classical and non-classical monocyte datasets in mouse but not human datasets (**Figure 1A**), which agrees with the Zawada et al. miRNome data (Panel 4).

Using OmicsNet, a web-based tool for the creation and visual analysis of biological networks (35), we uploaded the list of nine monocyte subset-specific miRNAs identified in our human analysis together with the list of 182 genes found overexpressed in classical or non-classical monocytes identified in five independent transcriptomic microarray datasets (36). We aimed to create and merge different types of biological networks that could provide a clue to the pathways involved in the functional heterogeneity of monocyte subsets. **Figure 4A** shows the 3D OmicsNet biological networks highlighting connections between the nine miRNAs and putatively targeted genes according to the TarBase software [**Table 2**; (37)]. Gene Ontology analysis with the Reactome pathway database (38) showed enrichment of biological process categories such as signal transduction, small GTPases of the Rho family (Rho GTPases), p75 NTR receptor-mediated and Sema4D in semaphorin signaling (**Figure 4B** and **Table 3**). The trafficking of monocytes into tissues requires the activation of integrins via signal transduction induced by Rho GTPases such as RHOA or RAP1, which results in cell adhesion to the blood-vessel wall (39). Rho-GTPases are key regulators of cellular actomyosin dynamics and are therefore considered pharmacological targets for restricting leukocyte motility, including monocytes, in inflammatory disorders (40). A comparison of protein expression based on cell maturity (from pro-monocyte to monocyte and to macrophage lineages) suggested that Rho proteins are readily available for signaling events in response to numerous activating cues (41). Human CD100/Sema4D belongs to a large family of membrane-bound proteins named Semaphorins that are involved in numerous functions, including axon guidance, morphogenesis, carcinogenesis, and immunomodulation; Sema4D in particular influences monocyte migration (42). Resident microglia and infiltrated peripheral monocytes are two main types of immune cells in the central nervous system that control the inflammation process. Recently, the p75 neurotrophin receptor (p75NTR) was found to play a role in the peripheral expansion and central nervous system trafficking of pro-inflammatory monocytes (43).

Although these pathways and putative target genes have not yet been validated and functionally studied, further investigating their implication will increase our understanding of the functional heterogeneity of monocyte subsets.

ROLE OF miRNAs IN MONOCYTE SUBSETS

Since the first description of blood monocytes in the early 2000s as a heterogeneous population of leukocytes displaying different phenotypic markers, homing properties, and immune

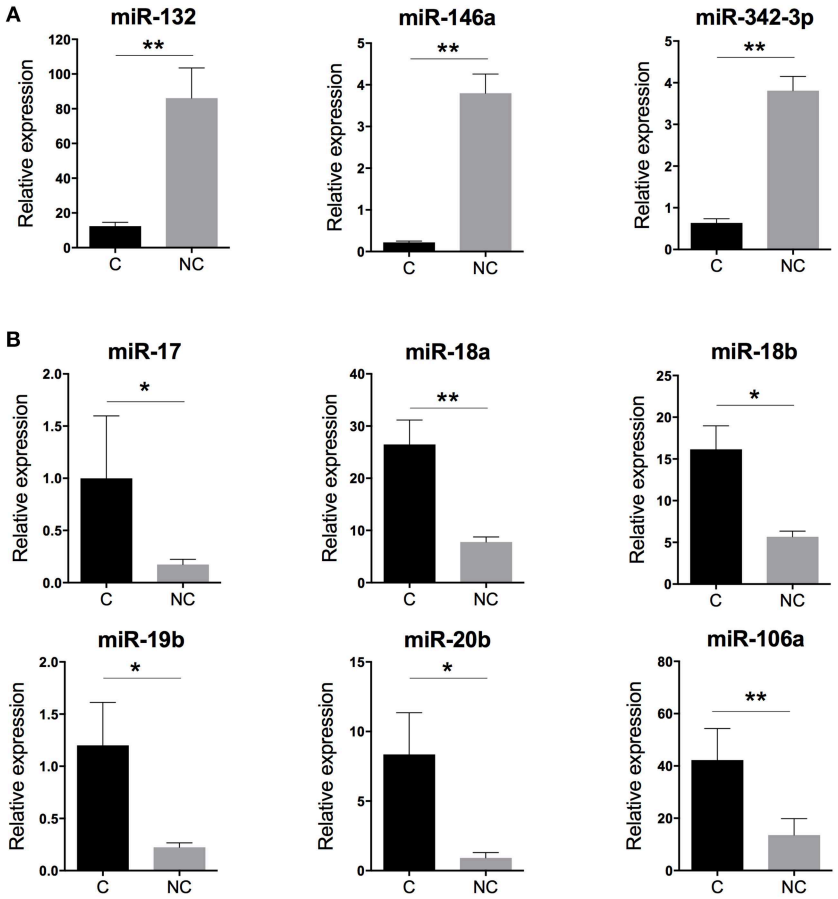

FIGURE 2 | Validation of the human monocyte subset-specific miRNA-based signature. Blood samples from healthy donors ($n = 7$) were collected from the French Blood Establishment (EFS). After Ficoll-Paque density gradient, classical (C) $CD14^{++}CD16^{-}$ and non-classical (NC) $CD14^{+}CD16^{++}$ monocyte subsets were FACS sorted with >97% purity (Montpellier RIO Cytometry platform). Total RNA was extracted from both monocyte subsets by using a miRNeasy kit and the automatized QIAcube procedure (QIAGEN). MiRNA expression was quantified by using multiplexed TaqMan RT-qPCR (Life Technology). **(A)** Quantification of the three miRNAs overexpressed in non-classical vs. classical monocytes. **(B)** Quantification of the six miRNAs overexpressed in classical vs. non-classical monocytes. Data are mean ± SD and differences were compared by non-parametric Mann-Whitney test (*$p < 0.05$, **$p < 0.01$).

functions (44), the scientific community has tried to dissect the role of individual subsets by identifying protein-encoding genes that specifically control the development and function of each sub-population. For example, the lineage-defining transcription factor nuclear receptor 4a1 (*Nr4a1*) was found essential for $Ly6C^{low}$ monocyte development because *Nr4a1*$^{-/-}$ mice lack $Ly6C^{low}$ monocytes (45). Because *Nr4a1* regulates inflammatory gene expression and differentiation of $Ly6C^{low}$ monocytes, the functions of $Ly6C^{high}$ monocytes can be studied independently *in vivo* by using *Nr4a1*$^{-/-}$ mice (46). The same expectations have been expressed for miRNA-encoding genes. However, few miRNAs have been identified (see previous section), and only three have been thoroughly studied *in vivo* by using genetic models. The first identified and most studied is miR-146a. In 2012, the group of Mikael Pittet showed that miR-146a is the highest differentially expressed miRNA between $Ly6C^{high}$ and $Ly6C^{low}$ monocytes (20). Also, until 2018, it remained the only miRNA described as regulating the functional heterogeneity of monocyte subsets.

Control of Innate Immune Response to Infections

For many years, miR-146a has been known as a negative regulator of inflammation in myeloid cells (26, 47). In-depth characterization of miR-146a$^{-/-}$ mice revealed decreased hematopoietic stem cell homeostasis during chronic inflammation, dysregulated hematopoietic stem cell differentiation toward myeloid cells, and abnormal myeloproliferation (48). The group of Pittet showed that miR-146a expression was inducible only in $Ly6C^{high}$ monocytes upon inflammatory stimuli, reaching levels comparable to those in $Ly6C^{low}$ monocytes in basal conditions. Lack of miR-146a in mice did not alter the development of monocyte subsets but markedly amplified the inflammatory response of $Ly6C^{high}$ monocytes upon bacterial challenge by targeting RelB, a non-canonical NF-κB family member highly conserved between mice and humans. This amplification of the inflammatory response is not due to more pro-inflammatory cytokine production per cell but rather to an expansion of $Ly6C^{high}$ monocytes in the

bone marrow and their increased trafficking to inflamed tissue during acute bacterial challenge because of high expression of CCR2 and responsiveness to monocyte chemoattractant protein 1-mediated chemoattraction. This interesting result parallels the fact that neither TNF receptor associated factor six nor interleukin 1 receptor associated kinase 1 expression was modified by miR-146a in Ly6C^{high} monocytes (20, 49), but they were modified in monocytic cell lines (47). Overall, by maintaining a low level of miR-146a, Ly6C^{high} monocytes can rapidly proliferate into the bone marrow to be the first mobilized cells to egress into the circulation and rejoin the site of bacterial attack. In contrast, Ly6C^{low} monocytes remain insensitive to this type of environmental danger because of constitutive high expression of miR-146a. Thus, the gradual increase of miR-146a expression in Ly6C^{high} monocytes upon stimulation acts as a negative feedback loop that represses proliferation and prevents overwhelming amplification of the inflammation by so-called inflammatory Ly6C^{high} monocytes within the injured tissue, which would be deleterious. However, this study does not answer the question of the role of miR-146a in Ly6C^{low} monocytes.

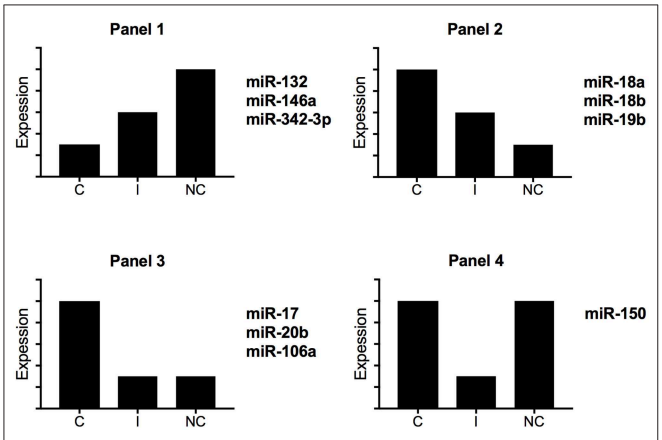

FIGURE 3 | Schematic representation of miRNA expression profiles for human monocyte subsets. By using miRNome data from the study of Zawada et al. (22), we identified four different expression profiles. C, classical monocytes CD14^{++}CD16^{-}; I, intermediate monocytes CD14^{++}CD16^{+}; NC, non-classical monocytes CD14^{+}CD16^{++}.

RelB can directly bind with the aryl hydrocarbon receptor (AHR) that supports the xenobiotic-detoxifying pathway, the AHR nuclear translocator like 1 (also named Bmal1) partner of Clock that regulates the circadian rhythm, and the bioenergy sensor sirtuin 1 (Sirt1) to integrate acute inflammation with changes in metabolism and mitochondrial bioenergetics. Finally, RelB is involved in chromatin modifications, and low RelB expression recapitulates the formation of silent heterochromatin upon endotoxin tolerance conditions, further halting inflammatory signaling (50). Although these functions have not all been investigated in terms of monocyte heterogeneity

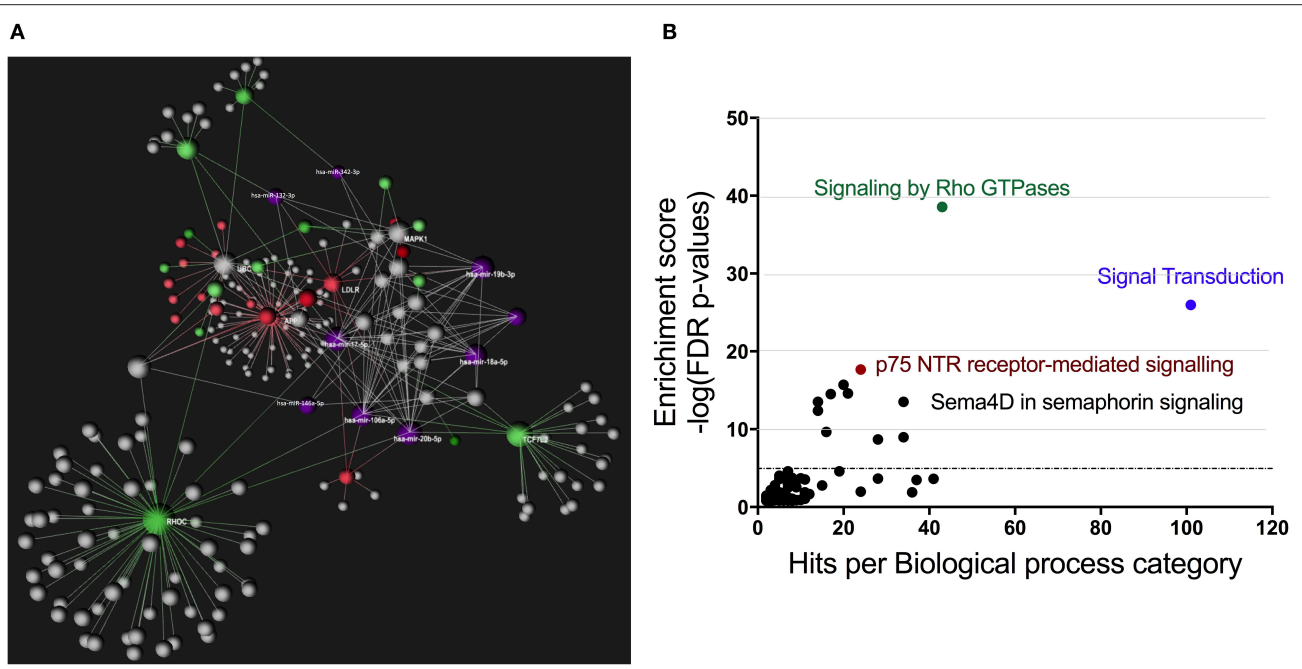

FIGURE 4 | Gene ontology analysis of genes putatively targeted by monocyte subset-specific miRNAs. **(A)** Using OmicsNet, a force-directed sub-network was constructed for the nine miRNAs with differential expression between classical and non-classical human monocytes (color violet) and their putative target genes extracted from a list of 182 genes with differential expression in classical and non-classical monocyte subsets. Red and green represent genes up- and downregulated, respectively, in classical vs. non-classical monocytes. Genes in gray are those that link genes putatively targeted by miRNAs or are associated in the network. **(B)** By using Reactome pathway data, we plotted genes with differential expression between monocyte subsets as the number of genes for the respective biological function category (x-axis) against the enrichment score for log10 of *p*-value (y axis).

TABLE 2 | List of genes putatively targeted by the nine monocyte subset-specific miRNAs.

miR-17	miR-18a	miR-18b	miR-19b	miR-20b	miR-106a	miR-132	miR-146a	miR-342-3p
AIB1	AIB1		AIB1	AIB1	AIB1	AIB1	AIB1	AIB1
APP					APP			
CCND1	CCND1	CCND1		CCND1	CCND1			
CRK				CRK	CRK	CRK		
DCAF8	DCAF8	DCAF8	DCAF8	DCAF8	DCAF8			
F2RL1				F2RL1	F2RL1			
FAS	FAS		FAS		FAS		FAS	
GIGYF1	GIGYF1	GIGYF1	GIGYF1	GIGYF1	GIGYF1			
ITGA2	ITGA2	ITGA2	ITGA2	ITGA2	ITGA2			
								INSIG1
LDLR			LDLR	LDLR	LDLR	LDLR		
MAPK1			MAPK1	MAPK1	MAPK1	MAPK1		
MAPK14			MAPK14		MAPK14	NAP1L1		
NAP1L1								
PTEN	PTEN		PTEN	PTEN	PTEN			
							RAC1	
RHOC				RHOC	RHOC			
RLIM	RLIM	RLIM	RLIM	RLIM	RLIM			
RORA	RORA	RORA	RORA	RORA	RORA			
SMAD4	SMAD4		SMAD4	SMAD4	SMAD4		SMAD4	
TCF7L2				TCF7L2	TCF7L2			
TNRC6B	TNRC6B	TNRC6B	TNRC6B	TNRC6B	TNRC6B			
UBC	UBC			UBC				
WAC	WAC	WAC	WAC	WAC	WAC			WAC

TABLE 3 | Gene ontology and functional pathway enrichment analysis.

Pathway	Total	Expected false positives	Hits	–log (FDR *p*-values)
Sema4D induced cell migration and growth-cone collapse	29	0.634	14	22.1
Sema4D in semaphorin signaling	34	0.743	14	18.8
NRAGE signals death through JNK	45	0.984	17	17.3
Rho GTPase cycle	123	2.69	43	16.0
Signaling by Rho GTPases	123	2.69	43	16.0
Cell death signaling via NRAGE NRIF and NADE	62	1.36	20	14.7
Lipoprotein metabolism	22	0.481	7	14.6
p75 NTR receptor-mediated signaling	85	1.86	24	12.9
G alpha (12/13) signaling events	80	1.75	21	12.0
Semaphorin interactions	72	1.57	16	10.2
Signaling by NGF	290	6.34	34	5.4
Axon guidance	292	6.38	28	4.4
Platelet activation signaling and aggregation	220	4.81	19	4.0
Developmental Biology	417	9.12	34	3.7
Signal Transduction	1,690	36.9	101	2.7

The top 10 terms were selected according to false discovery rate (FDR)-adjusted p-values.

and/or miRNA context, the miR-146/Relb axis might be the missing link with Bmal1-dependent regulation of Ly6C^{high} diurnal variations controlling their trafficking to sites of inflammation (51), AHR-dependent regulation of Ly6C^{high} monocyte-derived DC differentiation (52), and Sirt1-mediated inhibition of the pro-inflammatory macrophage activation (53). These are interesting areas to be addressed.

Control of the Inflammatory Response in the Context of Atherosclerosis

In 2015, the group of Robert Raffai showed that apolipoprotein E (ApoE) expression was higher in Ly6C^{low} than Ly6C^{high} monocytes (54). The expression of ApoE in monocytes had an anti-inflammation effect by enhancing the purine-rich PU-box binding protein 1-dependent miR-146a transcription, thereby

reducing Ly6C^{high} monocytosis, NF-κB–mediated inflammation, and atherosclerosis in the setting of hyperlipidemia. Thus, increasing miR-146a expression in Ly6C^{high} monocytes might have therapeutic application in atherosclerosis. Also, miR-146a may play a role in controlling the proliferation of Ly6C^{high} monocytes. This finding contradicts the general concept that monocytes are non-proliferating cells (55) but agrees with studies observing Ly6C^{high} monocytosis in bone marrow and blood, in different pathological contexts (20, 49, 54).

Role in Bone Erosion and Formation

RelB also promotes the differentiation of myeloid precursors into DCs and OCs and activates the transcription of pro-inflammatory genes in response to immune signals and environmental stressors. Monocyte subset-specific differences in miR-146a expression, together with the well-described role of miR-146a as a negative feedback regulator of inflammation and osteoclastogenesis in myeloid cells (56, 57) and reduced level of miR-146a expression in Ly6C^{high} monocytes, might explain why Ly6C^{high} monocytes are prone to egress from the bone marrow upon inflammatory stimuli and differentiate into DCs and OCs upon entry into the inflamed site. Also, in contrast, this information might also explain why by maintaining constitutively high levels of miR-146a, Ly6C^{low} monocytes are prevented from differentiating into DCs and OCs. Indeed, monocyte subsets have a differential contribution to osteoclastogenesis (16). The capacity of the Ly6C^{high} subset but not Ly6C^{low} subset to develop into OCs has been recently attributed to low miR-146a expression (49). Indeed, our group showed that classical monocytes display reduced miR-146a expression in both arthritic humans and mice as compared with healthy individuals; *in vivo* delivery of miR-146a mimics into Ly6C^{high} monocytes using a specific delivery system that spares Ly6C^{low} monocytes (58) rescued RelB expression in Ly6C^{high} monocytes, reduced their capacity to differentiate into OCs and reduced inflammatory-mediated bone erosion in an experimental model of arthritis. This is the first work to provide an *in vivo* proof of concept for a therapeutic strategy design targeting a subset-specific miRNA. Whether miR-146a plays other roles in Ly6C^{high} monocytes and investigating its Ly6C^{low}-specific function(s) remains for further investigation. Our transcriptomic analyses comparing both monocyte subsets sorted from miR-146a$^{+/+}$ and miR-146$^{-/-}$ mice showed that miR-146a modulates the expression of 1,000 genes in Ly6C^{high} monocytes but only 100 genes in Ly6C^{low} monocytes (49), which suggests that beyond osteoclastogenesis, miR-146a may play other roles in Ly6C^{high} functions but not many in Ly6C^{low} monocytes.

miRNA Function in Monocyte Subset Differentiation

Recently, the group of Stéphane Potteaux identified an miRNA critical for generating Ly6C^{low} monocytes. The authors observed increased expression of miR-21 in non-classical Ly6C^{low} monocytes of atherosclerotic ApoE$^{-/-}$ mice, which mediated their higher number and lifespan in this model (59). The frequency of Ly6C^{low} monocytes in blood, bone marrow and spleen was markedly reduced in ApoE$^{-/-}$ miR-21–deficient mice. Consequently, Ly6C^{low} monocyte numbers were reduced in the atherosclerotic aorta because of increased susceptibility to apoptosis. However, miR-21 deficiency did not affect trafficking of Ly6C^{high} monocytes nor their number in atherosclerotic aortas or the size of lesions but was associated with the presence of more pro-inflammatory macrophages in plaque, increased necrotic core, deficient efferocytosis, and increased macrophage death. This work reveals a role for miR-21 in atherosclerosis development and reveals the proof of concept that inhibiting miR-21 in monocytes might have relevant therapeutic application in atherosclerosis. However, many questions remain, including the target gene(s) that mediates the observed phenotype in ApoE$^{-/-}$ mice, whether miR-21 plays a role in Ly6C^{low} monocytes under non- ApoE$^{-/-}$ conditions, and which role it plays (if any) in the biology of Ly6C^{high} monocytes. In addition, because miR-21 controls macrophage polarization, apoptosis, and efferocytosis (60), determining which of these functions is affected in the context of monocyte subsets would be of interest.

In 2018, the group of Eric Solary identified miR-150 as overexpressed in Ly6C^{low} monocytes as compared with Ly6C^{high} monocytes and critical for promoting the terminal differentiation of classical monocytes into non-classical monocytes in both humans and mice (61). The authors found a defect of Ly6C^{low} monocytes in miR-150–deficient mice that did not affect the total number of monocytes in peripheral blood and bone marrow and was due to the un-repressed expression of Tet methylcytosine dioxygenase 3 (*Tet3*) gene. Tet3 is a dioxygenase that binds DNA and mediates demethylation but also promotes open chromatin independent of its catalytic function. Overall, Tet3 enhances transcription and gene expression, especially during changes in cellular identity (62). Thus, high *Tet3* expression in Ly6C^{high} monocytes due to low expression of miR-150 in this subset prevented their differentiation into Ly6C^{low} monocytes. This finding has important clinical implications because reduced expression of miR-150 was also found in peripheral-blood CD14^{+} monocytes, mostly classical monocytes, of patients with chronic myelomonocytic leukemia. Thus, monitoring the repartition of monocyte subsets is now an international recommendation as a diagnostic tool for patients with monocytosis to distinguish between chronic myelomonocytic leukemia and reactive monocytosis (63).

CONCLUSION

In this review, we give an overview of the state-of-the-art research of miRNAs that are differentially expressed between monocyte subsets and how they affect monocyte functional heterogeneity, with descriptions of functional and *in silico* studies of specific miRNAs. Three miRNAs miR-146a, miR-21, and miR-150 with differential expression in classical vs. non-classical monocyte subsets were all first identified as immune system regulators. The three miRNAs are inducible mediators of anti-inflammatory responses in the myeloid lineage acting via negative feedback loops and leading to the resolution of inflammation. Thus, they represent switches from pro- to anti-inflammatory responses of real therapeutic potential.

In terms of the functional heterogeneity of monocyte subsets, the few studies described bring new valuable insights into the role of the three miRNAs. Indeed, by identifying new target

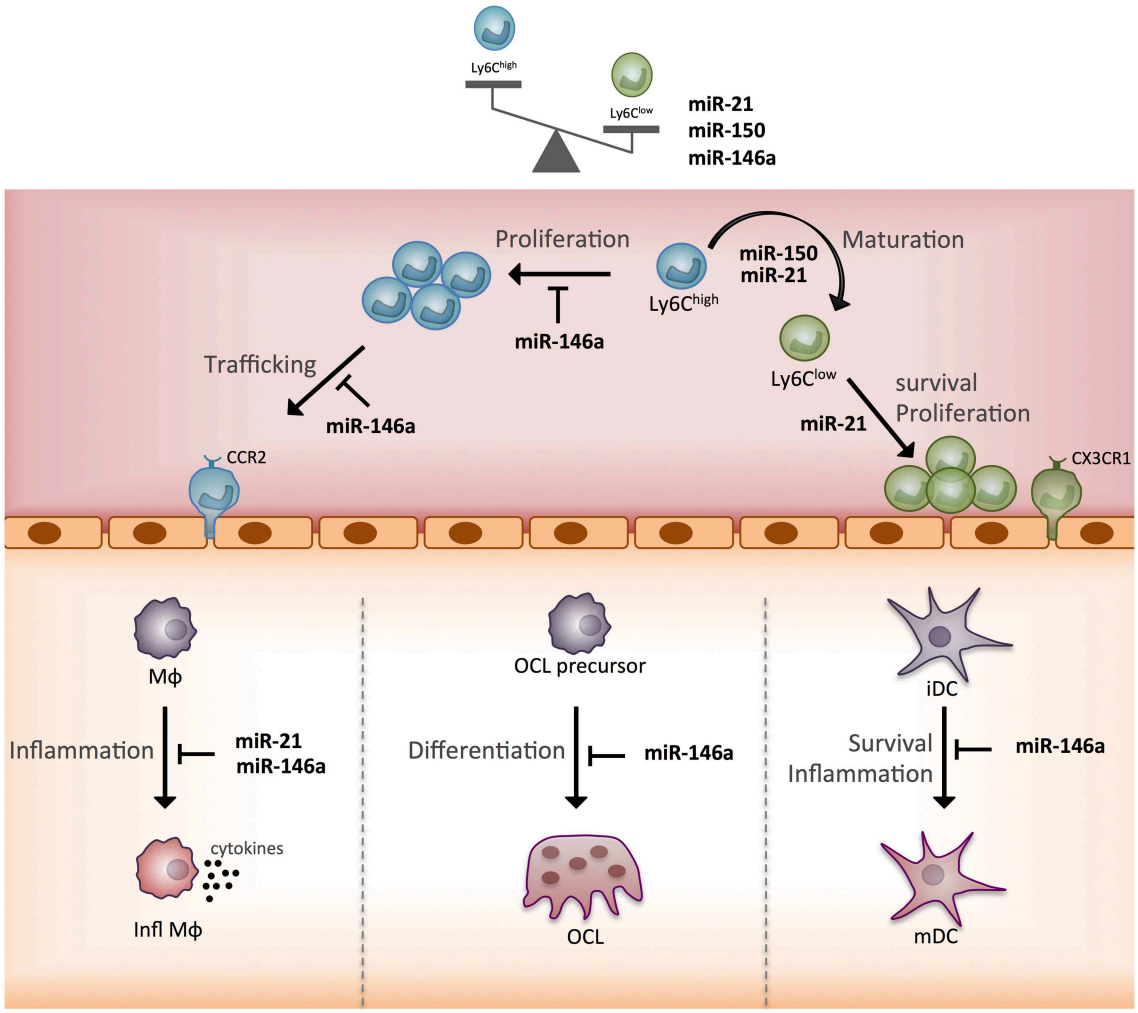

FIGURE 5 | Schematic representation of the function of three monocyte subset-specific miRNAs. With the three miRNAs showing differential expression in monocyte subsets that were functionally studied in mouse models, we propose a scheme outlining their role. MΦ, macrophage; Infl MΦ, inflammatory macrophage; OC, osteoclast; iDC, immature dendritic cell; mDC, mature dendritic cell; CCR2, C-C chemokine receptor type 2; CX3CR1, CX3C chemokine receptor 1.

genes and functions that discriminate Ly6C^{low} and Ly6C^{high} mouse monocytes, these studies helped specify miRNA-based mechanisms for the commitment of monocytes to a cellular subset fate and related functional specificity (**Figure 5**). Of note, miR-146a, miR-21, miR-150 all show greater expression in Ly6C^{low} rather than Ly6C^{high} monocytes, and their expression is increased by inflammation. The reported role for miR-146a concerns mainly Ly6C^{high} monocyte inflammatory functions, and that of miR-21 and miR-150 concerns the generation of Ly6C^{low} monocytes. However, so far, all studies have been performed with Ly6C^{high} monocytes, thus future studies need to work with Ly6C^{low} monocytes to address the role of miRNAs in the function specific to Ly6C^{low} monocytes. In addition, the role of miRNAs in the commitment of monocyte subsets to OCs remains poorly explored and deserves further investigations (64).

Finally, we must revisit this knowledge in light of recent works using single-cell RNA sequencing of human blood monocytes (65), high-dimensional mass cytometry (66), and ontogeny study (67), under steady state or pathological conditions (68), which broadens our perspectives by identifying new monocyte subsets and further underlines the control of monocyte plasticity by miRNAs and their target genes. Recently, 29 human immune cell types have been characterized by RNA sequencing and flow cytometry, and mRNA heterogeneity and abundance appeared to be cell type-specific (69). Most miRNAs act as rheostats, refining the expression of hundreds of genes to enhance cell differentiation. Thus, miRNA detection in single-cell monocytes is needed to understand the biology of the heterogeneity of monocytes and to propose new strategies for disease treatment and diagnosis.

DATA AVAILABILITY

The datasets analyzed for this study can be found in the NCBI gene expression omnibus, GSE137729 and GSE137730 for mouse and human data sets, respectively.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local human ethics committee (ID RCB 2008-A01087-48) and with the code of ethics of the world medical association. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ID-R contributed to the design of the work, analysis and interpretation of the data, wrote the review, and designed the figures and tables. MR and CP contributed to the acquisition and analysis of the data and revised the manuscript. FA contributed to the conception of the work and interpretation of the data, wrote the review, and designed the figures. All authors gave approval for publication of the content.

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REVIEW
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Inherited and Environmental Factors Influence Human Monocyte Heterogeneity

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Blood monocytes develop in the bone marrow before being released into the peripheral circulation. The circulating monocyte pool is composed of multiple subsets, each with specialized functions. These cells are recruited to repopulate resident monocyte-derived cells in the periphery and also to sites of injury. Several extrinsic factors influence the function and quantity of monocytes in the blood. Here, we outline the impact of sex, ethnicity, age, sleep, diet, and exercise on monocyte subsets and their function, highlighting that steady state is not a single physiological condition. A clearer understanding of the relationship between these factors and the immune system may allow for improved therapeutic strategies.

Keywords: monocyte, macrophage, inflammation, sex, age, diet, exercise, sleep

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INTRODUCTION

Adult blood leukocytes can be separated into two broad categories: lymphoid or myeloid. The lymphoid lineage consists of T, B, innate lymphoid and natural killer (NK) cells, while the myeloid compartment comprises of functionally and morphologically discrete cell types, including mononuclear phagocytes (monocytes and dendritic cells), granulocytes (neutrophils, basophils, and eosinophils), and platelets. Injured or infected tissue releases chemoattractants that rapidly recruit these myeloid cells to the site of injury. Once at the inflamed site, these cells coordinate and carry out key effector functions. Nearly 100 years ago, Alexander Maximow suggested that hematopoiesis was an extremely organized stepwise process arising from a common precursor cell (1). Indeed, hemopoietic stem cells that reside in the bone marrow undergo multiple differentiation stages, becoming progressively more restricted and eventually give rise to a heterogeneous population of leukocytes. Commitment to the myeloid lineage downstream of the common myeloid progenitor (CMP) (2) results in the generation of erythrocytes, platelets, granulocytes, monocytes, and dendritic cells. Nevertheless, several extrinsic factors influence leukocyte generation. Here, we will outline several studies that highlight how age, ethnicity, diet, exercise, sleep, and sex modulate human monocyte numbers under healthy homeostasis.

Circulating blood monocytes were initially believed to be a single population of cells with the potential to repopulate terminally differentiated resident mononuclear phagocytes in the periphery (3). In addition, blood monocytes act as an emergency squad recruited to sites of injury where they perform pro-inflammatory and pro-resolving functions (4–8). Blood monocytes were initially defined by their morphology. Later, with the introduction of flow cytometry, monocytes were shown to express high levels of the lipopolysaccharide (LPS) binding protein receptor, CD14 (9).

These $CD14^+$ monocytes were subsequently discovered to be a heterogeneous population that could be further divided into discrete subsets based on CD14 and CD16 (FcγRIII) expression in humans (10). Monocyte heterogeneity was later observed to be conserved among other species (11). Human $CD14^+$ $CD16^-$ monocytes, also known as classical monocytes, are analogous to the murine $Ly6C^{Hi}$ CX_3CR1^{int} classical monocytes. Intermediate and non-classical monocytes are identified as $CD14^+$, $CD16^+$, and $CD14^{lo}CD16^+$ cells, respectively, where the latter subset mirror $Ly6C^{Low}$ CX_3CR1^{Hi} non-classical monocytes in mice (12, 13). The expression of several membrane adhesion molecules and chemokine receptors can also be used to discriminate between these monocyte subsets (13–15).

Blood monocytes begin their life in the bone marrow, following a similar developmental fate to dendritic cells where they both arise from the macrophage/dendritic cell precursor (MDP) (16). In mice, the MDP was initially proposed to give rise to monocytes and dendritic cells but not granulocytes (17). Downstream of the MDP, the common monocyte progenitor (cMoP) was identified, which gives rise exclusively to classical monocytes (18). The human equivalent of the murine cMoP was recently identified within the bone marrow granulocyte-monocyte progenitors (GMP) fraction (19). This human cMoP gives rise directly to pre-monocytes, and subsequently, monocytes. Investigations into the developmental stages between the cMoP and mature monocytes uncovered in mice a $Ly6C^+$ $CXCR4^+$ monocyte subset that resides in the bone marrow termed pre-monocytes (20). These pre-monocytes show proliferative potential and downregulate CXCR4 upon entry into the circulation (20). The egression of murine classical monocytes from the bone marrow follows a circadian rhythm, regulated in part by CXCR4 (20) and the circadian gene *Bmal1* (21). It is widely accepted that classical and non-classical monocyte subsets are related developmentally, with classical monocytes having the potential to give rise to non-classical monocytes over time (14, 22–27) (**Figure 1**). While recent advances demonstrate monocyte development to be a highly regulated process under steady state, here we summarize the influence of inherited traits and lifestyle choices have on human monocyte homeostasis.

LIFESTYLE AND GENETIC FACTORS AFFECTING MONOCYTES

Our knowledge concerning the development and function of monocytes and monocyte-derived cells during pathology continues to expand. It is also necessary to appreciate the behavior of these cells under healthy physiological conditions. However, healthy homeostasis is not a solitary state, rather several factors—often overlooked—including sex, diet, exercise, and age influence the immune system. Whether genetic traits prevail over environmental cues regarding the immune response remains a matter of debate (28, 29).

The earliest accounts of the cell originate with Robert Hooke's seminal observations in 1665 (30). Cellular analysis began with microscopy, then progressed to flow cytometry and is currently enjoying a renaissance in the form of single-cell RNA analysis. Every advancing stride has led to a greater appreciation regarding the complexity and diversity of monocytes, their subsets, and

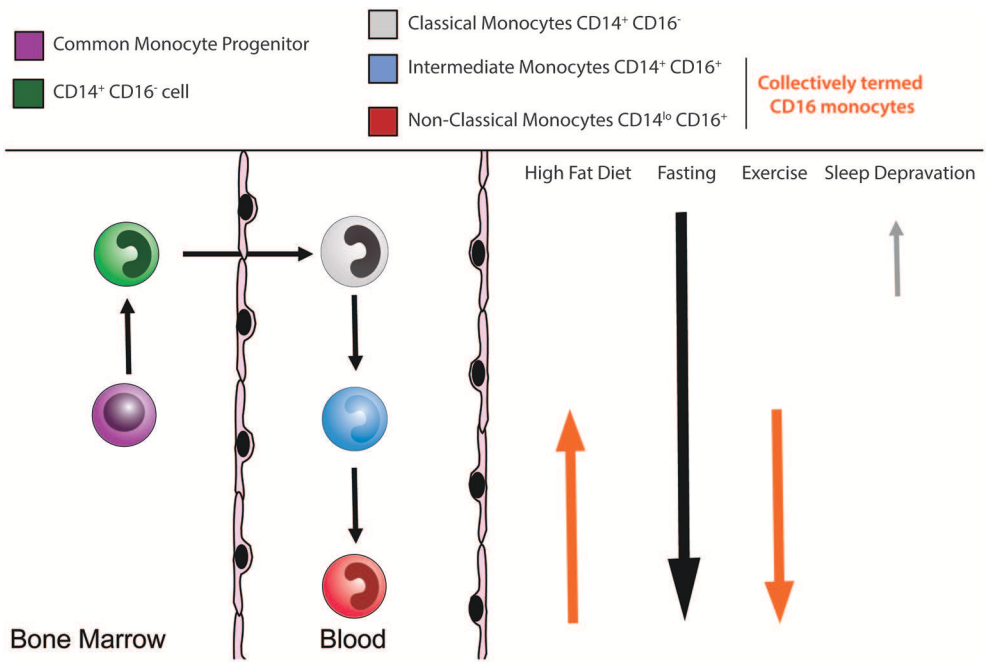

FIGURE 1 | Human monocyte subsets. Circulating monocytes arise in the bone marrow from a common monocyte progenitor (cMoP) before being released into the peripheral circulation. The circulating monocyte pool is composed of multiple subsets. Human $CD14^+$ $CD16^-$ classical monocytes (gray), $CD14^+$ $CD16^+$ intermediate monocytes (blue), and $CD14^{lo}CD16^+$ non-classical monocytes (red). Several external lifestyle factors can impact on these monocyte subsets.

function (31–34). Here, we consider if and how, lifestyle choices imprint on this diversity. This review will focus on our current understanding of human monocyte adaptations observed due to genetic and environmental factors; for a comprehensive review on monocyte biology, see Guilliams et al. (5) and Jakubzick et al. (6).

As a word of caution, the monocyte nomenclature was recently codified by Ziegler-Heitbrock et al. and approved by the International Union of Immunological Societies (35, 36). Nevertheless, complexity remains an issue within the historical literature and is further confounded by subsets conveyed sometimes as a proportion of total monocytes or in absolute numbers. Here, we have retold studies as originally described to avoid confusion.

SEX

Several physiological differences exist between the sexes, the most apparent being their role in reproduction. Another obvious difference is hormone concentration. Over three quarters of patients with autoimmune disease are female (37). Systemic lupus erythematosus (SLE), Sjögren syndrome, fibromyalgia, and rheumatoid arthritis afflict females more than males (38, 39), whereas ankylosing spondylitis, vasculitis, and Goodpasture syndrome predominantly occur in males (40). This sexual dimorphism of autoimmune-driven disease begs the question, do male and female immune systems differ?

Under physiological conditions, monocyte counts have consistently been reported to be elevated in males at all stages of life (41–43). This difference is most profound during adolescence (44). Curiously, one study examining over 400 individuals reported that monocytes are higher in Caucasian men than those in women; this difference was absent in the Afro Caribbean population (41). The proportion of non-classical monocytes has also been reported to be different amongst men and women (45). These differences in monocyte subsets may be attributed to the effect of estrogen and other sex hormones. To test this, researchers have turned to the menstrual cycle and menopause. During the menstrual cycle, 17 β-estradiol, and progesterone concentrations are consistently increased during the luteal phase in comparison to the follicular phase. Around 40 years ago, an elevated monocyte count was reported during the luteal phase (46). With our growing knowledge on monocytes, it would be worthwhile to revisit the impact of the menstrual cycle on monocyte subpopulations, especially as the identification of monocytes subsets remains inconsistent throughout the literature. Interestingly, postmenopausal women exhibit lower concentrations of estrogen and tend to have an increased blood monocyte count; nonetheless, following estrogen replacement therapy, monocyte numbers were restored to levels seen in younger females (47). These data suggest an increase in estrogen decreases monocyte numbers, supporting the observation that males tend to have a higher monocyte count. An exception to this would be the increase in monocytes observed during the luteal phase of the menstrual cycle. Together, this may suggest other endogenous factors affect monocyte composition.

Sex differences may become conspicuous in the disease setting. Both healthy males and females have equivalent number of intermediate monocytes; however, only male sarcoidosis patients exhibit an elevated number of intermediate monocytes compared to female sarcoidosis patients who had equivalent numbers to healthy female controls (48).

Sexual dimorphism has been reported in monocyte cytotoxic activity (49) and cytokine production. Male monocytes produce more inflammatory cytokines than females when stimulated with LPS (42); however, female sex hormones were not responsible for this effect as demonstrated *in vivo* by oral contraceptive use (50). It is important to note that these studies used LPS. PBMCs co-cultured with estrogen had altered expression of TLR3, TLR7, TLR8, TLR9, but not TLR2 and the LPS receptor, TLR4 (51). Therefore, the impact of estrogen on monocyte function may only become apparent in response to particular stimuli. These diverse responses on monocyte function between the sexes are discussed in detail (52). Further studies will help define the cytokines and/or hormones responsible for the divergence observed in monocyte count and function between males and females.

ETHNICITY

Ethnic diversity is reflected in disease susceptibility across different human populations (53). This has been identified in patients with tuberculosis (TB) infection (54), autoimmune hepatitis (55), and systemic lupus erythematosus (SLE) (56). A groundbreaking study by Nédélec et al. proposes that different environmental pressures on our immune system may explain why African ancestry is associated with a stronger inflammatory response compared to Europeans (57).

Ancestry has been shown to influence leukocyte counts, including neutrophils, lymphocytes, eosinophils, and monocytes (41, 58, 59). A trans-ethnic meta-analysis study revealed that those of a European ancestry tend to have a higher monocyte count compared to African-American and Japanese individuals (59). As expected, the interplay of ethnicity and other variables is likely to influence monocyte count. Caucasian males were observed to have a significantly higher count compared to African males, while no difference was noted for females (41), demonstrating the interaction of sex and ethnicity.

Regarding individual monocyte subsets, recently, a single study has shown that Caucasian populations tend to have a higher percentage of classical monocytes and conversely a lower percentage of non-classical monocytes than those sampled from an African population (58). In this study, Caucasians had a trend for increased CX_3CR1 expression; this could explain the difference in monocyte proportions. CX_3CR1 is involved in non-classical monocyte retention to the endothelium (60, 61), as well as a survival factor for this monocyte population (62); therefore, this increased expression might result in lower representation of free circulating non-classical monocytes due to their increased adherence. Of note, ethnic differences

are possibly reflected in monocyte function, as monocyte-derived cells from Filipino, Chinese, and non-Hispanic whites challenged with *Mycobacterium tuberculosis* produced varying quantities of cytokines from 137 volunteers (>44 from each background) (63). As ethnicity can influence the immune response, this may implicate the need for a more personalized take regarding therapeutics.

DIET

Diet varies from individual to individual—from what they eat to the quantity and frequency. A balanced diet is a basic requirement for a healthy lifestyle. This fine balance goes awry during surplus calorie intake, which contributes to many diseases, including atherosclerosis, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD). These western diet-related diseases are associated with systemic chronic inflammation (64).

Monocytes play a central role in several diet-related pathologies. In recent years, it has become evident that a high-fat western diet triggers a number of functional modifications in monocytes. Mice fed a western diet for 4 weeks led to an elevation in circulating and splenic monocytes compared to those fed on a standard chow diet (65). Further examination into how a western diet prompts myelopoiesis was described to be due to an increase in GMP in the bone marrow that also functionally reprograms myeloid cells through NLRP3. Upon reverting to a chow diet, monocyte numbers returned to baseline, although an increased activation status became imprinted in classical monocytes. Collectively, these data suggest that diet leads to innate immune training within the monocyte pool in mice. In a human study, 3 h after the consumption of a high-fat (McDonald's) meal resulted in an increased monocyte count, specifically an elevation in non-classical monocytes (66). Similarly, this observation is consistent with a study where $CD16^+$ monocytes (intermediate and non-classical) positively correlated with increased body weight (67). Interestingly, in these subjects, dietary intervention or gastric bypass surgery resulted in a decrease in the absolute number and percentage of these cells (67). Immediately following a high-fat meal, Khan et al. demonstrated a higher percentage of lipid-laden monocytes (66). These foamy monocytes increase their expression of CD11c, which is thought to lead to monocyte arrest *via* vascular cell adhesion protein (VCAM-1). While these are short-term effects, recurrent chronic exposure of a high-fat diet may lead to diet-related diseases.

Although a high-fat dietary intake increases peripheral monocyte numbers, the opposite is true in fasted individuals (68). Dietary restriction for 19 h in humans or 4 h in mice led to a significant reduction in both circulating classical and non-classical monocytes. This reduction in blood monocytes was due to the inhibition of monocyte egress from the bone marrow. This elegant study from the Merad group demonstrated that carbohydrate and protein consumption rescues monocyte numbers *via* the liver activated protein kinase-peroxisome proliferator-activated receptor alpha (AMPK-PPARα) pathway that regulates the monocyte chemoattractant protein, CCL2 (68). Furthermore, dietary restriction during experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, improved disease outcome and reduced myeloid cell infiltrate compared to animals with access to food *ad libitum* (68, 69). Similar findings have been observed in humans, where fasting diets have shown to improve the quality of life for patients with multiple sclerosis (69, 70).

In an experimental setting, a single macronutrient or micronutrient alters the monocyte composition and function. It is more realistic that it is a combination of several dietary nutrients in varying amounts that will alter the phenotype of monocytes.

SLEEP/WAKE CYCLE

Cortisol is the archetypical neuroendocrine anti-inflammatory hormone that regulates immune cell gene expression (71, 72). Cortisol follows a diurnal pattern where it peaks 30 min after waking in the morning and falls throughout the day. Cortisol is the endogenous member of the glucocorticoid family of immunosuppressive and anti-inflammatory drugs that acts on many leukocytes, including monocytes, where they induce a rapid decrease in circulating monocytes (73). Therefore, it would be interesting to know if monocytes also follow a diurnal pattern.

Meuret et al. performed to the best of our knowledge one of the earliest studies on monocyte kinetics. Meuret and colleagues observed a monocyte cycle occurring around every 5 days in humans. They proposed this due to a homeostatic loop within the bone marrow with transit time being the prevailing factor (74). Recently, we observed a population of $CD14^+$ $CD16^-$ cells resembling classical monocytes that reside in the human bone marrow (14); these cells exhibit a postmitotic dwell period of ~1.5–2 days before being released into the circulation (14, 75). It is possible that this maturation period, in addition to the time taken to differentiate into these cells, and the regulatory signals account for this monocyte cycle.

While long-term monocyte oscillations have been described, diurnal patterns are also present in both mice (20, 21) and humans, where monocyte numbers decrease during sleep and begin to gradually rise upon waking (76, 77). Cuesta et al. were able to stratify individuals into two categories, one where monocyte numbers peak during the morning, and another group where the monocyte count peaked in the evening (77). The reason behind the existence of these two groups remains unclear—all subjects except one were male, of a similar age who maintained comparable levels of activity, equivalent calorie intake, and exposure to light. In mice, CXCR4 and *Bmal1* regulate the circadian rhythm of circulating monocytes (20, 21).

Functional changes such as cytokine production, surface membrane protein expression, and phagocytic ability have been reported to follow a diurnal pattern (77–80). Cuesta et al. conclude that cytokine production follows a bimodal rhythm, where monocytes are more responsive at night, whereas during the day, a higher production results from the increased numbers of monocytes (77). In addition, while TLR1, 2, 4, and 9 expression does not differ throughout the day, activation of these receptors

results in the dampened expression of costimulatory molecules in the morning (78).

Sleep-deprived individuals who remain awake during the night gain a higher monocyte count compared to those who slept at this period (76). Congruent with these findings, mice with disrupted sleep also have an increase in peripheral blood classical monocytes (81). Uninterrupted sleep enables the release of hypocretin from the hypothalamus, which in turn regulates CSF1 production from bone marrow pre-neutrophils regulating monopoiesis (81). Although an increase in monocyte count occurs during sleep deprivation, a diurnal oscillation pattern remains (82). Furthermore, in night shift workers who are active at night, no phase shift was detected in monocyte numbers (77). Taken together, these studies establish that both internal and external body clocks influence monocyte behavior and emphasize the importance of appropriate time controls when conducting experiments.

Circannual or seasonal rhythms have also been detected in monocyte function. Monocyte counts remain stable throughout the year, yet phagocytosis, cytokine production, and prostaglandin metabolism vary (83, 84). Specifically, a higher proportion of monocytes phagocytose in winter compared to spring and autumn. In response to LPS, monocytes isolated during the autumnal period produce lower concentrations of both inflammatory [tumor necrosis factor (TNF)-α and interleukin (IL)-6] and anti-inflammatory (IL-10) cytokines. There are several possible explanations for the fluctuation in human monocyte function throughout the year; seasonal changes may be influenced by a myriad of factors including increased periods in crowded places (i.e., indoor contagion theory), reduced exposure to sunlight (vitamin D deficiency), or even temperature changes. Despite the reason for these changes, these seasonal changes may be relevant when performing long-term clinical trials and should be taken into consideration.

EXERCISE

Over 120 years ago, Schulte described that exercise impacts the human immune system and induces leukocytosis (85). Therefore, the question arises, do monocytes fluctuate during exercise? Studies have reported a rise in intermediate and non-classical monocytes immediately following exercise (86), while others have described a change in classical monocytes (80), classical and non-classical monocytes (87), and even all three populations (88, 89). At first glance, this can appear confusing however; not all exercise is equal as the intensity, duration, and the type of exercise (aerobic or anaerobic) influence monocytes (90) and may account for these different findings. Maximal oxygen consumption, a readout of an individual's fitness, negatively correlated with the percentage change in intermediate monocytes (89). Therefore, the larger the maximal oxygen consumption, the smaller the percentage change in intermediate monocytes. The majority of non-classical monocytes are constantly crawling on the endothelium in an LFA1-dependent manner (60, 61). This may be overcome during intense exercise by the increase in shear blood flow. Patrolling monocytes that previously were crawling on endothelial cells are now able to be isolated, providing a more genuine picture of blood monocyte subsets. While these effects are transient, long-term alterations in monocyte composition have also been observed following exercise. A 12-weeks exercise regime reduced the percentage of $CD16^+$ monocytes (91). This decline in intermediate monocytes could be associated with fat loss (67), as described above. In addition, TNF-α production was significantly reduced following this 12-weeks exercise program, while monocyte phagocytosis increased, suggesting that long-term exercise may promote a more anti-inflammatory monocyte while improving its phagocytic capacity.

AGE

At birth, monocytes are three times higher compared with those of adults (92). Christensen and colleagues analyzed over 63,000 hospital records and found that monocyte counts increase linearly with gestational age (93). This monocyte expansion continued into the first 2 weeks after birth. As an organism ages, so does its immune system. The term inflamm-aging was coined by Franceschi 20 years ago (94) to describe the progressive increase in the organism's proinflammatory status as it matures. Inflamm-aging includes adaptive immunity, immunosenescence, and dysregulation of the innate immune response. While some studies have reported no changes in the mononuclear phagocyte count in different age cohorts (43, 95), others have noticed a decrease in dendritic cells (96) and an increase in monocyte subsets in the older aged cohort (45, 96–99), particularly in intermediate and non-classical monocytes. Plasma CCL2 is elevated in older individuals (99), which may result in monocyte mobilization from the bone marrow, resetting the dynamic equilibrium of blood monocyte subsets. However, in advanced old age (81–100 years), fewer classical and intermediate monocytes have been detected (100).

Coincidently, $CD16^+$ monocytes have been reported to expand in inflammatory conditions, also increased in older individuals. These monocytes, in particular, non-classical monocytes, produce higher basal levels of TNF-α and is thought to account for the increase in plasma TNF-α levels in aged individuals (45, 97, 101). A consequence of this inflammatory environment results in dysregulated innate immunity, such as impaired phagocytosis (45). The pharmacological blockade of TNF-α in aged mice improved bacterial clearance and returned classical monocytes to levels in young mice (102). Therefore, the accumulation of monocytes in the elderly may account for age-related inflammatory conditions. While plasma TNF-α changes with age, hormonal changes also occur. Therefore, it is important to consider age and environmental and genetic factors, as all interact with the immune system. There are as many "young" 85-year-olds running marathons as "old" sedentary 75-year-olds.

CONCLUSION

Recently, new monocyte subsets have been described in mice (35, 103–105) and humans (32, 34). The identification of

these new populations demonstrates how recent technological advances have changed the mononuclear phagocyte landscape. Future insight to these subsets will provide therapeutic strategies to enhance these cells when they are beneficial and block the detrimental effects. In the quest for novel therapeutics, it is critical to remember how sex and environmental and lifestyle choices lead to physiological variations within a leukocyte population as discussed here for monocytes. It is important to examine these variables from a holistic stance using defined objective criteria to avoid bias that may have crept into previous studies. Historically, research into the impact of lifestyle choices was performed on a limited cohort with certain subjective evaluations. The adoption of electronic health records will provide greater insight into how sex and environmental and lifestyle choices impact monocytes and additional leukocyte populations on a previously unimaginable scale.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Pleiotropic Effects of the GM-CSF Rheostat on Myeloid Cell Differentiation and Function: More Than a Numbers Game

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Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) is a myelopoietic growth factor that has pleiotropic effects not only in promoting the differentiation of immature precursors into polymorphonuclear neutrophils (PMNs), monocytes/macrophages (MØs) and dendritic cells (DCs), but also in controlling the function of fully mature myeloid cells. This broad spectrum of GM-CSF action may elicit paradoxical outcomes—both immunostimulation and immunosuppression—in infection, inflammation, and cancer. The complexity of GM-CSF action remains to be fully unraveled. Several aspects of GM-CSF action could contribute to its diverse biological consequences. Firstly, GM-CSF as a single cytokine affects development of most myeloid cells from progenitors to mature immune cells. Secondly, GM-CSF activates JAK2/STAT5 and also activate multiple signaling modules and transcriptional factors that direct different biological processes. Thirdly, GM-CSF can be produced by different cell types including tumor cells in response to different environmental cues; thus, GM-CSF quantity can vary greatly under different pathophysiological settings. Finally, GM-CSF signaling is also fine-tuned by other less defined feedback mechanisms. In this review, we will discuss the role of GM-CSF in orchestrating the differentiation, survival, and proliferation during the generation of multiple lineages of myeloid cells (PMNs, MØs, and DCs). We will also discuss the role of GM-CSF in regulating the function of DCs and the functional polarization of MØs. We highlight how the dose of GM-CSF and corresponding signal strength acts as a rheostat to fine-tune cell fate, and thus the way GM-CSF may best be targeted for immuno-intervention in infection, inflammation and cancer.

Keywords: GM-CSF, macrophages, dendritic cells, differentiation, function

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INTRODUCTION

The Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) is a small glycoprotein that is able to stimulate generation of polymorphonuclear neutrophils (PMNs) as well as mononuclear monocytes, macrophages (MØs) and dendritic cells (DC) (1–3). When added to mouse bone marrow precursors *in vitro*, GM-CSF acts in two phases: an early differentiating phase of PMNs and $CD11b^{+}$ mononuclear cells from progenitors, and a late phase of MØs and monocyte-derived

DCs (moDC) from $CD11b^+$ mononuclear cells. For several decades it has been known that the outcome of such cultures is greatly influenced by a number of factors, including cell density, the presence of stromal cells, co-stimulatory signals, the serum quality and the concentration of GM-CSF (1). Despite this, the molecular mechanisms underpinning the heterogeneity of the myeloid cells produced in these GM-CSF induced cultures are still ill defined. For example, while cytokines such as IL-4, IL-13, TNF-α, TLR ligands or even GM-CSF concentration could alter dramatically the ratio of generated myeloid cells (1, 4–6), the nature of this bias under different conditions has not been fully resolved at a molecular level. It has also not been fully resolved whether differentiation fate under these conditions is the result of either plasticity between MØ and moDC, or the selective expansion of a committed precursor under favorable conditions of culture. While GM-CSF is extensively used in supporting myelopoesis *in vitro*, the role of GM-CSF *in vivo* remains obscure. GM-CSF deficiency has little impact on myeloid cells except for the impairment of alveolar MØs *in vivo* (7–10). Nevertheless, in transgenic mice harboring high levels of GM-CSF (GM-CSF-Tg), myelopoiesis is substantially increased (11, 12).

While the importance of GM-CSF for myelopoiesis *in vivo* remains a matter of debate, there is cogent evidence that GM-CSF is an important mediator in inflammatory conditions such as during infection and tumor immunity (13–16). These studies suggest a role for GM-CSF in regulating biological functions of fully mature cells. Studies on GM-CSF have mainly focused on its pro-inflammatory role. Nevertheless, GM-CSF has also been linked to immuno-suppression, particularly in tumor setting. Thus, exposure of myeloid cells to GM-CSF can lead to sharp opposite extremes, and these contrasting effects of GM-CSF on myeloid cells remains hitherto unexplained.

The GM-CSF receptor (GM-CSFR) is composed of a ligand-specific alpha chain and a beta chain common with IL-3 and IL-5. Despite sharing this signaling beta chain, IL-3 or IL-5 engagement leads to distinct signaling events and myeloid cell outcomes (17). For example, IL-3 is mostly associated with differentiation of mast cells/basophils, while IL-5 is associated with differentiation of eosinophils (17). GM-CSFR is found on most myeloid cells including their precursors. Upon engagement, GM-CSFR elicits JAK2 phosphorylation, which triggers multiple intracellular signaling pathways, including STAT5, PI3K, and MAPK (15, 18). Of note, GM-CSF can selectively turn on signaling modules in a dose-dependent fashion, and can therefore differentially impact cell survival, proliferation, and differentiation at different doses (15, 18–20). GM-CSF has been shown to activate and/or upregulate many transcriptional factors such as the STAT proteins, PU.1 and interferon regulatory factors (IRFs) (18). Such factors have been implicated in the differentiation and function fate determination of myeloid cells, but it is not clear how induction and function of these transcription factors are linked to GM-CSF signaling strength.

Apart from GM-CSF abundance, GM-CSF signaling strength can be influenced by multiple factors, including post-translational modification. For example, glycosylated GM-CSF has less immunogenicity and greater *in vivo* pharmacokinetic availability than its non-glycosylated form Gribben et al. (21). Nevertheless, glycosylation of GM-CSF is not required for its biologic activity *in vitro* (22). In contrast, the GM-CSF receptor α subunit requires N-glycosylation for binding and signaling (23, 24). Thus, it has been speculated that glycosylation of the α subunit may modulate cellular responsiveness to GM-CSF (24). In addition, GM-CSF receptor signaling can also be regulated by the suppressors of cytokine signaling proteins (SOCS family members). However, the consequences of SOCS signaling in controlling GM-CSFR signaling strength and therefore myeloid cell differentiation and/or function have been little explored.

In this review, we will highlight the dynamic changes in GM-CSF quantity in different pathological situations and dose-dependent differences in the biological response to GM-CSF, ranging from immunostimulating to immunosuppressive. We dissect the differential impact of GM-CSF on the main types of myeloid cells. As the upstream events of GM-CSF signaling and the inflammatory biological outcomes have been reviewed elsewhere (17, 20), we will highlight the potential role for negative feedback regulators on GM-CSF signal strength and downstream transcriptional factors that influence myeloid differentiation trajectory and function (**Figure 1**). Furthermore, we will discuss the contribution of PI3K and downstream NFκB pathways upon GM-CSF engagement in controlling not only myeloid cell survival (19) but also macrophage polarization via the differential involvement of Akt1 and Akt2 subunits (25). Finally, we also discuss the role of GM-CSF in regulating end-cell function, particularly functional polarization of MØs. In the process, we aim to shed some light on the paradoxical role of GM-CSF in immune regulation and facilitate the agonistic and antagonistic targeting of GM-CSF as an immuno-intervention in infection, inflammation, and cancer. As this review covers mouse and human studies, we have indicated the species when human GM-CSF is discussed.

DYNAMIC PRODUCTION OF GM-CSF: HOW MUCH IS PRODUCED *IN VIVO*?

The amount of GM-CSF is likely to be a key factor in determining its biological activity (19, 26). Thus, we will briefly describe the main sources of GM-CSF. A diverse set of hematopoietic and non-hematopoietic cells have been shown to secrete GM-CSF. They include T cells (27–30), human natural killer cells (31), mast cells (32), monocytes/MØs (33), human endothelial cells (34), and human fibroblasts (35). The relative contribution of each individual subset to the overall GM-CSF produced under steady-state or inflammatory conditions has not been determined. In the lung, production of GM-CSF by endothelial cells in the steady state was instrumental in the differentiation of alveolar MØs from fetal monocytes (10, 36). Under inflammatory conditions, such as collagen induced arthritis and experimental autoimmune encephalomyelitis (EAE), the production of GM-CSF by T cells has been reported to promote disease progression (28–30), although there is contention about the role of GM-CSF in EAE pathology (37). On the other hand, GM-CSF derived from radio-resistant wild-type cells in $GM\text{-}CSF^{-/-}$ bone

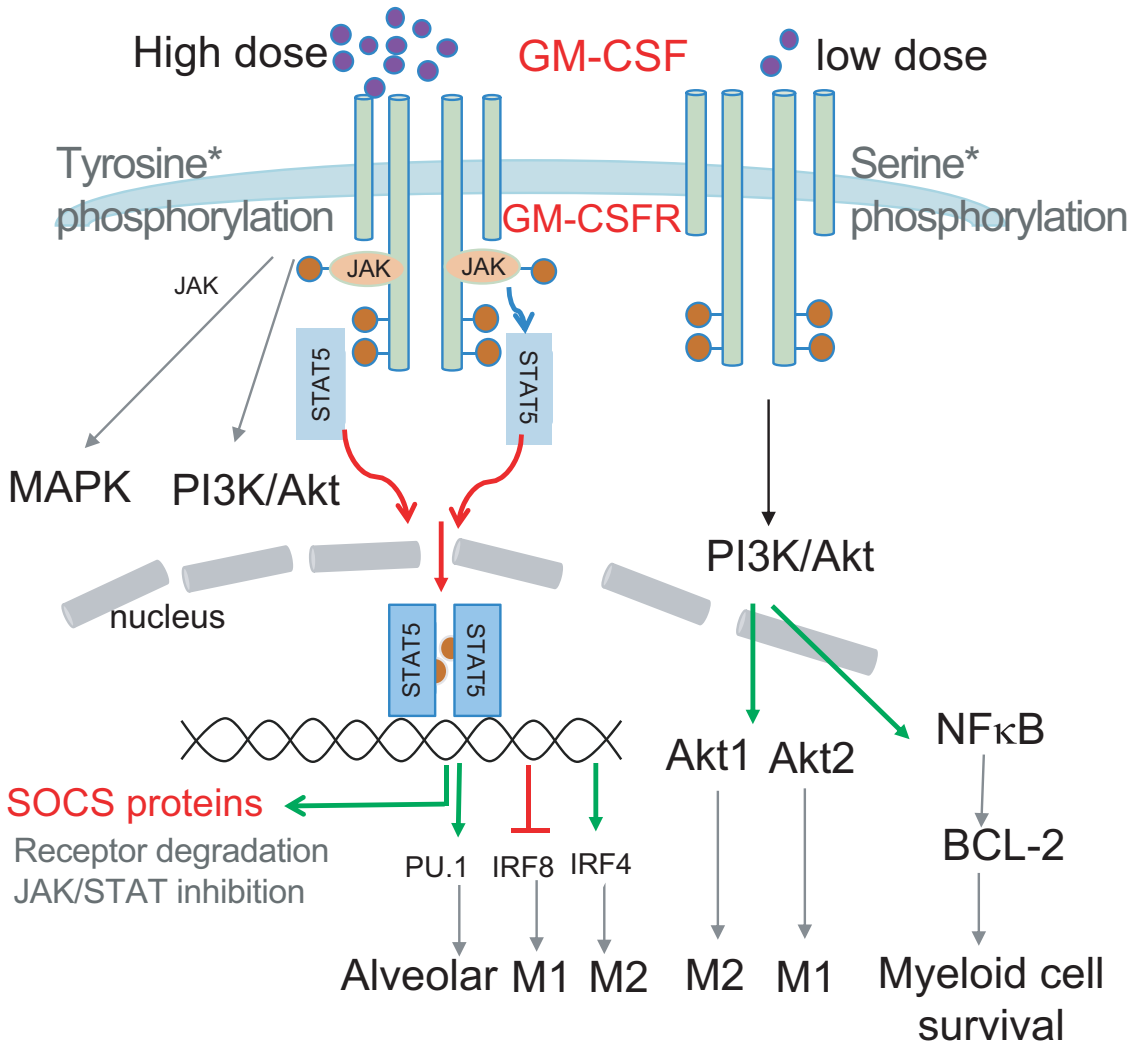

FIGURE 1 | Schematic illustrating how GM-CSF dose selects the signal modules to be activated. Low dose GM-CSF signaling triggers Ser phosphorylation of the GM-CSFR beta chain, PI3K/Akt activation and subsequent BCL-2 enhanced survival. High dose signaling triggers Tyr phosphorylation of the GM-CSFR beta chain resulting in JAK/STAT5 activation, leading to upregulation of transcription factors PU.1 and IRF4, and downregulation of IRF8 to impact differentially on myeloid cell differentiation and function. JAK2/STAT5 activation by GM-CSF could also induce transcription of SOCS proteins to negatively regulate signaling to form a signaling regulatory loop. PI3K activation can also contribute to MØ polarization via preferential activation of Akt1 and Akt2.

marrow reconstituted irradiation chimera was sufficient to confer resistance to infection with *Mycobacterium tuberculosis* (38). GM-CSF is often used in the range of 10–20 ng/ml for *in vitro* myeloid cell differentiation (2–4, 39). It raises the question—what levels of GM-CSF can be reached *in vivo*? In physiological situations, concentrations of around 20 pg/mL of GM-CSF could be detected in human serum (40). Under pathological conditions, human GM-CSF was found to be significantly elevated in the serum and tissues in inflammatory diseases such as rheumatoid arthritis and colitis (41–43). GM-CSF increase was also observed in mice following LPS administration (44) and during bacterial infection (45). Notably, GM-CSF quantity can reach and persist at >10 ng per lung of mice infected with *M. tuberculosis* (38). When human GM-CSF was used for myeloid recovery after chemotherapy and bone marrow transplantation, patients were given with >32 μg/kg body per day for 14 days (46).

SOLID TUMOR DERIVED GM-CSF: COMMON FEATURE?

It has long been appreciated that tumor cells can produce a variety of cytokines and chemokines (47). The Broad Institute cancer cell line encyclopedia database (https://portals.broadinstitute.org/ccle) shows that a broad spectrum of solid tumor cell lines express human GM-CSF mRNA. For example, tumor cells from the kidney, pancreas and gastrointestinal tract displayed prominent GM-CSF transcription. Concordantly,

an early study showed that about a third of the 75 human tumor lines tested secreted GM-CSF; this comprised a large proportion of lines from renal, prostate and colon cancers and a modest proportion of breast, cervical, ovarian and melanoma cancers (47). Indeed, 10^5 W-RCC renal cancer cells produced a remarkable 39 ng/mL after 16 h in culture (47). A mouse renal tumor line RenCa also produced about 0.5 ng GM-CSF/10^6 cells/24 h (48). In another study, a panel of mouse pancreatic ductal adenocarcinoma (PDA) tumor cell lines all produced GM-CSF (60–500 pg/mL) while benign pancreatic ductal cells did not (49). These results indicate that GM-CSF production by human and mouse tumor cells may not be uncommon.

ROLE OF GM-CSF IN TUMOR, AUTOIMMUNITY/INFLAMMATION AND INFECTION: STIMULATING OR SUPPRESSIVE?

Several reviews have described that GM-CSF has a profound immune regulatory role in health and disease (13–17). Here we briefly discuss the role of GM-CSF in tumor, autoimmunity/inflammation, and infection, with the aim to contrast the opposite roles of GM-CSF in immune regulation.

GM-CSF Promotes and Suppresses Tumor Immunity

The use of murine tumor cells genetically modified to secrete cytokines has established GM-CSF as a strong immune adjuvant for vaccination to promote anti-tumor immunity (50). In a vaccination setting, Zarei et al. showed that tumor derived GM-CSF was sufficient to recruit DCs to the vaccination site in murine tumor models, thereby promoting a strong anti-tumor response and protecting from further tumor challenge (48). Hence, clinical trials using human GM-CSF as an immune adjuvant in cancer patients have been conducted with some promising outcomes (51–53). However, the use of human GM-CSF at high doses may lead to advert events such as immunosuppression (54). In mouse models, tumor derived GM-CSF has also been shown to promote the development of myeloid derived suppressor cells (49). Consequently, neutralization of GM-CSF has also been shown to reduce suppressive cells and limit tumor growth (49). Furthermore, tumor derived GM-CSF can also act in an autocrine manner to sustain tumor growth (55). Thus, GM-CSF secretion within the cancerous tissue may have very contrasting effects on either promoting anti-tumor immunity, suppressing anti-tumor immunity or promoting tumor growth directly. It is likely that the temporal and spatial abundance of GM-CSF, together with the machinery controlling GM-CSF signal strength including receptor expression and regulatory circuitry would dictate the cellular and biological outcome of tumor derived GM-CSF.

GM-CSF Promotes and Suppresses Autoimmunity

Evidence that GM-CSF is pro-inflammatory in several autoimmune diseases comes from various studies: (1) treatment with human GM-CSF to correct neutropenia results in flare-ups of rheumatoid arthritis (56, 57); (2) human GM-CSF was present in lesions of rheumatoid arthritis (41); and the cerebrospinal fluid of MS patients (58); (3) GM-CSF deficiency confers resistance to experimental collagen induced arthritis (59) and EAE (60) in mouse models. In line with the above studies, anti-GM-CSF mAb treatment was found to be effective at ameliorating the ensuing disease in mouse models, partly by reducing myeloid cell infiltration (61, 62). In clinical trials, anti-human GM-CSF mAb namilumab and MOR103 demonstrated evidence of efficacy in active rheumatoid arthritis (63, 64). Similarly, human trials of anti-GM-CSF receptor α mAb Mavrilimumab on rheumatoid arthritis had also been shown to reduce disease activity (65, 66).

However, GM-CSF is not always detrimental in autoimmune settings and has also been shown to be beneficial via the suppression of undesired immune responses (67). The supporting evidence includes: (1) treatment with human GM-CSF ameliorates Crohn's disease (68); (2) GM-CSF prevents diabetes development in NOD mice by promoting immature tolerogenic DCs and controlling the number of regulatory T cells (69); (3) GM-CSF deficiency in mouse results in the development of lupus-like disorder (70) while combined deficiency of GM-CSF and IL-3 results in the development of autoimmune diabetes (71). The cellular and molecular basis for these beneficial effects of GM-CSF is not clear. As discussed in a recent review (67), there are at least two potential mechanisms for GM-CSF to suppress autoimmunity. Firstly, GM-CSF can induce DCs and macrophages to activate antigen-specific Tregs and suppresses experimental autoimmune disease in autoimmune thyroiditis (72). GM-CSF-autoantigen conjugates had been found to be particularly effective to expand Tregs in an EAE model (73). GM-CSF can even directly expand *in vitro* induced Tregs to suppress disease development in a cell transfer model of type 1 diabetes (74). Secondly, GM-CSF can induce the production of monocytes with suppressive functions that dampen disease induction and severity in an IRF1 dependent fashion (75). Beyond autoimmunity, MØs can also be detrimental or beneficial to graft tolerance in organ transplantation (76). In such a context, it is interesting to note that GM-CSF mediates graft-vs. -host disease but not graft-vs. -leukemia responses, suggesting an intervention opportunity targeting GM-CSF in allogenic hematopoietic cell transplantation (77).

GM-CSF Promotes Immunity and Mediates Immunopathology During Infection

Studies in mice deficient in GM-CSF and GM-CSFR have highlighted the critical role for GM-CSF and its receptor in maintaining alveolar MØs in the lung (7, 10, 36, 78). Many studies have established that GM-CSF has a non-redundant role in promoting anti-pathogen immunity. Deficiency in GM-CSF reduced emergency myelopoiesis and reduced *Listeria* and *M. tuberculosis* protection in mice (79, 80). Concordantly, GM-CSF treatment enhanced protective immunity against infection with *M. tuberculosis* and *Salmonella typhimurium* (81, 82). GM-CSF also promoted resistance against various parasite infections including blood-stage malaria (83), trypanosomiasis (84), and

leishmaniasis (85). Interestingly, the combined blockade of GM-CSF and IL-3 prevented the development of cerebral malaria (86). Notably, infection in human and mouse models can also lead to immunosuppression (87–91). Unfortunately, although these studies indicated an association with the generation of immunosuppressive myeloid cells, full understanding on how GM-CSF shapes immunosuppression remains elusive.

DOSE-DEPENDENT DIFFERENTIATION OF PMNS AND MØs?

The exposure of bone marrow progenitors to GM-CSF leads to the production of two functionally distinct myeloid cells: PMNs and MØs. What determines the deviation to PMN vs. MØ pathway? In early studies using *in vitro* agarose cultures, high GM-CSF concentrations favored PMNs differentiation, whereas low concentrations favored MØ differentiation (26, 92); this effect was termed "differentiation downgrading." Interestingly, a recent article has provided a mathematical interpretation for this observation, enabling the reproduction of the concentration dependent pattern of GM-CSF induced differentiation based on induction of key transcriptional factors controlling lineage commitment (93). However, when GM-CSF signaling strength that is represented by both GM-CSF quantity and receptor density is high over time, monopoiesis is favored over granulopoiesis (93). In line with this predictive model, our recent data showed that high dose GM-CSF favored monopoiesis over granulopoiesis *in vitro* (5). Similarly, GM-CSF transgenic mice had preferential expansion of MØs in multiple organs (5, 11, 12). Consistent with the findings above, van Nieuwenhuijze et al. described increased MØs compared to PMNs in transgenic mice expressing high level of GM-CSF (12). Conceivably, GM-CSF signal strength is not only reflected by the ratio GM-CSFR:GM-CSF but also by intracellular mechanism controlling GM-CSF signaling. We contend that all these factors ultimately play a critical role in determining myeloid cell differentiation.

DIFFERENTIAL REQUIREMENT OF GM-CSF FOR SURVIVAL OF MØs AND PMNS?

Human PMNs rapidly lose viability in culture (94). Human GM-CSF but not G-CSF, IL-6, and IL-8 prevented apoptosis of PMNs, prolonging *in vitro* survival (94). Of note, despite sharing the βc receptor with GM-CSF, IL-3 did not improve cell survival, likely due to low expression of IL-3 receptor on mature PMNs (94, 95). We observed that the addition of small quantities of GM-CSF *in vitro* (80 pg/mL) can lead to substantially increased survival of murine blood PMNs (5). Interestingly, a detailed analysis of the signaling pathway induced by such low levels of GM-CSF have shown that it was sufficient to activate Ser585 of the GM-CSFR, thereby promoting downstream signaling events, in particular the PI3K-Akt pathway, that led to increased cell survival (19, 96). As pro-survival members of the BCL-2 family including BCL-2, BCL-xL, A1, MCL-1, and BCL-w have a key role in maintaining the viability of most immune cells (97), the precise contribution of individual molecules to PMN survival, specifically GM-CSF enhanced PMN survival, is unclear. Human GM-CSF has been shown to increase expression of BCL-2 but not BCL-xL in one study (96) while it increased BCL-xL transcription in another study (98). Functionally, antagonism of BCL-2 or BCL-xL has had some effects on mouse and human neutrophil count *in vivo* (99, 100). Similarly, A1, identified as a GM-CSF induced molecule (101), showed a pro-survival role for PMN in some studies (5, 102) but not in the most definitive study where all the functional A1 genes were ablated (103). In addition, human GM-CSF could promote granulocyte survival by maintaining MCL-1 stability (104). It is somewhat puzzling that human GM-CSF can also induce the expression of the pro-apoptotic BCL-2 family member Bim in human and mouse PMNs via a PI3K dependent fashion (105). Compared to PMNs, monocytes/MØs had better spontaneous survival in culture, and survival enhancement by GM-CSF was less remarkable than the effect observed on PMNs (5). The loss of either MCL-1 or A1 has a limited effect on murine monocyte/ MØ survival (103, 106). Overall, GM-CSF has a prominent role in promoting survival of myeloid cells. However, the molecular events responsible for the differential survival properties observed for PMNs and monocytes/MØs, with or without GM-CSF remain ill explained. Furthermore, there is little known about the role of GM-CSF in regulating multiple non-BCL-2 regulated cell death pathways including death-receptor regulated apoptosis, necroptosis and autophagy.

DIFFERENTIAL IMPACT OF GM-CSF ON DIFFERENTIATION OF MØs AND MODCS: PLASTICITY OR SELECTIVE EXPANSION?

GM-CSF is routinely used to generate large numbers of dendritic cells from mouse bone marrow or human monocyte cultures (2, 3, 107). Yet recently, $CD11c^+$ mononuclear cells generated in the former culture were found to contain two main populations: $CD11c^+MHCII^{int}CD11b^{hi}$ $CD115^{hi}Flt3^-$ MØs and a $MHCII^{hi}CD11b^{int}$ cell fraction enriched for $Ftl3^+$ DCs (4). MØs and DCs within $CD11c^+$ mononuclear cells not only differ in their gene signature but also function (4). MØs have a high capacity for producing proinflammatory cytokines while DCs have a high capacity for presenting antigens (4). In addition, recent evidence highlighted that the inflammasome activity of such cultures was due to MØs, not DCs (108).

Ontogeny analyses elegantly showed that macrophage-dendritic precursors, common monocyte progenitors, common dendritic cell progenitors, and $Ly6C^{high}$ monocytes can all become MØs or DCs, with different expansion and differentiation rates (4). Of note, $Flt3^+CD11c^-$ $MHCII^+$ $PU.1^{hi}$ cells within the $Ly6C^+$ monocyte subset have been identified as precursors of GM-CSF dependent moDCs (109). Notwithstanding, there are still many unanswered questions regarding the conditions determining the differentiation fate of MØs and DCs.

GM-CSF Signaling Strength

GM-CSF signal strength is the net result of GM-CSF quantity, GM-CSFR expression level and positive/negative regulatory circuitry controlling GM-CSF signaling. Most *in vitro* studies use a range of 5–20 ng/mL GM-CSF to drive DC differentiation, with variation in cell density and culture duration. It had been shown that low dose of GM-CSF promotes the development of immature DCs featuring tolerogenic function (110). Using the recent definition of MØs and DCs within $CD11c^+$ cells generated in GM-CSF culture (4), we and others noticed that an intermediate dose of GM-CSF favored moDC differentiation while higher doses of GM-CSF favored macrophage differentiation (5, 111). As alluded to earlier, the GM-CSFR could work as a binary switch: low doses of GM-CSF led to Ser phosphorylation, whereas high doses led to Tyr phosphorylation and STAT5 activation (19). However, it remains unclear on how this binary switch contributes to DC and MØ differentiation.

In addition to the interpretation of the abundance of the ligand, the GM-CSF induced signaling cascade can be regulated by negative regulators of cytokine signaling. One such example is the degradation of GM-CSFR through SOCS1 mediated by ubiquitination (112). Yet, the consequences of SOCS1-mediated GM-CSFR downregulation has not been examined in the context of DC differentiation. In response to GM-CSF, myeloid cells are induced to express another member of the SOCS family, CISH (113–115). CISH knockdown by shRNA was shown to impede GM-CSF-induced DC development and DC function (115). However, as authors demonstrated that CISH knockdown suppressed precursor cell proliferation, it is still unclear if CISH knockdown can directly impact on the differentiation of MØs and DCs.

Taken together, we speculate that GM-CSF induced signaling strength dictates cellular outcome, with moderate GM-CSF signaling strength enabling DC differentiation while strong GM-CSF signaling strength favors MØ differentiation.

Promotion of DC Differentiation by IL-4 and Other Stimuli: Fate Plasticity?

Even at the monocyte stage when cell proliferation is very limited (4), human and mouse GM-CSF, particularly with IL-4, can differentiate human and mouse monocytes into DCs (4, 107, 109). It raises the question of whether IL-4 alters the differentiation fate for cells destined to become MØs in its absence, implying a certain degree of fate plasticity within that compartment. Consistent with the idea of a certain degree of plasticity, IL-4, through the activation of the transcription factor STAT6, has recently been shown to induce demethylation of genes favoring DC differentiation and enforced STAT6 activation in the absence of IL-4 also favors DC differentiation (116). Interestingly, the transcription factor PU.1 has been shown to be required for the induction of STAT6-mediated transcription (117) and to promote DC generation from monocytes while inhibiting MØ production (109). Thus, PU.1 and STAT6 could abet terminal DC development. However, individual STAT proteins seldom act in isolation such that functional balance between multiple STAT proteins is important to determine cell differentiation (118). Interestingly, the effects of IL-4 on GM-CSF induced DC differentiation was shown to be dependent on the dose of both IL-4 and GM-CSF (119), suggesting that differentiation trajectories are dependent on the signal strength of both cytokines. Of note, IL-4 not only altered the differentiation trajectory under GM-CSF but also increased APC function of generated dendritic cells (120). IL-4 induced the expression of IRF4 that was not only critically required for DC differentiation, but also for their antigen cross-presentation capacity and the expression of costimulatory molecules (120).

An IL-4 related Th2 cytokine IL-13 has also been shown to enhance GM-CSF stimulated DC differentiation from mouse bone marrow cells (119) and human monocytes (121, 122), although the potency and action of IL-4 and IL-13 may differ. Furthermore, TNF-α and LPS added at a late stage of bone marrow cell culture with GM-CSF have also been shown to promote DC differentiation/maturation (2, 3). At least for TNF, multiple STAT proteins including STAT6 can be activated upon stimulation. Overall, there is considerable plasticity for GM-CSF induced differentiation of mononuclear cells, subject to the conditions that activate signaling modules favoring either DC or MØ differentiation.

Importance of GM-CSF for *in vivo* moDC Differentiation

Despite the strong potency of GM-CSF to induce DC differentiation *in vitro*, GM-CSF and its receptor are redundant for the differentiation of moDCs *in vivo*, at least during acute infection and inflammation (9, 123, 124). It could be that infection and inflammation induce high levels of many cytokines including M-CSF and TNF-α that could influence moDC differentiation and therefore mask the role of GM-CSF. In situations where GM-CSF concentration increase is more selective (e.g., GM-CSF overexpression or engraftment of a GM-CSF-producing tumor) (109, 125), GM-CSF seems to have a positive role in inducing moDC differentiation. In an EAE model with Th17 transfer, $GM\text{-}CSFR^{-/-}$ moDC infiltrates in CNS tissue were significantly reduced in a competitive scenario (126). Our view is that GM-CSF is sufficient but not essential for production of moDCs *in vivo*. Its importance on moDCs *in vivo* may instead be more critical for their effector function (see below).

Impact of GM-CSF on Non-moDCs

Many decades of work have established that the dendritic cell network is heterogenous and consists of many subsets with different phenotypic and functional features (127–129). DCs, excluding moDCs, have recently been categorized into three groups: cDC1s (for both $CD8^+$ and $CD103^+$ DCs), cDC2s (for $CD11b^+$ and $CD172\alpha^+$), and pDCs (130). Despite the differentiation of these cells being largely independent of GM-CSF, GM-CSF has pleiotropic impacts on all these DC subsets. In Flt3L-supplemented cultures of bone marrow cells, inclusion of low dose GM-CSF (0.3 ng/mL) increased the production of cDC1s, cDC2s, and pDCs, while neutralization of endogenous GM-CSF reduced all DC generation (131). Similar findings have

also been derived *in vivo*, particularly in mice with combined loss of GM-CSF and Flt3L (132). Enhancement of overall DC differentiation by GM-CSF is likely due to the positive effect of GM-CSF on progenitor commitment to myeloid lineages and expansion of such progenitors. However, at high doses of GM-CSF, development of cDC1s and pDCs under Flt3L stimulation was severely hampered (133, 134). At least for pDCs, it was shown that strong GM-CSF signaling leads to strong STAT5 activation and suppression of IRF8 transcription, which is critical for pDC differentiation (134). cDC1s include both lymphoid $CD8^+$ DCs and tissue $CD103^+CD207^+$ migratory DCs (130). Even though $CD8^+$ DCs were reduced in GM-CSF transgenic mice, the number of $CD103^+$ DCs was increased in GM-CSF transgenic mice (135), indicating subtle differences in the two types of cDC1s differentiated at different locations. Apart from the impacts on differentiation and DC cell survival discussed above, GM-CSF has also been shown to increase the cross-presentation properties of cDC1s both *in vitro* and *in vivo* (131, 136). Functional enhancement of cDC1s by GM-CSF is also associated with an increase in CD103 expression (131, 136). However, expression of CD103 *per se* is not sufficient for acquisition of cross-presentation capacity as TGF-β increased CD103 expression but not cross-presentation of cDC1s (131). Together, GM-CSF has a broad impact not only on the processes driving DC differentiation but also affects DC effector function at the mature state. Once again, the nature and the extent of these GM-CSF induced changes may be greatly affected by the relative abundance of GM-CSF, the state of maturity and the microenvironment encountered by the cells.

PRIMING END CELL FUNCTION BY GM-CSF: MORE THAN A NUMBERS GAME?

Despite GM-CSF seeming to be redundant in the development of moDCs *in vivo* (9, 123, 124), GM-CSF is still required for function of monocytes/MØs in the induction and progression of EAE (123, 124). Here we will discuss the different aspects of impact on MØ function by GM-CSF with the caveats of certain degrees of ambiguity surrounding the definition of monocytes, moDCs and MØs *in vivo*, and the difficulty delineating the impact of GM-CSF on cell survival and function *per se* in some studies.

Production of Cytokines and Chemokines: Priming Effect by GM-CSF

Both GM-CSF and M-CSF can generate MØs in bone marrow cultures. However, after LPS stimulation, the two factors elicit different functions. Human GM-CSF facilitates the differentiation of $CD14^+$ monocytes into IL-23 producing M1 like MØs while M-CSF promotes differentiation of M2 like MØs (137). In murine systems, GM-CSF differentiated bone marrow derived MØs (GM-BMMØs) also produce more IL-12, IL-23, TNF-α, and IL-6 than M-CSF differentiated MØs (BMMØs) (138). Moreover, GM-BMMØs preferentially activated NFκB while BMMØs preferentially activated the IRF3-STAT1 axis (138, 139). From the cytokine pattern elicited, it was proposed that GM-BMMØs is "M1-like" ($IL\text{-}12^{hi}$, $IL\text{-}23^{hi}$, $IL\text{-}10^{lo}$) while BMMØs is "M2-like" ($IL\text{-}12^{lo}$, $IL\text{-}23^{lo}$, $IL\text{-}10^{hi}$) (138). An adoptive transfer study supported this proposal in that GM-BMMØs but not BMMØs induced a Th1 response via IL-12 production and transferred resistance to parasite infection (140). In EAE, GM-CSF responsiveness in $CCR2^+Ly6C^{hi}$ monocytes/moDCs was critical for disease pathogenesis, whereas GM-CSF responsiveness in cDCs or PMNs was deemed unimportant (123, 124). Moreover, GM-CSF responsiveness in $CCR2^+$ cells was required for IL-1β production (124), likely from MØs but not DCs (108). Overall, these studies highlight the importance of GM-CSF in priming MØs for production of proinflammatory cytokines under TLR and NLR stimulation and provides an explanation for the adjuvant effect of GM-CSF in cancer, inflammation, and infection, even when numbers of myeloid cells are not affected.

Antigen-Presenting Cell (APC) Function and Costimulation

An early study showed that GM-CSF enhanced APC function by increasing IL-1β production and MHC expression (141). We and others had demonstrated that GM-CSF was required for acquisition of cross-presentation capacity by cDC1s (131, 136). Bone marrow precursors cultured with GM-CSF generated $CD11c^+$ cells with modest levels of CD86 and MHC II, particularly in low density cultures, whereas late addition of IL-4 dramatically increased expression of CD86 and MHC II (6). Of note, *in vivo* treatment with human GM-CSF needed co-administration of IL-4 to enhance APC function (142). These observations suggest that GM-CSF by itself has a limited capacity to up-regulate costimulatory molecules. Consequently, $CD11c^+$ cells derived from GM-CSF cultures alone have a weak capacity to induce T cell proliferation compared with those derived from IL-4 supplemented cultures (6). To complicate the issue, moDCs could also suppress the APC function of cDCs (125). Overall, although GM-CSF promotes APC survival and differentiation fate, it may have limited direct effect on APC function.

Effector Function

In the steady state, a deficiency in GM-CSF or its receptor GM-CSFR led to defective terminal differentiation of alveolar MØs, resulting in impaired surfactant catabolism and pulmonary alveolar proteinosis in both human and mice (8, 143). GM-CSF activated PU.1 to drive this differentiation pathway (144); local delivery of GM-CSF restored PU.1 and corrected the disease (144–146). In GM-CSF transgenic mice, MØs showed increased phagocytic activity and increased production of oxygen degradation products (11, 147). *In vitro*, GM-CSF primed GM-BMMØs for TLR-stimulated increased nitric oxide and lipid mediator LTB4 production but a reduction in PGE2 (148). In the absence of GM-CSF, MØs had reduced capacity for up-taking apoptotic cells (70).

PRIMING END CELL FUNCTION BY GM-CSF: WHAT DETERMINES M1 MØ OR M2 MØ DEVIATION?

Although GM-CSF has been viewed predominantly as a pro-inflammatory cytokine and promotes differentiation of M1-like MØs that produce proinflammatory cytokines (137, 138, 149), GM-CSF has also been associated with development of M2-like MØs (47, 49). What then determined the M1-like MØ vs. M2-like MØ fate under GM-CSF stimulation? Evidence from tumor settings indicated that GM-CSF abundance was a key factor in determining cell fate. Production of high levels of GM-CSF by tumor cells led to increased M2 like MØ accumulation within the cancerous tissues, thereby inhibiting T cell response in mouse models of melanoma and pancreatic cancer (47, 49). Conversely, GM-CSF blockade reduced the development of M2 like MØs (49). It remains unclear how GM-CSF drives M2 like MØ differentiation. A study showed that GM-CSF could activate JAK2/STAT5 which in turn suppressed IRF8 transcription (150). Functionally, IRF8 could suppress M2 like MØ differentiation since IRF8 deficiency promoted M2-like MØs differentiation in tumors, while overexpression of IRF8 reduced M2-like MØ accumulation (150). Other transcription factors influenced by GM-CSF signaling in M2-like MØ activity include C/EBPbeta (151) and RORC1 (152). Interestingly, IL-3, a cytokine sharing the signaling receptor with GM-CSF, also promoted prostaglandin E2-producing M2 like MØs *in vitro* (153).

Apart from difference in cytokine production, mouse M2 MØs express high levels of characteristic markers such as Arginase 1 (Arg1), Chitinase-like 3 (Chil3, YM1), and transglutaminase 2 (Tgm2) (149, 154). These molecules had been demonstrated to mediate immunosuppression, tumor metastasis and tumor growth (155, 156). While excess GM-CSF has been associated with development of M2 like MØs (47, 49), IL-4 is also known for its potent role in shaping M2 MØ differentiation and confers many functional characteristics of M2 MØs (149). However, when IL-4 was dosed in combination with GM-CSF, M2 MØs could also differentiate into fully functional APCs (47). The coordinate action of GM-CSF and IL-4 in promoting myeloid cell fate decisions remains puzzling. We reasoned that GM-CSF and IL-4 likely instruct distinct signaling modules leading to M2 MØ differentiation. As alluded to above, GM-CSF activated STAT5 which in turn suppressed IRF8, the transcription factor suppressing M2 MØ differentiation (150). On the other hand, IL-4 promoted M2 MØ differentiation via STAT6 activation and IRF4 induction in M-CSF differentiated MØs (157, 158). To complicate the issue, GM-CSF can also induce IRF4 expression in MØs (159). IRF4 also played an important role in deciding DC vs. MØ fate, as a recent study showed that IRF4 deficiency favored MØ differentiation over DC differentiation of monocytes in the presence of IL-4 and GM-CSF (120). Overall, the signaling events emanated from GM-CSF and IL-4, leading to the differentiation of functionally distinctive DCs, M1-like MØs and M2-like MØs, have not been fully defined. In addition to IL-4, another Th2 cytokine IL-13 has been shown to suppress the production of proinflammatory cytokines (160, 161). It seems that both IL-4 and IL-13 acted in a similar fashion via STAT6 activation to modulate MØ function (162).

GM-CSF can activate PI3K and NFκB pathways promoting myeloid cell survival (19) and contributing to lung inflammation (163). However, activation of PI3K pathway can also polarize MØs (25, 75, 164). Of note, the downstream signaling molecules associated with PI3K activity, Akt1, and Akt2, have been shown to have contrasting effects in controlling MØ polarization; while the latter promotes M2 MØs, Akt1 was shown to induce M1 MØ polarization (25). An unanswered question is whether and how GM-CSF, and in particular its signaling strength, promotes differential activation of Akt1 vs. Akt2.

Finally, GM-CSF can also mediate immunosuppression indirectly via promoting Treg induction (165). GM-CSF induces the expression of milk fat globule EGF8 (MFG-E8) that promotes uptake of apoptotic cells by MØs, inducing their production of TGFβ and thereby controlling Treg development (165). Interestingly, TLR stimulation or uptake of necrotic cells was shown to downregulate MFG-E8 expression and to reduce the impact of GM-CSF on MFG-E8 expression, thus preserving the pro-inflammatory action of GM-CSF in tumor immunity (165), suggesting a pathway countering GM-CSF mediated immunosuppression.

Beyond tumors, the influence of GM-CSF on M2 like MØs extends to several inflammatory situations such as autoimmunity (67), infection (166), and transplantation (167). In general, research into the impact of GM-CSF has so far mainly focused on its property to expand myeloid cells. It still remains unclear how GM-CSF steers macrophage function to M1 MØs vs. M2 MØs. While M2 MØs may be detrimental in the context of tumor immunity, they may also be beneficial in damping autoimmunity, transplant rejection and infection-associated immunopathology and therefore it is of importance to be better define GM-CSF and its signaling components as this may avail therapeutic targets during M2 MØs development and function.

CONCLUDING REMARKS

GM-CSF is produced by many cells and its receptor is broadly expressed by hematopoietic cells. Engagement of GM-CSFR activates multiple signal pathways in a dose dependent manner to impact on multiple cellular processes including survival, proliferation, differentiation and function of multiple myeloid cells. Due to its promiscuous properties, GM-CSF roles in controlling pro-inflammatory or anti-inflammatory processes in healthy or diseased individuals are often complex and paradoxical. We opine that GM-CSF signaling strength likely determines biological outcome (**Figure 2**). At the cellular level, it drives differentiation of different cell subsets by activating different signaling modules. At the functional level, it programs antigen presentation capacity, proinflammatory function and suppressive function. Ultimately, these cellular

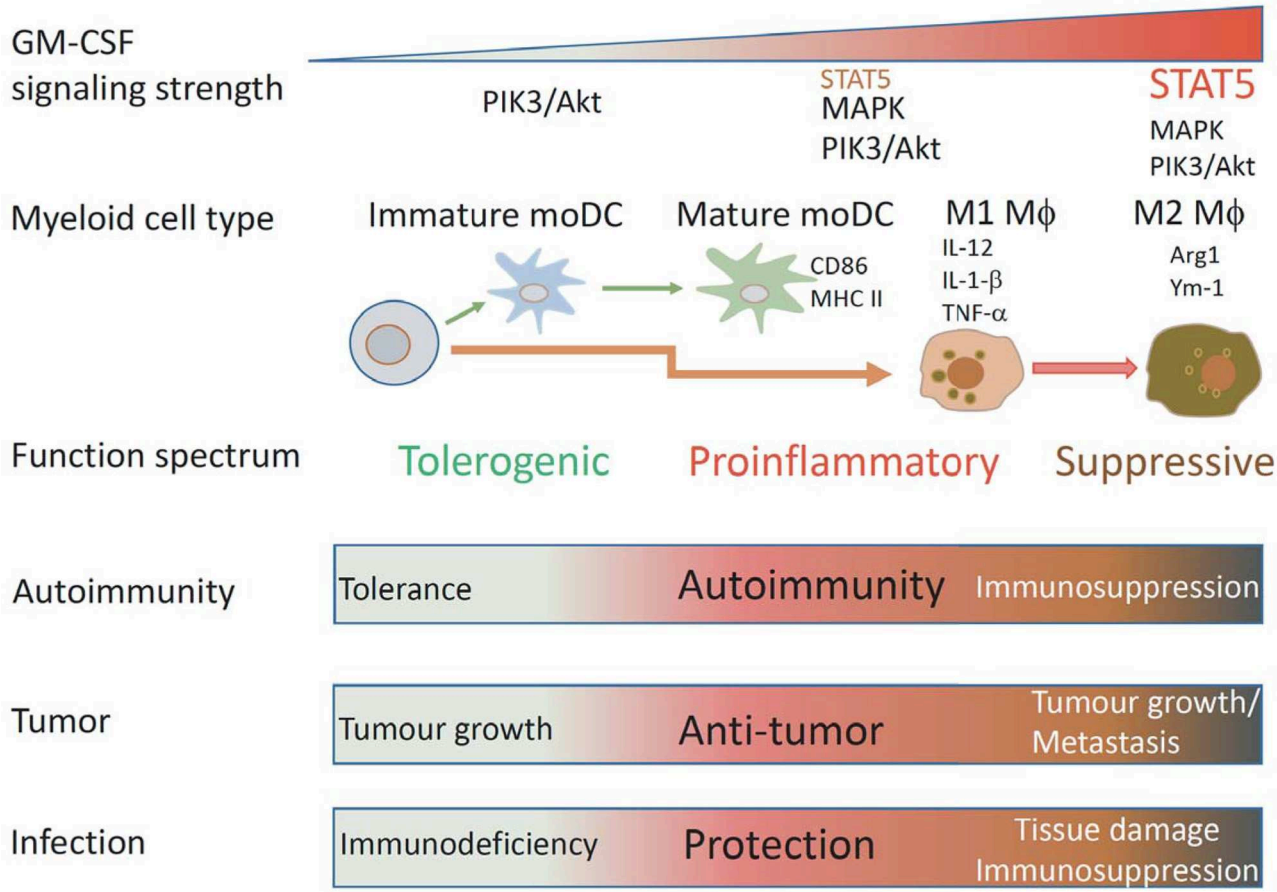

FIGURE 2 | Schematic illustrating how GM-CSF signaling strength affects mononuclear myeloid cell differentiation and function. Under different GM-CSF signaling strength, different types of mononuclear myeloid cells with different functional properties are differentiated. Low GM-CSF signaling strength favors development of immature DCs, intermediated signaling strength favors development of MHCII^{hi}CD86^{hi} mature DCs, high signaling strength favors development of proinflammatory M1 MØs, ultra-high signaling strength favors development of suppressive M2 MØs. According to these properties, these cells could have particular impacts on immunity to autoantigens, tumors, and infection.

changes will impact immunity and immunopathology in different disease settings.

In the tumor setting, relatively low to moderate doses of GM-CSF favored the immune adjuvant activity, while high doses of endogenous tumor-derived or exogenous GM-CSF could expand M2 like suppressor cells (54). GM-CSF also directly or indirectly expanded Tregs (67). For the latter, blocking GM-CSF could improve anti-tumor immunity (49). As GM-CSF mediated graft-vs.-host disease but not graft-vs.-leukemia response (77), blocking GM-CSF and receptor signaling could be also beneficial. Beyond ligand abundance, downstream signaling responsible for different cell fates should also be explored as intervention targets. Individual IRF members and Akt subunits have differential impacts on DCs, M1, and M2 MØs. SOCS family members naturally act as negative regulators as a brake on cytokine signaling. Their action can be potentially targeted to modify monocytic cell differentiation and function. Furthermore, directly targeting suppressive function of M2 MØs may also be considered. Both Arginase 1 and Chil3 are critical for arginine metabolism while arginine availability is key to an optimal T cell immune response (168). Arginase 1 inhibitor L-Norvaline and iNOS2 inhibitor L-NMMA had been found to enhance T cell proliferation (125, 169). It would be interesting to test whether selective targeting of these effector molecules of M2 MØs could enhance the beneficial anti-tumor effect of GM-CSF. In addition, IL-4 and IL-13 can dramatically change the differentiation trajectory of immune cells and their function. Therefore, their potential should also be considered when immune intervention strategies are explored.

In the autoimmune setting, anti-human GM-CSF mAb (63, 64) and anti-human GM-CSF receptor α mAb (65, 66) have also been shown to ameliorate rheumatoid arthritis in clinical trials, reinforcing the work of several decades that GM-CSF is a key proinflammatory cytokine. Yet, it remains unknown whether the tolerogenic roles of GM-CSF including expansion of Tregs (74) and induction of suppressive MØs (75) could also be harnessed. In addition, immunosuppression also occurs in

chronic infections in which high levels of GM-CSF can persist (38). Perhaps, antagonism of GM-CSF in such settings could also be beneficial.

In summary, GM-CSF has pleiotropic effects on myeloid cell differentiation and function. The complexity of GM-CSF action provides a challenge but also an opportunity for tailored immune intervention. To fully capitalize on the agonistic and antagonistic effects of GM-CSF as in cancer, inflammation and infection, the differential impact of GM-CSF signaling strength on different target cells should be considered.

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YZ, AL, and MC wrote the paper.

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S100A12 Expression Is Modulated During Monocyte Differentiation and Reflects Periodontitis Severity

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S100A12 is a calcium-binding protein of the S100 subfamily of myeloid-related proteins that acts as an alarmin to induce a pro-inflammatory innate immune response. It has been linked to several chronic inflammatory diseases, however its role in the common oral immunopathology periodontitis is largely unknown. Previous *in vitro* monoculture experiments indicate that S100A12 production decreases during monocyte differentiation stages, while the regulation within tissue is poorly defined. This study evaluated S100A12 expression in monocyte subsets, during monocyte-to-macrophage differentiation and following polarization, both in monoculture and in a tissue context, utilizing a three-dimensional co-culture oral tissue model. Further, we explored the involvement of S100A12 in periodontitis by analyzing its expression in peripheral circulation and gingival tissue, as well as in saliva. We found that S100A12 expression was higher in classical than in non-classical monocytes. S100A12 expression and protein secretion declined significantly during monocyte-to-macrophage differentiation, while polarization of monocyte-derived macrophages had no effect on either. Peripheral monocytes from periodontitis patients had higher S100A12 expression than monocytes from controls, a difference particularly observed in the intermediate and non-classical monocyte subsets. Further, monocytes from periodontitis patients displayed an increased secretion of S100A12 compared with monocytes from controls. In oral tissue cultures, monocyte differentiation resulted in increased S100A12 secretion over time, which further increased after inflammatory stimuli. Likewise, S100A12 expression was higher in gingival tissue from periodontitis patients where monocyte-derived cells exhibited higher expression of S100A12 in comparison to non-periodontitis tissue. In line with our findings, patients with severe periodontitis had significantly higher levels of S100A12 in saliva compared to non-periodontitis patients, and the levels correlated to clinical periodontal parameters. Taken together, S100A12 is predominantly secreted by monocytes rather than by monocyte-derived cells. Moreover, S100A12 is

increased in inflamed tissue cultures, potentially as a result of enhanced production by monocyte-derived cells. This study implicates the involvement of S100A12 in periodontitis pathogenesis, as evidenced by increased S100A12 expression in inflamed gingival tissue, which may be due to altered circulatory monocytes in periodontitis.

Keywords: monocytes, monocyte-derived cells, S100A12 protein, periodontal diseases, gingiva, saliva

INTRODUCTION

Monocytes are mononuclear phagocytes, which after birth derive from hematological precursors in the bone marrow and enter the blood circulation (1). In blood, human monocytes are divided into three main subsets based on the expression of CD14 and CD16. Classical monocytes have high CD14 expression and no CD16 ($CD14^{hi}CD16^{-}$), intermediate monocytes show high CD14 and low CD16 ($CD14^{hi}CD16^{+}$), and non-classical monocytes have low expression of CD14 together with high CD16 ($CD14^{+}CD16^{hi}$) (2). These subsets present different transcriptional profiles with $CD16^{+}$ monocytes being at a more advanced stage of myeloid differentiation (3, 4). The intermediate subset has been suggested to correspond to an activation and/or differentiation state of $CD14^{+}$ monocytes (5), though a closer relationship to the non-classical subset has been reported (6).

Following injury, infection or inflammation, monocytes are rapidly recruited to the tissues where they become activated, alter their phenotype and can mature into inflammatory macrophages. This monocyte-to-macrophage transition occurs in a non-synchronized manner, and these cells are highly heterogeneous in the tissue (1). *In vitro*, macrophage diversity has been functionally classified into two groups: "classically activated" (M1) or "alternatively activated" (M2) macrophages. Macrophages undergo activation to M1 or M2 phenotype in response to toll-like receptor (TLR) ligands/interferon (IFN)-γ or interleukin (IL)-4/IL-13, respectively. M1 macrophages are generally efficient producers of reactive oxygen and nitrogen intermediates and inflammatory cytokines, while M2 macrophages have high levels of scavenger, mannose and galactose-type receptors, being fundamental for tissue remodeling, repair and healing (7). In tissue however, a plethora of stimuli occurs at the same time, inducing a spectrum of activation rather than the black and white scenario that occurs with M1/M2 polarization *in vitro*.

S100A12 is a member of the S100 family of low molecular weight proteins, which are characterized by two calcium binding EF-hand motifs connected by a central hinge region (8, 9). S100A12 is mainly expressed in the cytoplasm of myeloid cells and, upon release, acts as a proinflammatory alarmin (9, 10). Interestingly, its gene expression is higher in classical than in non-classical monocytes (3, 6), and it decreases during monocyte to macrophage maturation (11). S100A12 binds to receptor for advanced glycation end products (RAGE) and TLR4, it is chemotactic for leukocytes and induces a strong inflammatory response in monocytes (12, 13). Its expression is markedly increased at sites of inflammation, and the levels in circulation might be useful as a measure of disease activity in chronic inflammatory diseases (14–17).

Periodontitis is a chronic inflammatory disease characterized by destruction of the tooth-supporting structures, which can lead to tooth loss and contribute to the systemic inflammatory burden (18). Its pathogenesis involves a complex interplay between the dysbiotic microbiota and the host immune-inflammatory response (19), where myeloid cells play a critical role in the periodontal injury. These cells infiltrate the gingival tissue during disease, undermine oral mucosa integrity through the production of matrix metalloproteinase (MMP)-12 (20) and contribute to the alveolar bone resorption (21). It has been shown that S100A12 levels in saliva are associated with periodontal disease (22). However, the role of monocyte-produced S100A12 in periodontal inflammation has not been investigated yet.

Therefore, the present study aimed to evaluate S100A12 dynamics during monocyte-to-macrophage differentiation and polarization. Further, we aimed to explore its involvement in periodontitis using a 3D co-culture tissue model, and by analyzing S100A12 expression in tissue, peripheral circulation, and saliva from patients with periodontitis.

MATERIALS AND METHODS

Human Subjects

Healthy participants and patients with periodontitis were recruited at the Department of Dental Medicine, Karolinska Institutet, Stockholm, and at the specialist clinic Danakliniken, Danderyd, Sweden. Periodontitis patients were included based on the presence of at least 4 teeth with probing pocket depths $\geq$ 6 mm and radiographic bone loss. Control participants had no periodontal pockets $\geq$ 4 mm and no history of periodontitis. Participants were excluded if they had chronic inflammatory diseases that could affect the periodontium, or had used antibiotics or corticosteroids 3 months before the inclusion. Additionally, a cohort of individuals living in the Kalmar county, Sweden, was recruited. They were randomly selected, orally examined and provided a stimulated saliva sample. Participants answered a questionnaire containing data about age, sex, smoking habits, and presence of diseases. Smoking was recorded as current smokers or never/former smokers.

This study was carried out in accordance with the recommendations of the regional ethics committee in Stockholm, Sweden and of the regional ethics committee at the Lund University, Sweden with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the regional ethics committee in Stockholm, Sweden (Dnr. 2012/1579-32 and 2017/1333-32) and by the

regional ethics committee at the Lund University, Sweden (Dnr. 2011/366).

Monocyte Isolation, Differentiation, and Polarization

Buffy-coated blood from anonymous healthy donors were used for the *in vitro* experiments. To study monocytes in periodontitis, peripheral blood was also collected in EDTA-containing vacutainers from periodontitis patients and periodontally healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient centrifugation (BD Diagnostics, San Jose, CA, USA), after which monocytes were isolated using the EasySep Human monocyte enrichment kit without CD16 depletion (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's instructions. Monocytes from healthy donors were cultured in 6-well plates (5×10^5 monocytes/well) in complete RPMI 1640 medium (10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FCS) supplemented with CSF-1 (50 ng/ml; BioLegend, San Diego, CA, USA) for 1, 3, and 8 days at 37°C, 5% CO_2, to assess the monocyte-to-macrophage differentiation. After 8 days in culture, macrophages were polarized with LPS (50 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and IFN-γ (50 ng/ml; BioLegend, San Diego, CA, USA) or IL-4 and IL-13 (50 ng/ml each; BioLegend, San Diego, CA, USA) for another 24 h. Non-polarized macrophages were used as controls. PBMCs from periodontitis patients and periodontally healthy individuals were stored frozen after collection. The PBMCs where thawed in complete RPMI, and used for flow cytometry staining or monocyte isolation followed by *in vitro* culture. The monocytes were cultured (37°C, 5% CO_2) in 24-well plates in complete RPMI with CSF-1 (50 ng/ml; Biolegend, San Diego, CA, USA) at a concentration of 3×10^5 cells/ml and incubated for 24 h.

3D Oral Tissue Culture

A 3D oral tissue model was set up containing epithelial cells (TERT-immortalized normal human oral keratinocyte line OKF6/TERT-2, kindly provided by J. Rheinwald) (23), primary fibroblasts (24), and monocytes as previously described (20). Briefly, 3-μm pore size transwell inserts were placed in 6-well plates and coated with a mixture of bovine type I collagen (PureCol, Cell Systems, Troisdorf, Germany) and DMEM (GE Healthcare Life Sciences, Uppsala, Sweden). Fibroblasts (7.5×10^4 cells/model) were suspended in complete DMEM and diluted in a PureCol and DMEM suspension with addition of media after 2 h and cultured for 7 days in 5% CO_2 at 37°C. Monocytes (4×10^5/model) in complete RPMI were then added on top of the fibroblast layer and incubated for 1.5 h in 5% CO_2 at 37°C, after which complete DMEM was added for a 24 h incubation. Epithelial cells (4×10^5/model) in complete K-SFM were added on top of the fibroblast and monocyte layers. After a 1.5 h incubation in 5% CO_2 at 37°C, complete K-SFM was added for a 48 h incubation. The models were air-exposed by removing the media, followed by the addition of complete K-SFM supplemented with an additional 0.3 mM $CaCl_2$ only to the outer chamber. To assess time-dependent secretion of S100A12, models were cultured for 3, 5, and 7 days after monocytes were implanted and supernatants were collected. Similar models without the addition of monocytes were also set up.

Oral tissue cultures were repeatedly stimulated with K-SFM containing *E. coli* LPS (100 ng/ml; Invivogen, San Diego, CA, USA) and IFN-γ (50 ng/ml; BioLegend, San Diego, CA, USA). Complete K-SFM only was used as a control. The stimulations were added in the upper compartment at day 2, 3, and 5 after monocyte implantation in conjunction with media change in the bottom compartment. Supernatants were collected at day 7 after monocyte implantation. Additionally, conditioned medium from oral tissue models without monocytes were used to stimulate monocytes and supernatants were collected after 24 h.

Real-Time PCR

RNA from *in vitro* cultured cells was isolated using the Quick-RNA MiniPrep kit (Zymo Research Corp, Irvine, CA, USA), and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. S100A12 mRNA expression was determined using SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA) in the 7500-fast-real-time detection system (Applied Biosystems, Foster City, CA, USA) using specific primers (forward: CACATTCCTGTG CATTGAGG/reverse: GGTGTCAAAATGCCCCTTC, Eurofins, Ebersberg, Germany) related to the housekeeping gene GAPDH (forward: TCCACTGGCGTCTTCACC/reverse: GGCAGAGA TGATGACCCTTTT) by the ΔΔCt method.

Gingival Tissue Collection and Digestion

Gingival tissue was harvested during periodontal surgery from an inflamed site with a persistent periodontal pocket ≥6 mm in periodontitis patients ($n = 6$). In the controls, gingival tissue was harvested from a site without periodontal pocket in conjunction with an implant surgery or tooth extraction performed for reasons other than periodontitis ($n = 6$). Tissues were placed in complete RPMI 1640 media (GE Healthcare Life Sciences, Uppsala, Sweden). The tissues were cut into small pieces and incubated with 0.3 mM $CaCl_2$ in phosphate buffered saline (PBS) at 37°C with magnetic stirring for 10 min. After that, they were incubated with Collagenase II (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and DNase I (0.25 mg/ml; Roche, Basel, Switzerland) in RPMI 1640 without FCS at 37°C with magnetic stirring for 40 min. The suspension was filtered through a 70-μm mesh filter (Falcon, Corning, NY, USA), washed with complete RPMI, and centrifuged at 400 g for 5 min. Cell suspensions were then stained and analyzed by flow cytometry.

Immunohistochemistry and Immunofluorescence

Gingival tissue from periodontitis patients ($n = 4$) and healthy controls ($n = 5$) were harvested during periodontal surgery and immediately placed in tissue transport solution (Histocon; Histolab Products AB, Gothenburg, Sweden), after which they were embedded in optimal cutting temperature compound (OCT; Histolab Products AB, Gothenburg, Sweden). 3D oral tissue cultures were incubated in 2M sucrose for 1 h, and then the

membranes and attached models were removed from the insert and embedded in OCT. The embedded tissues and 3D cultures were stored at −80°C until sectioning.

Seven micrometers of sections were fixed in acetone and endogenous peroxidase was blocked in methanol and H_2O_2. Avidin and biotin solutions (Vector Laboratories, Burlingame, CA, USA) and the appropriate serum were used for blocking of the sections. Tissues were incubated with a monoclonal anti-S100A12 antibody (clone OTI1D1, ThermoFisher, Waltham, MA, USA), a monoclonal anti-CD68 antibody (clone PG-M1, Dako, Glostrup, Denmark) or their respective isotype controls (Abcam, Cambridge, UK) overnight at 4°C. An incubation with the biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) was performed, followed by the ABC complex (Vector Laboratories, Burlingame, CA, USA), and then developed in diaminobenzidine (DAB) solution (Vector Laboratories, Burlingame, CA, USA). Slides were dehydrated, mounted with Pertex (Histolab Products AB, Gothenburg, Sweden) and visualized and photographed using an Olympus BX43 light microscope equipped with an Olympus SC50 camera (Olympus Corporation, Tokyo, Japan).

Double labeling was performed on gingival tissue using the following antibodies: mouse anti-S100A12 (clone OTI1D1) and rat anti-CD206 (clone 309210, R&D Systems, Minneapolis, MN, USA) or isotype controls. Specific staining was detected by flourochrome-labeled goat anti-mouse (AF647) and anti-rat (AF488) antibodies. Cell nuclei counterstain was performed with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, ThermoFisher, Waltham, MA, USA) and sections were mounted in Prolong Gold Antifade Mountant (ThermoFisher, Waltham, MA, USA). Immunofluorescence staining was visualized using a Nikon Eclipse E600 Fluorescence Microscope equipped with the Nikon digital camera DMX1200 (Nikon, Tokyo, Japan).

Western Blot

Total protein from gingival tissue from periodontitis patients ($n = 6$) and controls ($n = 6$) was isolated using a protein extraction buffer (T-Per, ThermoFisher, Waltham, MA, USA) supplemented with a protease inhibitor cocktail (Roche Molecular Systems Inc., Pleasanton, CA, USA). Fifteen μg of total protein per lane was separated by gradient Mini-PROTEAN® TGX™ 4–20% gels (Bio-Rad Laboratories, Hercules, CA, USA) and then transferred to nitrocellulose membranes (GE Healthcare Life Science, Uppsala, Sweden). After incubation with blocking buffer [Tris buffered saline, 0.1% Tween 20 (TBS-T) and 5% non-fat milk powder] for 1 h at RT, membranes were washed with TBS-T and incubated with the S100A12 antibody (clone OTI1D1, ThermoFisher, Waltham, MA, USA) overnight at 4°C. Membranes were then incubated with the secondary horseradish-peroxidase-conjugated IgG (Cell Signaling Technology, Inc., Beverly, MA, USA) at RT for 1 h. As a loading control, β-actin (Cell Signaling Technology, MA, USA) was used. Membranes were developed with Amersham ECL select Western Blotting Detection Reagent (GE Healthcare Life Science, Uppsala, Sweden). The blots were visualized and quantified with the ChemiDoc™ XRS (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Flow Cytometry

Cell preparations from blood or digested tissues, were incubated with BD Horizon Fixable Viability Stain 510 (BD Bioscience, Franklin Lakes, NJ, USA) diluted in PBS. The cells were then incubated with human Trustain FcX (BioLegend, San Diego, CA, USA) in FACS buffer (2% FCS, 1 mM EDTA in PBS) for 10 min in RT followed by the addition of fluorochrome-conjugated antibodies and an additional 30 min incubation on ice. The following antibodies were used in different combinations: CD14 (clone M5E2, PerCP; BioLegend), CD14 (clone MϕP9, APC; BD Bioscience), CD16 (clone 3G8, BV421; BioLegend), CD90 (clone 5E10, PE; BioLegend), CD45 (clone HI30, BV421; BioLegend), HLA-DR (clone L243, APC or PE/Cy7; BioLegend), CD64 (clone 10.1, PE/Cy7; BioLegend), and CD206 (clone 15-2, APC/Cy7; BioLegend). Cells were fixed and permeabilized with BD Cytofix/Cytoperm, after which they were incubated with the following intracellular antibodies for 30 min on ice: S100A12 (clone 161205, AF488) or IgG2b (AF488), both from R&D Systems (Minneapolis, MN, USA). Cells were analyzed with BD FACSVerse™ (BD Bioscience). Data analysis was performed using the FlowJo software, version 10 (Tree Star, Ashland, OR, USA).

Plasma Collection

The blood collected from periodontitis patients ($n = 5$) and controls ($n = 5$) was diluted with equal amount of PBS followed by a Ficoll-Hypaque gradient centrifugation (BD Diagnostics). The upper layer was collected and stored at −80°C until analysis.

Saliva Collection

A clinical oral examination was performed in 336 individuals (51.4 [±17.7] years; 50.6% women) including plaque index, bleeding on probing (BOP) and probing pocket depth (PPD). The degree of alveolar bone loss was determined through radiographic examination. The association between the different periodontal parameters and S100A12 levels in saliva was evaluated by categorizing the patients within the following groups: BOP ≤ 20% or >20% (22); presence or not of pathological periodontal pockets (PPD ≥ 4 mm); and loss of supporting bone tissue <1/3 of the root length (PD-group), ≥1/3 of the root length in <30% of the sites (PD group), and horizontal bone loss ≥1/3 of the root length in >30% of the sites (PD+ group) (25). In addition, the participants were diagnosed according to the new classification of periodontal diseases (26, 27). Stimulated saliva samples were collected during 5 min of chewing on 0.5 g paraffin and the salivary flow was measured. Saliva was immediately frozen at −20°C and then centrifuged and supernatants were stored at −80°C until analysis. Total amount of protein was determined by the Bradford assay.

ELISA

S100A12 levels in supernatants, plasma, and saliva were analyzed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The assay range of detection is 7.81–500 pg/ml. The assay was previously validated for saliva by performing spike and recovery tests (22). Saliva and plasma samples were

diluted 1:1,000 and 1:5, respectively. Readings were made using a microplate spectrophotometer at 450 nm with wavelength correction set to 540 nm to subtract background (SpectraMAX 340, Sunnyvale, CA, USA).

Statistical Analysis

Data analysis was performed with GraphPad Prism, version 8 (GraphPad Software, La Jolla, CA, USA). Group comparisons were performed using Mann–Whitney, Kruskal–Wallis or Friedman test with a Dunn's post-test when appropriate. Correlations were analyzed by Spearman rank correlation coefficient. Statistical significance was set at $p \leq 0.05$.

RESULTS

S100A12 Expression Is Higher in Classical Monocytes and Decreases During Monocyte-to-Macrophage Differentiation

To gain insight into S100A12 modulation in monocytes and macrophages, we assessed its expression in human monocyte subsets (**Figure 1A**) and the results showed that classical monocytes displayed higher expression and were more frequently positive for S100A12 than non-classical monocytes (**Figure 1B**). Next, S100A12 expression and secretion during monocyte-to-macrophage differentiation in culture with CSF-1 for 8 days were evaluated and we found that both mRNA expression and protein secretion were significantly higher in monocytes (day 1) than in macrophages (day 8). No significant difference in mRNA expression or protein secretion was observed in comparison with day 3. In fact, S100A12 secretion was on average 19 times higher in monocytes in comparison with macrophages (**Figure 1C**). Moreover, when macrophages were classically (LPS/IFN-γ) or alternatively activated (IL-4/IL13) no significant difference was seen in the expression and secretion of S100A12 between unpolarized and "M1" or "M2" subsets (**Figure 1D**).

S100A12 Expression Is Altered in Circulating Monocytes From Periodontitis Patients

Previous studies have reported an increase in the proportion of non-classical monocytes in periodontitis (28, 29). We investigated whether peripheral monocytes from periodontitis patients (52.6 ± 5.1 years old; 2 females and 3 males) present differential S100A12 expression and secretion when compared with monocytes from periodontally healthy participants (42.4 ± 15.9 years old; 3 females and 2 males), and whether this difference was related to a particular subset. The monocyte counts did not differ significantly between periodontitis patients and controls (**Figure 2A**). However, the frequency of non-classical monocytes was lower in periodontitis patients (**Figure 2B**). The classical subset displayed a tendency toward higher frequency in periodontitis ($p = 0.055$). Mean counts (±SD) of the classical, intermediate, and non-classical subsets in the controls were 3.9 (±1.4), 0.4 (±0.1), and 0.3 (±0.1) × 10^8/L, respectively. In periodontitis, they were 5.2 (±1.9), 0.4 (±0.1), and 0.2 (±0.1) × 10^8/L. The percentage of S100A12$^+$ monocytes was higher in periodontitis than in controls (**Figures 2C,D**). In comparison to healthy controls, a greater proportion of intermediate and non-classical monocytes from periodontitis patients were S100A12$^+$ (**Figure 2E**). Classical monocytes in periodontitis patients were more frequently S100A12$^+$, although this did not reach significance ($p = 0.055$). After 24 h in culture, monocytes from periodontitis patients secreted significantly higher levels of S100A12 than control monocytes (**Figure 2F**). However, plasma levels of S100A12 did not differ significantly between the groups (**Figure 2G**).

S100A12 Secretion Is Increased by Inflammatory Stimuli in 3D Oral Tissue Cultures

To investigate the modulation of S100A12 production by monocytes in a setting associated with tissue inflammation, we used a 3D oral tissue culture resembling the oral tissue containing epithelial cells, fibroblasts, and monocytes from healthy volunteers. CD68$^+$ cells were present in the cultures showing the engraftment and differentiation of monocytes (**Supplementary Figure 1**). Oral tissue cultures with monocytes showed significantly higher S100A12 levels than those without ($p < 0.0001$). Further, a significant time-dependent increase in the secretion of S100A12 was seen in the cultures with monocytes, however no significant difference was found in cultures without monocytes (**Figure 3A**). When stimulated with a combination of LPS and IFN-γ there was a significant increase in S100A12 levels in cultures with monocytes, whereas no significant difference was seen in cultures without (**Figure 3B**). To assess whether factors produced by epithelial cells and/or fibroblasts would lead to S100A12 secretion by monocytes, we stimulated them with conditioned medium from cultures without monocytes, however no significant effect was observed (**Figure 3C**), which suggests S100A12 secretion might be dependent on direct interaction within the model. A representative immunohistochemical analysis showed S100A12 staining in a 3D oral tissue culture with monocytes (**Figure 3D**).

S100A12 Expression Is Higher in Gingival Tissue Affected by Periodontitis

Since inflammatory stimuli modulates S100A12 levels in oral tissue cultures, the expression of S100A12 in gingival tissue affected by periodontitis was evaluated. Immunohistochemical analysis showed strong S100A12 staining in the connective tissue mainly in conjunction with the inflammatory infiltrate in periodontitis, whereas in the controls a weak staining was seen in the connective tissue (**Figure 4A**). The presence of CD68$^+$ cells in gingival tissue is presented in a **Supplementary Figure 1**. Immunofluorescence staining of gingival tissue showed S100A12 expression can be seen in proximity of CD206-expressing cells (**Figure 4B**). Western blot evaluation of tissue protein extracts revealed a significantly higher expression of S100A12 in periodontitis compared to controls (**Figure 4C**).

Further, S100A12$^+$ monocyte-derived cells (MCs) in gingival tissue from periodontitis patients and controls were characterized. Digested tissues were stained and live,

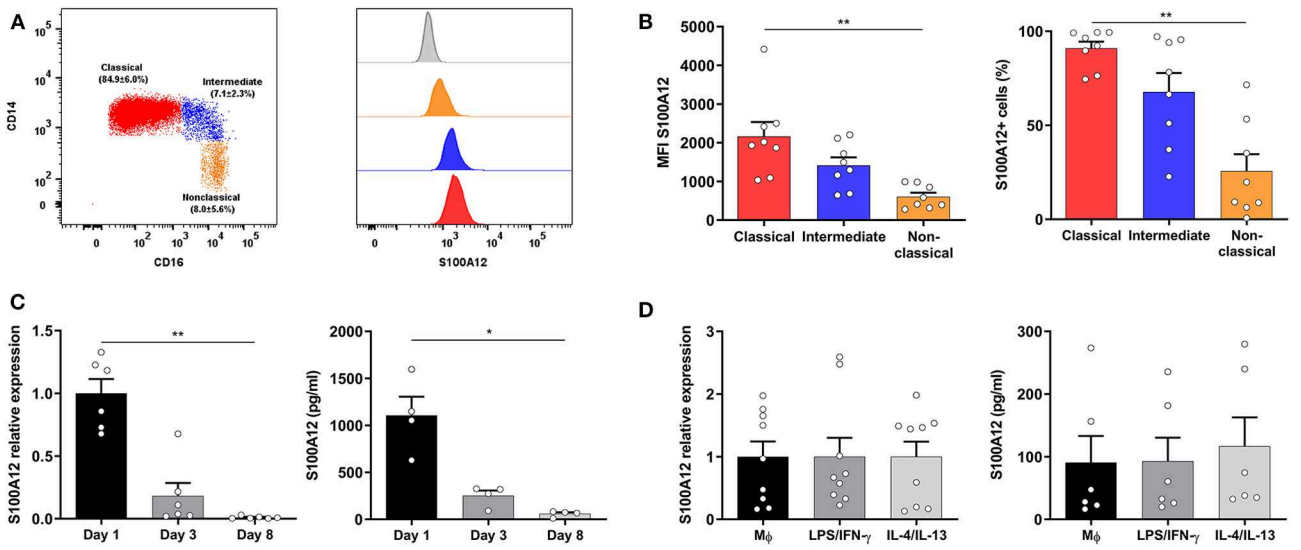

FIGURE 1 | S100A12 expression and secretion during monocyte-to-macrophage differentiation and polarization. **(A)** Representative dot plot showing the gating strategy of human monocyte subsets based on CD14 and CD16 expression after doublet and dead cells exclusion, and HLA-DR expression, as well as mean (±SD) frequency of monocyte subsets. Histogram depicting the S100A12 median fluorescence intensity (MFI) in classical (red), intermediate (blue), and non-classical monocytes (orange), as well as in the isotype control (gray histogram). **(B)** S100A12 MFI and percentage of S100A12$^+$ cells in classical, intermediate, and non-classical monocytes ($n = 8$). **(C)** S100A12 relative mRNA expression (normalized to GAPDH) and secretion in monocytes cultured with CSF-1 for 1, 3, and 8 days ($n = 4$–6). **(D)** S100A12 relative mRNA expression (normalized to GAPDH) and secretion in macrophages polarized by LPS/IFN-γ and IL-4/IL-13 for 24 h ($n = 6$–9). Data are presented as mean ± SEM. Differences were calculated with Friedman test with Dunn's *post-hoc* test, $^*p < 0.05$, $^{**}p < 0.001$.

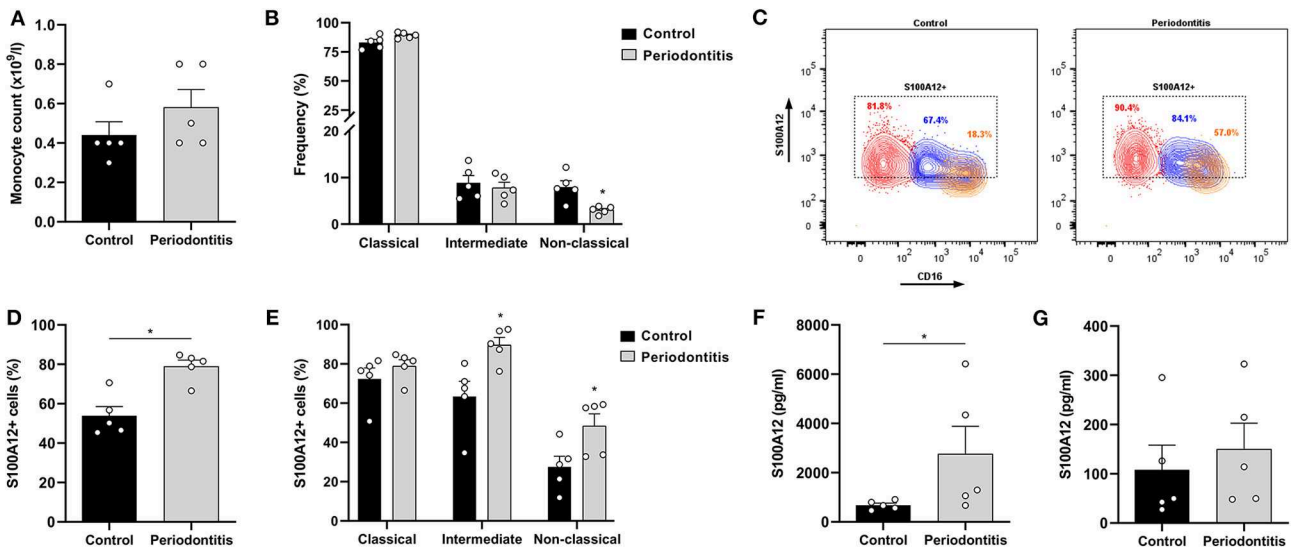

FIGURE 2 | S100A12 expression in peripheral monocytes from periodontitis patients and healthy controls. **(A)** Monocyte counts (x10^9/l) in participants with periodontitis ($n = 5$) and controls ($n = 5$). **(B)** Frequency of classical, intermediate, and non-classical monocytes in participants with periodontitis ($n = 5$) and controls ($n = 5$). **(C)** Representative contour plots of S100A12$^+$ classical (red), intermediate (blue), and non-classical (orange) monocytes in periodontitis and control. Proportion of S100A12$^+$ cells in each subset is included in the plot. **(D)** Percentage of S100A12$^+$ monocytes in periodontitis patients ($n = 5$) and controls ($n = 5$). Monocytes were identified based on the expression of CD14, CD16, and HLA-DR. **(E)** Percentage of S100A12$^+$ cells in classical, intermediate, and non-classical monocytes from periodontitis patients ($n = 5$) and controls ($n = 5$). **(F)** Secretion of S100A12 in monocytes from periodontitis patients ($n = 5$) and controls ($n = 5$) after 24 h in culture. **(G)** S100A12 levels in plasma from periodontitis patients ($n = 5$) and controls ($n = 5$). Data are presented as mean ± SEM. Differences were calculated with Mann–Whitney test, $^*p < 0.05$.

single, CD45$^+$ cells expressing CD14, and CD64 were gated (**Figure 4D**). These cells also expressed HLA-DR. No significant difference in the proportion of CD14$^+$CD64$^+$ cells was observed between periodontitis and control participants (**Figure 4E**). However, a significant increase in the proportion of S100A12$^+$ MCs was found in periodontitis (**Figure 4F**), and

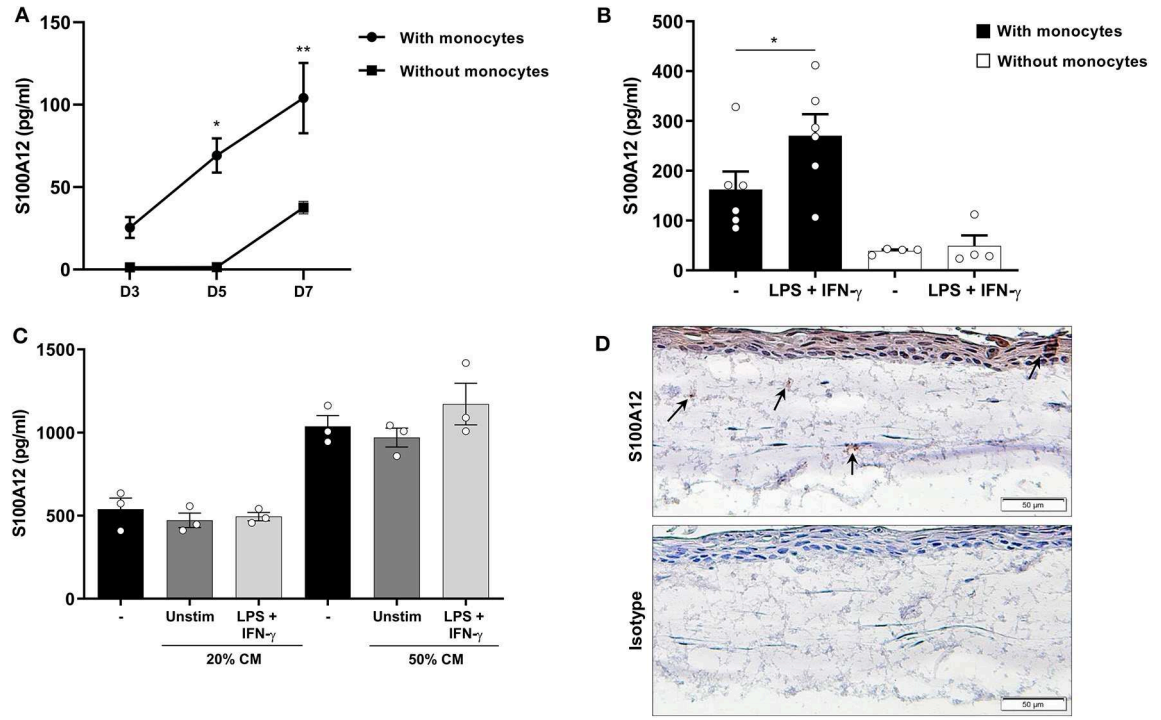

FIGURE 3 | Secretion of S100A12 in a 3D oral tissue culture with and without monocytes after inflammatory stimulation. **(A)** Time-dependent secretion of S100A12 in cultures with ($n = 4$) and without healthy human monocytes ($n = 3$) after 3, 5, and 7 days in culture. **(B)** S100A12 secretion on day 7 after monocyte implantation after repeated stimulation with LPS and IFN-γ in cultures with ($n = 6$) and without monocytes ($n = 4$). **(C)** S100A12 secretion by monocytes ($n = 3$) after 24 h stimulation with conditioned medium (CM) from cultures without monocytes ($n = 3$). **(D)** Immunohistochemical staining of S100A12 or isotype control in an oral tissue culture with monocytes. Arrows indicate S100A12$^+$ cells. Data are presented as mean ± SEM. Differences were calculated with Wilcoxon test or Friedman test with Dunn's *post-hoc* test, $^*p < 0.05$, $^{**}p < 0.001$.

the periodontitis-associated S100A12$^+$ MCs were less frequently CD206$^+$, a marker of alternatively activated macrophages (**Figure 4G**). A significant, negative correlation was seen between the proportion of S100A12$^+$ and CD206$^+$ cells in gingival tissue (**Figure 4H**).

Salivary Levels of S100A12 Are Elevated in Severe Periodontitis

Lastly, the levels of S100A12 in saliva from a large cohort of orally examined participants ($n = 336$) were measured to investigate whether S100A12 is a potential biomarker for periodontal disease. Each participant was diagnosed according to the new classification of periodontal diseases (26, 27). Clinical characteristics are presented in **Table 1**. Participants having periodontitis stages III/IV were significantly older than healthy/gingivitis and periodontitis stages I/II participants. No significant difference was observed among the groups regarding the frequency of smokers. Participants with periodontitis stages III/IV had the worst periodontal status as evidenced by lower number of teeth and increased plaque index, bleeding, and number of periodontal pockets. Comparing the levels of S100A12, participants with BOP > 20% showed significantly higher levels than those with BOP ≤ 20% (**Figure 5A**). The presence of PPD ≥ 4 mm was also related to significantly increased levels of S100A12 in saliva (**Figure 5B**), whereas no significant difference was seen based on the amount of bone loss (**Figure 5C**). S100A12 correlated positively with plaque index, BOP, the number of PPD ≥ 4 mm, and the total amount of protein. On the contrary, it correlated negatively with the salivary flow rate (**Figure 5D**). S100A12 levels were significantly increased in patients with more severe stages of periodontitis (III and IV) in comparison with healthy/gingivitis participants. Patients having periodontitis stages I and II exhibited a tendency toward higher S100A12 levels than the healthy/gingivitis individuals ($p = 0.06$; **Figure 5E**).

DISCUSSION

S100A12 is a potent trigger of inflammatory processes and a potential biomarker for chronic inflammatory diseases. However, a detailed characterization of S100A12 kinetics in mononuclear myeloid cells in homeostasis vs. inflammation is lacking. The present study investigated S100A12 dynamics in monocyte subsets, during monocyte maturation stages and polarization, as well as its association with periodontitis. We found that the expression of S100A12 was higher in classical than in non-classical monocytes, decreased during monocyte-to-macrophage differentiation, and was not influenced by macrophage polarization. Peripheral monocytes from periodontitis patients showed altered expression of S100A12.

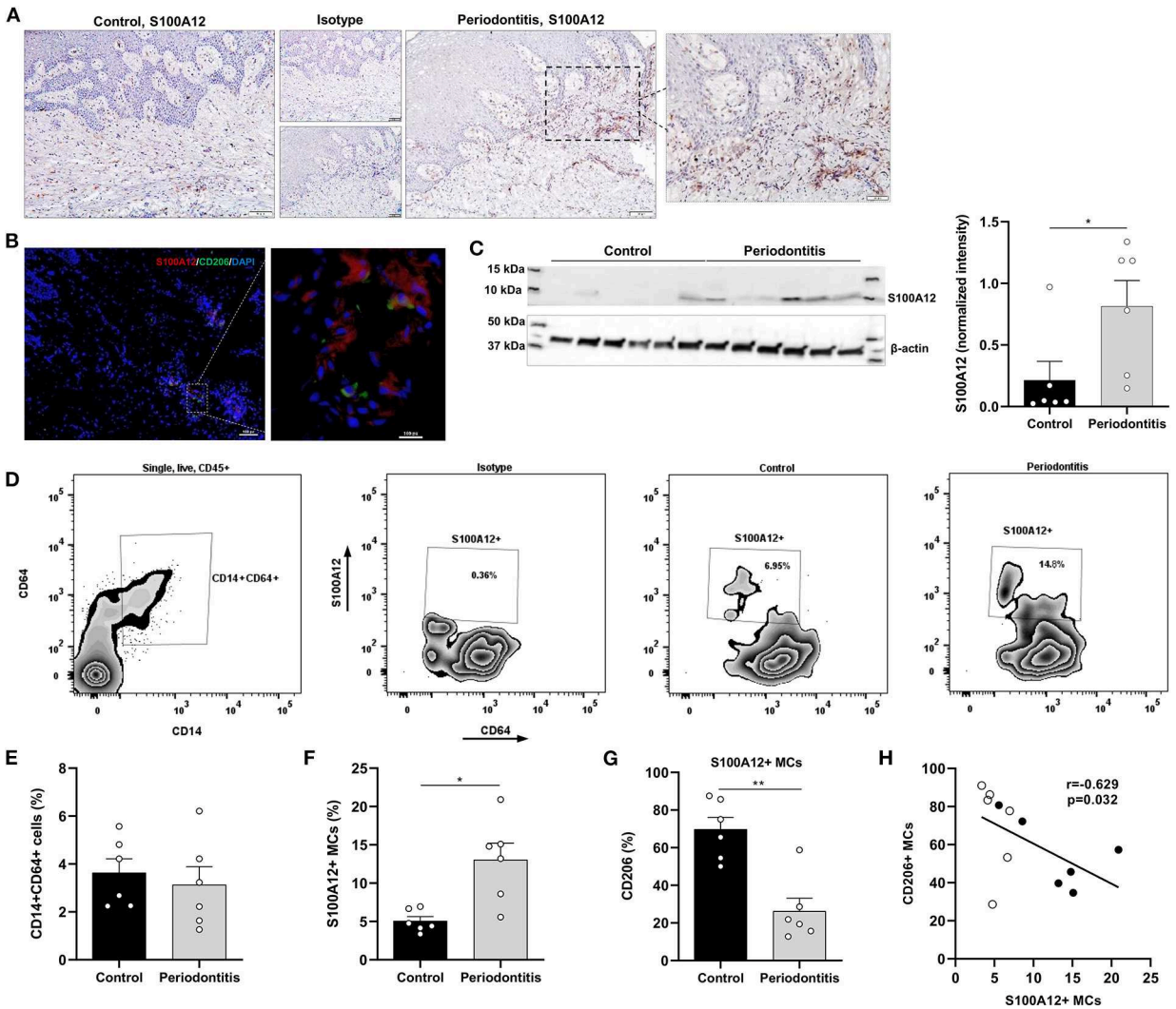

FIGURE 4 | S100A12$^+$ monocyte-derived cells in gingival tissue from periodontitis patients and healthy controls. **(A)** Representative immunohistochemical staining of S100A12 in gingival tissue from a periodontitis patient and control. **(B)** Immunofluorescent staining of gingival tissue from periodontitis patient showing the presence of S100A12 (red) and CD206 (green). Nuclei was counterstained with DAPI (blue). **(C)** Western blot analysis of S100A12 monomer in gingival tissue from periodontitis patients ($n = 6$) and controls ($n = 6$), and S100A12 expression presented as normalized intensity to β-actin. **(D)** Representative zebra plot showing the gating strategy of monocyte-derived cells (MCs) in gingival tissue based on CD14 and CD64 expression on single, live, CD45$^+$ cells, and zebra plots depicting the S100A12$^+$ MCs in health and in periodontitis. Proportion of S100A12$^+$ cells is included in the plot. **(E)** Percentage of CD14$^+$CD64$^+$ cells within the CD45$^+$ compartment in gingival tissue from periodontitis patients ($n = 6$) and controls ($n = 6$). **(F)** Percentage of S100A12$^+$ cells within the CD14$^+$CD64$^+$ compartment in gingival tissue from periodontitis patients ($n = 6$) and controls ($n = 6$). **(G)** Percentage of CD206$^+$ cells in S100A12$^+$ MCs in gingival tissue from periodontitis patients ($n = 6$) and controls ($n = 6$). **(H)** Spearmen correlation between the percentage of S100A12$^+$ MCs and the percentage of CD206$^+$ MCs. White and black circles indicate controls and periodontitis participants, respectively. Data are presented as mean ± SEM. Differences were calculated with Mann–Whitney test, $^*p < 0.05$, $^{**}p < 0.001$.

Moreover, in a 3D oral tissue culture inflammatory stimuli modulated S100A12 expression in the presence of monocytes, and the frequency of S100A12$^+$ monocyte-derived cells was increased in inflamed gingival tissue affected by periodontitis. Levels of S100A12 in saliva were higher in patients with severe stages of periodontitis. These findings indicate that S100A12 is involved in periodontitis pathogenesis.

We found that classical monocytes (CD14^{hi}CD16$^-$) exhibited increased S100A12 expression in comparison with non-classical monocytes (CD14$^+$CD16^{hi}), and the intermediate subset (CD14^{hi}CD16$^+$) had an expression more similar to the classical subset. This finding is in agreement with previous studies showing higher S100A12 gene expression in the classical subset (3, 6, 30), highlighting its ability to support inflammation (6, 12). Conflicting results have been reported regarding a closer relationship of the intermediate subset to the classical or non-classical subset (5, 6). In our study, S100A12 protein expression was 1.16 times higher in classical monocytes than in intermediates and 1.92 times higher in intermediates than in non-classical monocytes, though no significant difference was seen between the intermediate and the non-classical subsets. Wong et al. (6) have shown that S100A12 gene expression is

13.76 times higher in the classical subset and 2.35 lower in the non-classical subset both in relation to the intermediate subset.

Monocyte-to-macrophage differentiation is a complex process, where major changes occur to the cell global transcriptome (31). Here we show that S100A12 was downregulated during this process under CSF-1 and its expression was not significantly affected when macrophages were later classically or alternatively activated. In agreement with our findings, Sander et al. (32) have shown that monocytes differentiated by CSF-1 had lower expression of S100A12 in comparison with $CD14^+$ monocytes. Similarly, Shah et al. (11) have found decreased expression of S100A12 during maturation of monocytes to macrophages along with unaltered expression following polarization. Thus, S100A12 is part of the genes that are rapidly regulated during differentiation, maintained in mature macrophages, and refractory to polarization (31). We have now added that in concordance with gene expression, S100A12 secretion is also reduced during monocyte-to-macrophage differentiation. A decrease in the S100A12 expression during monocyte to macrophage maturation goes in line with lower expression in non-classical monocytes, since both macrophages and circulating non-classical monocytes represent a more advanced stage of differentiation (3, 4).

Interestingly, we found that circulating monocytes from periodontitis patients have an altered expression of S100A12, which was mainly seen in the intermediate and non-classical subsets. The monocytes from periodontitis patients also secreted significantly higher levels of S100A12 after 24 h in monoculture than monocytes from periodontally healthy participants.

TABLE 1 | Clinical characteristics of the study groups.

	Healthy/gingivitis ($n = 133$)	Periodontitis stage I/II ($n = 95$)	Periodontitis stage III/IV ($n = 108$)	*p*-value*
Age (years)	45.9 (±19.1)	47.7 (±15.1)	61.5 (±13.3)[a,b]	<0.001
Males, *n* (%)	55 (41.4)	50 (52.6)	61 (56.5)	0.041
Females, *n* (%)	78 (58.6)	45 (47.4)	47 (43.5)	
Smokers, *n* (%)	15 (11.3)	12 (12.6)	17 (15.7)	0.596
N° teeth	25.5 (±4.2)	26.3 (±2.6)	24.0 (±4.0)[a,b]	<0.001
Plaque index (%)	18.9 (±26.0)	17.1 (±16.8)	22.4 (±23.1)[a]	0.011
BOP (%)	5.5 (±13.5)	10.4 (±8.1)[a]	14.3 (±14.6)[a]	<0.001
PPD 4–5 mm (*n*)	–	7.0 (±6.7)	19.2 (±18.8)	<0.001
PPD ≥ 6 mm (*n*)	–	–	1.3 (±2.2)	–

*BOP, bleeding on probing; PPD, probing pocket depth. *Kruskal–Wallis/Mann–Whitney test or Chi-square test. [a]$p < 0.05$ in comparison to healthy/gingivitis group. [b]$p < 0.05$ in comparison to periodontitis stage I/II group.*

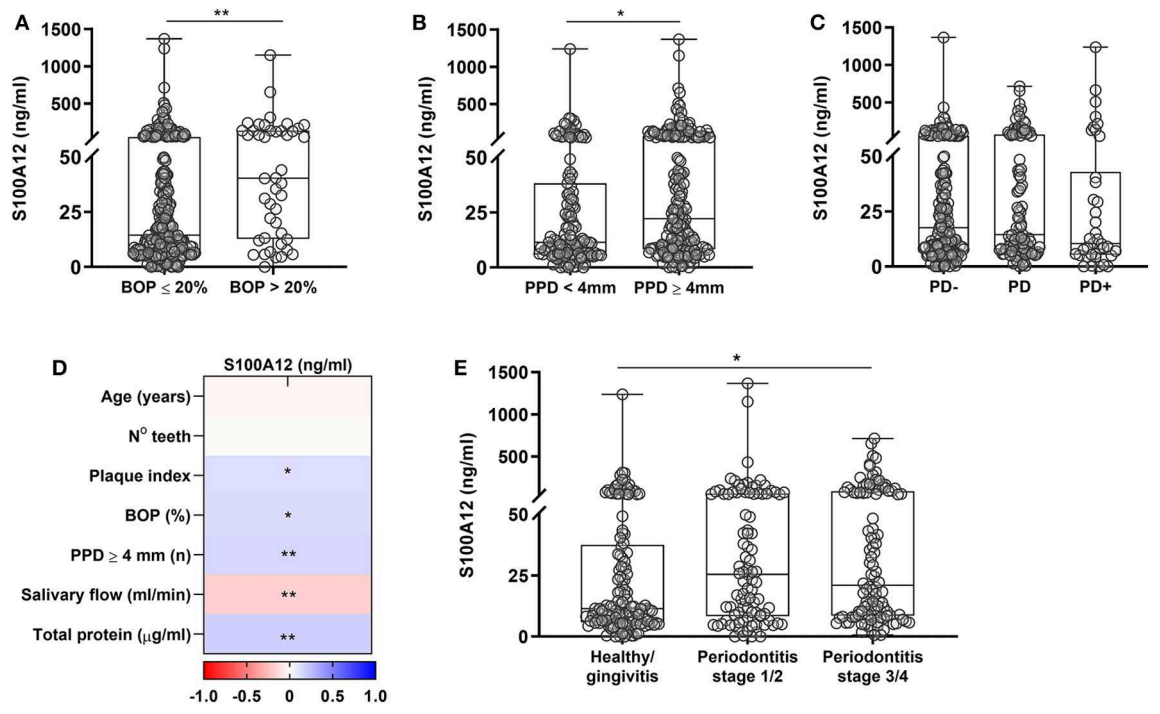

FIGURE 5 | Salivary levels of S100A12 in relation to periodontal disease. **(A)** S100A12 levels in saliva from participants having bleeding on probing (BOP) ≤ 20% ($n = 292$) and those having BOP > 20% ($n = 44$). **(B)** Salivary levels of S100A12 in participants having ($n = 201$) or not probing pocket depth (PPD) ≥ 4 mm ($n = 135$). **(C)** Levels of S100A12 in saliva from participants without bone loss (PD–; $n = 175$), with localized bone loss (PD; $n = 103$), and generalized bone loss (PD+; $n = 44$). Fourteen radiographs were missing. **(D)** Spearman correlation heat map between S100A12 and clinical parameters. **(E)** Salivary levels of S100A12 in healthy/gingivitis participants ($n = 133$) and those with periodontitis stages I/II ($n = 95$) or stages III/IV ($n = 108$). Data are presented as median and quartiles. Differences were calculated with Mann–Whitney test or Kruskal–Wallis with Dunn's *post-hoc* test, *$p < 0.05$, **$p < 0.001$.

Similarly, increased expression of S100A12 has been reported in PBMCs from patients with pre-mature coronary artery disease (33). Higher release of prostaglandin E2 and IL-8 has been shown in LPS-stimulated whole blood cell cultures from periodontitis patients in comparison with healthy participants, indicating an intrinsic characteristic or differential priming of the monocytes in periodontitis (34). As S100A12 gene expression is very sensitive to low levels of LPS (12), we speculate that higher exposure to LPS in periodontitis might be at least partially responsible for the enhanced S100A12 expression seen in this study. In fact, transcriptome analysis of circulating monocytes in periodontitis evidenced they might be more functionally active, with the identification of several genes that interact with invading microorganisms or respond to LPS stimulation (35).

Despite being downregulated during monocyte differentiation, a time-dependent increase in S100A12 levels was seen in a 3D oral mucosa culture in the presence of monocytes, which was further increased by LPS and IFN-γ stimulation. Such an increase was not seen in the absence of monocytes, suggesting monocytes are needed for the S100A12 response to inflammatory stimuli in the model. Furthermore, conditioned medium from cultures lacking monocytes did not increase S100A12 secretion by monocytes. It is worth noting that the secreted levels in the cultures were somewhat similar to that of differentiated macrophages in monoculture. Our group previously reported that monocyte-derived cells in the model are $CD64^+$ and display increased expression of CD14, CD68, and CD163 during their differentiation, evidencing a macrophage-like phenotype (20). Previous reports have shown that both TLR ligands and IFN-γ induce S100A12 expression in monocytes and/or macrophages (12, 36, 37). S100A12 produced by stimulated monocytes/macrophages might be important for leukocyte recruitment during inflammation (12).

In consonance with the increased expression of S100A12 in peripheral monocytes in periodontitis, we also found higher expression of S100A12 as well as increased frequency of $S100A12^+$ monocyte-derived cells in gingival tissue affected by periodontitis. This could be a reflection of increased recruitment of $S100A12^+$ monocytes to the inflamed tissue, especially intermediate and non-classical, already altered in peripheral circulation. S100A12 expression is higher in several chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease (14–16). S100A12 is a chemoattractant to monocytes (12) and induce a strong inflammatory response in these cells acting as an endogenous TLR4 ligand, which could contribute to the amplification of the inflammatory response (13). Furthermore, $S100A12^+$ cells in periodontitis showed a lower expression of CD206 than in controls, and the percentage of $S100A12^+$ cells correlated negatively with that of $CD206^+$ cells. Viniegra et al. (38) have shown that the expression of CD206, a marker of alternatively activated macrophages, increased during the healing phase of periodontal disease and that the induction of resolving macrophages through a peroxisome proliferator-activated receptor γ (PPAR-γ) agonist reduced the alveolar bone loss and increased CD206 expression in periodontal tissues. Interestingly, a PPAR-γ agonist was shown to inhibit S100A12 expression by macrophages (39), indicating that S100A12 is probably part of the network of inflammatory mediators orchestrating the destruction of periodontal tissues.

Lastly, we found that salivary levels of S100A12 were related to the degree of gingival inflammation and the presence of pathological periodontal pockets. Also, S100A12 levels were significantly higher in patients having periodontitis stages III and IV than in non-periodontitis participants. It is worth mentioning we used radiographic bone loss in the clinical classification instead of clinical attachment loss, which is the primary stage determinant (27). These results are partially in agreement with a previous report from our group, showing that S100A12 alterations were related to gingival bleeding, but not to periodontal pockets, which could be due to the different clinical categorizations used in both cohorts. S100A12 was also related to increased burden of periodontal inflammation (22). According to these reports bleeding seems to be the main determinant of S100A12 levels in saliva and this might be related to the increased density of inflammatory cells in sites with bleeding (40). Taken together, these results highlight S100A12 as a possible biomarker of periodontal inflammation. Whether it has the ability to predict future periodontal destruction deserves further investigation.

In conclusion, S100A12 is predominantly secreted by monocytes rather than by differentiated macrophages. However, S100A12 accumulates in inflamed tissue, potentially as a result of increased production by monocyte-derived cells. This study implicates the involvement of S100A12 in the pathogenesis of periodontitis, and both gingival tissue and circulatory monocytes are altered in periodontitis. S100A12 levels in saliva reflect the severe stages of periodontitis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the regional ethics committee in Stockholm, Sweden and of the regional ethics committee at the Lund University, Sweden with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by regional ethics committee in Stockholm, Sweden (Dnr. 2012/1579-32 and 2017/1333-32) and by the regional ethics committee at the Lund University, Sweden (Dnr. 2011/366).

AUTHOR CONTRIBUTIONS

RL-J, SH, and EB designed the study, collected and analyzed data, and wrote the manuscript. RC, SZ, and MM collected and analyzed data, and revised the manuscript. GJ, BA, and SÅ collected and interpreted data, and revised the manuscript. MS supervised the experiments. MS and BK contributed to the

study design and revised the manuscript. All authors gave final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00086/full#supplementary-material

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frontiers in Immunology

ORIGINAL RESEARCH
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Transcriptomic Analysis of Monocyte-Derived Non-Phagocytic Macrophages Favors a Role in Limiting Tissue Repair and Fibrosis

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Monocyte-derived macrophages are readily differentiating cells that adapt their gene expression profile to environmental cues and functional needs. During the resolution of inflammation, monocytes initially differentiate to reparative phagocytic macrophages and later to pro-resolving non-phagocytic macrophages that produce high levels of IFNβ to boost resolutive events. Here, we performed in-depth analysis of phagocytic and non-phagocytic myeloid cells to reveal their distinct features. Unexpectedly, our analysis revealed that the non-phagocytic compartment of resolution phase myeloid cells is composed of Ly6C^{med}F4/80^{-} and Ly6C^{hi}F4/80^{lo} monocytic cells in addition to the previously described Ly6C^{-}F4/80^{+} satiated macrophages. In addition, we found that both Ly6C^{+} monocytic cells differentiate to Ly6C^{-}F4/80^{+}macrophages, and their migration to the peritoneum is CCR2 dependent. Notably, satiated macrophages expressed high levels of IFNβ, whereas non-phagocytic monocytes of either phenotype did not. A transcriptomic comparison of phagocytic and non-phagocytic resolution phase F4/80^{+} macrophages showed that both subtypes express similar gene signatures that make them distinct from other myeloid cells. Moreover, we confirmed that these macrophages express closer transcriptomes to monocytes than to resident peritoneal macrophages (RPM) and resemble resolutive Ly6C^{lo} macrophages and monocyte-derived macrophages more than their precursors, inflammatory Ly6C^{hi} monocytes, recovered following liver injury and healing, and thioglycolate-induced peritonitis, respectively. A direct comparison of these subsets indicated that the non-phagocytic transcriptome is dominated by satiated macrophages and downregulate gene clusters associated with excessive tissue repair and fibrosis, ROS and NO synthesis, glycolysis, and blood vessel morphogenesis. On the other hand, non-phagocytic macrophages enhance the expression of genes associated with migration, oxidative phosphorylation, and mitochondrial fission as well as anti-viral responses when compared to phagocytic macrophages. Notably, conversion from phagocytic to satiated macrophages is associated with a reduction in the expression of extracellular matrix constituents that

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were demonstrated to be associated with idiopathic pulmonary fibrosis (IPF). Thus, macrophage satiation during the resolution of inflammation seems to bring about a transcriptomic transition that resists tissue fibrosis and oxidative damage while promoting the restoration of tissue homeostasis to complete the resolution of inflammation.

Keywords: inflammation, macrophages, efferocytosis, transcriptional profiling, fibrosis

INTRODUCTION

Acute inflammation is the protective response of the host to damaging events that may interrupt tissue homeostasis, such as physical or chemical injury, as well as microbial infections. A successful response eliminates the threat locally, repair the affected tissue, and restore its structure and function without deleterious fibrosis. Inflammation initiates with the production of soluble mediators by resident cells in the injured/infected tissue that promote the exudation of defense and/or signaling proteins, reinforced by the influx of granulocytes from the blood. Upon the arrival of these leukocytes, mostly neutrophils, they primarily function to phagocytose and eliminate foreign microorganisms via distinct intracellular killing mechanisms, resulting in neutrophils undergoing programmed cell death (apoptosis) (1, 2). This occurs alongside monocyte influx and their maturation into inflammatory macrophages upon infiltration of the inflamed tissue. Macrophages engulf apoptotic polymorphonuclear neutrophils (PMN) in a nonphlogistic process termed efferocytosis (1, 3). This clearance process initiates an active anti-inflammatory and pro-resolution phase that blocks excessive neutrophil recruitment and eliminates the early inflammatory elements and, in turn, results in clearance of these macrophages by either *in situ* apoptosis or egression via the lymphatic system (4, 5). Inflammatory macrophages polarize to distinct subpopulations following exposure to different bioactive molecules and environments. These subpopulations compose a wide spectrum of phenotypes that range from classically (M1) to alternatively (M2) activated macrophages—two commonly used myeloid measuring sticks generated during responses to bacterial or helminth infections and support Th1 or Th2 development, respectively (6). Recent molecular studies indicate that macrophage differentiation at different tissues and activation under different settings is associated with substantial shifts in gene expression patterns (hundreds of genes) depending on the specific stimuli (7–9). Nevertheless, most of these patterns define a distinct activation state of macrophages that cannot be confined to an M1 or M2 phenotype. As a result, the current literature promotes the usage of marker combinations or inducing agents to ascribe macrophage phenotypes rather than the M1 and M2 extremes (6, 10, 11).

Engulfment of apoptotic cells evokes signaling events that block the release of pro-inflammatory mediators from macrophages stimulated by microbial moieties, a phenomenon termed immune silencing. This process is accompanied by the production of cytokines that can promote the resolution of inflammation and wound repair (e.g., TGFβ and IL-10) (12, 13) with the production of pro-resolving lipid mediators, such as resolvin (Rv) E1 and RvD1 that block PMN infiltration and promote their clearance (1). Notably, the uptake and processing of high amounts of biopolymers, such as the ones expressed by apoptotic cells, require coping with large amounts of reactive oxygen species (ROS) generated in metabolic processes. Therefore, mitochondrial ROS production is limited in high-burden efferocytic macrophages, by means such as mitochondrial fission and lowering mitochondrial membrane potential (14, 15), to allow continued engulfment and avoid oxidative damage. Recent studies in spontaneously resolving, zymosan A-induced murine peritonitis characterized macrophages from resolving peritonitis into two distinct subtypes based on differing surface expression of the adhesion molecule CD11b that also composes complement receptor 3 (CR3) that mediates apoptotic cell engulfment by human macrophages (16). Compared to their $CD11b^{high}$ counterparts, the $CD11b^{low}$ macrophages are characterized by lower levels of pro-inflammatory mediators (e.g., TNFα, IL-1β, CCL2, 3, and 5) and proteins (e.g., iNOS and COX2), and pro-fibrotic factors (e.g., arginase-1). However, they display a higher secretion of the anti-infammatory/pro-resolving cytokine TGFβ and higher expression of the pro-resolving enzyme 12/15-lipoxygenase (LO). $CD11b^{low}$ macrophages migrate out of inflamed sites and, compared to $CD11b^{high}$ cells, exert decreased phagocytic activity despite containing higher numbers of PMNs previously engulfed. Hence, they were termed satiated or non-phagocytic macrophages (17, 18). A similar series of phenotypic transitions by monocyte-derived macrophages was previously reported in acute liver injury, where $Ly6C^{hi}$ monocytes infiltrate the liver, clear apoptotic neutrophils, and convert to $Ly6C^{lo}$ macrophages that express 12/15-LO (19–21).

Recently, IFNβ expression by non-phagocytic macrophages, and the novel roles of this cytokine as an effector in resolving bacterial inflammation were reported (17). We aimed to identify the satiated macrophage subset within the non-phagocytic macrophage population, determine the transcriptomic origin of resolution phase macrophages (of both the phagocytic and non-phagocytic phenotypes), and identify the unique gene clusters expressed by non-phagocytic/satiated macrophages. Furthermore, we sought to determine whether these unique clusters support key effector functions of satiated macrophages. Such functions include loss of phagocytic/efferocytic capacity while maintaining low ROS burden, deviation from the M2-like/reparative/pro-fibrotic phenotype to a pro-resolving phenotype, and metabolic shifts between various metabolic pathways. Here, we report that resolution phase non-phagocytic myeloid cells are composed of two distinct subsets, in addition to satiated macrophages. However, the $Ly6C^{+}$ subsets are

not becoming phagocytic prior to differentiation and do not express high levels of IFNβ as the satiated macrophages. We also found that both phagocytic and non-phagocytic resolution phase macrophages express a transcriptome that is more similar to the one expressed by monocytes than to RPM and more similar to reparative Ly6C^{lo} monocyte-derived macrophages than inflammatory Ly6C^{hi} monocytes from liver injury or peritoneal thioglycolate challenge. In addition, we found non-phagocytic macrophages to display a satiation-associated transcriptome with a significant change in expression patterns between phagocytic and satiated macrophages that attest to a complete phenotype switch in satiated macrophages that involves phagocytic properties, tissue repair and fibrosis, and metabolic programs.

MATERIALS AND METHODS

Mice

C57BL/6 WT male mice were purchased from Harlan Laboratories. All mice that were used at the age of 8–15 weeks and did not undergo previous procedures. All mice were housed under a 12-h:12-h light–dark cycle and specific pathogen-free conditions, up to five mice per cage. Mice were fed standard pellet chow and reverse osmosis water *ad libitum*. Animal experiments were approved by the Committee of Ethics, University of Haifa (authorization no. 246/14).

Murine Peritonitis

Male C57BL/6 mice were randomly assigned to experimental groups. Mice were injected I.P. with zymosan A (1 mg/ml in PBS, 1 ml per mouse). PKH2-PCL green (0.25 mM; 0.5 ml; Sigma-Aldrich) was injected I.P. at 20, 44, 62, or 68 h, and peritoneal exudates were collected 4 h later. Peritoneal cells were stained with PE- or Brilliant violet-conjugated rat anti-mouse F4/80, PerCP-conjugated rat anti-mouse CD11b, Pacific Blue- or PerCP-conjugated rat anti-mouse Ly6C, PE-conjugated rat anti-mouse CD115, and PE/Cy7-conjugated mouse anti-mouse CX_3CR1 (Biolegend) and analyzed by flow cytometry as in **Supplemental Figure 1**. F4/80$^+$macrophages were sorted according to PKH2-PCL green signal intensity as in (17) using the FACSaria III sorter (Beckton-Dickinson) to give distinct F4/80$^+$/PKH2^{hi} and F4/80$^+$/PKH2$^{lo/neg}$ macrophage populations. Ly6C^{med}F4/80^{neg} and Ly6C^{hi}F4/80^{lo} monocytic cells were sorted using the SH800 sorter (Sony). In some experiments, flow cytometry analysis using the FlowJo software (Treestar) was performed to identify distinct leukocyte populations as detailed in the results section.

Monocytic Ablation

To ablate monocyte migration to the peritoneum, mice received 400 μl of anti-mouse CCR2 mAb (clone MC-21, generously given by Prof. Mack, Regensburg, Germany) conditioned media (29 μg Ab/ml, I.P.) concomitantly with zymosan A peritonitis onset (0 h) and at 24 h PPI.

RNA Isolation

RNA extraction was performed as previously described (17). Briefly, all RNA species from sorted cells were extracted using the Aurum Total RNA kit (Bio-Rad Laboratories, Inc.). RNA integrity was scored by Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Pico kit (Agilent Technologies). Samples were prepared for Illumina sequencing using NEB's Ultra Directional RNA Library Prep Kit for Illumina (NEB#7420). Libraries were sequenced with a 50 bp SR run on Illumina HiSeq 2500 using a V3 flow cell.

Data Processing and Analysis

Sequenced reads were compared to available murine Ensembl 70 genes using mouse genome build (GRCm38), and expression was compared between PKH2^{hi} and PKH2^{lo} macrophages using two separate analysis pipelines: RSEM/EdgeR and TopHat2/cuffdiff. Depending on the pipeline, between ∼3,300 and 3,400 genes were found to be differentially expressed (FDR $\leq$ 0.05), with a wide overlap in results between the two pipelines. Significance values presented were from the TopHat2/cuffdiff analysis. Differentially expressed genes with statistical significance were filtered and visualized through a volcano plot, where F4/80$^+$/PKH2^{hi} cells served as a reference sample. Genes with FDR adjusted *p*-value (*q* value) $\leq$ 0.05 were considered as genes exhibiting differential expression between the two macrophage subsets and were selected for enriched gene ontology (GO) analysis. GO enrichment analysis was performed on the differentially up- or downregulated genes with the DAVID Bioinformatics Resources 6.7 software using the annotation categories of GOTERM_BP_5 and KEGG_PATHWAY, similarity threshold 0.7, and EASE score 0.25. For HeatMap analyses, expression values of genes were rescaled to a mean of 0 and a standard deviation of 1, and hierarchical clustering was performed using the R package Superheat with Euclidean distance and complete linkage methods (22). Published datasets were obtained in the form of gene raw counts or CPM-TMM normalized values at GREIN (23). For principal component analysis, resolution phase peritoneal PKH2^{hi} and PKH2^{lo} macrophage datasets were normalized to the resident macrophage RNAseq dataset from Lavin et al. (7) or to ImmGen OpenSource (24) using rlog utility of DESeq2 package (25). Alternatively, the same datasets were normalized to liver macrophage microarray datasets from Zigmond et al. (19), processed with robust multi-array average (RMA) of oligo package (26) and followed by quantile normalization. Combined datasets were corrected for batch effects using ComBat utility of SVA package (27). Data analysis was performed using the R program (https://www.r-project.org/). The accession number for the RNA-seq reported in this manuscript is BioProject: PRJNA390886.

RESULTS

Resolution Phase Non-phagocytic Myeloid Cells Contain Two Subsets of Ly6C$^+$ Monocytic Cells in Addition to Satiated Macrophages

Ly6C$^+$F4/80$^-$ monocytes infiltrate the peritoneal cavity during the onset of resolution (12–24 h post peritonitis) and differentiate gradually to Ly6C$^-$F4/80^{hi} macrophages that are highly

phagocytic/efferocytic (17, 28). These phagocytic peritoneal macrophages express high levels of the macrophage surface marker CD11b, in addition to high F4/80. However, following extensive efferocytosis, they lose their phagocytic capacity and convert to a state of satiation. This phenotype conversion is accompanied by a reprogramming process and a reduction in both aforementioned surface markers (18). Recently, it was shown that non-phagocytic F4/80^{+} macrophages express high levels of IFNβ that upon secretion promotes bacterial clearance and the resolution of inflammation (17). IFNβ expression by resolution phase macrophages was also upregulated by the uptake of apoptotic cells (17). Therefore, we sought to determine whether non-phagocytic macrophages are exclusively satiated. To this end, we injected the phagocytic dye PKH2-PCL green to mice during different phases of zymosan A-induced peritonitis and analyzed the phagocytic capacity of the various myeloid phenotypes in the exudates. Our results in **Figures 1A–C** show that Ly6C^{med}CD11b^{med}F4/80^{-} monocyte-like cells are infiltrating the peritoneum at 24 h and convert at 72 h, at least in part, to Ly6C^{-}CD11b^{hi}F4/80^{hi} macrophages. This conversion is associated with a transition of a F4/80^{-}PKH2^{lo} monocyte subset to an F4/80^{+}PKH2^{hi} macrophage subset reflecting improved phagocytosis upon maturation. Unexpectedly, we observed that a significant portion of the Ly6C^{+} cells remain undifferentiated and phagocytosis reluctant even at the later phase of resolution (72 h). Also notable is the presence of a small Ly6C^{hi}F4/80^{lo} subset (purple dots), commonly regarded as classical inflammatory monocytes, that is sustained during resolution but does not acquire phagocytic capacity and remains PKH2^{neg} (**Figure 1C**). As expected from previous reports (17, 18, 28), a subset of F4/80^{hi}/PKH2$^{lo/neg}$ cells corresponding to satiated macrophages was also evident in this analysis and distinguishable from non-phagocytic Ly6C^{hi}F4/80^{lo} monocytes. Since PMN-like cells can also be part of the Ly6C^{med}F4/80^{neg} population, we performed an additional analysis of CD11b^{+} cells based on Ly6C and Ly6G expression. Our results (**Figures 1D–F**) show that the Ly6C^{med} subset is composed of both monocytes (Ly6G^{-}F4/80$^{lo/neg}$ cells, 23.5% of Ly6C^{med}) and PMN-like cells ((Ly6G^{+}F4/80^{neg} cells, 76.5% of Ly6C^{med}), whereas the Ly6C^{hi}F4/80^{lo} subset did not contain any PMNs.

To better understand the phagocytic properties of F4/80^{+} myeloid subsets, we further analyzed these samples by gating on PKH2^{hi}, PKH2^{lo}, or PKH2^{neg} cells and analyzing their F4/80, Ly6C, and CD11b expression. Our results in **Figures 2A–C** show that phagocytic PKH2^{hi} cells (red dots) were initially Ly6C^{+}F4/80^{+} immature monocytes, but at 72 h, they completely

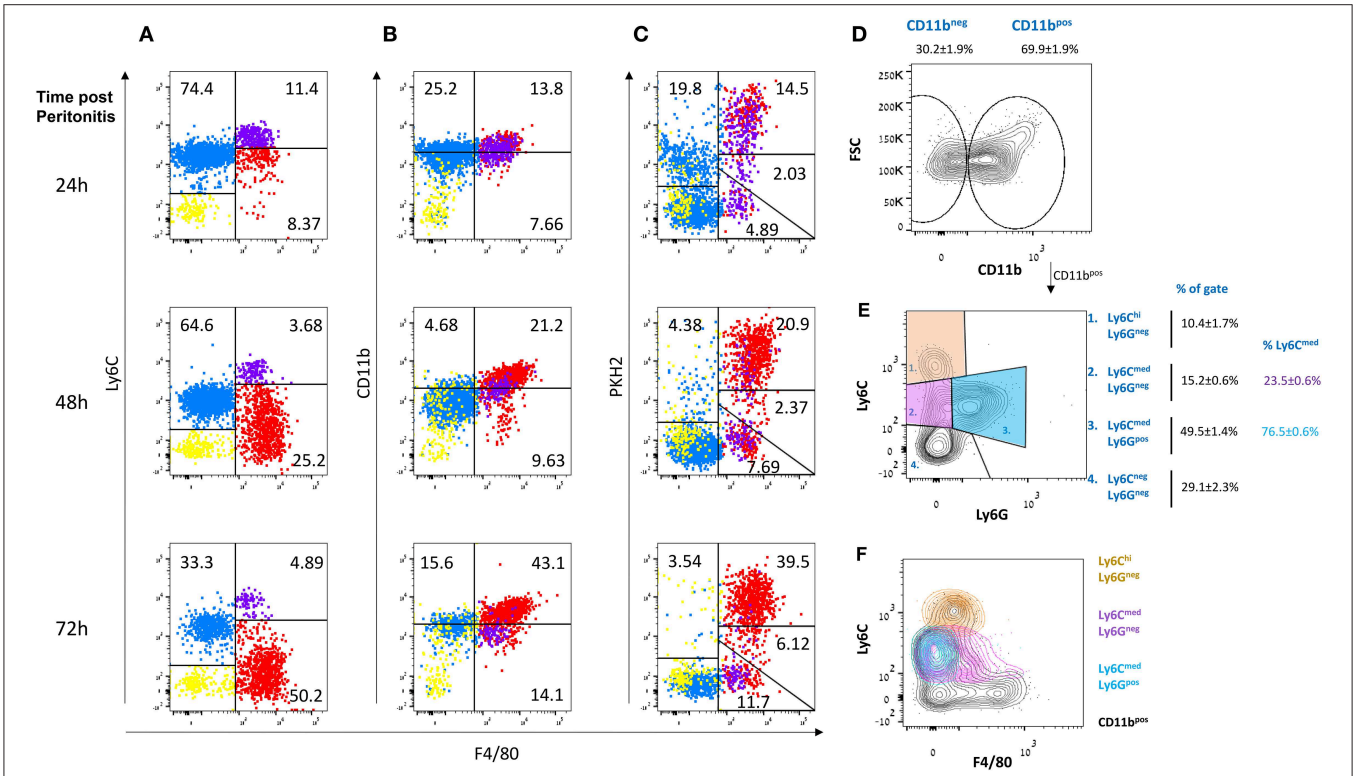


FIGURE 1 | Non-phagocytic myeloid cells in peritoneal exudates contain monocytes and macrophages. Zymosan A (1 mg/mouse) was injected intraperitoneally to male mice. After 20, 44, or 68 h, these mice were injected I.P. with the phagocyte-specific dye PKH2-PCL green. Four hours later, the peritoneal cells were recovered and immunostained for F4/80 and CD11b. Dot plot analysis was performed for the expression of Ly6C (**A**, Y axis), CD11b **(B)**, and PKH2-PCL acquisition **(C)**, relative to F4/80 expression (X axis) by various exudate cells. Results are representatives from n = 8 mice for 24 h, six mice for 48 h, and seven mice for 72 h. **(D–F)** Peritoneal cells were recovered 66 h PPI and immunostained for CD11b, Ly6C, Ly6G, and F4/80 and analyzed by flow cytometry. Results are representative plots and means ± SEM (n = 12) showing CD11b^{+} gating **(D)**, Ly6G vs. Ly6C (identifying monocytes and neutrophils, **(E)**, and Ly6C vs. F4/80 **(F)**.

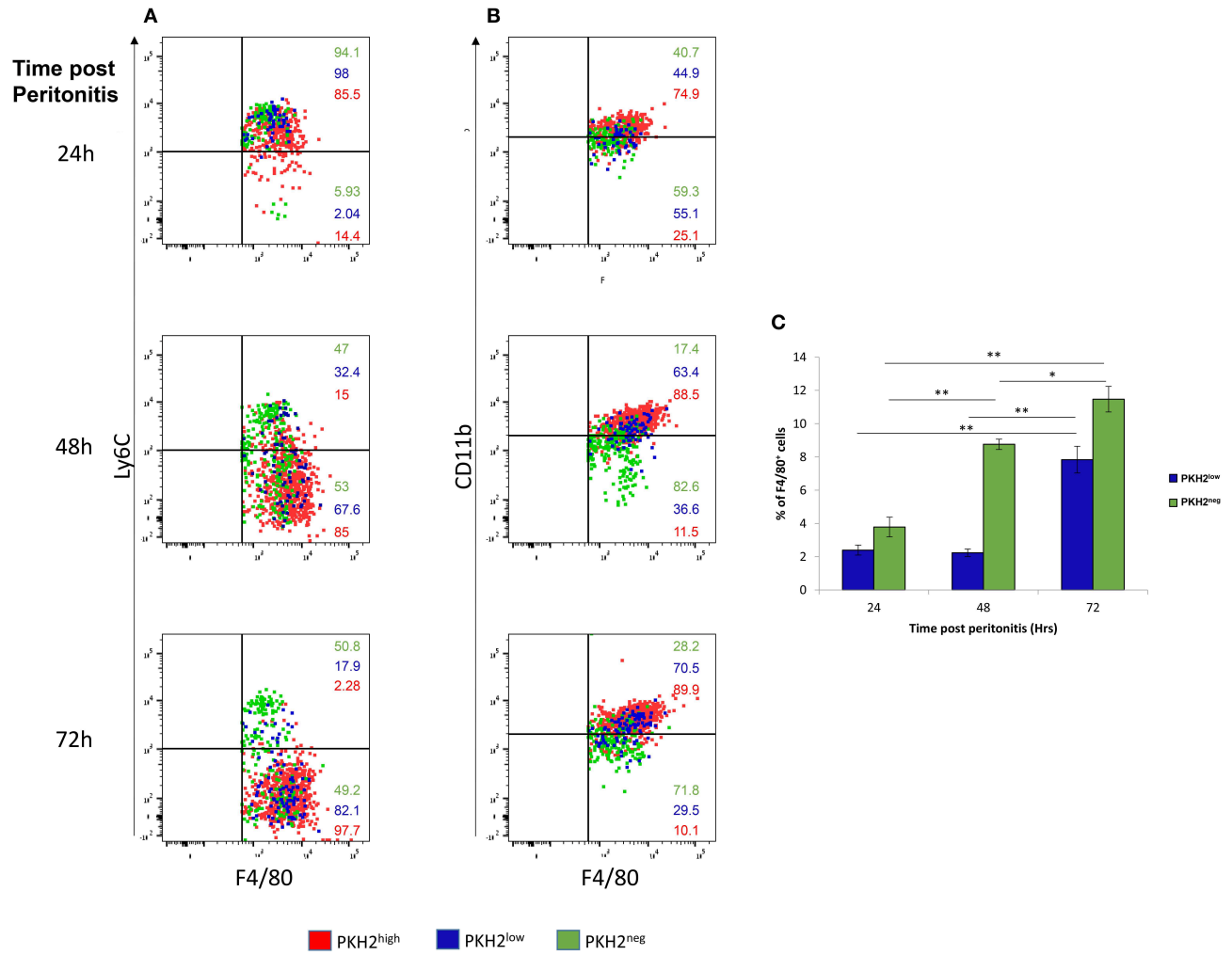


FIGURE 2 | Non-phagocytic monocytes and satiated macrophages show different kinetics during the resolution of peritonitis. **(A,B)** Dot plots of F4/80$^+$ PKH2-PCL high (red), low (blue), and negative (green) cells are presented relative to Ly6C **(A)** or CD11b **(B)**. **(C)** Percentage of F4/80$^+$PKH2^{low} and PKH2^{neg}cells at 24–72 h PPI. Results are means ± SEM (n = 8 mice for 24 h, six mice for 48 h, and seven mice for 72 h). *P < 0.05, **P < 0.05 (Tukey's HSD).

matured to Ly6C$^-$F4/80$^+$ macrophages. The satiated PKH2^{lo} macrophages (blue dots) followed a similar maturation path to the phagocytic ones, suggesting that they are indeed generated following complete maturation and loss of phagocytosis. Interestingly, the phagocytosis-reluctant F4/80^{lo} monocytes showed a very different expression of maturation markers than the other subsets. They also expressed an Ly6C$^+$F4/80$^+$ phenotype at 24 h, but at 72 h, only half of these cells expressed the Ly6C$^-$F4/80$^+$ mature phenotype. Notably, the frequency of the PKH2^{neg} cells in the exudates increased gradually with time, while the frequency of the PKH2^{lo}/satiated macrophages increased only at 72 h (**Figure 2C**), and as previously reported, these cells contained a distinct population of CD11b^{low} macrophages (**Figure 2B**). Thus, non-phagocytic F4/80$^+$ cells contain, in addition to satiated macrophages, phagocytosis-reluctant Ly6C^{hi}F4/80^{lo}monocytes.

Ly6C^{med}F4/80^{neg} and Ly6C^{hi}F4/80^{lo} Cells Both Convert to Ly6C^{neg}F4/80$^+$ Macrophages

The inflammatory monocytic subsets in the peritoneum during early resolution can differentiate to F4/80$^+$ macrophages while being replaced by Ly6C$^+$ cells that infiltrate from the circulation at later times. Therefore, we aimed to determine the extent of conversion of these monocytic cells to Ly6C^{neg}F4/80$^+$ macrophages. To this end, we sorted Ly6C^{med}F4/80^{neg} (of both neutrophilic and monocytic origin) or Ly6C^{hi}F4/80^{lo} cells from peritoneal exudates at 48 h PPI, labeled them with CFSE, and transferred them to the peritoneum of mice at the same phase of peritonitis. After an additional 24 h, the peritoneal cells were recovered, and the expression of maturation markers in the labeled population was examined. Our results (**Figures 3A–D**) show that both the Ly6C^{med}F4/80^{neg} and

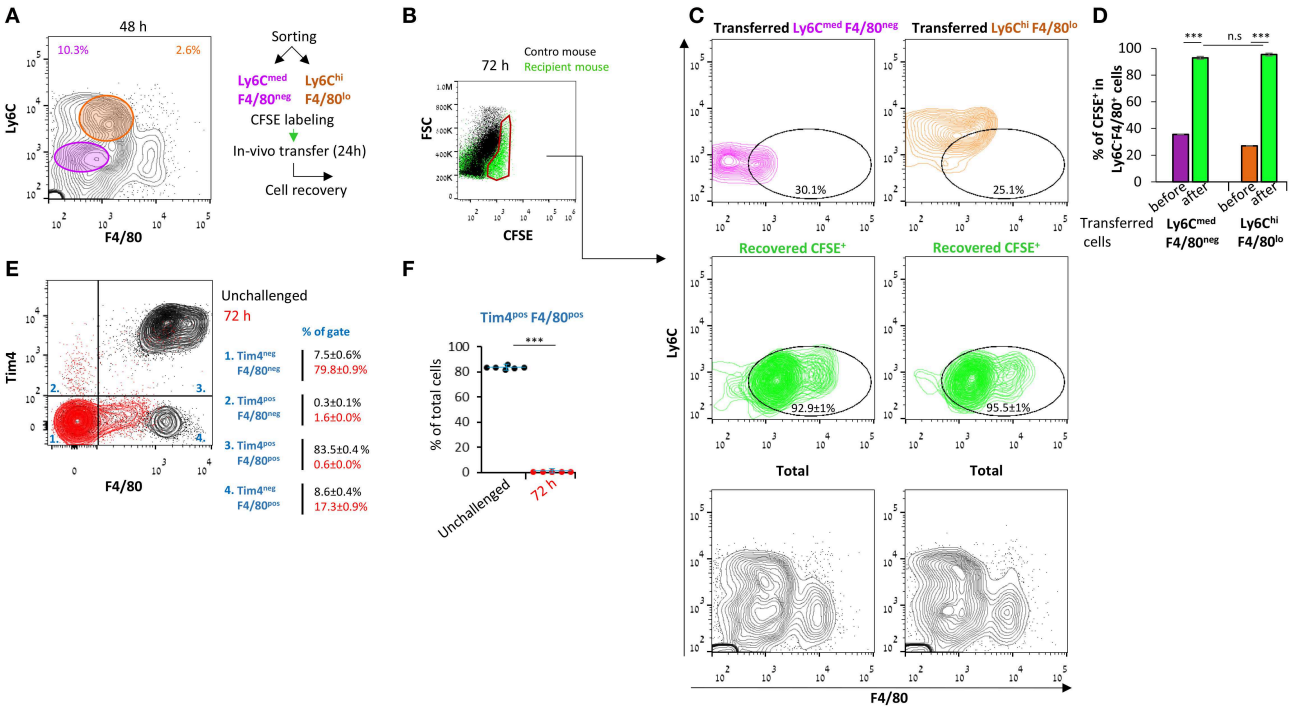


FIGURE 3 | Ly6C^{med}F4/80^{neg} and Ly6C^{hi}F4/80^{lo} cells both convert to Ly6C^{neg} F4/80^{+} macrophages. Peritoneal exudates were recovered from WT mice 48 h PPI. **(A)** Monocytic cells were sorted into Ly6C^{med}F4/80^{neg} and Ly6C^{hi}F4/80^{lo} populations. **(B–D)** Sorted cells were labeled with CFSE and transferred to recipient mice with ongoing peritonitis at 48 h. At 72 h, peritoneal cells were recovered, immunostained for Ly6C and F4/80, and CFSE^{+} cells **(B)** were analyzed by flow cytometry **(C, D)**. Results are stacked contour plot from six mice **(C)** and means ± SEM ($n = 6$). $P < 0.001$ (Student's t-test). **(E,F)** Peritoneal exudates were recovered from unchallenged mice or at 72 h PPI, immunostained for F4/80 and Tim4 and analyzed by flow cytometry. Results are stacked contour plots from six mice **(E)** and percentage means ± SEM of F4/80^{+} Tim4^{+} cells **(F)**.***$P < 0.001$(Student's t-test).

Ly6C^{hi}F4/80^{lo} subsets almost completely converted to the Ly6C^{neg}F4/80^{+} phenotype. Importantly, no contribution of F4/80^{hi}Tim4$^{+/-}$ resident peritoneal cells to the F4/80^{+} macrophage subset was observed (**Figures 3E,F**) at this time, as previously reported for 48 h (17). Notably, the PMN-like cells were almost eliminated 24 h post transfer (**Figure 3C**), suggesting these cells underwent apoptosis and were engulfed by macrophages. Thus, both Ly6C^{med}F4/80^{neg} and Ly6C^{hi}F4/80^{lo} cells seem to be monocytes that differentiate *in vivo* to Ly6C^{neg}F4/80^{+} macrophages during the resolution of inflammation.

All Resolution Phase Monocytic/Macrophage Subsets Are CCR2 Dependent

CCR2 ligation was previously shown to be essential for monocyte recruitment and differentiation to macrophages during low-grade (0.1 mg/mouse) zymosan A-induced peritonitis (29). Therefore, we aimed to determine whether it is also essential for the recruitment of either Ly6C^{med}F4/80^{neg} or Ly6C^{hi}F4/80^{lo} monocytes during medium-grade peritonitis and whether its blockage during inflammation will abrogate the generation of Ly6C^{-}F4/80^{+} resolution phase macrophages. To distinguish the monocytic/macrophages from PMN-like cells, we stained the cells with the monocytic markers CX$_3$CR1 and CD115 (30). Our results show (**Figures 4A–C**) that the anti-CCR2 antibody MC-21 significantly reduced the percentages and/or peritoneal cell counts of most CX$_3$CR1^{+} myeloid cells, including the Ly6C^{med}F4/80^{neg}CD115^{lo}, Ly6C^{hi}F4/80^{lo}, Ly6C^{neg}F4/80^{hi}, and Ly6C^{neg}F4/80^{lo} subsets. Notably, the percentages of the CX$_3$CR1^{-}Ly6C^{med}F4/80^{neg}PMN-like cells were not significantly changed, but their cell counts did reduce by 50%. The reduction in numbers of Ly6C^{med}F4/80^{neg} PMN-like cells suggests resolution phase monocytic cells or macrophages also enhance the recruitment or delay the apoptotis/clearance of PMN-like cells during the resolution of inflammation. A comparison of the CD115 and CX$_3$CR1 surface expression levels revealed similar expression of CD115 in both monocytic subsets that is increased upon maturation to macrophages and reduced following conversion to satiated Ly6C^{neg}F4/80^{lo} macrophages (**Figure 4H**). CX$_3$CR1 expression was similar on all myeloid subsets except Ly6C^{hi}F4/80^{lo} monocytes that expressed significantly lower levels than all other myeloid cells (**Figure 4I**). Thus, our results suggest that all resolution phase monocytic cells are recruited through CCR2 or derived from CCR2-recruited precursors.

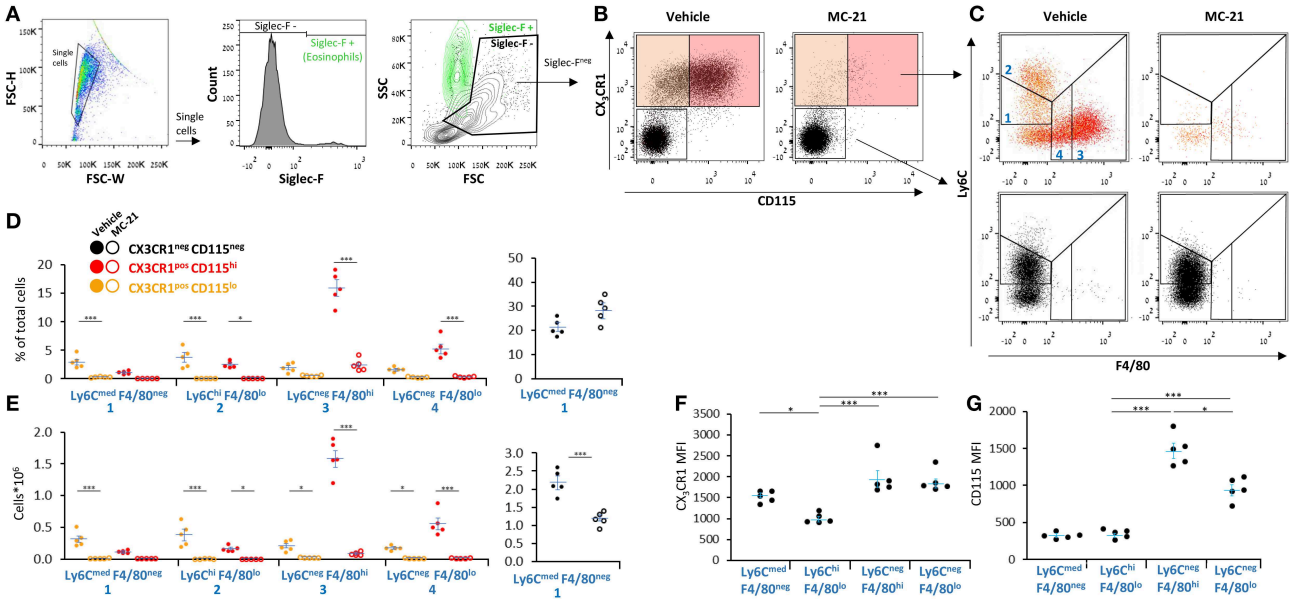


FIGURE 4 | All resolution phase monocytic/macrophage subsets are CCR2-dependent. WT mice undergoing peritonitis were treated I.P. with anti-mouse CCR2 mAb (clone MC-21) or vehicle (control) at peritonitis initiation (0 h) and 24 h PPI. At 72 h, peritoneal cells were collected, immunostained for Ly6C, F4/80, CD115, CX_3CR1, and Siglec–F and analyzed by flow cytometry. **(A)** The gating strategy excluded Siglec-F^+ eosinophils (green). **(B–D)** Samples were analyzed according to CX_3CR1 vs. CD115 **(B)** and CX_3CR1^+ (top) or CX_3CR1^- (bottom) cells were analyzed according to F4/80 vs. Ly6C and $CD115^{hi}$ (red dots) vs. $CD115^{lo}$ subsets **(C)**. Analysis of the percentages **(D)** and cell numbers **(E)** of the indicated subsets is presented. Results are stacked dot plots **(B,C)** and means ± SEM **(D,E)** from $n = 5$. **(F,G)** CD115 **(F)** and CX_3CR1 **(G)** expression by various CX_3CR1^+ myeloid subsets. Results are means ± SEM of MFI from $n = 5$. $^*P < 0.05$, $^{***}P < 0.001$ (Student's *t*-test or Tukey's HSD).

Satiated Macrophages Are the Highest Producers of IFNβ Among Resolution Phase Leukocytes

It was previously shown that non-phagocytic $F4/80^+$ macrophages express higher IFNβ mRNA and protein levels in comparison with their phagocytic counterparts (17). Therefore, we aimed to determine whether this expression is exclusive to satiated $F4/80^{hi}PKH2^{lo}$ macrophages or also takes place in phagocytosis-reluctant $F4/80^{lo}PKH2^{neg}$ monocytes, or other resolution phase leukocytes. Our flow cytometry analysis (**Figures 5A–D**) shows that satiated $F4/80^{hi}PKH2^{lo}$ macrophages indeed express the highest amount of IFNβ of all the analyzed leukocyte subsets. $F4/80^{lo}PKH2^{neg}$ monocytes/macrophages express significantly lower levels of IFNβ than satiated macrophages, whereas eosinophils and $F4/80^-PKH2^+$ monocytes express even lower amounts of this cytokine. Surprisingly, we found phagocytic $F4/80^{hi}PKH2^{hi}$ macrophages to express high levels of IFNβ protein, but not as high as their satiated counterparts (**Figure 5E**). Thus, satiated macrophages seem to be the major producer of IFNβ in resolution phase exudates.

The Transcriptome of Resolution Phase Macrophages Is More Similar to Monocytes Than to Resident Peritoneal Macrophages

Previous studies have debated regarding the contribution of monocyte-derived inflammatory macrophages and their yolk sack-derived resident peritoneal counterparts in spontaneously resolving zymosan A-induced peritonitis (9, 17, 18, 28, 31). In order to improve our understanding of the transcriptomic origin of resolution phase macrophages and the changes that take place during the satiation process, mice were injected I.P. with PKH2-PCL at 62 h post zymosan A-induced peritonitis. After an additional 4 h, peritoneal macrophages were sorted, using flow cytometry, based on their phagocytic uptake of PKH2-PCL (17). The RNA from sorted $PKH2^{hi}$ and $PKH2^{lo/neg}$ macrophages was sequenced, and a total of 31,727 genes were annotated. A volcano plot was generated from the obtained data in order to assess the 3,442 differentially expressed genes ($q \leq 0.05$, 10.9% of annotated genes) (**Figure 6A**). The produced differential gene list is presented across all the samples as a HeatMap (**Figure 6B**), in which hierarchical clustering generated two lists: 1,690 upregulated genes and 1,752 downregulated genes in $PKH2^{lo}$ relative to $PKH2^{hi}$ macrophages.

We previously indicated that select genes from RPM are barely expressed in either phagocytic or non-phagocytic resolution phase macrophages from zymosan A-induced peritonitis, while monocyte markers are abundantly expressed in these cells (17). Since macrophages are able to change their transcriptome in an environment-specific manner (7), we aimed to further characterize the transcriptomes of phagocytic and satiated macrophages to determine whether genes expressed by peritoneal macrophages are also substantial in resolution phase macrophages. To this end, we designated 30 genes with the highest specific expression in either RPM or monocytes based on Lavin et al. (7) and compared their expression to phagocytic

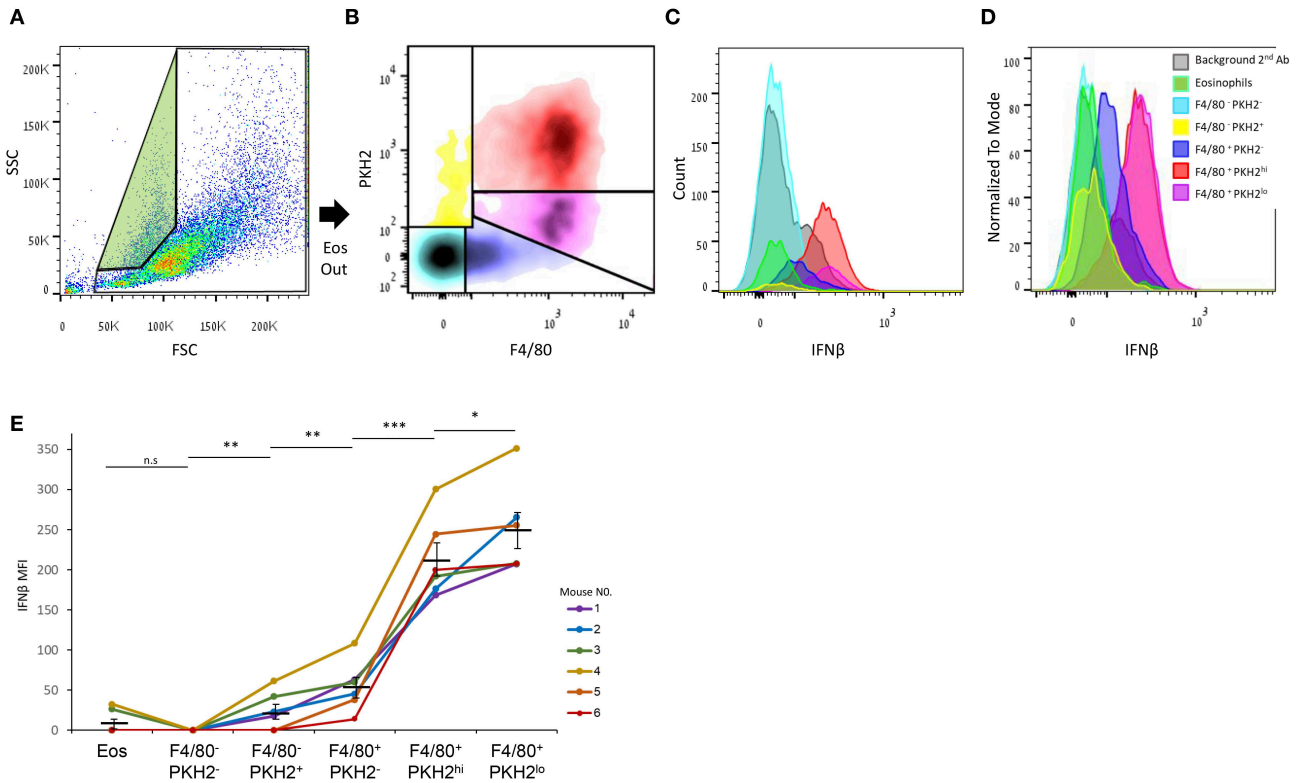


FIGURE 5 | Satiated macrophages express the highest level of IFNβ of all resolution phase leukocytes. Peritoneal exudates were recovered from WT mice 66 h PPI, and the cells were immunostained for F4/80, fixed, permeabilized, and immunostained for IFNβ. **(A)** The gating strategy for eosinophils (green) and other immune cells. **(B)** Density plot analysis of F4/80 vs. PKH2 staining resulted in five distinct populations: F4/80$^-$PKH2$^-$ (cyan), F4/80$^-$PKH2$^+$ (yellow), F4/80$^+$PKH2$^-$ (blue), F4/80$^+$PKH2^{hi} (red), and F4/80$^+$PKH2^{lo} (purple). These populations were then analyzed for IFNβ expression, and results were presented as counts **(C)** and normalized to mode **(D)**. MFI means ± SEM from six independent mice are shown **(E)**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). Data for second antibody alone and eosinophils were previously reported in (17).

and non-phagocytic macrophages. Our results (**Figure 6C**) indicate that some genes (i.e., rap1b, saa3, and nfkbia) highly expressed by RPM are also abundantly expressed by phagocytic and non-phagocytic resolution phase macrophages. However, neither phagocytic nor non-phagocytic macrophages expressed notable mRNA levels of markers of RPM, such as *timd4* [3.47 and 1.26 reads per kilobase million (RPKM) for phagocytic and non-phagocytic macrophages, respectively], *vsig4* (2.58 and 4.39 RPKM, respectively), *nt5e* (1.31 and 2.24 RPKM, respectively), and *cd209b* (0 and 0.22 RPKM, respectively). Unexpectedly, although the canonical RPM transcription factor GATA6 is not expressed in resolution phase macrophages (17), some genes regulated by this transcription factor (8, 9), such as cd9 (139.19 and 266.25 RPKM for phagocytic and satiated macrophages, respectively), cd24a (68.35 and 1198.62 RPKM, respectively), and cd93 (44.35 and 12.37, respectively) were expressed by both phagocytic and non-phagocytic resolution phase macrophages and their levels were significantly modulated upon phenotype conversion.

Overall, analysis of our transcriptomic data against the 30th highest expressed genes in RPM and monocytes indicated a significantly increased median RPKM value for resolution phase macrophages of both phenotypes toward monocyte genes (**Figure 6D**) than toward their RPM counterparts. Moreover, analysis of the percentage of genes that were expressed at 10 RPKM or lower levels revealed a significantly higher percentage in RPM than in monocyte genes (**Table 1**). Principal component analysis (PCA) and calculation of PCA Euclidian distances revealed that PKH2^{hi} (phagocytic) and PKH2^{lo} (non-phagocytic) macrophages are positioned closer to one another, as well as to the small intestine and large intestine macrophages than to any other myeloid subset presented (**Figures 6E,F**). Moreover, both phagocytic and satiated resolution phase macrophages were positioned closer to monocytes than to RPM (**Figures 6E,F**). Of interest, intestinal macrophages that have many common features with monocytes (7) were the closest resident macrophage subset to resolution phase macrophages (**Figures 6E,F**). Notably, non-phagocytic macrophages were found to increase the expression of monocyte genes, in comparison to phagocytic macrophages (14 of 15 genes that were modulated in a statistically significant manner), whereas the expression of RPM genes was decreased in these cells (15 of 21 genes) (**Figure 6C**). Thus, our transcriptomic analysis indicates

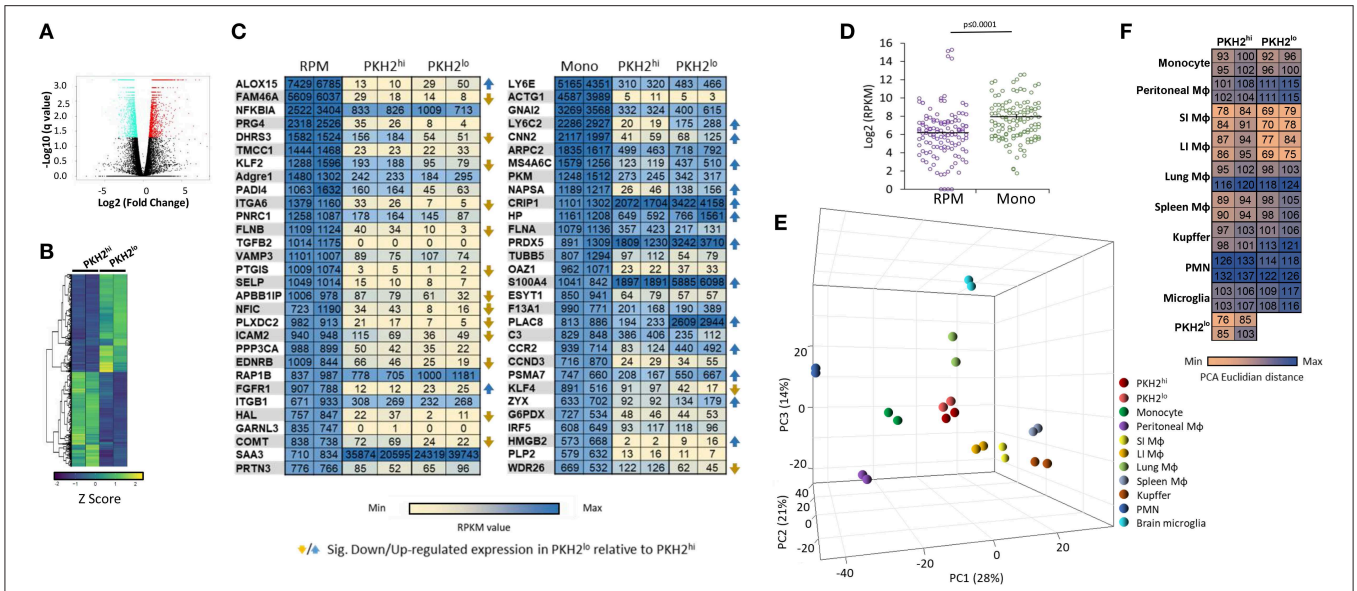


FIGURE 6 | Transcriptomic analysis of PKH2^{hi}/phagocytic and PKH2$^{lo/neg}$/non-phagocytic resolution phase macrophages. Male C57BL/6 mice were injected intraperitoneally with zymosan A (1 mg/mouse) followed by an injection of PKH2-PCL at 62 h. Four hours later, the peritoneal cells were recovered and immunostained for F4/80 and CD11b. Then, F4/80^{+} macrophages were sorted based on the extent of PKH2-PCL acquisition (PKH2^{hi} vs. PKH2$^{low/neg}$ populations; >98% purity) using the FACSAria II sorter [as reported in (17)]. The collected cells were immediately used for RNA extraction (with RNA integrity value above 7.5), and a gene expression microarray analysis was performed using Illumina hiSeq 2500. Annotated genes were plotted using a volcano plot to identify the significant differentially expressed genes comparing PKH2^{hi} and PKH2^{lo} macrophages with significance depicted at $q \leq 0.05$ values **(A)**. Differentially expressed genes were examined across samples and hierarchically clustered into HeatMap of two lists: 1,690 up- and 1,752 down-regulated genes in PKH2^{lo} relative to PKH2^{hi}. Data presented are Z score normalized **(B)**. Annotated genes were examined in comparison to various resident murine macrophage populations, as well as monocytes and PMNs [database from (7)]. The 30 highest expressed genes (on CPM-TMM scale) from either resident peritoneal macrophages (out of 282 exclusive genes) or monocytes (out of 272 exclusive genes) were compared to PKH2^{hi} and PKH2$^{lo/neg}$ macrophages by RPKM values **(C)** and by distribution around the expression median values of each sample **(D)**. Differential distances of PKH2^{hi} and PKH2$^{lo/neg}$ macrophages from resident peritoneal macrophages and from monocytes were visualized on a 3D PCA plot **(E)** and enumerated as PCA Euclidian distances **(F)**.

TABLE 1 | RPM and monocyte genes under-represented in resolution phase macrophages.

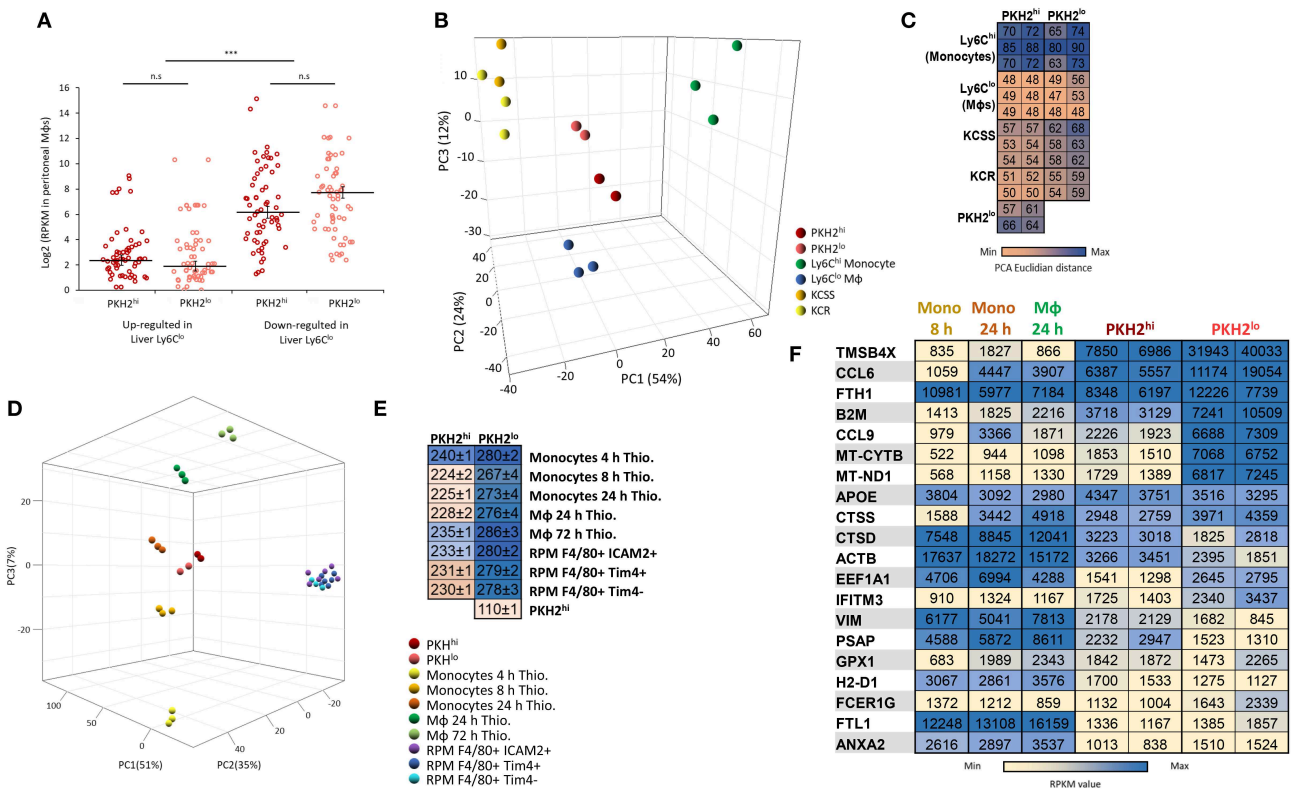
	PKH2^{hi} (%)	PKH2^{lo} (%)
RPM genes < 10 RPKM	8.33	21.67
Monocyte genes < 10 RPKM	1.67	3.33

The percentage of genes from phagocytic (PKH2^{hi}) and (PKH2^{lo}) macrophages that were expressed at lower than 10 RPKM values in the 30th highest expressed genes in RPM and monocytes.

that both phagocytic and non-phagocytic resolution phase macrophages are monocyte-derived with similarities to resident peritoneal and intestinal macrophages.

Resolution Phase Macrophages Resemble Liver Reparative Ly6C^{lo} Macrophages and Peritoneal Monocyte-Derived Macrophages Elicited by Thioglycolate

Acetominophen-induced liver injury, like zymosan A-induced peritonitis, is hallmarked by inflammatory Ly6C^{hi} monocyte differentiation to reparative Ly6C^{lo} macrophages and the clearance of apoptotic neutrophils (19–21). Therefore, we compared the transcriptome of these liver-associated, monocyte-derived cells to peritoneal phagocytic and non-phagocytic macrophages. Our results show that in the 50 highest-fold changed genes downregulated in liver Ly6C^{lo} macrophages, there is a significantly higher expression in both peritoneal phagocytic and non-phagocytic macrophages, compared to the upregulated genes (**Figure 7A**). Interestingly, the PCA and Euclidian distance analysis revealed an increased similarity of both phagocytic and non-phagocytic macrophages to Ly6C^{lo} macrophages rather than to their Ly6C^{hi} precursors or Kuppfer cells (**Figures 7B,C**), thus suggesting that the 50 highest expressed genes are less indicative of transcriptomic changes in this analysis. In addition, comparison of resolution phase macrophages and thioglycolate-elicited monocytes/macrophages analyzed by the ImmGEN consortium (24) revealed that both phagocytic and non-phagocytic macrophages show the highest resemblance to monocytes and macrophages elicited at 8–24 h post thioglycolate administration (PTA). These macrophages showed lower similarity to monocytes or macrophages recovered at 4 or 72 h PTA, respectively, or to various subsets of resident peritoneal macrophages (**Figures 7D–F**). Notably, phagocytic and non-phagocytic macrophages showed a significantly higher resemblance to one another (two fold) than to any other monocyte/macrophage subset, thus, underscoring their common



	Mono 8 h	Mono 24 h	Mϕ 24 h	PKH2hi		PKH2lo	
TMSB4X	835	1827	866	7850	6986	31943	40033
CCL6	1059	4447	3907	6387	5557	11174	19054
FTH1	10981	5977	7184	8348	6197	12226	7739
B2M	1413	1825	2216	3718	3129	7241	10509
CCL9	979	3366	1871	2226	1923	6688	7309
MT-CYTB	522	944	1098	1853	1510	7068	6752
MT-ND1	568	1158	1330	1729	1389	6817	7245
APOE	3804	3092	2980	4347	3751	3516	3295
CTSS	1588	3442	4918	2948	2759	3971	4359
CTSD	7548	8845	12041	3223	3018	1825	2818
ACTB	17637	18272	15172	3266	3451	2395	1851
EEF1A1	4706	6994	4288	1541	1298	2645	2795
IFITM3	910	1324	1167	1725	1403	2340	3437
VIM	6177	5041	7813	2178	2129	1682	845
PSAP	4588	5872	8611	2232	2947	1523	1310
GPX1	683	1989	2343	1842	1872	1473	2265
H2-D1	3067	2861	3576	1700	1533	1275	1127
FCER1G	1372	1212	859	1132	1004	1643	2339
FTL1	12248	13108	16159	1336	1167	1385	1857
ANXA2	2616	2897	3537	1013	838	1510	1524

FIGURE 7 | Resolution phase macrophages resemble liver reparative Ly6C^{lo} macrophages and peritoneal monocyte-derived macrophages elicited by thioglycolate. Annotated genes were compared to the database of monocyte/macrophage populations from acute liver injury induced by overdose of N-acetyl-p-aminophenol (APAP) [Zigmond et al. (19)]. These subsets include inflammatory Ly6C^{hi} monocytes and their descendants, Ly6C^{lo} monocytes, as well as Kupffer cells from the steady state (KCSS) and recovered (KCR) phases. The 50 highest up- or downregulated genes in the liver Ly6C^{lo} differentiated macrophages were compared to PKH2^{hi}/phagocytic and PKH2^{lo}/^{neg}/non phagocytic macrophages **(A)**. Differential distances of PKH2^{hi} and PKH2$^{lo/neg}$ macrophages from liver macrophages and monocytes were visualized on a 3D PCA plot **(B)** and enumerated as PCA Euclidian distances **(C)**. Alternatively, annotated genes were compared to the database of resident tissue macrophages and thioglycolate-elicited peritoneal monocyte/macrophage populations from the ImmGEN consortium (OpenSource mononuclear phagocyte project). The peritoneal resident populations were designated as RPM F4/80$^+$ICAM2$^+$ (F4/80$^+$ICAM2$^+$CD3$^-$CD19$^-$Ter119$^-$) and RPM F4/80$^+$ Tim4$^+$/Tim4$^-$ (B220$^-$Ly6C$^-$F480$^+$CD11b$^+$CD64$^+$Tim4$^+$/Tim4$^-$). The peritoneal thioglycolate-elicited populations were designated as follows: monocytes 4 and 8 h Thio (CD45$^+$CD11b$^+$CD115$^+$Ly-6C$^+$ICAM2$^-$CD226$^-$), monocytes 24 h Thio (CD45$^+$CD11b$^+$CD115$^+$Ly-6C$^+$CD36^{lo}ICAM2$^-$CD226$^-$), and macrophages 24 and 72 h Thio (CD45$^+$CD11b$^+$CD115$^+$Ly-6C^{lo}CD36$^+$ICAM2$^-$CD226$^-$). Differential distances of PKH2^{hi} and PKH2$^{lo/neg}$ macrophages from peritoneal resident, and thioglycolate-elicited monocytes/macrophages were visualized on a 3D PCA plot **(D)** and enumerated as PCA Euclidian distances presented as group to group mean ± SEM **(E)**. The 20 highest expressed genes (on CPM-TMM scale) from either PKH2^{hi} or PKH2$^{lo/neg}$ macrophages were compared to monocytes 4 and 8 h Thio and macrophages 24 h Thio by RPKM values **(F)**.

origin. Together, this analysis suggests that the transcriptomic profile of both phagocytic and non-phagocytic macrophages resembles reparative macrophages from liver injury, and peritoneal monocyte-derived macrophages, which might contain or mature into both subsets. These results also suggest that the Ly6C^{hi}F4/80^{lo} monocytic subset does not contribute significantly to the transcriptome of non-phagocytic macrophages that is rather dominated by satiated macrophages.

Transcriptomic Modulation in Non-phagocytic/Satiated Macrophages Supports a Role in Limiting Tissue Repair and Fibrosis

In order to analyze the nature of the differential gene clustering and the potential variation in the properties of non-phagocytic macrophages, both upregulated and downregulated gene lists were separately analyzed by GO enrichment for biological processes and KEGG pathways at DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases (NIAID), NIH. Enrichment output was clustered into 88 upregulated and 143 downregulated biological processes together with three upregulated and 10 downregulated KEGG pathways. The 23 select clusters from the upregulated and downregulated genes (**Figure 8A**) represent fundamental shifts in cell metabolism, phagocytic activity, tissue interaction and repair, and paracrine modulation of inflammatory processes progress. Based on the above and in order to better understand the genes involved in macrophage phenotype acquisition in terms of modulation of phagocytosis, tissue repair, metabolism, and immune activity, a supervised search toward GO pathways was conducted based on MGI (32). Our results in **Figure 8**

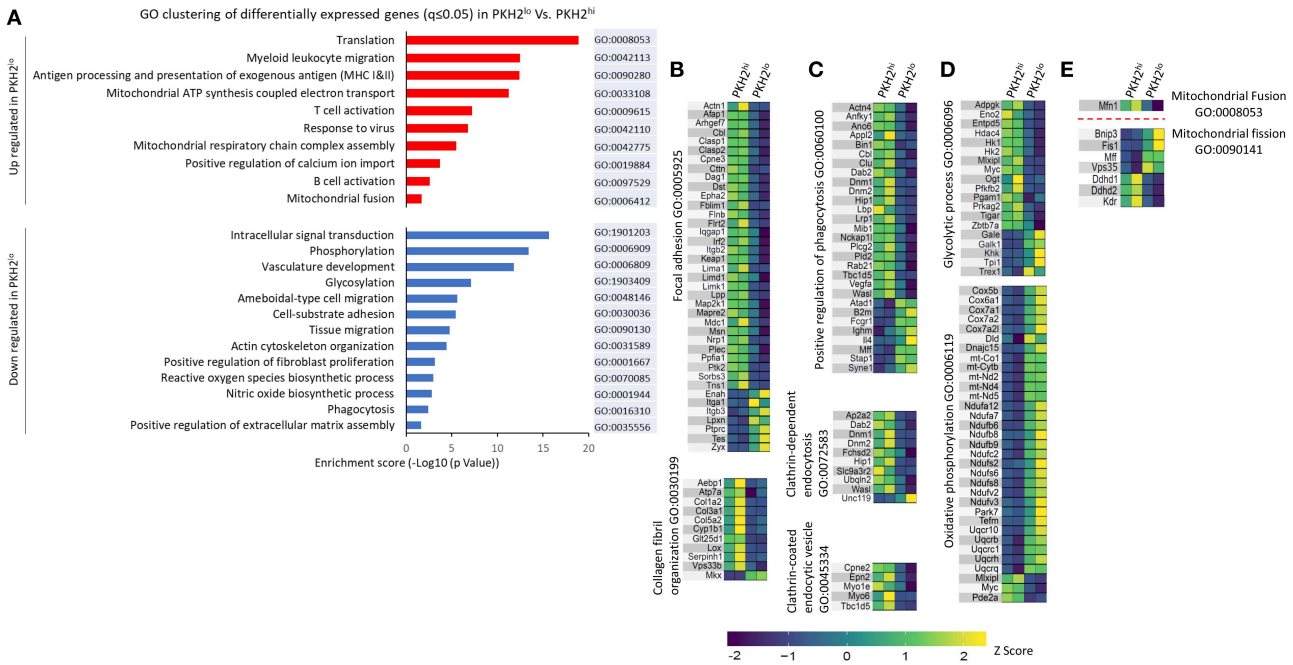


FIGURE 8 | Select functional GO pathways skewed in non-phagocytic macrophages. Analysis of gene enrichment for biological processes and KEGG pathway was performed on the differential up- and down-regulated gene clusters **(A)**. A search for GO pathways was performed to examine skewed functions comparing PKH2^{hi} and PKH2^{lo} macrophages in terms of tissue repair and fibrosis **(B)**, phagocytic activity **(C)**, bioenergetics **(D)**, and mitochondrial dynamics **(E)**. Data presented are differentially expressed (twofold change) genes from each GO term category with $q \leq 0.05$.

show several phenotypic shifts at the transcriptomic level that are associated with macrophage loss of phagocytosis. Satiated macrophages show a significant reduction in the expression of gene clusters involved in intracellular signal transduction, vascular development, cell–substrate adhesion, actin cytoskeleton organization, and positive regulation of both fibroblast proliferation and extracellular matrix organization. These changes suggest a shift from an M2-like/reparative phenotype to a pro-resolving phenotype. Moreover, satiated macrophages express reduction in gene clusters involving collagen organization and focal adhesion (**Figure 8B**). These are two important gene clusters for macrophages that mediate tissue repair and wound healing, but also tissue fibrosis and scarring that leads to organ failure (33). Bitterman and colleagues previously indicated that fibrotic ECM can initiate a pro-fibrotic cycle in fibroblasts that leads to idiopathic pulmonary fibrosis (IPF) (34). Notably, of the 28 genes that were both significantly changed in IPF patients and significantly downregulated in satiated macrophages (two fold), 26 were upregulated, while two were downregulated in IPF patients (**Table 2**). These findings support the notion that resolution phase macrophages deviate from their M2/pro-fibrotic phenotype upon conversion from phagocytic to satiated macrophages and that M2-like resolution phase macrophages might promote tissue fibrosis by directly producing ECM components in addition to regulating fibroblast proliferation and ECM deposition.

Previous reports have indicated that satiated macrophages lose their phagocytic potential upon conversion from their phagocytic counterparts and migrate to remote sites (17, 18). Our results in **Figure 8A** show that satiated macrophages downregulate gene clusters, such as phagocytosis, actin cytoskeleton organization, and ameboidal-type cell migration, while increasing clusters like myeloid leukocyte migration. Moreover, our GO analysis indicates that satiated macrophages mostly downregulate positive regulation of phagocytosis, clathrin-dependent endocytosis, and clathrin-coated endocytic vesicles (**Figure 8C**). These findings support the notion that phagocytic macrophages undergo a process of satiation that results in a loss of their phagocytic properties and their controlled departure of the injury site during the resolution of inflammation.

Previous studies in the last 20 years have indicated that a broad metabolic switch takes place during macrophage differentiation to M1- and M2-like phenotypes. While bacterial and inflammatory stimuli induce glycolytic pathways in macrophages that acquire M1-like features, oxidative phosphorylation and the TCA cycle are the preferred metabolic processes in M2-like macrophages (35). Our results reveal a similar dichotomy in phagocytic and satiated macrophages during the resolution of inflammation. **Figure 8D** shows increased expression of genes involved in mitochondrial ATP synthesis-coupled electron transport and respiratory chain complex assembly that compose an oxidative phosphorylation cluster, while genes included in the glycolytic process are downregulated. Moreover, genes associated with NO biosynthesis, a hallmark of M1 macrophages, are downregulated in satiated macrophages (**Figure 8A**). Notably, additional mitochondrial processes seem to take place on the transcriptomic level during satiation. Only one gene, mitofusin-1 (Mfn1), is significantly downregulated in the

TABLE 2 | Comparative analysis of genes modulated in satiated macrophages and IPF patients.

Description	Symbol	Fold reduction ($-log_2$)
Laminin B1	Lamb1	5.73342
Collagen, type I, alpha 2	Col1a2	3.72915
Collagen, type III, alpha	Col3a1	3.65418
Secreted acidic cysteine rich glycoprotein	Sparc	3.26993
Lysyl oxidase-like 1	Loxl1	3.17508
Collagen, type V, alpha 2	Col5a2	3.16363
Cysteine rich protein 61	Cyr61	2.999
Bone morphogenetic protein 1	Bmp1	2.37722
Serine (or cysteine) peptidase, inhibitor, clade G, member 1	Serping1	2.85737
Collagen, type VI, alpha 2	Col6a2	2.60818
Laminin, beta 2	Lamb2	2.51054
Syntaxin 1A (brain)	Stx1a	2.36814
Follistatin-like 1	Fstl1	2.35297
Vasorin	Vasn	2.27123
Collagen, type VI, alpha 1	Col6a1	2.23102
Cysteine rich transmembrane BMP regulator 1 (chordin like)	Crim1	2.22188
Lysyl oxidase-like 3	Loxl3	1.92259
Niemann Pick type C1	Npc1	1.75604
Collagen, type I, alpha 1	Col1a1	1.63809
Scavenger receptor cysteine rich domain containing (5 domains)	Ssc5d	1.53205
platelet-derived growth factor C polypeptide	Pdgfc	1.48094
Tissue inhibitor of metalloproteinase 2	Timp2	1.38781
Phospholipid transfer protein	Pltp	1.29273
Latent transforming growth factor beta binding protein 3	Ltpb3	1.24257
C-type lectin domain family 11, member a	Clec11a	1.17277
Filamin, alpha	Flna	1.16859
Elastin microfibril interfacer 2	Emilin2	1.13144
Laminin, gamma 1	Lamc1	1.10757

A list of all the genes that were significantly down-regulated in satiated macrophages and significantly changed in samples for IPF patients. Genes labeled in yellow were up-regulated while genes labeled in gray were down-regulated in IPF patients.

mitochondrial fusion cluster. However, this is a key regulator of mitochondria fusion (36). On the other hand, four genes associated with mitochondrial fission were upregulated in satiated macrophages (**Figure 8E**). Unexpectedly, the other three genes involved in mitochondrial fission were downregulated in satiated macrophages. However, these genes are also involved in other processes that are downregulated in these macrophages, such as inhibition of oxidative phosphorylation and blood vessel morphogenesis. ROS production is also downregulated in satiated macrophages by reducing the expression of this gene cluster specifically (**Figure 8A**). Thus, satiated macrophages seem to regulate the expression of various gene clusters involved in important functions that these cells execute highlighted by limiting excessive tissue repair and fibrosis.

DISCUSSION

The emergence of satiated Ly6C$^-$F4/80$^+$CD11b^{low} macrophages that contained high numbers of apoptotic cell nuclei but engulfed low levels of the phagocytosis-acquired dye PKH2-PCL *in vivo* was previously reported during the resolution phase of murine peritonitis (17, 18). These macrophages were converted from phagocytic Ly6C$^-$F4/80$^+$CD11b^{high} that contained low numbers of apoptotic cell nuclei. The expression and secretion of IFNβ by non-phagocytic F4/80$^+$ macrophages was recently reported (17), and therefore, it was of interest to determine whether satiated macrophages are the only non-phagocytic myeloid subset. Surprisingly, our results revealed, in addition to the satiated F4/80$^+$PKH2^{lo} macrophage subset, two other subsets of Ly6C$^+$ monocytes in resolving exudates. One subset was characterized as Ly6C^{med}F4/80^{neg} monocytes that initially displayed low phagocytic capacity (at 24 h PPI). However, at 72 h PPI, the low phagocytic monocytes seem to differentiate to Ly6C$^-$F4/80$^+$ macrophages with high phagocytic capacity. Notably, a significant portion of these monocytes do not become mature and phagocytic even at 72 h PPI, suggesting that these phagocytosis-reluctant monocytes are key regulators of the resolution of inflammation on site. The second population of non-phagocytic monocytes is characterized as Ly6C^{hi}F4/80^{lo}monocytes. The frequency of these F4/80^{lo}PKH2^{neg} cells is increasing continuously during the transition from the inflammatory to the resolving phases of peritonitis (**Figure 2C**) without acquiring any phagocytic activity. At 72 h PPI, only 50% of these cells are Ly6C$^-$F4/80$^+$, while transfer experiments showed that almost all of these cells become Ly6C$^-$F4/80$^+$ within 24 h of peritoneal maturation. Thus, this non-phagocytic Ly6C^{hi}F4/80^{lo}CXC$_3$CR1$^+$CD115^{lo} population also seems to be supplemented by blood-borne precursors, while maturing *in vivo* to an Ly6C$^-$F4/80$^+$ phenotype without acquiring phagocytic capacity. Importantly, these PKH2^{neg} monocytes contain a higher percentage of CD11b^{low} cells than their PKH2^{lo} satiated counterparts (18) (**Figure 2B**) at 48 and 72 h, suggesting that modulation of CD11b expression is important for both acquisition and loss of phagocytosis capacity. It is important to note that the aforementioned changes in macrophage phenotypes should take into account the migration of young monocytes to the peritoneum that replenishes the non-phagocytic populations and the emigration of mature macrophage to remote sites that diminishes the frequency of phagocytic and/or satiated macrophages.

Since the expression of IFNβ by non-phagocytic macrophages was performed using a gating strategy that did not discriminate F4/80$^+$ satiated and phagocytosis-reluctant monocytes, we used flow cytometry to directly evaluate IFNβ expression by each resolution phase leukocyte subset. We found (**Figure 5**) that F4/80$^-$PKH2$^+$ and F4/80^{lo}PKH2^{neg} monocytes expressed very low amounts of IFNβ. Phagocytic (F4/80^{hi}PKH2^{hi}) and satiated (F4/80^{hi}PKH2^{lo}) macrophages, however, expressed high levels of this cytokine with the latter being significantly superior to all other leukocyte subsets. Notably, while phagocytic macrophages were found to express low levels of IFNβ mRNA and non-secreted isoforms of this protein, they did

express higher levels of the secreted isoform (17), which could explain the relatively high detection of IFNβ protein by flow cytometry.

The big disparity in IFNβ expression between satiated and phagocytosis-reluctant monocytes suggests that the former are the major contributors to the transcriptome of non-phagocytic macrophages, especially considering the many IFN-responsive genes upregulated in non-phagocytic macrophages (17). These findings are also supported by the lack of difference in F4/80 expression between phagocytic and non-phagocytic macrophages (237.6 and 239.5 RPKM, respectively), whereas flow cytometry shows a twofold difference between $Ly6C^{hi}F4/80^{lo}$ and $Ly6C^{-}F4/80^{hi}$ cells (data not shown, $N = 6$). In addition, the relative similarity of the transcriptomes of both resolution phase macrophage subsets and resolution phase reparative $Ly6C^{lo}$ macrophages from liver injury, compared to their $Ly6C^{hi}$ monocyte counterparts, underscores the contribution of mature satiated macrophages rather than immature monocytes to the transcriptome of non-phagocytic macrophages. Notably, both resolution phase macrophage subsets had a higher transcriptomic similarity to monocytes and monocyte-derived macrophages rather than RPM. These findings are in accord with previously published results (17) and further support the notion that resolution phase macrophages in this zymosan A-induced inflammation are monocyte-derived. Non-phagocytic macrophages showed some increased transcriptomic similarity to monocytes than their phagocytic counterparts (**Figure 6C**). Therefore, we cannot exclude some contribution of $Ly6C^{hi}F4/80^{lo}$ cells to their transcriptome. Nevertheless, it seems that the transcriptome of non-phagocytic macrophages is dominated by the satiated subset, and we will further discuss the function of these cells as satiated macrophages.

A comparison of the transcriptomes of phagocytic and satiated macrophages suggest that satiation is associated with an M1-to-M2 metabolic shift, namely, from glycolysis to oxidative phosphorylation, that is maintained during the resolution sequel, while satiated macrophages transition from a pro-fibrotic phenotype to a pro-resolving one. The increase in mitochondria fission and the reduction in ROS biosynthetic clusters seems linked to the high oxidative burden (from apoptotic debris) (15) that satiated macrophages need to tolerate, possibly by reducing their production of ROS. Thus, satiated macrophages seem to adjust to the balance between loss of the phagocytic machinery and the need to degrade cellular constituents and control ROS production.

Notably, we found satiated macrophages to upregulate gene clusters associated with T- and B-cell activation as well as responses to viruses. The unique IFNβ-associated gene signature previously observed in these macrophages (17) and the role of some inflammatory cytokines and chemokines in the resolution phase of inflammation (37–40) can partially account for this gene regulation. However, it is also documented that inflammatory cytokines, like TNFα, play a role in limiting muscle fibrosis by promoting the death of fibro/adipogenic progenitors in affected tissues (41). Resolution phase macrophages can also play a significant role in bridging the gap between innate and acquired immunity by attracting various myeloid subsets to the resolving site and affecting lymphoid responses (29).

In conclusion, we have shown that the resolution of inflammation yields several species of phagocytosis-reluctant and satiated myeloid cells, as well as phagocytic macrophages. The comparative analysis of the transcriptomes of satiated macrophages and their phagocytic precursors reveals a distinct shift in gene clusters that correspond to phagocytic, metabolic, and inflammatory properties. These genes and pathways are highlighted in the current report, suggesting a tissue repair and fibrosis-limiting role for satiated macrophages, and serving as a prelude to further studies that will decipher the intricate properties of resolution phase macrophages in various organs and inflammatory models.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the BioProject repository with accession number PRJNA450293.

ETHICS STATEMENT

The animal study was reviewed and approved by the committee of Ethics in Animal Experimentation, University of Haifa.

AUTHOR CONTRIBUTIONS

SB and SS isolated macrophages extracted RNA and performed bioinformatics analysis of the sequences obtained. SB also performed the transfer, monocytic ablation, and MDSC characterization experiments, and wrote the manuscript. SS and SA performed the myeloid cell characterization. SS-Z and NS assisted in RNA isolation and data analysis. DB assisted in data analysis and discussion. AA designed the study and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00405/full#supplementary-material

Supplementary Figure 1 | Gating strategy for analysis of monocyte/macrophage subsets. Immunostained peritoneal cells from 24–72 h PPI were plotted as FSC vs. SSC, and small apoptotic cells and lymphocytes, as well as granulocytic eosinophils were excluded from the analysis **(A)**. Then, single cells were gated for further analysis **(B)** according to Ly6C vs. F4/80 **(C)**, CD11b vs. F4/80 **(D)**, and PKH2 vs. F4/80 **(E)**.

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Corrigendum: Transcriptomic Analysis of Monocyte-Derived Non-Phagocytic Macrophages Favors a Role in Limiting Tissue Repair and Fibrosis

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Following a reassessment of the contribution of Dr. Dalit Barkan to discussions on the database presented in the manuscript, the authors came to the conclusion that her name should be included in the author list.

AUTHOR CONTRIBUTIONS

SB and SS isolated macrophages extracted RNA and performed bioinformatics analysis of the sequences obtained. SB also performed the transfer, monocytic ablation, and MDSC characterization experiments, and wrote the manuscript. SS and SA performed the myeloid cell characterization. SS-Z and NS assisted in RNA isolation and data analysis. DB assisted in data analysis and discussion. AA designed the study and wrote the manuscript.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Myeloid-Derived Suppressor Cells in Kidney Transplant Recipients and the Effect of Maintenance Immunotherapy

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Myeloid-derived suppressor cells (MDSC) represent a heterogeneous group of myeloid regulatory cells that were originally described in cancer. Several studies in animal models point to MDSC as important players in the induction of allograft tolerance due to their immune modulatory function. Most of the published studies have been performed in animal models, and the data addressing MDSCs in human organ transplantation are scarce. We evaluated the phenotype and function of different MDSCs subsets in 38 kidney transplant recipients (KTRs) at different time points. Our data indicate that monocytic MDSCs (Mo-MDSC) increase in KTR at 6 and 12 months posttransplantation. On the contrary, the percentages of polymorphonuclear MDSC (PMN-MDSC) and early-stage MDSC (e-MDSC) are not significantly increased. We evaluated the immunosuppressive activity of Mo-MDSC in KTR and confirmed their ability to increase regulatory T cells (Treg) *in vitro*. Interestingly, when we compared the ability of Mo-MDSC to suppress T cell proliferation, we observed that tacrolimus, but not rapamycin-treated KTR, was able to inhibit $CD4^+$ T cell proliferation *in vitro*. This indicates that, although mTOR inhibitors are widely regarded as supportive of regulatory responses, rapamycin may impair Mo-MDSC function, and suggests that the choice of immunosuppressive therapy may determine the tolerogenic pathway and participating immune cells that promote organ transplant acceptance in KTR.

Keywords: kidney transplantation, mTOR inhibition, myeloid-derived suppressor cells, tacrolimus, immunosuppression

INTRODUCTION

Kidney transplantation is a treatment option for patients with end-stage renal disease (ESRD). Although immunosuppressive protocols have clearly reduced the incidence of acute rejection, transplant patients continue at high risk of treatment side effects, and long-term allograft survival has not improved significantly (1). As a consequence, the main goals in transplantation are to predict the risk of developing rejection and to find biomarkers of tolerance to allow immunosuppression withdrawal in order to minimize the adverse effects of the currently available immunosuppressive regimens.

An increasing field of research is focused on the study of immune cells with regulatory and/or suppressive function. Among them, myeloid-derived suppressor cells (MDSCs) have gained attention in the last years. The MDSCs are a heterogeneous group of myeloid cells able to suppress adaptive and innate immune responses and have been suggested as potential biomarkers for allograft tolerance. They were initially described in cancer, and several studies have pointed out MDSC to play an important role in the regulation of immune responses in other clinical setting, such as organ transplantation, infection, and autoimmune diseases (2–4).

Myeloid-derived suppressor cells were first described in mice as $CD11b^+$ $Gr1^+$ cells, and experimental transplant models demonstrated that MDSCs have an important role in the induction of tolerance (5). On the contrary, evidence on their role in human transplantation is scarce and non-conclusive. In renal transplant patients, Luan et al. observed MDSC, defined as $CD33^+$ $CD11b^+HLA\text{-}DR^-$, able to expand T regulatory cells (Treg) *in vitro* and correlate with Treg cell numbers *in vivo* (6). These data were confirmed by Meng et al. who associated MDSC numbers with less tissue injury and longer allograft survival (7). Human MDSCs are divided into three main subsets: monocytic MDSC (Mo-MDSCs: $CD33^+CD11b^+CD14^+HLA\text{-}DR^-$), polymorphonuclear MDSC (PMN-MDSCs: $CD33^+CD11b^+CD15^+HLA\text{-}DR^-$), and a population lacking both differentiation surface markers classified as early-stage MDSC (e-MDSCs: $CD33^+HLA\text{-}DR^-CD15^-CD14^-$) (8). Since these phenotypic markers are not exclusive of MDSCs and they are present in other myeloid cells such as monocytes, macrophages, and granulocytes, MDSC cells are further defined upon demonstration of their suppressive function (9).

Due to the paucity of the MDSC data in clinical organ transplantation and that different immunosuppressants may have a distinct effect on MDSC, we monitored circulating MDSC subset frequencies in kidney transplant recipients (KTRs). The main goal of the study was to compare transplant recipients receiving standard triple therapy to those maintained on a regimen including rapamycin and evaluate the effect of each therapeutic arm on MDSC in relation to kidney transplant outcomes.

Abbreviations: 7AAD, 7-amino-actinomycin D; CNI, calcineurin inhibitors; e-MDSC, early-stage MDSCs; HC, healthy controls; KTRs, kidney transplant recipients; MDSCs, myeloid-derived suppressor cells; Mo-MDSCs, monocytic MDSCs; mTOR, mammalian target of rapamycin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMN-MDSCs, polymorphonuclear MDSCs.

MATERIALS AND METHODS

Study Design

A total of 38 consecutive KTRs were enrolled in the study after giving consent while they were listed for kidney transplantation in the Hospital Universitario Marqués de Valdecilla in 2016. The study was approved by the Hospital Universitario Marqués de Valdecilla Ethics Committee. The mean follow-up time was 459 days. The clinical and immunological features of the KTR are summarized in **Table 1**. Clinical data were collected from patient records, and blood was drawn at baseline/day 0, 180, and 360 days after transplantation. The clinical and immunological features of the KTR are summarized in **Table 1**.

Monoclonal Antibodies and Flow Cytometry Analysis

The PBMCs or isolated MDSCs were stained with the following monoclonal antibodies: anti-CD33-APC (clone D3HL60.251), anti-CD3-FITC (clone UCHT1), anti-CD14-ECD (clone RMO52), and anti-CD11b-PE-cyanin (clone Bear1) (Beckman Coulter, Marseille, France); anti-CD16-APC-Cy7 (clone 3G8) and anti-CD56-FITC (clone HCD56 and anti-HLA-DR-Brilliant Violet 510 (clone L243) (Biolegend, San Diego, CA, United States); anti-CD19-FITC (clone 4G7), anti-CD14-FITC (clone MφP9), anti-CD25-PE (clone 2A3), and anti-FoxP3-Pacific Blue (clone 206D) (BD Biosciences); anti-CD15-CF Blue (clone MCS-1) (Inmunostep, Salamanca, Spain); and anti-CD4-APC-Vio770 (clone REA623) from Miltenyi Biotech. The cells were incubated for 20 min, washed with phosphate-buffered saline (PBS), and acquired in a Cytoflex® flow cytometer (Beckman Coulter). MDSCs were quantified by flow cytometry following the gating strategy proposed by Bronte et al. (8) to characterize MDSC subsets: Mo-MDSCs ($CD33^+CD11b^+HLADR^-$ $CD14^+$ $CD15^-$), PMN-MDSC ($CD33^+CD11b^+HLADR^-$ $CD15^+$ $CD14^-$), and e-MDSC Lin^- ($CD14^+CD56^+CD3^+CD19^+$) $CD33^+CD11b^+HLADR^-$ $CD14^-CD15^-$. Total MDSCs were defined as $CD33^+CD11b^+HLADR^-$ cells. Fluorescence minus one control was used to identify $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ cells.

Isolation and Sorting of MDSC

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy donors and from KTR by Ficoll density gradient centrifugation. To isolate $CD33^+$ $HLA\text{-}DR^-$ and $CD33^+$ $HLA\text{-}DR^-$ $CD14^+$ cells (Mo-MDSC), the $CD33^+$ cells were first sorted by magnetic-automated cell sorting using CD33-positive separation microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Further isolation of $CD33^+HLA\text{-}DR^-$ cells and $CD33^+HLA\text{-}DR^-$ $CD14^+$ was performed by sorting enriched cells on a FACS-ARIA II (BD Biosciences, San Jose, CA, United States). The purity of the cell sorting was tested after

TABLE 1 | Main features of study population (*N* = 38).

Recipients: Age, mean, years	51.88 (SD13.23)
Donors: Age, mean, years	49.61 (SD12.63)
Healthy controls: Age, mean, years	46.17 (SD11.85)
Recipient Sex (% female)	18 (47.37%)
Donor sex (% female)	19 (50%)
Dialysis post kidney transplant	10 (26%)
Preexisting anti-HLA antibodies	13 (34.21%)
Class I antibodies	10 (26%)
Class II antibodies	8 (21.05%)
Rejection	6 (15.78%)
RT	11 (28.94%)
Induction treatment	
None	21 (55.26%)
ATG	12 (31.57%)
Basiliximab	5 (13.15%)
Both	0 (0.00%)
Immunosupressive protocol	
Calcineurin inhibitor	33 (86.84%)
mTOR inhibitor	0 (0.00%)
Both	5 (13.15%)
ABDR mismatches	
>3	24 (63.15%)
=3	14 (36.84%)
Class II mismatches	
0	8 (21.05%)
1	17 (44.73%)
2	13 (34.2%)
Renal disease	
Glomerular	11 (28.94%)
Others	1 (2.63%)
Congenital	7 (18.42%)
Sistemic	10 (26.31%)
Vascular	2 (5.26%)
Interstitial	5 (13.15%)
Unknown	2 (5.26%)
Peripheral blood creatinine	
Cr 7 days post trasplant	2.28 (SD1.70)
Cr 30 days post transplant	1.90 (SD1.39)
Cr 120 days post transplant	1.40 (SD0.45)
Cr 180 days post transplant	1.40 (SD0.48)

SD, standard deviation; ESRD, end stage renal disease; 1stT, first transplant; RT, retransplant patients.

each experiment, and >98% efficiency was considered acceptable for the study. The experimental conditions were replicated at least four times.

Whole Blood Cultures

Whole blood culture was performed as follows: fresh blood anticoagulated with lithium-heparin was diluted 1:4 in Gibco^{TM} DMEMF/12 GlutaMAX^{TM} supplement medium (Thermo Fisher Scientific) containing 100 U/ml penicillin (Lonza) and 100 mg/ml streptomycin (Lonza). Cells were stimulated throughout the cultures with 5 ng/ml recombinant human monocyte colony stimulating factor (rhM-CSF; R&D Systems, Wiesbaden-Nordenstadt). For some experiments, human CD14$^+$ monocytes were isolated from Ficoll density gradient centrifugation of PBMC followed by positive selection using anti-CD14 microbeads (Miltenyi, Bergisch-Gladbach, Germany). Isolated CD14$^+$ monocytes were stained with Cell Tracker^{TM} Green CMFDA Dye (Thermo Fisher Scientific) at 2 nM and then added back into whole blood cultures at 10^5 cells/tube (Falcon® 5 ml round bottom polystyrene test tube) diluted 1/4 in Gibco^{TM} DMEMF/12 GlutaMAX^{TM} supplement medium (Thermo Fisher Scientific) and supplemented with 100 U/ml penicillin (Lonza), 100 mg/ml streptomycin (Lonza), and rhM-CSF (R&D Systems, Wiesbaden-Nordenstadt) at 5 ng/ml carried on 0.1% human albumin. Purity of sorted cells was tested after isolation, and >95% efficiency was considered acceptable for the study. Cells were collected, and location was analyzed at baseline and 1 and 2 days after culture.

In vitro Evaluation of MDSC Suppressor Function

CD4$^+$ T cells were isolated from healthy donors or KTR PBMC by immunomagnetic depletion using EasySep^{TM} Human CD4$^+$ naive T Cell Isolation Kit (Stemcell Technologies, Grenoble, France) and incubated with carboxyfluorescein succinimidyl ester (CFSE). The CFSE-labeled T CD4$^+$ cells (5×10^5) were stimulated with Dynabeads human T-activator CD3/CD28 (Life Technologies AS, Oslo, Norway) in U-bottomed 96-well plates with complete Roswell Park Memorial Institute (RPMI) media supplemented with 10% human AB + serum. Proliferation was determined using flow cytometry. Autologous Mo-MDSCs were added to the culture at 1:2 ratio (CD4$^+$ T cells: MDSCs), and proliferation was determined at day 5. Proliferation assays from blood donors were performed five times. These same functional assays were also carried out with MDSC from four renal transplant receptors: four patients under calcineurin inhibitor (tacrolimus) and four patients under mTOR inhibitor treatment (rapamycin) with at least 24 months of IS treatment.

In vitro Expansion of Treg Generation

peripheral blood mononuclear cells were obtained from KTR under maintenance immunosuppression with tacrolimus. CD4$^+$ T cells were sorted from the PBMC as described above. CD4$^+$ T cells (5×10^5) were polyclonally stimulated and cultured with CD33$^+$HLA-DR$^-$CD14$^+$ (Mo-MDSC) at different concentrations. Treg generation was determined at day 5 by staining with the monoclonal antibodies indicated above and flow cytometry analysis.

Western Blot

Gel electrophoresis and immunoblotting were performed as described elsewhere (10).

Statistical Analysis

Non-parametric Mann–Whitney *U* test and Student's *t*-test were used to compare two groups, as appropriate. More than

two groups were compared using the parametric analysis of variance (ANOVA), the non-parametric Kruskal–Wallis (not matching), or Friedman (repeated measures) test. Comparisons between two paired groups were performed using the Student's *t*-test for paired data or the Wilcoxon signed-rank test when data were or not normally distributed, respectively. Multiple comparisons were assessed using Dunn or Tukey's tests. Statistical analyses were performed using GraphPad software version 6.01 (GraphPad Inc., San Diego, CA, United States). To examine the relationship between bivariate variables, the Pearson correlation was calculated using SPSS Statistics version 24.

RESULTS

Monitoring MDSC in Kidney Transplant Patients

We hypothesized that MDSC subset frequencies might serve as useful biomarkers of clinical outcome after kidney transplantation. Therefore, we first quantified Mo-MDSC, PMN-MDSCs, and e-MDSC in peripheral blood from KTRs at 0, 180, and 360 days after transplantation. We found an increase in total $CD33^{+}HDL\text{-}DR^{lo}$ MDSC frequency at 180 days after transplantation [median, 11.5%; interquartile range (IQR), 6.2–17.0%] (**Figures 1B**, **2A**) in comparison with patients on the day of transplantation (median, 8.8%; IQR, 5.0–16.4%) (**Figures 1A**, **2A**). MDSC frequency at 360 days posttransplant was also increased but not significantly (median, 11.2%; IQR, 4.9–17.8%; **Figures 1C**, **2A**). Next, we examined changes in MDSC subset distribution after transplantation (**Figure 2** and **Supplementary Figures S1, S2**). Mo-MDSC frequencies were significantly increased at 180 and 360 days posttransplant (median, 22.71%; IQR, 6.75–57.56% and median, 25.48%; IQR, 8.85–56.58%) in comparison to patients on the day of transplantation (median, 10.56%; IQR, 3.18–37.55%) (**Figures 1A–C**, **2B**). PMN-MDSC and e-MDSC frequencies were lower at 180 days after transplantation (median, 41.71%; IQR, 12.67–62.79% and median, 5.5%; IQR, 1.9–10.87%) compared to patients on the day of transplantation (median, 54.6%; IQR, 29.4–84.95% and median, 6.15%; IQR, 3.9–13.5%), and they remained lower 360 days posttransplantation (median, 43.14%; IQR, 10.28–63.02% and median, 4.09%; IQR, 2.11–8.2%) (**Figures 1A–C**, **2C,D**). Despite these changes, we did not find any association between the MDSC subsets, and the clinical data are summarized in **Table 1** for patients included in the present work. Importantly, all the KTRs were receiving tacrolimus (**Table 1**) as main immunosuppressant during the first 360 days after transplantation shown.

MDSC From Transplant Patients Induce the Production of Tregs *in vitro*

Treg expansion is one of the main mechanisms by which MDSCs exert suppressive function (11, 12). Hence, we evaluated the capacity of Mo-MDSC from healthy donors and KTR to boost Tregs *in vitro*. We observed a significant increase in the frequency of Tregs recovered from the culture when $CD4^{+}$ T cells were stimulated with Mo-MDSC from cells from KTR at 360 days after transplantation, confirming their suppressive function (**Figure 3**).

MDSC From Tacrolimus Treated KTR Effectively Suppress T Cell Proliferation *in vitro*

The T-cell-suppressive capacity of Mo-MDSC from healthy controls, tacrolimus, and rapamycin-treated KTR was compared using an *in vitro* assay of polyclonally activated T cell proliferation. Sorted Mo-MDSC were added at a 1:2 ratio to autologous CD3/CD28-stimulated $CD4^{+}$ T cells. Four patients under long-term tacrolimus treatment and four patients under long-term rapamycin maintenance therapy were analyzed (**Figure 4**). Results indicate that Mo-MDSC obtained from tacrolimus treated KTR were significantly suppressive in comparison with rapamycin treated KTR. This suggests that Mo-MDSC from transplant patients exhibit different suppressive function *in vitro*, according to the immunosuppressive therapy that KTRs receive.

Rapamycin Inhibits the Function of *in vitro* Generated Myeloid Suppressor Cells

Following-up our observation of Mo-MDSC obtained from rapamycin-treated KTRs, we next investigated the effect of rapamycin on myeloid suppressor cells that were generated *in vitro* from whole blood cultures. First, we developed a flow cytometry panel that allowed us to reliably detect Mo-MDSC from human whole blood cultures according to their $CD45^{+}$ $CD33^{+}$ Lin^{-} $HLA\text{-}DR^{lo}$ $CD14^{+}$ $CD15^{-}$ phenotype (**Figure 5A**). Using whole blood cultures, we next investigated whether CSF1-stimulated human monocytes acquire a Mo-MDSC phenotype ($CD33^{+}$ Lin^{-} $HLA\text{-}DR^{lo}$ $CD14^{+}$ $CD15^{-}$) *in vitro*. When cultured for 48 h, we observed an increase in Mo-MDSC frequency in whole blood cultures from healthy donors (**Figure 5B**). Next, we investigated the effect of rapamycin on Mo-MDSC in whole blood cultures and observed that rapamycin led to accumulation of $HLA\text{-}DR^{lo}$ $CD14^{+}$ Mo-MDSC over 48 h (**Figure 5C**). This suggests that mTOR inhibition promotes Mo-MDSC development. Surprisingly, we found that rapamycin exposure substantially reduced the T-cell-suppressive capacity of Mo-MDSC (**Figure 5D**). It has been previously shown that T cell suppression by human-monocyte-derived Mo-MDSC is in part mediated by the expression of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) (13). Our results confirm that rapamycin blocked the expression of IDO (**Figure 5E**), suggesting that the suppressive effect of Mo-MDSC from rapamycin-treated KTR may be compromised due to the impaired expression of IDO.

DISCUSSION

Myeloid-derived suppressor cells represent a varied group of myeloid regulatory cells that were originally studied in cancer

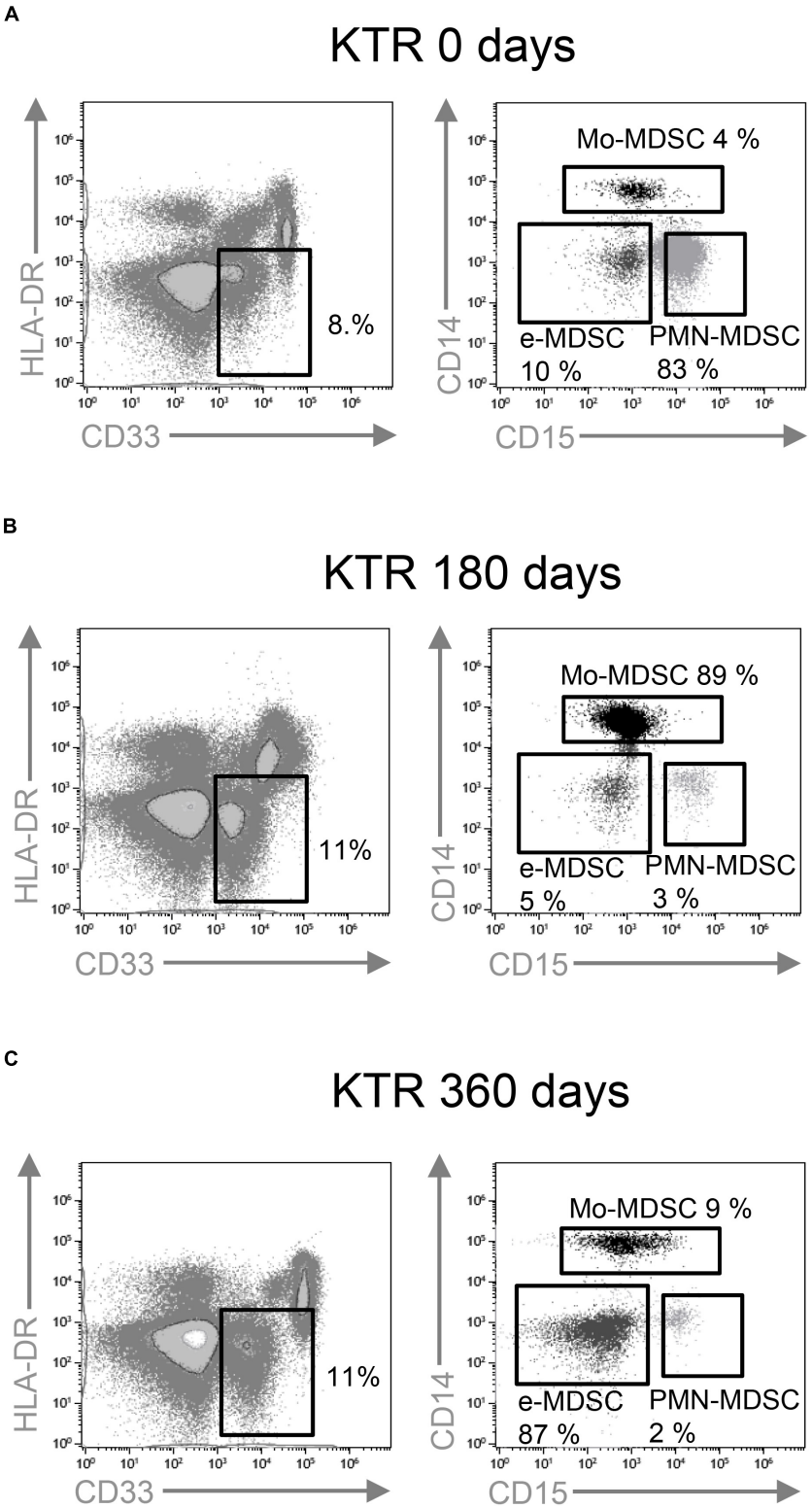

FIGURE 1 | Characterization of myeloid-derived suppressor cell (MDSC) subsets by flow cytometry. $CD33^{+}$ $HLA\text{-}DR^{-}$ myeloid cells were selected from live cells after doublets and debris exclusion. To define monocytic (Mo-MDSC), early-stage (e-MDSC), and polymorphonuclear (PMN-MDSC) MDSC, the CD14 and CD15 expression was analyzed on cells selected from $CD33^{+}HLA\text{-}DR^{-}$ MDSC. Representative flow cytometry data of MDSC from **(A)** patients on the day of transplantation (day 0), **(B)** kidney transplant recipients on day 180, and **(C)** day 360 posttransplantation is shown.

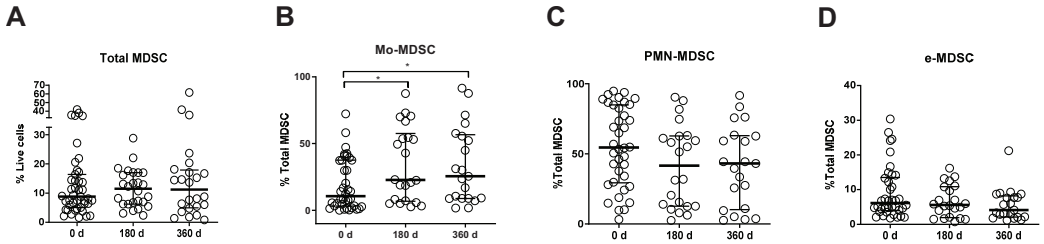

FIGURE 2 | Myeloid-derived suppressor cell (MDSC) frequencies in kidney transplant recipients (KTRs). **(A)** Frequencies of total myeloid-derived suppressor cells (MDSC) in peripheral blood mononuclear cells (PBMC); **(B)** monocytic MDSC (Mo-MDSC); **(C)** early-stage MDSC (eMDSC); and **(D)** polymorphonuclear MDSC (PMN-MDSC) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney *U* test. (*$p < 0.05$).

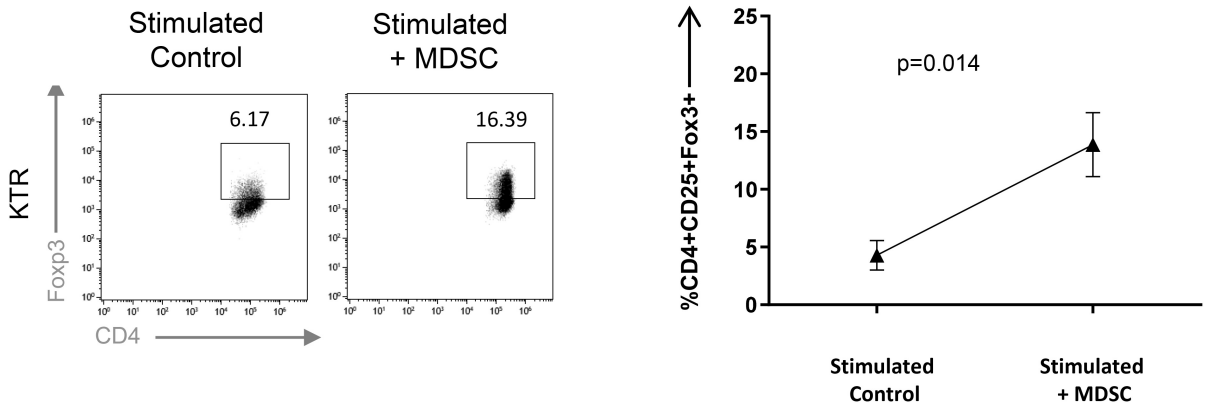

FIGURE 3 | Monocytic myeloid-derived suppressor cell (Mo-MDSC) from kidney transplant recipients (KTRs) expand Treg *in vitro*. MDSC dependent $CD4^+FoxP3^+$ Treg expansion was analyzed by flow cytometry. Naive $CD4^+$ T cells cocultured under polyclonal activation with autologous Mo-MDSC obtained KTR at day 360 are shown ($n = 3$, unpaired *t*-test).

(14). Several studies demonstrating their immunoregulatory action in animal models point to a potential role of MDSC in the induction of tolerance after transplantation (2). As most of the published studies were performed in animal models, there is a paucity of data addressing MDSC features and their role in human transplantation. We found that absolute numbers of circulating total MDSC were increased in KTR and in the short term after transplantation, whereas they declined to baseline levels 1 year after transplantation. We also observed an increase in Mo-MDSCs frequencies in the short term after transplantation and 1 year after transplantation. Luan et al. found that peripheral blood MDSCs were increased in KTR (6). Hock et al. also reported that renal transplant recipients had elevated frequencies of circulating MDSC (15), but they further found that MDSC numbers had returned to normal levels 12 months posttransplantation (16). However, in their previous study, long-term KTR had increased MDSC numbers, suggesting that MDSC recover and even expand in the long term, as graft acceptance progresses. These observational studies suggest that MDSC numbers increased rapidly and peaked following immunosuppressive therapy. Whether these increases are the result of potential differences between the two immunosuppressive regimens used (tacrolimus and mTOR inhibitors) or whether MDSC subsets are differentially regulated by local conditions or treatments is still a matter of debate.

Studies developed in mice suggest that MDSCs have an important role to induce T regulatory cells (Treg) after transplant (11, 12), but their role in human transplantation is still unclear. In KTR, Luan et al. observed that $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ MDSC are capable of expanding Treg, and they correlate with Treg increases *in vivo* (6). Consistent with this view, Meng et al. (7) found that MDSCs isolated from transplant recipients were also able to expand regulatory T cells and were associated with longer allograft survival. Okano S. et al. also found a positive correlation between MDSC and Treg in intestinal transplant patients (17), and we report here an increase in Treg expansion after Mo-MDSC coculture. However, there was no significant linear association between MDSC absolute numbers and percentage Treg when we examined the relationship between total MDSC subsets and $CD4^+CD25^+Foxp3^+$ Treg *in vivo*.

Myeloid cell surface markers define potential MDSC, but the lack of unique phenotypic markers obliges to perform functional studies to identify MDSC subsets. We tested the suppressive capacity of MDSCs from KTR under calcineurin (tacrolimus) or mTOR (rapamycin) inhibition at 360 days of immunosuppressant maintenance therapies. Our results demonstrate that MDSC from healthy donors display marginal

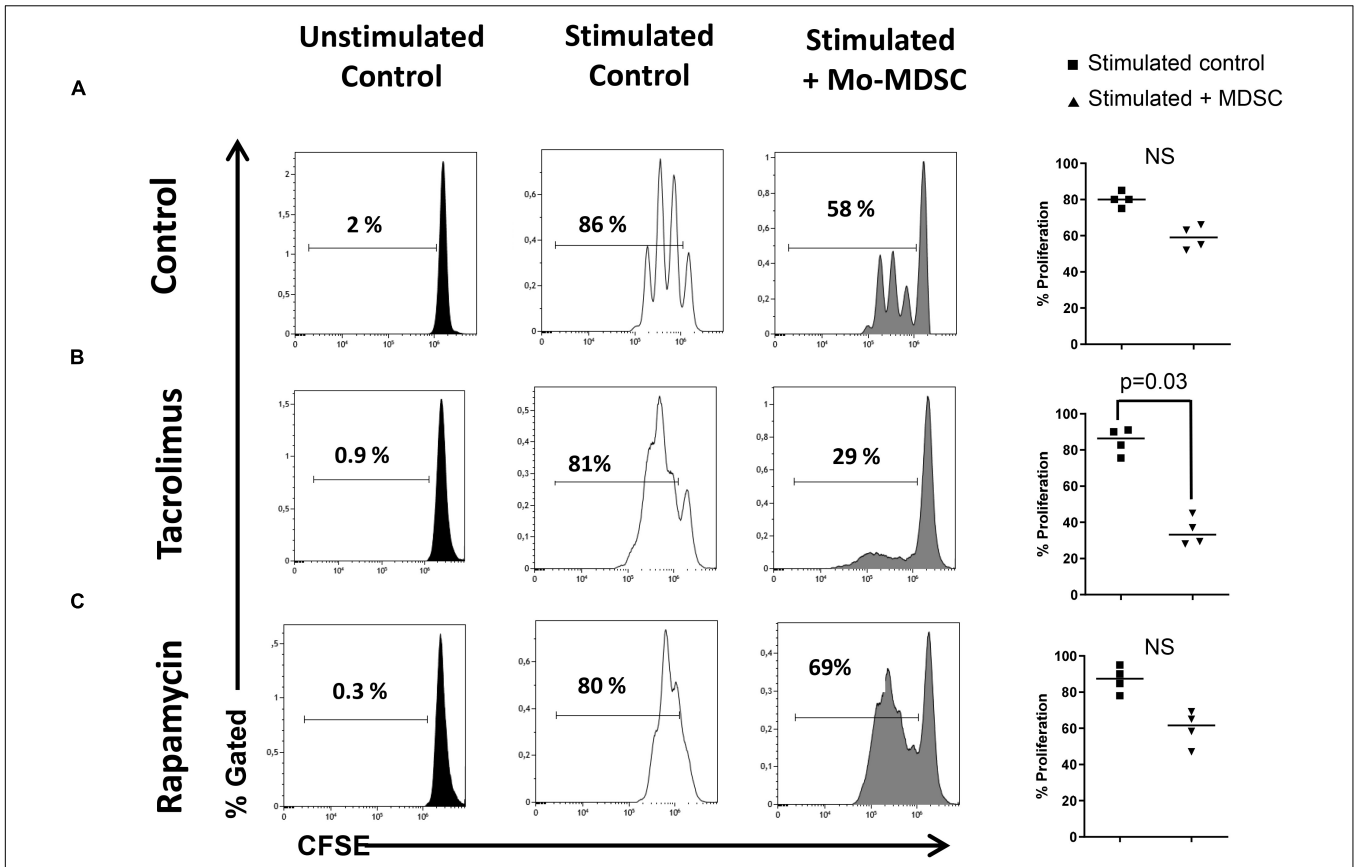

FIGURE 4 | Suppressive function of myeloid-derived suppressor cells (MDSCs). Sorted $CD4^+$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and cultured under polyclonal activation alone or with autologous monocytic myeloid-derived suppressor cells (Mo-MDSCs). Representative flow cytometry plots of four independent experiments with Mo-MDSCs from healthy volunteers; kidney transplant patients under tacrolimus treatment and rapamycin are shown. Individual data of experiments are displayed in the right plot graphs where stimulated control cells are represented as black squares and stimulated cells + Mo-MDSC are represented as black triangles. Differences between groups were assessed by Mann–Whitney test and only indicated if differences were significant.

suppression of polyclonal T $CD4^+$ responses. In contrast, Mo-MDSCs from KTR exhibit potent suppressive function. The results are consistent with previous data demonstrating that $CD11b^+CD33^+HLA\text{-}DR^-$ myeloid cells from human KTR inhibit T cell proliferation, but they found no inhibition when $CD11b^+CD33^+HLA\text{-}DR^-$ cells were obtained from healthy donors (6). Moreover, we observed that Mo-MDSC from KTR under tacrolimus treatment had increased suppressive activity compared to rapamycin, and this immune inhibitory function may be related to the upregulation of inducible nitric oxide synthase (iNOS) (18).

On the other hand, rapamycin downregulates iNOS expression in MDSC, and the suppressive activity and MDSC numbers are significantly reduced after rapamycin treatment in an allogeneic skin transplant model (19). Our results are consistent with this hypothesis, and we attribute loss of suppressive function to diminished IDO expression in rapamycin-exposed Mo-MDSC. However, other studies demonstrated that rapamycin prolongs cardiac allograft survival through the enhancement of MDSC migration and suppressive activity (20). Chen X. et al. showed that mTOR signaling is a negative determinant of MDSC function in immune-mediated hepatic injury (IMH) diseases. In the context of IMH, the blocking of mTOR with rapamycin or mTOR-deficient $CD11b^+Gr1^+$ MDSC mediated the protection against IMH (21). Another study addressing the murine MDSC response to acute kidney injury demonstrated that MDSC reduced the injury, and the effect was potentiated by MDSC induction and enhancement of the immunosuppressive activity promoted by mTOR (22). More recently, a previously unrecognized mechanistic pathway associated with organ rejection identifies the expression of mTOR by graft infiltrating macrophages at the center of epigenetic and metabolic changes that correlate with graft loss (23). This novel functional mechanism involves non-permanent reprogramming of macrophages and has been termed "trained immunity" (24). Therefore, it seems that, while mTOR inhibition may prevent trained immunity and inflammatory pathways in myeloid cells (25, 26), it may also interfere with tolerogenic programming and the ability of myeloid cells to expand Treg and suppress T-cell-mediated immune responses. This dual effect of mTOR inhibition (immunogenic vs. tolerogenic) and the resulting

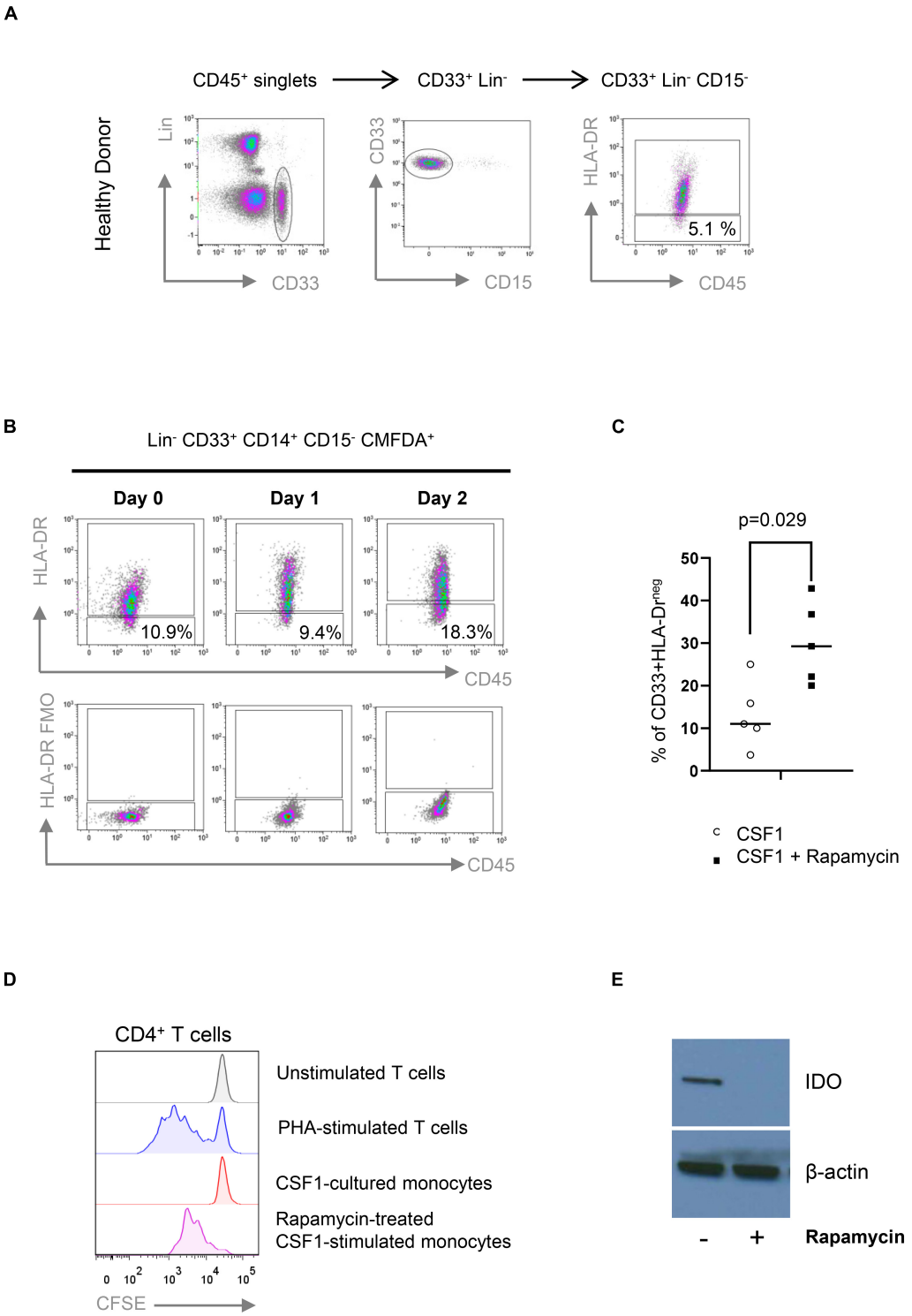

FIGURE 5 | Rapamycin prevents the suppressive function of CD33$^+$HLA-DR$^-$/low myeloid cells. **(A)** Gating strategy for the identification of CD33$^+$HLA-DR$^-$/low myeloid cells obtained from healthy control (HC). Fluorescence minus one (FMO) controls were used to define HLA-DR expression (not shown). **(B)** Colony stimulating factor 1 (CSF1) induces the accumulation of CD33$^+$HLA-DR$^-$/low myeloid cells *in vitro*. CD14$^+$ cells were isolated from peripheral blood, labeled with CFDMA and cocultured with CSF1 for 2 days. CD33$^+$HLA-DR$^-$/low phenotype was analyzed in CFDMA$^+$ cells at day 0, 1, and 2 after culture. FMO controls were used to define HLA-DR expression. **(C)** CD33$^+$HLA-DR$^-$/low myeloid cell frequencies after 48 h in WB cultures treated with or without rapamycin. Differences between groups were assessed by paired *t*-test. **(D)** Rapamycin-treated CSF1-stimulated monocytes are less effective than untreated monocytes in suppressing phytohemagglutinin (PHA)-stimulated proliferation of allogeneic human CD4$^+$ T cells in 1:1 direct cocultures (n = 3). **(E)** Western Blot analyses indicate that rapamycin-treated CSF1-derived CD33$^+$HLA-DR$^-$/low myeloid cells prevents the expression of IDO.

dominant pathway *in vivo* is likely to determine the outcome of the transplanted organ. Taken together, the effects of distinct immunosuppressive drugs on MDSC development and function need to be better characterized in KTR.

Understanding the effect of immunosuppressive drugs on MDSC in clinical transplantation is important to develop strategies to promote tolerance. While there are many unanswered questions regarding the development and function of MDSC human transplantation, we conclude that MDSCs are increased in KTR early after transplantation and that Mo-MDSC subsets from KTR are able to suppress immune responses *in vitro*. How immunosuppressive therapy may enhance or impair MDSC numbers and function is not clear, and additional prospective studies in KTR are required to establish if the long-term transplant tolerance by immune modulation is dependent on MDSC.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Hospital Universitario Marqués de Valdecilla Ethics Committee (CEIC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MI-E: data acquisition, analysis, interpretation, investigation, methodology, writing, and original draft. DS-A and PR: conceptualization, formal analysis, supervision, writing, and review. DM-F: data acquisition, analysis, interpretation, investigation and methodology. SG-F, CP, and PL-P: investigation, and methodology. RV, JR, and ER: patient recruitment and clinical data analysis. JH, JO, and ML-H: conceptualization, project administration, funding acquisition, formal analysis, writing, reviewing, and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00643/full#supplementary-material

FIGURE S1 | Comparison of MDSC subsets: Mo-MDSC, PMN-MDSC, and e-MDSC at day 0 and 180 days after transplant **(A)** and at day 0, day 180, and 360 after transplant **(B).** Levels of Mo-MDSC 180 days after transplant were significantly increased compared to day 0. The central number is the difference (in percent) between the means of the two time points **(A)** and the three time points **(B)**. Differences between time points were calculated using the following formula: (mean posTx-mean preTx)/mean preTx.

FIGURE S2 | MDSC absolute numbers in KTR. **(A)** Frequencies of total myeloid-derived suppressor cells (MDSCs) in peripheral blood mononuclear cells (PBMCs); **(B)** monocytic-MDSCs (Mo-MDSCs); **(C)** early stage-MDSC (eMDSCs), and **(D)** polymorphonuclear MDSCs (PMN-MDSCs) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney *U* test. (*$p < 0.05$).

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REVIEW
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Redefining Tumor-Associated Macrophage Subpopulations and Functions in the Tumor Microenvironment

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The immunosuppressive status of the tumor microenvironment (TME) remains poorly defined due to a lack of understanding regarding the function of tumor-associated macrophages (TAMs), which are abundant in the TME. TAMs are crucial drivers of tumor progression, metastasis, and resistance to therapy. Intra- and inter-tumoral spatial heterogeneities are potential keys to understanding the relationships between subpopulations of TAMs and their functions. Antitumor M1-like and pro-tumor M2-like TAMs coexist within tumors, and the opposing effects of these M1/M2 subpopulations on tumors directly impact current strategies to improve antitumor immune responses. Recent studies have found significant differences among monocytes or macrophages from distinct tumors, and other investigations have explored the existence of diverse TAM subsets at the molecular level. In this review, we discuss emerging evidence highlighting the redefinition of TAM subpopulations and functions in the TME and the possibility of separating macrophage subsets with distinct functions into antitumor M1-like and pro-tumor M2-like TAMs during the development of tumors. Such redefinition may relate to the differential cellular origin and monocyte and macrophage plasticity or heterogeneity of TAMs, which all potentially impact macrophage biomarkers and our understanding of how the phenotypes of TAMs are dictated by their ontogeny, activation status, and localization. Therefore, the detailed landscape of TAMs must be deciphered with the integration of new technologies, such as multiplexed immunohistochemistry (mIHC), mass cytometry by time-of-flight (CyTOF), single-cell RNA-seq (scRNA-seq), spatial transcriptomics, and systems biology approaches, for analyses of the TME.

Keywords: tumor microenvironment, tumor-associated macrophages, multiplexed immunohistochemical staining, single-cell sequencing, spatial transcriptomics

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INTRODUCTION

The tumor microenvironment (TME), which refers to the structure of tumor tissue containing stromal cells (including immune cells, connective tissue cells, and vascular components), is crucial in tumor progression and metastasis. A close association between inflammation and the TME has been established in recent years, although the link was first noted in the nineteenth century (1). Currently, inflammation in the TME is generally considered a hallmark of cancer (2), reflecting

that inflammatory cells interact with tumor cells to influence the progression of tumors. Among the diverse inflammatory cells infiltrating the TME, macrophages, which are termed tumor-associated macrophages (TAMs), including both resident macrophages and circulating monocytes recruited to the TME, are predominant elements (3).

The role of TAMs in the TME, which is critical to current TAM-targeted strategies, remains to be uncovered due to the intricate heterogeneity of macrophages. Preclinical and clinical data show a close relationship between high infiltration of TAMs and a poor prognosis in most types of tumors (4), such as pancreatic ductal adenocarcinoma (PDAC) (5), glioblastoma (6), and bladder cancer (7). On the other hand, TAM infiltration has also been found to be associated with a favorable prognosis in some cases, such as in ovarian cancer (8) and colorectal cancer (9). Such different outcomes can be attributed to not only the distinct cancer types but also some intra-tumoral factors, such as the TAM distribution in the TME. For example, some studies on non-small cell lung cancer (NSLSC) reported that increased infiltration of TAMs in tumor islets was associated with a good prognosis, whereas increased levels of TAMs in the tumor stroma were found to be associated with a poor prognosis (10, 11). These findings may indicate the inter- and intra-tumoral heterogeneity of TAMs, which may relate to the ontogeny, activation status, and localization of TAMs in the TME. To discriminate the distinct roles of TAMs among various conditions, TAM subsets and their functions in the TME urgently need to be redefined.

In this review, we will summarize the current understanding of the dual roles that TAMs play in the TME and highlight the inter- and intra-tumoral heterogeneity of TAMs, thus emphasizing the necessity of further investigating and redefining TAM subpopulations with distinct functions. The integration of some novel and powerful technologies as a work flow to analyze the heterogeneity of TAMs will also be discussed, including multiplexed immunohistochemistry (mIHC), mass cytometry by time-of-flight (CyTOF), single-cell RNA-seq (scRNA-seq), and spatial transcriptomics.

CHARACTERISTICS OF TAMs

Signals in the TME may impact the diversity and function of TAMs, leading to dual roles for TAMs in tumor progression that can be summarized as tumor-promoting and tumor-suppressing activities (12). In accordance with the commonly accepted theory proposed by Mills' team, TAMs can be mainly classified into the antitumor M1 phenotype (classically activated state) and the pro-tumor M2 phenotype (alternatively activated state), reflecting the Th1-Th2 polarization of T cells (13, 14). Once TAMs derived from peripheral blood monocytes are recruited to the TME by tumor-secreted attractants, they undergo M1-like or M2-like activation in response to various stimuli (**Figure 1**).

Induced by interferon-γ (IFN-γ) (15), tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (16, 17), M1-like TAMs are involved in activating Th1-type immune responses as they have a high capacity for antigen presentation. They produce nitric oxide (NO), reactive oxygen species (ROS) and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12, IL-23, C-X-C motif chemokine (CXCL) 9, CXCL10, TNF-α, and major histocompatibility complex (MHC) molecules (18–24). The expression of surface proteins, including CD68, CD80, and CD86 (25), and the intracellular protein suppressor cytokine signaling 3 (SOCS3) can also be upregulated (26). Through secretion of the described factors, M1-like TAMs function as the main forces in innate host defense and kill tumor cells, thus suppressing tumors.

In contrast, M2-like TAMs, which are generated under the influence of several cytokines such as IL-10 and transforming growth factor (TGF)-β, activate Th2-type immune responses and promote tumorigenesis and development (27). They may mainly promote upregulation of the expression of anti-inflammatory cytokines and chemokines, including IL-10, TGF-β, CC chemokine ligand (CCL) 17, CCL18, CCL22, and CCL24 (24). Such secretion is involved in tumor invasion and metastasis. Surface proteins, such as CD206 (mannose receptor-1), CD204 and CD163 (macrophage scavenger receptors), are also overexpressed (28). These M2-like TAMs have critical roles in facilitating epithelial-mesenchymal transition (EMT), angiogenesis and immunosuppression (29, 30). Moreover, M2-like TAMs are one of the factors hampering the efficacy of chemotherapy and radiotherapy through suppression of $CD8^+$ T cell function, leading to tumor progression and poor outcomes (28, 31, 32). Additionally, we summarize several current markers linked with clinical outcomes that appear in studies of TAMs in different tumor types (**Table 1**).

However, the characteristics of TAMs summarized in **Figure 1** mostly correspond to conditions *in vitro*, and TAMs are not precisely divided into the M1 and M2 phenotypes *in vivo*, reflecting the insufficiency of this principle in understanding the comprehensive functions of TAMs due to the heterogeneity defined to date. Markers of the M1 and M2 phenotypes can be co-expressed on an individual cell (55). These markers also show defects when applied to differentiate the antitumor M1-like and pro-tumor M2-like phenotypes. For example, CD163 and CD206 are common M2-associated markers, but TAMs highly expressing CD163 and CD206 in gastrointestinal tumors or ovarian ascites were found to be functionally equivalent to M1-like TAMs with regard to stimulating T cell activity (56). The explanation for the link between TAM definition and function relies heavily on the M1-M2 paradigm, which may have greatly distorted our perception. Using current widely accepted biomarkers, whether TAMs actually exert pro-tumor or antitumor functions is unclear (57). Our understanding of functional markers may be far too simple to decipher the complex activation of TAMs in the TME.

EXPLORING THE MARKED DIVERSITY OF TAMs

Ontogeny of Monocytes and TAMs

Historically, macrophages in the TME were thought to originate from circulating monocyte precursors in the bone marrow (BM), responding to various tissue damage signals. In the

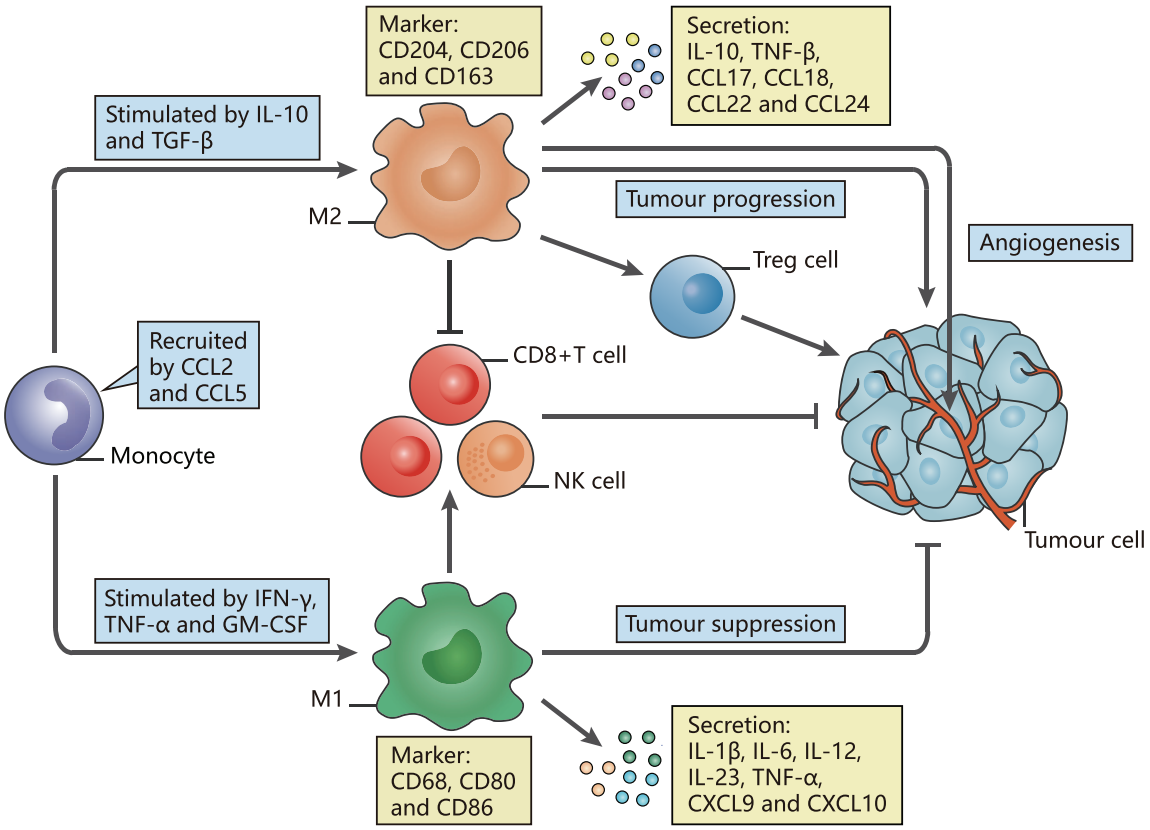

FIGURE 1 | The polarization of TAMs and their characteristics. The figure displays a general principle of polarized M1-like and M2-like phenotypes. M1-like and M2-like phenotypes represent two extremes of TAM polarization and display distinct functions. In response to different stimuli in the TME, TAMs undergo M1-like, or M2-like activation. M1-like TAMs are stimulated by IFN-γ, TGF-α, or GM-CSF, express CD68, CD80, and CD86, secrete IL-1β, IL-6, IL-12, IL-23, CXCL9, and CXCL10, and exert anti-tumor effects. In contrast, M2-like TAMs are activated by IL-10 or TGF-β, express CD163, CD204, and CD206, secrete IL-10, TNF, CCL17, CCL18, CCL22, and CCL24 and promote tumor progression.

search for TAM progenitors in mouse mammary tumors, studies show that tumor-infiltrating monocytes are almost exclusively distinguished by high expression of Ly6C, which serves as a principal marker of mouse monocytes. These Ly6C^{high} monocytes contribute to TAMs continuously and renew all non-proliferating TAM populations, constituting a heterogeneous myeloid fraction including M1-like MHC-II^{high} and M2-like MHC-II^{low} TAM subpopulations (58–60). Recent studies argued that recruited macrophages originated from both the BM and the spleen and suggested a minor splenic contribution to the main proportion of TAMs derived from the BM by utilizing a lineage-tracing analysis of fluorescent spleen- and BM-derived monocytes (61, 62). However, several studies have revealed that a group of macrophages reside in tissues beginning in the early embryonic phase (63, 64), further validating the coexistence of macrophages with different origins (65).

The origin and maintenance of these tissue-resident macrophages (TRMs) is controversial. TRMs were initially thought to originate from circulating monocytes. Recently, adult TRMs have been shown to derive from the continuous wave of embryonic and adult haematopoiesis, and the contribution to each TRM population is tissue specific (66, 67). Using parabiosis and genetic fate-mapping methods, studies have reported that TRMs in some tissues, such as the brain, are maintained locally and continue to undergo self-renewal throughout adult life with minimal contributions from circulating monocytes, while research on BM-derived mononuclear cells has indicated that TRMs in other tissues may have a relatively high monocyte contribution characterized by distinct increases at a tissue-specific speed under steady- and inflammatory-state conditions (66, 68–70). These observations suggest that the origin of TRMs is controlled under both inflammatory and stable conditions, exhibiting tissue-specific and inflammation-specific characteristics. To further identify the monocytic source of TRMs in various conditions, a study used a fate-mapping model with Ms4a3 as the specific gene expressed by granulocyte/monocyte progenitor cells to effectively track monocytes and granulocytes but not lymphocytes or tissue dendritic cells. As a result, the contribution of monocytes to the TRM pool was quantified, showing variations during homeostasis and inflammation in different models (71).

TABLE 1 | TAMs markers correlated with clinical outcomes and functions.

TAM marker	Tumor type	Level	Overall function	Clinical outcome	Function	References
CD68	Breast	High	Pro-tumor	Reduced OS Increased tumor stage and size	Promote invasion and lymphatic metastasis of breast cancer	(33)
CD68	Gastric	High	Pro-tumor	Reduced OS Lymph node metastasis Higher TNM stage	Enhance tumor growth and aggressiveness	(34)
CD68	Colorectal	High	Anti-tumor	Improved OS Reduced tumor budding	Counter the aggressive tumor budding phenotype	(35)
CD68	Prostate	High	Anti-tumor	Improved DFS Lower TNM stage	Express NOS2 and TNF-α Contribute to tumor cell cytotoxicity	(36)
CD163	Breast	High	Pro-tumor	Reduced RFS and DSS	Promote cancer cells migration and intravasation into both blood and lymphatic vessels	(37)
CD163	HNSCC	High	Pro-tumor	Poor OS and PFS	Promote tumor progression	(38)
CD163	Pancreatic	High	Pro-tumor	Reduced OS	Upregulate CD59 expression on cancer cells Protect cancer cells from complement-dependent cytotoxicity	(39)
CD163	Colorectal	High	Anti-tumor	Lower tumor grade Reduced lymph node metastasis	Counter cancer cell invasion	(35)
CD204	Breast	High	Pro-tumor	Poor OS, RFS and DMFS	Promote tumor cell proliferation, migration and invasion	(40)
CD204	LADC	High	Pro-tumor	Reduced DFS Advanced tumor stage Lymphovascular invasion Lymph node metastasis	Associated with tumor aggressiveness	(41)
CD204	Oesophageal	High	Pro-tumor	Reduced OS	Elevate the PD-L1 expression in cancer cells Promote tumor cell invasion and migration	(42)
CD206	Ovarian	High	Pro-tumor	Lymphatic invasion	Upregulate expressions of MMP-2, MMP-9 and MMP-10 Enhance ovarian cancer cells invasion via TLRs signaling pathway	(43)
CD206	OSCC	High	Pro-tumor	Reduced DSS and PFS Higher clinical stage Cervical nodal metastasis	Promote proliferation and invasion in OSCC via EGF production	(44)
Folate receptor β	Pancreatic	High	Pro-tumor	Reduced OS	Promote angiogenesis, hematogenous metastasis Upregulate expression of VEGF	(45)
Wnt5a+CD68+/ CD68+	Colorectal	Ratio high	Pro-tumor	Reduced RFS and OS Higher TNM stage	Secrete IL-10 to induce M2 polarization Promote tumor proliferation, migration and invasion (Wnt5a+CD68+ macrophages)	(46)
Galectin-9 and CD68	Bladder	High coexpression	Pro-tumor	Poor OS and RFS	Correlated with increasing numbers of Tregs and decreasing numbers of CD8+T cell Related to reduced cytotoxic molecules, enhanced immune checkpoints or immunosuppressive cytokines.	(47)
CD163+CD204+	OSCC	High	Pro-tumor	Reduced PFS	Promote T-cell apoptosis and immunosuppression via IL-10 and PD-L1	(48)
CD68++CD163+	Gastric	High*	Anti-tumor	Increased OS and RFS	Clear dead cells and remodel tissue	(49)
CD68 and HLA-DR	NSCLC	High coexpression	Anti-tumor	Increased survival time	Prevent progression of NSCLC	(50)

(Continued)

TABLE 1 | Continued

TAM marker	Tumor type	Level	Overall function	Clinical outcome	Function	References
CD68 and HLA-DR	NSCLC	High coexpression	Anti-tumor	Increased DSS	Exhibit antitumoral functions	(51)
CD68 and NOS2	Gastric	High coexpression	Anti-tumor	Preferent survival	Immuno-stimulatory	(52)
CD86	ICC	High	Anti-tumor	Longer median overall OS	Promote tumor cytotoxicity Amplify Th1 responses	(53)
NOS2	Colorectal	High	Anti-tumor	Increased RFS Improved survival in a stage dependent manner	Provide a positive feedback loop in anti-tumor response Tumor prevention	(54)

Wnt5a, Wnt family member 5A; NOS2, nitric oxide synthase-2; HNSCC, head and neck squamous cell carcinoma; LADC, lung adenocarcinoma; OSCC, oral squamous cell carcinoma; NSCLC, Non-small cell lung cancer; ICC, intrahepatic cholangiocarcinoma; OS, overall survival; TNM, tumor-node-metastasis; DFS, disease-free survival; RFS, relapse free survival; DS S, disease-specific survival; PFS, progression-free survival; DJ'v:IFS, distant metastasis survival.

**Only in the effective density (effective density: the number of TAM that had a tumor cell within a 10 f!m radius).*

The relative contributions of monocyte-derived TAMs and TRMs in tumor models have also been revealed. Recent cell tracking studies have illustrated at the molecular level that recruited macrophages predominate in the TME because a significant decrease in TAM abundance is often followed by blockade of CC chemokine receptor 2 (the receptor for CCL2, which plays a pivotal role in the recruitment of TAMs) in most cases (72). In research on glioblastoma, inflammatory monocytes/macrophages have been revealed to express both CCR2 and CX3CR1, but microglia express only CX3CR1. Due to such distinct molecular identification, multiparameter flow cytometry analyses have further validated that the relative proportion of more than 85% of the TAMs within tumors are BM-derived macrophages, whereas resident microglia account for the remaining approximate proportion of 15% (73). For the purpose of future functional studies, more evidence for definitive identification of monocyte-derived TAMs and TRMs is required.

Moreover, attention has been gradually drawn to the questionable dictation of functions driven by multiple origins of TAMs. The interplay between TAMs with different origins remains to be elucidated, but studies in mouse models can provide evidence to some extent. Notably, several studies have discussed TRMs and monocyte-derived TAMs in terms of distinct overall functions and genomic differences. TRMs in a mouse model of PDAC were shown to promote PDAC progression with fibrosis-modulating functions, while impairment of circulating monocytes alone had limited impacts (74). Monocyte-derived TAMs have also been suggested to play a stronger role in antigen presentation, whereas TRMs exhibit a profibrotic transcriptional signature, indicating their role in the production and remodeling of molecules in the extracellular matrix (74). Such distinct functions were confirmed in another case of lung cancer, where TRMs were significantly correlated with tumor cell growth *in vivo*, while accumulation of monocyte-derived TAMs led to enhanced tumor dissemination (75). To evaluate differences at the genomic level, a study on glioma using transgenic mice models validated that glioma TAMs expressed Arg1 (an M2 marker) shortly after trafficking into tumors, the expression of which was stimulated relatively later by microglia (76), which may reveal different responses of TAMs with distinct origins to tumor growth. Collectively, these findings may jointly suggest a potential influence of TAM origin on functional changes, which requires further exploration.

However, the differences between mouse and human monocytes, such as the repertoire of surface receptors, must be emphasized (14). The classification of mouse monocyte subsets relies on the differential expression of Ly6C, while human monocytes can mainly be characterized by the expression of CD14 and CD16 into distinct subpopulations of $CD14^{+}CD16^{-}$and $CD14^{lo}CD16^{+}$ monocytes (60, 77, 78). $CD14^{+}CD16^{-}$ monocytes have been proposed to share similarities with $Ly6C^{hi}$ mouse monocytes in the expression patterns of certain molecules. Meanwhile, $CD14^{lo}CD16^{+}$ human monocytes are counterparts to $Ly6C^{lo}$ mouse monocytes (79). A study compared the gene expression profiles of monocyte subsets and found some conversely expressed molecules between matched subsets of the two species, such as CD36, CD9, and TREM-1. Peroxisome proliferator activated receptor (PPAR) has also been validated to be prominently expressed in mouse monocytes, which is absent in humans (80). Additionally, specific cytokines regulating cells vary in mice and humans, which also contribute to the differences between the two species (81). Considering the functional differences in monocyte subsets in phagocytic capacity, which is regarded as one of the cardinal features of blood monocytes, patterns of receptors involved in the uptake of apoptotic cells and other phagocytic cargo were also shown to differ in monocyte subsets of humans and mice (80). Most of our current knowledge depends on mouse models, and whether the origins of human TRMs match those of mice remains to be considered. Comparisons of distinct types of monocyte-derived macrophages with TRMs in humans revealed a lack of specific markers indicative of the subset of origin, resulting in incomplete knowledge of their functions among various cancer types (24, 82, 83), which may indicate the need for further investigation to address the possible different monocyte lineages in humans.

Plasticity of TAMs

TAMs can further display remarkable plasticity within the TME and switch from one phenotype to another (84). However, the M1/M2 paradigm represents two extreme activation states of TAMs, which may neglect that the adaption driven by environmental signals in the TME is flexible rather than static. These environmental cues are mainly stimulated by tumor cells, immune cells, and the extracellular matrix (85). TAMs show the ability to reversibly respond to specific stimuli in the TME, transforming antitumor M1-like and pro-tumor M2-like phenotypes during the immune response under certain conditions. Such plasticity also results in diverse subpopulations of TAMs.

The pro-inflammatory M1-like phenotype in the TME may evolve into the M2-like phenotype following tumor progression, thus exerting a tumor-supporting influence (86). Chemokines such as CXCL12 can be highly secreted from monocytes during tumor progression and then facilitate the transition from M1-like to M2-like TAMs, forming a proangiogenic and immunosuppressive response with upregulation of M2 inducers (87). Recently, the adaptative ability of TAMs has been further validated in pre-clinical models and clinical trials, and the regulation of TAM polarization to enhance antitumor functions has been successfully stimulated. The PI3K-γ pathway and colony-stimulating factor 1 (CSF-1)/colony-stimulating factor 1 receptor (CSF-1R) expression are generally considered important in the polarization of M2-like TAMs (88, 89). A study reported that dual blockade of the PI3K-γ pathway and CSF-1/CSF-1R resulted in a switch from an M2-like state to an M1-like state in PDAC models (90), leading to a reduction in immunosuppressive macrophage numbers and stimulation of a $CD8^+$ T cell response. Inhibition of CSF-1/CSF-1R alone has achieved similar effects in other models of glioblastoma (91), melanoma (92), and rhabdomyosarcoma (93). A CD40 agonist has also been reported to stimulate the transformation of a pro-tumor M2-like state into an antitumor M1-like state in a PDAC mouse model, enhancing antitumor immune responses (94). Signal transducer and activator of transcription (STAT) 3/STAT6 can reportedly direct tumor-promoting macrophage polarization. A small-molecule inhibitor of STAT3 significantly reduced M2-like polarization in a case of malignant glioma, while TAMs in STAT6-deficient mice displayed an M1-like phenotype, enhancing antitumor immunity (95). Additional targets, such as CCL5-CCR5, IL-12, histone deacetylases (HDACs), and tyrosine-protein kinase receptor 2 (TIE2), have been explored to reprogramme TAMs to suppress tumor growth (96). Taken together, these results suggest that suppressing the tumor-promoting functions of TAMs can elevate antitumor activities and reverse the immunosuppressive status in the TME.

The plasticity of macrophages highlights macrophage reprogramming as an attractive therapeutic strategy to inhibit tumor progression, enabling these cells to adapt their function to meet the needs of antitumor defense. To better understand the activated status of TAMs within the TME, further studies on specific markers to differentiate the distinct functions of antitumor and pro-tumor TAMs are in high demand.

Intra-Tumoral Heterogeneities of TAMs

In addition to inter-tumoral heterogeneity, several factors may contribute to the intra-tumoral heterogeneity of TAMs, especially tumor hypoxia and the distribution of TAMs in the TME.

Hypoxia, which often develops within the TME due to an imbalance between oxygen supply and demand caused by abnormal vasculature, acts as a powerful attractant of TAMs (97). TAMs can be continuously elicited to hypoxic regions through elevated expression of hypoxia-inducible factor (HIF)-1α, a key transcription factor that regulates hypoxia-induced gene expression. HIF-1α upregulates CXC receptor 4 (CXCR4) in monocytes/macrophages and the specific ligand CXCL12 and also induces the chemotactic responsiveness among these reactants (98, 99). Moreover, TAM migration may be inhibited under the influence of hypoxia, resulting in TAM accumulation (98). Consequently, TAMs are recruited and maintained in hypoxic compartments with increased expression of chemoattractants, thus fostering tumor progression (100). Strong tumor hypoxia and high-density hypoxic TAMs have been associated with poor survival, highlighting the clinical significance of hypoxia (101).

Notably, hypoxia contributes significantly to the pro-tumoral functions of MHC-II^{lo} M2-like TAMs by altering gene expression profiles rather than directly influencing TAM differentiation (102). TAMs are prone to develop a pro-angiogenic phenotype under the influence of hypoxia, which is involved in metabolism, angiogenesis, and metastasis. When assessing intra-tumoral localization depending on the level of hypoxia, a study selectively labeled MHC-II^{lo} M2-like TAMs and MHC-II^{hi} M1-like TAMs and found that MHC-II^{lo} TAMs predominated in hypoxic regions, while MHC-II^{hi} TAMs resided in less hypoxic areas. These hypoxia-oriented TAMs achieve a proangiogenic response not only by directly upregulating angiogenic molecules, especially VEGF-A, which is a potent pro-angiogenic factor (60), but also through upregulation of angiogenic modulators such as matrix metalloproteinase (MMP) 7 (103). Additionally, hypoxia-oriented TAMs may also suppress T cell activation through upregulation of IL-10 and negative checkpoint regulators such as PD-L1 (104). A recent study also showed an increased level of indoleamine 2, 3-dioxygenase (IDO) when co-culturing macrophages with hepatoma cells under hypoxic conditions, limiting the proliferation of cytotoxic T cells as well as expanding Treg cells (105). In contrast, impeding TAM migration to hypoxic areas may result in a more antitumoral macrophage phenotype and reduced tumor growth. A study established that the Sema3A/Neuropilin-1 signaling axis controlled the entry of TAMs into hypoxic regions, and that specifically blunting this pathway enhanced antitumor immunity and alleviated angiogenesis, thus inhibiting tumor growth and metastasis (104). This phenomenon demonstrates the interaction between TAMs and tumor hypoxia, highlighting the partial intra-tumoral heterogeneity determined by hypoxia.

Similar to hypoxia, different histological distributions of TAMs have also been correlated with distinct tumor progression according to a large number of experimental studies in mice, which are usually divided into the tumor nest (TN),

tumor stroma (TS) and invasive tumor margin (TM). In a study evaluating the distribution of TAMs and the associated survival rate in gastric cancer (GC), increased $CD163^+$ TAM accumulation in the TS and TM was found to be closely related to tumor progression, whereas the relationship between $CD163^+$ TAMs in the TN and tumor progression was not as close as that between $CD163^+$ TAMs and the TS or TM (106). The prominent role that TAMs in the TS play in tumor progression over those in the TN has also been validated in other types of tumors (107, 108). Moreover, specific localization of TAMs may also impact how they affect tumor growth; for instance, TN-associated macrophages are more pro-angiogenic than macrophages in the TS in breast cancer (109). These functional variations of TAMs may be attributed to their histological locations within the TME and the intra-tumoral heterogeneity of TAMs.

Overall, the functions of TAM subsets exhibit significant intra-heterogeneity in the TME. TAM subsets with distinct functions in the TME must be redefined. An improved understanding of how TAM subsets are influenced by intraregional conditions such as hypoxia and histological distributions will certainly benefit related therapeutic approaches.

EMERGING TECHNOLOGIES FOR ANALYZING FUNCTIONAL BIOMARKERS IN SUBSETS OF TAMs

To further analyze functional biomarkers of TAMs in the TME, high-resolution information is needed to investigate distinct TAM subtypes, which can be obtained by utilizing some distinctive, newly emerging technologies, including mIHC, CyTOF, high-throughput scRNA-seq, and spatial transcriptomics (28).

mIHC

Along with downstream quantitative image analysis, mIHC represents a powerful tool to visualize and analyze complex cell-to-cell and cell-to-stroma interactions within tumors (110). An increasing number of automated digital pathology systems are being designed for mIHC analysis, such as HALO from Indica Labs (Corrales, NM), which enables tissue segmentation with artificial intelligence and quantifies various histopathological changes as an outstanding image analysis platform (111). Updated information regarding recent technological advances can be found in some reviews (112).

Mass Cytometry

CyTOF is a next-generation platform for single-cell assessment that overcomes the spectral limitation by replacing fluorophores with metal isotope labels for probes (such as antibodies and RNA probes) (113). Despite its dependence on preselected markers and loss of spatial information, CyTOF contributes substantially to a more comprehensive understanding of cellular phenotypic signatures with the quantification of multiple surface and intracellular proteins, which has been widely applied to explore the phenotypic complexity of microenvironments, such as those of lung adenocarcinoma (114) and diffuse astrocytomas (115). Useful protocols for CyTOF analysis can be referenced in several detailed reviews (116, 117).

Bulk RNA-seq and scRNA-seq

Compared with bulk RNA-seq, scRNA-seq focuses more on cell heterogeneity, which may address TAM complexity via an unbiased analysis of cells based on transcriptomic profiles and greatly revolutionizes transcriptomic studies. To date, numerous scRNA-seq technologies have been developed for single-cell transcriptomic studies, which are specifically designed for full-length transcripts and the 3′-end or 5′-end of the transcripts. Advanced scRNA-seq methods can provide unprecedented opportunities to comprehensively explore the expression dynamics of both protein-coding and non-coding RNAs at the single-cell level (118). In addition to sequencing, subsequent computational data analyses are critical because scRNA-seq is associated with higher dropouts and nosier data than bulk RNA-seq due to a lower amount of initial material (119). Some reviews have summarized distinct software and applications available for various research purposes (120, 121).

Spatial Transcriptomics

Recently developed spatial transcriptomics can be complementary to provide detailed visualized spatial information (122). This method can not only offer high-resolution *in situ* gene expression profiles and reveal the molecular genealogy of tissue lineages but also define continuous temporal and spatial pluripotency states, thus identifying the networks of molecular determinants driving lineage specification and tissue organization. Relevant protocols are available in some references (123), and several applications are specialized for spatial transcriptomics, such as ST Spot Detector (124) and ST viewer (125).

INTEGRATED STRATEGIES TO REDEFINE THE SUBPOPULATIONS AND FUNCTIONS OF TAMs

To overcome the limitations of applying a single method, the integration of novel methods may provide a preferable solution that may be complementary when characterizing TAMs. The use of multiple techniques has already been applied to study cell heterogeneity and highlight the utility of integrated strategies, such as assessing all haematopoietic cells by scRNA-seq and CyTOF (126), revealing the landscape of immune cells in hepatocellular carcinoma with two different kinds of scRNA-seq (127), and studying the infiltration of cells in PDAC with scRNA-seq and spatial transcriptomics (128). We will further describe a more detailed methodology in proteomic, transcriptomic, spatial, and functional dimensions to examine TAM diversity at the single-cell level, thus generating a complete landscape of TAMs in the TME (**Figure 2**).

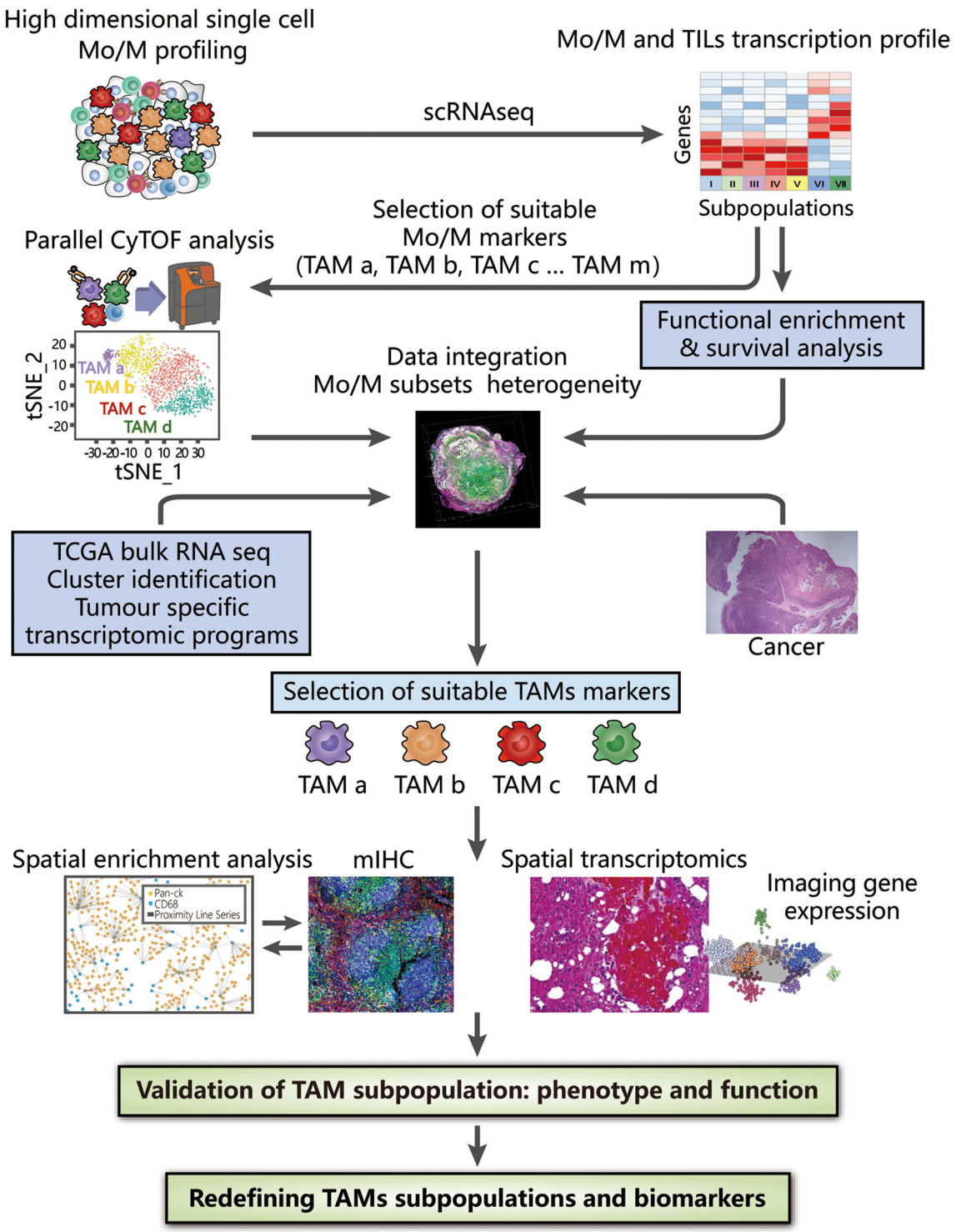

FIGURE 2 | Integrated strategies to redefine the classification of TAMs. High-dimensional analysis of TAMs supported by CyTOF and scRNA-seq, along with bioinformatic approaches (including dimension reduction tools and cluster analysis), provides an overview of surface protein and gene expression, thus contributing to the identification of TAM subsets at the proteomic and transcriptomic levels. Clusters of interest can then be selected depending on either different compositions or distinct functions among identified TAM subpopulations, which are associated with their histopathological characteristics in tissue samples and clinical significance confirmed by survival analysis. By combining bulk RNA-seq data obtained from TCGA and tumor-specific transcriptomic programme, the heterogeneity of TAMs can be further analyzed to provide evidence for the selection of suitable TAM markers. Based on these markers, the spatial distribution in the TME obtained by mIHC and spatial transcriptomics facilitate subsequent generation of the complete landscape in tumor tissues and deconvolution of cell-state relationships, benefiting a deeper understanding of the associations between the functions and phenotypes of TAMs. The integrated use of these technologies strongly reveals the inter- and intra-tumoral heterogeneity of TAMs, potentially redefining TAMs with valuable biomarkers.

Identifying TAM Subtypes at the Proteomic and Transcriptomic Levels With CyTOF and scRNA-seq

To identify TAM subtypes at either the proteomic or transcriptomic level, using CyTOF and scRNA-seq together can reveal the diversity of TAMs in detail (126). The gene expression profile generated by scRNA-seq can guide the selection of specific markers of monocytes/macrophages for CyTOF analyses to explore TAM subpopulations at the proteomic level following similar data processing procedures. This combined application also serves as an alternative to further investigate whether any correlation exists between protein and gene expression. The complementary integration of CyTOF and scRNA-seq may potentially show an overlap, thus generating meticulous and complete profiles of both the phenotypes and transcriptomes of TAMs.

Analyzing Different Compositions or Overall Functional Differences in Identified TAM Subpopulations Among Varying Histopathological Conditions

To distinguish TAM subpopulations with distinct functions, analyses can be conducted based on either different compositions or overall functions through functional enrichment analysis, differential gene expression analysis and survival analysis (126, 129).

Functional enrichment analysis annotates possible immunosuppressive or antitumor functions and pathways of the proteins or genes in highlighted clusters (130). An analysis can be easily performed through the DAVID website, which is one of the most frequently employed enrichment analysis approaches (131). With identified survival-related subpopulations of TAMs through survival analysis, we can then highlight the markers of these clusters and conduct more specific functional studies on them.

Examining the possible overall difference in functions among diverse conditions is usually most feasible and may lead to the discovery of clusters enriched in specific pathways associated with functions of either tumor promotion or suppression. Moreover, we may then explore and discuss the differentially expressed biomarkers among these identified clusters, thus contributing to research on potential biological differences.

Associating With Bulk RNA-seq Profiles From TCGA and Tumor-Specific Transcriptomic Programme

Notably, scRNA-seq serves as a powerful tool to reveal TAM heterogeneity but may be limited by insufficient specimens. Bulk RNA-seq profiles provided by The Cancer Genome Atlas (TCGA), which contains large cohorts of samples and reports the averaged gene expression across a broad range of cells in various tumor types, can supplement previous results obtained by scRNA-seq, enabling further identification of TAM subpopulations and validation of bulk transcriptomic data. Scrutinizing previous findings with a wider range of statistics in TCGA is essential to reach more reliable conclusions regarding functional associations (132).

Moreover, a tumor-specific transcriptomic programme can be applied to identify relationships between somatic mutation alterations, which are considered a main cause of cancer and differentially expressed genes within tumors. Such a programme can also focus on networks of tumor-specific genes to measure the activation status of corresponding pathways, further indicating the inter- and intra-tumoral heterogeneity together with previous approaches.

Mapping Distinct TAM Subpopulations and Deconvolving Cell-State Relationships Within the TME

Supplementary application of mIHC or spatial transcriptomics can be employed to further determine how the local microenvironment may impact cell functions and the cell state. A computer-supported workflow can be generally followed to quantitatively characterize the spatial heterogeneity of TAMs in the TME with different metrics, including cell density, cell/cluster, the mean distance, and the cluster area. In this workflow, image analysis algorithms are first employed to identify and locate TAMs in the patient tissue sample, mapping out the coordinates of each cell of interest and facilitating successive analysis of spatial point patterns and morphology. On the one hand, intra-tumoral heterogeneity can be determined directly through application of the spatial point process model in the full-point mode sliding window. On the other hand, morphological analysis extracts information to illustrate inter-cellular interactions and related geometric properties of the cell clusters. Moreover, calculated indicators of each cluster further guide the establishment and validation of systems biology models for immune-oncology research as well as their associated prognostic outcomes.

Meanwhile, spatial transcriptomic technology is a favorable alternative to spatial techniques designed to evaluate the gene expression profile of single cells, which complements missing spatial information in scRNA-seq. Using histological tissue sections and spatial barcoding to analyze gene expression, as well as downstream analysis, we can generate sample clusters that correspond to well-defined morphological features and unbiasedly detect the spatial distributions of marker genes in tumor tissue samples.

Overall, the integrated application of high-throughput techniques overcomes the limitations of each method and results in a complementary profile on the basis of phenotype, transcriptome, and infiltration status (133). Although TAMs have been proposed as novel therapeutic targets and several treatments to eradicate or modulate TAMs are being evaluated (134), a major gap exists in our current understanding of diverse TAM subsets, biomarkers, and their functions. This methodological strategy focuses on examining specific TAM heterogeneity in primary tumors as well as their metastases in multiple dimensions and benefits researchers studying how ontogeny, activation status and localization dictate macrophage biomarkers, undoubtedly showing a promising ability to discriminate TAM subsets with

specific biomarkers and to establish firm correlations between particular TAM populations and clinical outcomes. Such research into heterogeneous TAM biology will be highly relevant to the design of new and specific antitumor therapies targeting TAMs to achieve therapeutic effects as well as possible.

SUMMARY AND OUTLOOK

TAMs, the key components in the TME, are related to tumor invasion and metastasis and have shown emerging potential as new targets for cancer immunotherapy (135). As thoroughly discussed, the ontogeny, plasticity, and inter- and intra-tumoral heterogeneity of TAMs have complicated defining the exact function of TAMs from the currently limited understanding of TAM phenotypes, emphasizing the necessity of redefining the subtypes and functions of TAMs in the TME.

In this review, we provide a framework to decipher differences among TAM functional phenotypes and their composition in the TME, establishing an all-encompassing analysis that includes the phenotypes, transcriptomes, spatial distributions, and functions of TAM subsets with the integration of novel techniques to offer more detailed and complementary information. This approach may show unique advantages that bypass the limitations of each technique in studies.

Through our strategy, we (1) identify TAM subpopulations at the phenotypic and transcriptomic levels in a single-cell solution; (2) associate TAM subpopulations with histopathological and clinical characteristics, identifying TAM subtype-specific markers for spatial studies; (3) generate a complete landscape of the TME and map TAM subpopulations to deconvolve cell-state relationships; and (4) highlight valuable TAM subpopulations. This review offers important insight into redefinition of TAMs with functional biomarkers. From a therapeutic standpoint, our strategy offers the possibility of precisely targeting TAM subpopulations with distinct antitumor functions. However, more joint efforts are warranted to generate a common vision of TAM heterogeneity.

AUTHOR CONTRIBUTIONS

DX designed the structure of this article. KW, KL, and DX wrote the manuscript. XY reviewed the manuscript. XL, PX, and PN made substantial and intellectual contributions to the work. All authors approved the article for publication.

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