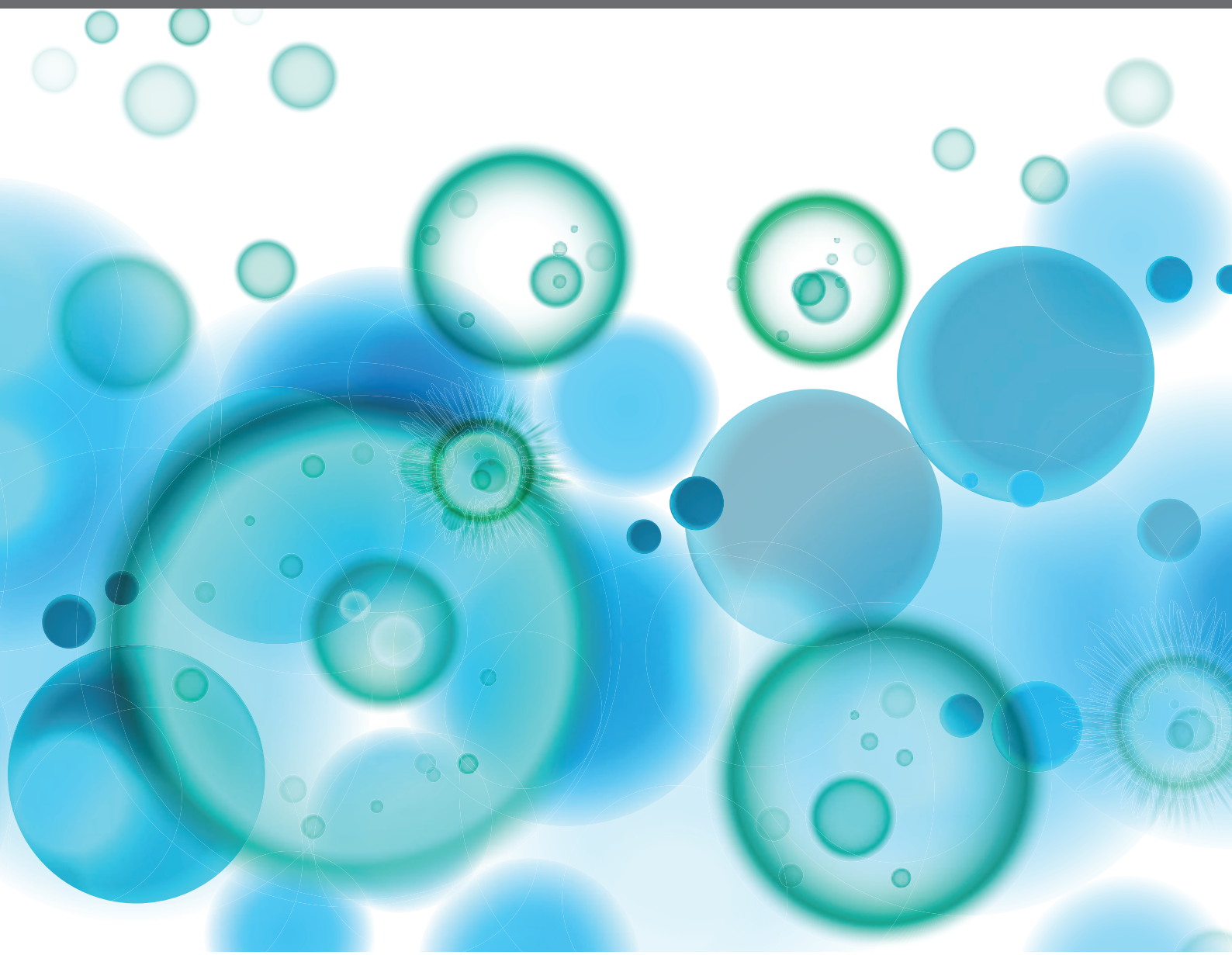
TARGETING MYELOID CELLS TO FIGHT CANCER

EDITED BY: Maija Hollmén, Wei Zheng and Jeffrey W. Pollard
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-393-7
DOI 10.3389/978-2-88963-393-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

TARGETING MYELOID CELLS TO FIGHT CANCER

Topic Editors:
Maija Hollmén, University of Turku, Finland
Wei Zheng, Icahn School of Medicine at Mount Sinai, United States
Jeffrey W. Pollard, University of Edinburgh, United Kingdom

Citation: Hollmén, M., Zheng, W., Pollard, J. W., eds. (2020). Targeting Myeloid Cells to Fight Cancer. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-393-7

Table of Contents

frontiers in Immunology

EDITORIAL
published: 03 December 2019
doi: 10.3389/fimmu.2019.02835

Editorial: Targeting Myeloid Cells to Fight Cancer

Maija Hollmén[1]*, *Wei Zheng*[2] *and Jeffrey W. Pollard*[3,4]

[1] MediCity Research Laboratory, University of Turku, Turku, Finland, [2] Icahn School of Medicine at Mount Sinai, New York, NY, United States, [3] MRC Centre for Reproductive Health, The University of Edinburgh, Edinburgh, United Kingdom, [4] Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, NY, United States

Keywords: tumor microenvironment, monocyte, macrophage, neutrophil, immunosuppression, immunotherapy

Editorial on the Research Topic

Targeting Myeloid Cells to Fight Cancer

Refractory and relapsed cancer patients often develop resistance mechanisms associated with an immunosuppressive tumor microenvironment (TME). Indeed, the TME is critically involved not only in tumor growth, invasion and dissemination, but in immune editing and resistance to different therapies both conventional and biological. Innate myeloid cells such as monocytes, macrophages, neutrophils, and a diverse set of cells, sometime called myeloid derived suppressor cells (MDSCs) may drive both local and systemic immunosuppression. These cells, however, possess novel molecules and functions that could be targeted to fight against cancer making them potential candidates for development of second-generation immunotherapy approaches. The aim of this article collection is to provide a comprehensive overview of the different myeloid subsets in the TME, and to describe recent developments and approaches targeting myeloid cells to enhance anti-tumor immunity and the clinical efficacy of standard-of-care cancer drugs.

HETEROGENEITY OF MYELOID POPULATIONS

Neutrophils exhibit diverse and sometimes contradictory roles in the TME. The review by Jeong et al. summarizes the regulation of neutrophils that determines the reciprocal dynamics between them and tumor cells. This continuous interaction has expanded to a formidably complicated network, including tumor types, tumor stages, subtypes of neutrophils, various signaling molecules mediating crosstalk, and spatial-temporal interactions with other cell types. This ever-growing complexity as reviewed by Jeong et al. has revealed how much more there is still to be learned to be able to achieve effective and consistent therapeutic effects based upon targeting neutrophils. This diversity is a point emphasized in the mini-review by Granot who suggests that simple binary classifications are insufficient and that the context defines neutrophil types and functions. Mackey et al. in their minireview address a particular aspect of the contextual regulation of neutrophils in the TME, i.e. neutrophil maturation outside the bone marrow. This atypical maturation and notably the promotion of immature neutrophils by tumors underlie many of the different responses of neutrophils to cancer.

Similar to neutrophils, monocytes also show significant diversity in the TME. In their review, Canè et al. support the notion of a "monocyte continuum" instead of classification of stepwise differences between the different subtypes. They also maintain that monocytes are more than precursors of macrophages and dendritic cells but also make a direct contribution to cancer development themselves. An additional feature of this review is the summary of current monocyte-targeting drugs and their mechanisms of actions. Along these lines, Mengos et al. describes the documented clinical findings on a $CD14^{+}HLA\text{-}DR^{lo/neg}$ monocyte population

Edited by:
Fabrizio Mattei,
National Institute of Health (ISS), Italy

Reviewed by:
Ekaterina Jordanova,
Center for Gynaecologic Oncology Amsterdam, Netherlands
Viktor Umansky,
German Cancer Research Center (DKFZ), Germany

***Correspondence:**
Maija Hollmén
maijal@utu.fi

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *28 October 2019*
Accepted: *18 November 2019*
Published: *03 December 2019*

Citation:
Hollmén M, Zheng W and Pollard JW (2019) Editorial: Targeting Myeloid Cells to Fight Cancer. Front. Immunol. 10:2835. doi: 10.3389/fimmu.2019.02835

that has emerged as an important mediator of tumor-induced immunosuppression. Different from MDSCs, these cells arise from the regular circulating monocyte pool, which lose HLA-DR expression and get deactivated after an acute or chronic inflammatory trigger leading to immune paralysis. The immunosuppressive monocytes can negatively affect responses to immune checkpoint inhibitors and could be potentially used as biomarkers to understand disparate responses to immune activating drugs, in addition to their potential targeting to increase immunotherapy responses.

SEEING IS BELIEVING

Cellular processes as analyzed by tissue sections at a certain time point may be missed, impossible to obtain or even misinterpreted. With new technologies, intravital optical imaging is providing an unprecedented opportunity to look at cancer cells and stromal cells in their native environment and thus yields arguably the most reliable results in interpreting the TME. Laviron et al. examine the pros and cons of intravital imaging as compared to other approaches, such as cell sorting and sequencing, and suggest that an integrative approach offers the most valuable information.

COMPOUNDS AFFECTING MYELOID FUNCTIONS IN THE TME

The mode of action of several FDA approved drugs such as bisphosphonates, trabectedin, imatinib, and sunitinib has been shown to involve the modulation of macrophage functions in addition to their respective targets. Whether their macrophage targeting effects are significantly contributing to drug efficacy remains to be investigated. In their article Tanita et al. shed light on the mode of action of bexarotene, a third-generation retinoid that has been used for decades in the treatment of both early and advanced cutaneous T-cell lymphomas (CTCL). The authors show that bexarotene decreases CCL22 production by $CD163^+$ macrophages in CTCL patients. Since CCL22 attracts $CCR4^+$ lymphocytes, such as Tregs, Th2 cells, and also CTCL cells to support tumor progression, this study proposes the potential of targeting tumor associated macrophages (TAMs) in CTCL for improved patient outcome (Tanita et al.)

Another interesting concept is reported by Willebrand et al. where they studied the effect of high salt intake on the functional modulation of MDSCs in the TME. Excess salt intake has been previously shown to balance various innate and adaptive immune cells toward a pro-inflammatory state, and believed to be associated with several autoimmune disorders. Therefore, an interesting question that the authors raised was whether high salt conditions can promote anti-tumor immunity to inhibit tumor growth? Surprisingly, the authors find that, while high-salt diet altered T cell populations, the delay in tumor growth was largely mediated by an impairment in MDSC suppressive functions (Willebrand et al.) Thus, high salt-induced molecular changes could be potentially utilized during immunotherapy.

POSSIBILITIES TO TARGET MYELOID CELLS FOR IMPROVED CANCER CARE

As a general assumption, macrophages are mostly considered to have an unfavorable role in cancer since they are effective suppressors of anti-tumor immune responses and can contribute to tumor progression in multiple ways. Tumor growth is often compared to the wound healing process and is metaphorically described as a "wound that does not heal." Hua and Bergers focus on two common components in these two intricately related processes—blood vessels and immune cells. They explain how tumor cells hijack the immunosuppressive and angiogenic programs that occur during the resolution phase of wound healing toward their own ends. The authors thus suggest targeting both myeloid cells and angiogenesis to revert the hijacked wound-healing process.

In a mini review Bercovici et al. discuss the plasticity of macrophages and focus on macrophage regulated intratumoral T cell migration and activation. The authors emphasize that T cell focused treatments should be systematically replaced by rational combination treatments stimulating both innate and adaptive arms of the immune system. When appropriately stimulated macrophages can effectively induce anti-tumor responses in cooperation with T cells. To date, only a few myeloid cell targeting strategies under clinical development have yielded promising results and many have been terminated due to toxicities. In their review Jahchan et al., indicate the potential of targeting TAMs in cancer immunotherapy. They make a persuasive case for such approaches through the targeting of particular functions of TAMs or by altering their differentiation to anti-tumoral responses either directly or through the enhancement of immunotherapy. They also provide an authoritative update of all current clinical trials using these approaches.

As shown by this collection of articles, a tremendous diversity of myeloid cells orchestrate tumor-stromal and stromal-stromal interactions to regulate the final outcome of the disease. Elucidation of the detailed mechanisms and systemic rational evaluation of therapeutic targeting of these cells may be the key to fighting cancer.

AUTHOR CONTRIBUTIONS

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

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

frontiers in Immunology

REVIEW
published: 22 May 2019
doi: 10.3389/fimmu.2019.01147

The CD14^{+}HLA-DR$^{lo/neg}$ Monocyte: An Immunosuppressive Phenotype That Restrains Responses to Cancer Immunotherapy

*April E. Mengos, Dennis A. Gastineau and Michael P. Gustafson**

Nyberg Human Cellular Therapy Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic, Phoenix, AZ, United States

OPEN ACCESS

Edited by:
Maija Hollmén,
University of Turku, Finland

Reviewed by:
Eyad Elkord,
University of Salford, United Kingdom
Rolf Kiessling,
Karolinska Institute (KI), Sweden

***Correspondence:**
Michael P. Gustafson
gustafson.michael@mayo.edu

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *19 March 2019*
Accepted: *07 May 2019*
Published: *22 May 2019*

Citation:
Mengos AE, Gastineau DA and Gustafson MP (2019) The CD14^{+}HLA-DR$^{lo/neg}$ Monocyte: An Immunosuppressive Phenotype That Restrains Responses to Cancer Immunotherapy. Front. Immunol. 10:1147. doi: 10.3389/fimmu.2019.01147

Recent successes in cancer immunotherapy have been tempered by sub-optimal clinical responses in the majority of patients. The impaired anti-tumor immune responses observed in these patients are likely a consequence of immune system dysfunction contributed to by a variety of factors that include, but are not limited to, diminished antigen presentation/detection, leukopenia, a coordinated network of immunosuppressive cell surface proteins, cytokines and cellular mediators. Monocytes that have diminished or no HLA-DR expression, called CD14^{+}HLA-DR$^{lo/neg}$ monocytes, have emerged as important mediators of tumor-induced immunosuppression. These cells have been grouped into a larger class of suppressive cells called myeloid derived suppressor cells (MDSCs) and are commonly referred to as monocytic myeloid derived suppressor cells. CD14^{+}HLA-DR$^{lo/neg}$ monocytes were first characterized in patients with sepsis and were shown to regulate the transition from the inflammatory state to immune suppression, ultimately leading to immune paralysis. These immunosuppressive monocytes have also recently been shown to negatively affect responses to PD-1 and CTLA-4 checkpoint inhibition, CAR-T cell therapy, cancer vaccines, and hematopoietic stem cell transplantation. Ultimately, the goal is to understand the role of these cells in the context of immunosuppression not only to facilitate the development of targeted therapies to circumvent their effects, but also to potentially use them as a biomarker for understanding disparate responses to immunotherapeutic regimens. Practical aspects to be explored for development of CD14^{+}HLA-DR$^{lo/neg}$ monocyte detection in patients are the standardization of flow cytometric gating methods to assess HLA-DR expression,

an appropriate quantitation method, test sample type, and processing guidances. Once detection methods are established that yield consistently reproducible results, then further progress can be made toward understanding the role of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in the immunosuppressive state.

Keywords: immunosuppression, monocytes, MDSCs, cancer, immunotherapy, biomarker

INTRODUCTION

Exquisitely and carefully modulated immune responses coordinate the balance between preventing microbial onslaught and preventing autoimmune attack. Too little immune activation results in insufficient clearance of foreign invaders, and too much immune activation results in the targeting of self-antigens and potentially devastating autoimmune syndromes. This finely choreographed tightrope act is accomplished, in part, by a specialized array of immune cells which patrol the body and exert immunomodulatory roles. While in the larger context these cells exert the beneficial tempering of immune over-responsiveness, they can also by similar mechanisms negatively impact anti-cancer immunotherapy efficacy.

The goal of cancer immunotherapy is to successfully stimulate anti-tumor responses and overcome tumor-mediated immunosuppression. Generation of anti-tumor immunity has been accomplished through different modalities including cellular immunotherapy, vaccines, monoclonal antibodies, cytokine administration, and oncolytic virotherapy. These multi-faceted approaches have yielded tremendous clinical successes in the past few years. Even so, there have been significant difficulties in generating durable responses in a majority of cancer patients. As such, further understanding of immune dysfunction and the identification of predictive biomarkers are required so that methods may be developed to increase the efficacy of immunotherapeutic agents.

Recent data reveal that the potential exists to utilize the assessment of $CD14^+HLA\text{-}DR^{lo/neg}$ monocyte abundance as a biomarker to predict which patients may or may not respond to immunotherapy regimens. For $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in particular, an extensive array of studies involving immunotherapy demonstrate that high baseline levels of these immunosuppressive monocytes were associated with diminished anti-tumor responses and/or poor clinical outcomes. As $CD14^+$ monocytes lose HLA-DR expression and thus convert from an inflammatory to an anti-inflammatory phenotype, they play a role in subverting effective anti-tumor responses, and their abundance in patient blood inversely correlates with favorable outcomes.

The purpose of this review is to highlight the documented findings which demonstrate this correlation. In the context of cancer immunotherapy, the abundance of these cells may guide patient selection and/or provide patient monitoring capabilities for understanding clinical responses on the individual level. Since there are considerable differences in the biology of these cells in animal models compared to human studies, this review will focus mainly on published data from human studies and clinical trials. However, in some cases, examples may be provided from animal models where the observations appear to be congruent with human data.

$CD14^+HLA\text{-}DR^{lo/neg}$ MONOCYTES ARE IMMUNOSUPPRESSIVE CELLS THAT RESPOND TO SYSTEMIC PRO-INFLAMMATORY CONDITIONS

Myeloid cells that suppress the immune system have been described by a variety of names including myeloid derived suppressor cells (MDSCs), M2 monocytes/macrophages, tumor associated macrophages/myeloid cells, and regulatory myeloid cells (1–3). They are a heterogeneous population comprised of precursors of granulocytes, macrophages, and dendritic cells (DC). However, their characterization and classification into different subsets remains to be resolved as there are considerable inconsistencies in the way these subsets are defined and reported (4). Monocytes that have low or no HLA-DR expression have been most commonly referred to as $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes or monocytic MDSCs. HLA-DR is one of three MHC class II glycoproteins expressed on antigen-presenting cells whose function is to present peptides derived from antigens ingested by the cell to T-cell receptors (TCR) resulting in T-cell activation. As such, these cells have a diminished capacity to present antigens to T cells and a large body of work has demonstrated these cells to be immunosuppressive. Since the functional capacities related to the immunosuppressive mechanisms of these cells have been reviewed elsewhere (5–7), this subject will not be discussed here. Although, there is still considerable debate over the origins of human MDSCs (8), several lines of evidence that will be discussed in this review suggest that $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes should be best understood in terms of arising from the normal circulating monocyte pool and not from an early precursor cell independent of monocytes. As for other MDSCs, lineage negative ($CD3^-CD19^-CD56^-CD14^-$) $LIN^-CD33^+HLA\text{-}DR^-$ cells have been described as immature MDSCs (iMDSCs) and $CD33^+CD15^+HLA\text{-}DR^-$ cells as polymorphonuclear MDSCs (PMN-MDSCs). $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes also express high levels of CD33 and CD11b on their surface. As will be discussed later, CD33 expression is greater on monocytes compared to other myeloid cells. CD11b is expressed on nearly all myeloid cells but also is expressed on human natural killer NK cells (9) and therefore is not an appropriate marker for human MDSCs. For the sake of brevity, $CD33^+CD11b^+CD14^+HLA\text{-}DR^{lo/neg}$ immunosuppressive

monocytes throughout this review will be referred to as $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes/cells. It should also be noted that there has been another type of immunosuppressive monocyte described as $CD1c/BDCA1^+CD14^+$ (10). These $CD1c^+$ monocytes are a mix of classical and intermediate monocytes and are functionally distinct from $CD1c^+$ dendritic cells (11). These cells express HLA-DR but not to the same extent as dendritic cells (10, 11).

Monocytes play a critical role in the response to infection. Sepsis results when the initial strong pro-inflammatory phase [referred to as systemic inflammatory response syndrome (12, 13)] then switches to an anti-inflammatory phase. At first glance, it appears that these conditions act sequentially, but there are likely elements of both pro- and anti- inflammatory mediators throughout the entire process. Monocytes are highly sensitive in the transition to the immunosuppressive state and become deactivated, resulting in a phase known as "immunoparalysis" (14, 15) or compensatory anti-inflammatory response syndrome (16, 17). Immunoparalysis is defined by a decrease in the level of HLA-DR expression on monocytes during the course of sepsis.

One group has defined immunoparalysis in patients with septic shock as having occurred when <30% of the monocyte pool expresses HLA-DR (14). In the early stage of sepsis in these patients, pro-inflammatory cytokines such as TNFα, IL-1, GM-CSF, and IL-6, drove the deactivation of monocytes by down regulating HLA-DR through IL-10 and TGF-β mediated pathways and diminished capacity for pro-inflammatory cytokine production (16, 18). These observations have been confirmed by many other studies. For example, in patients with injuries from blunt trauma, those that had low levels of HLA-DR on monocytes after the second day of admission were significantly more likely to develop sepsis than those patients that had high levels (19). Monneret et al. demonstrated the relationship of low HLA-DR levels to survival in patients with sepsis (20). In the early stages of septic shock, the expression of HLA-DR on monocytes was not different between survivors and non-survivors. However, after 48 h post onset, survivors had significantly higher expression of HLA-DR on monocytes than those that did not survive the event.

In contrast, there are some reports that have not found a relationship between the loss of monocyte HLA-DR expression and septic shock (21, 22). These apparent contradictory reports perhaps may result from differences in the timing of sample procurement, clinical settings, and measurement parameters for HLA-DR expression or other uncharacterized variables. However, a recent review has confirmed the regulatory role of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in both normal and pathological responses to a diverse array of microbial infections (23).

The loss of HLA-DR on monocytes has been reported in other non-malignant conditions with an inflammatory component. $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes have been described in patients with severe burns (24, 25), acute and chronic liver inflammation (26–28), pancreatitis (29–31), amyotrophic lateral sclerosis (32), and immediately after surgical procedures (33). Although the precise mechanisms of monocyte deactivation and HLA-DR loss have yet to be elucidated in each of these diseases, a familiar pattern of either acute or chronic inflammation tends to be an initial event triggering the development of immunosuppressive monocytes. Taken together, the overall data in non-malignant conditions demonstrate that the loss of HLA-DR is a well-established marker of functional deactivation of monocytes and that it associates with poor clinical outcomes in critically ill patients.

HLA-DR can be down regulated through a variety of mechanisms. Under normal physiological conditions, HLA-DR is under the transcriptional control of the MHC Class II transactivator (CIITA) (34, 35). HLA-DR expression can be induced by IFN-γ through transcriptional activation via CIITA (36) and also by GM-CSF possibly through post-transcriptional mechanisms (33). Conversely, several cytokines can down-regulate monocytic HLA-DR expression. IL-1β and TGF-β directly down-regulate transcription of HLA-DR through CIITA and/or prevent IFN-γ induction of HLA-DR (16, 37, 38). IL-10 also strongly decreases surface HLA-DR expression but it's mechanism of action is to increase intracellular sequestration of MHC Class II molecules (39) via ubiquitination by inducing the membrane-associated RING-CH (MARCH) ubiquitin ligase (40). Glucocorticoids and steroid hormones can also negatively regulate HLA-DR transcription by decreasing CIITA mRNA levels. Down-regulation of HLA-DR on monocytes has been observed in response to cortisol (41), prednisolone (42), and dexamethasone (43, 44). Overall, many of these mechanisms that regulate HLA-DR expression have been shown to be critical mediators of immune paralysis in both sepsis and in malignant settings.

Soon after the role of monocyte deactivation was observed in sepsis patients, reports began appearing in the literature that cancer patients also exhibit monocytes with low HLA-DR expression. Patients with glioblastoma (45), ovarian cancer (46), and melanoma (47) were some of the first cancer patients discovered to have low monocytic HLA-DR expression. In previous studies with our colleagues at Mayo Clinic Rochester, we found high levels of these cells in a variety of different cancer patient groups including those with glioblastoma (43), non-Hodgkin lymphoma (48), chronic lymphocytic leukemia (49), and renal cell carcinoma (50). In order to understand the severity of immunosuppression in cancer patients, we compared the presence of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in cancer patients to those patients with acute lung injury with or at risk for sepsis (51). Many of the cancer patients had levels of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes equally high as patients with sepsis.

In vitro experiments demonstrated that monocytes isolated from healthy volunteers can lose HLA-DR expression through co-culture with tumor-derived exosomes (47), exposure to conditioned media from cultured tumor cells (52, 53), or even incubation with cytokines like TGF-β (37). Furthermore, Ribechini et al. have identified a potentially unique pathway in which GM-CSF can license $CD14^+$ monocytes such that upon later exposure to INF-γ, the monocytes would switch to an immunosuppressive phenotype through the upregulation of indolamine 2,3-dioxygenase (IDO) (54). Bergenfeltz et al. found that monocytes isolated from breast cancer patients exhibited gene expression profiles similar to monocytes isolated from

sepsis patients (55). Specifically, TNFα, IL-1β, HLA-DR, and CD86 genes were significantly down-regulated in monocytes from breast cancer patients compared to controls suggesting that some of the mechanisms that convert monocytes to the immunosuppressive state are identical in both septic and malignant conditions.

The implications of these findings for cancer immunotherapy are significant. The presence of high levels of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes suggests that many of these cancer patients had reached a point of immunoparalysis prior to treatment and thus may not be very responsive to immunotherapeutic approaches. On the other hand, many cancer patients have been observed with normal levels of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes. The timing of onset, progression and intensity of immunoparalysis in cancer patients compared to patients with sepsis will certainly involve both similar and unique mechanisms. As such, further work is needed to understand how these cells respond and contribute to tumor development.

IMPACT ON IMMUNOTHERAPY

Checkpoint Inhibitors

The impact of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes on CTLA-4 inhibition with ipilimumab has most clearly been demonstrated in melanoma patients with advanced disease. Meyer et al. reported that $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were elevated in melanoma patients. While $CD14^+HLA\text{-}DR^{lo/neg}$ monocyte populations were not affected by ipilimumab treatment, patients that responded to ipilimumab treatment had significantly less pre-treatment frequencies of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes than those patients that did not respond to treatment (56). In another study, lower pre-treatment frequencies of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were associated with overall patient survival (57). The percentages of $CD14^+HLA\text{-}DR^{lo/neg}$ cells of total monocytes appeared to be more predictive of survival than absolute cell counts (cells/μl). The authors also reported that after 6 weeks of ipilimumab treatment, lower percentages of $CD14^+HLA\text{-}DR^{lo/neg}$ cells were associated with higher changes in absolute T cell counts, suggesting that the $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes restricted $CD8^+$ T cell response. These data were confirmed to some extent by Tarhini et al. (58), Martens et al. (59) and Gebhardt et al. (60). Gebhardt et al. found that decreased $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were related to declines in nitric oxide production in response to ipilimumab treatment. Finally, de Coaña et al. found that in melanoma patients PMN-MDSCs decreased upon ipilimumab treatment whereas $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes did not change (61). However, in patients who received a clinical benefit, $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes decreased after treatment whereas this was not the case in patients who progressed. While the frequency of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes was not compared to healthy volunteers, baseline levels of these cells were similar between patients with progressive disease and those that had a clinical benefit. Taken together, the results from these studies present an interesting dynamic. Clearly, lower baseline frequencies of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes are predictive of outcome and therefore these monocytes may interfere with the efficacy of ipilimumab treatment. However, in some patients, particularly for those who do respond to treatment, there is evidence that immunosuppressive monocytes decline after treatment. Further studies are needed to confirm and delineate the mechanisms behind these observations.

Data are also emerging that demonstrate the role of monocytes in altered responses to anti-PD-1 therapy. In a study of stage IV melanoma patients receiving anti-PD-1 therapy, single cell mass cytometry was used to investigate peripheral blood biomarkers (62). The pre-treatment frequency of classical monocytes ($CD14^+CD16^-$) that express high levels of HLA-DR was predictive of overall survival in these patients. The majority of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes are classical monocytes so the higher expression of HLA-DR in these patients likely reflects lower levels of immunosuppressive monocytes. The authors of this study did report that a population similar to $CD33^{lo}CD11b^+HLA\text{-}DR^{lo}$ cells was not different between responders and non-responders. However, the gating strategy for isolating $CD33^{lo}CD11b^+HLA\text{-}DR^{lo}$ cells could very well include both monocytic and granulocytic populations. In mixed lymphocyte reaction proliferation assays using PBMCs from healthy donors, it was shown that proliferation of nivolumab-treated T cells improved in the absence of monocytes (63). Additionally, blockade of CSF-1R signaling on monocytes improved T cell proliferation. Interestingly, the authors of this study also report that activation of T cells by nivolumab treatment induced the release of M-CSF from T cells thereby increasing the immunosuppressive functions of monocytes through adenosine production and upregulation of PD-L1 on monocytes. PD-1 and CSF-1R blockade in combination was also found to augment the cytolytic capacity of tumor infiltrating lymphocytes (TILs) in co-cultures of $CD3^+$ TILs and $CD11b^+$ tumor infiltrating myeloid cells from patients with glioblastoma (64). $CD14^+HLA\text{-}DR^{lo/neg}$ monocyte levels also appear to distinguish responders from non-responders in nivolumab treated metastatic melanoma patients who progressed after ipilimumab therapy (65). Since there have not been many mechanistic insights garnered from these human studies, further investigation is needed to determine whether the impact of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes on checkpoint inhibition reflects a general immunosuppressive environment or to what degree the expression of monocytic PD-1 and/or PD-L1 disrupts the efficacy of checkpoint blockade (66–68).

Cancer Vaccines

Data from studies and clinical trials demonstrate that immunosuppressive monocytes impact cancer vaccines through several mechanisms that limit optimal anti-tumor responses. They have been shown to adversely affect responses to direct injection of peptides/whole tumor cells as well as to *ex-vivo* differentiated dendritic cell (DCs) vaccines. In a therapeutic cancer vaccine study using multiple tumor-associated peptides (TUMAPs) in renal cell carcinoma (RCC) patients, Walter et al. looked at six populations of MDSCs (among other immune parameters) to determine whether these cells influenced the survival outcome of patients treated with the vaccine (69). Although five of the six types of MDSCs were

elevated in RCC patients prior to treatment, $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes demonstrated the strongest association with overall survival whereby their presence in high numbers was inversely correlated with survival. In non-small cell lung cancer (NSCLC) patients receiving a telomerase peptide vaccine, high levels of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were found to be associated with poorer progression free survival (70). Prostate cancer patients with low frequencies of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes prior to receiving prostate/GVAX vaccine in combination with ipilimumab had a mean survival time of 52 months compared to 20 months mean survival for those with high pre-treatment frequencies (71). These cells did increase during the course of treatment but the increase did not correlate with response to treatment. In a trial testing DCs for patients with primary recurrent glioblastoma, both $CD15^+SSC^{lo}$ cells and $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were found to be elevated in patients that progressed but only the $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were found to be both higher as a percent of parent populations and also in cell counts (cells/μl) (72). Poschke et al. demonstrated in stage IV melanoma patients that the presence of $CD14^+HLA\text{-}DR^{lo/neg}$ cells purified with elutriated monocytes negatively impacted the maturation, migration, antigen uptake, and cytokine production of DCs (73). In another trial with melanoma patients, $BDCA1^+CD14^+$ cells were shown to inhibit T cell proliferation in an antigen-dependent fashion resulting in impaired responses to monocyte-derived DCs (10). In pre-clinical studies, our group has found that monocytes purified by immunomagnetic selection from a variety of cancer patients have deficiencies in DC maturation and that the method of cell culture can influence their maturation (7). Additionally, we found that $CD14^+HLA\text{-}DR^{lo/neg}$ cells inversely correlated with the maturation marker CD83 on dendritic cells. Taken together, the data from these studies and clinical trials demonstrate that immunosuppressive monocytes impact cancer vaccines through several mechanisms that limit optimal anti-tumor responses.

Hematopoietic Progenitor Cell Transplantation

The functional consequences of immunosuppressive monocytes in hematopoietic progenitor cell transplantation appear to be dependent on the type of transplant. In autologous transplants, higher levels of $CD14^+HLA\text{-}DR^{lo/neg}$ cells in leukapheresis products were independent predictors of adverse outcomes both in terms of overall survival and progression free survival in patients with non- Hodgkin lymphoma (74). However, in the allogeneic setting, $CD14^+HLA\text{-}DR^{lo/neg}$ cells may confer protection against acute graft vs. host disease (aGVHD). Myeloid cells, including $CD14^+HLA\text{-}DR^{lo/neg}$ cells, are some of the first cells to recover after transplantation (75, 76). Mougiakakos et al. demonstrated that transplant patients had both elevated monocytes and $CD14^+HLA\text{-}DR^{lo/neg}$ cells at 1–3 months post-transplant (77). They also demonstrated that higher frequencies in peripheral blood also associated with higher grades of aGVHD. The induction of these cells was likely in response to circulating levels of G-CSF and IL-6, along with other pro-inflammatory cytokines. The administration of G-CSF alone to human donors was sufficient to expand both $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes and PMN-MDSCs in both phenotype and function (78). Similarly, the presence of high $CD14^+HLA\text{-}DR^{lo/neg}$ monocyte cell counts in the G-CSF mobilized graft was associated with lower risks of developing aGVHD in recipients without affecting the relapse rate or the transplant-related mortality rate (79). So whereas immunosuppressive monocytes negatively affect outcomes in autologous transplant patients, they conversely may provide a benefit of a reduced risk of aGVHD in allogeneic transplant recipients.

Adoptive and Chimeric Antigen Receptor (CAR) T Cell Therapies

$CD14^+HLA\text{-}DR^{lo/neg}$ monocytes may negatively impact the effectiveness of adoptive and Chimeric Antigen Receptor (CAR) T cell therapies. While the data from clinical trials is too limited to show this conclusively, the ability of suppressive monocytes to inhibit T cells in human cancers has been well documented (43, 48, 80–83) and has been associated both with dysfunctional antigen-specific T cells and negative outcomes in melanoma patients (84). CD19-CAR T cell expansions from mononuclear cell collections that contained high percentages of monocytes produced poorer cell yields in children with acute lymphocytic leukemia and non-Hodgkin lymphoma (85). While they did not specifically measure $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes, when they depleted monocytes by adherence to plastic techniques, cell expansion improved, and typical expected yields were achieved. While data has yet to be published from clinical trials monitoring immunosuppressive monocytes in those receiving CAR-T cell therapies, reports from animal model studies suggest that rodent MDSCs are expanded after treatment with CAR-T cells in a GM-CSF dependent fashion, and that this limited the anti-tumor activity of the infused cells (86). Other data from animal models suggest that although transferred T cells likely induce myeloid derived suppressor cells (87), there may be some conditions in which T cell therapy can be successful despite immunosuppression caused by myeloid cells (88). As data emerges in the human setting, it is not unreasonable to expect that $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes will be shown to play some role in reducing the anti-tumor efficacy of CAR T cells because the nature of the cytokine release syndrome involves pro-inflammatory cytokines which have previously been demonstrated to induce these cells under other conditions.

EFFICACY OF THERAPEUTIC APPROACHES TARGETING IMMUNOSUPPRESSIVE MONOCYTES

As evidence for the role of immunosuppressive monocytes in inhibiting anti-tumor responses continues to build, it becomes readily apparent that therapeutically targeting these cells should improve responses to immunotherapy. Agents designed to interfere with MDSCs have generally been classified into four categories: (1) inhibition of the conversion, appearance and/or expansion of MDSCs, (2) inhibition of MDSC immunosuppressive functions, (3) interference of MDSC

trafficking to tumors, and (4) direct removal and cytotoxic approaches (89–91). Several agents that interfere with these mechanisms have shown promise in pre-clinical animal studies and have been reviewed elsewhere (89–93). As these drugs move into clinical trials, it will be very important understand how they will affect each of the different MDSC populations as well as total myeloid cells.

Several examples in the literature which are summarized in **Table 1** highlight how drugs targeted to MDSCs affect the subpopulations in a differential manner. For example, gemcitabine has been shown to reduce MDSC accumulation in tumors in animal models (94) but appears to preferentially decrease PMN-MDSCs and total monocytes but not monocytic MDSCs when tested in pancreatic cancer patients (95). In another case, the treatment of solid tumor cancer patients with an agonistic TRAIL-R2 antibody resulted in the decline of different MDSC populations while not affecting other myeloid populations (96). Tyrosine kinase inhibitors (TKI) also demonstrate differential effects on MDSCs. In chronic myeloid leukemia patients treated with imatinib, nilotinib, or dasatinib, all three TKIs decreased PMN-MDSCs but only dasatinib reduced $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes (97). Furthermore, the decline in $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes correlated with positive patient molecular responses. Another TKI, sunitinib, was shown to preferentially inhibit the suppressive activity of $CD14^+CD16^+$ monocytes and reductions in these cells were associated with sunitinib responders (98).

There have been a few clinical trials that have specifically targeted monocytes and/or immunosuppressive monocytes. Nywening et al. hypothesized that pharmacological prevention of monocyte trafficking to tumors via a small molecule CCR2 inhibitor improves anti-tumor immunity (99). They tested the CCR2 inhibitor PF-04136309 in combination with FOLFIRINOX chemotherapy vs. chemotherapy alone in patients with pancreatic ductal adenocarcinoma. The CCR2 inhibitor prevented monocyte egress from bone marrow and subsequently reduced monocyte infiltration into tumors. The reduced monocyte infiltrate resulted in an increase of intra-tumoral lymphocytes and improved anti-tumor immunity. Patients receiving the combination of PF-04136309 and chemotherapy had higher than expected response rates. One potential caveat is that monocyte blockade may result in increased infiltration of granulocytes and, consequently, dual blockade of both monocytes and granulocytes has been proposed (100). While this study did not measure immunosuppressive monocytes *per se*, there is ample evidence that immunosuppressive monocytes also migrate to tumors via the CCL2/CCR2 pathway (43, 50, 101, 102).

In other clinical trials, investigators have tested the effectiveness of blocking CSF-1R signaling in cancer patients. Myeloid differentiation, monocytic commitment, trafficking, survival, and proliferation of monocytes/macrophages are all influenced by CSF-1R signaling (103). It is hypothesized that blocking the signaling function of this receptor will result in the reduction of monocyte/macrophage infiltration into tumors and consequently limit the immunosuppressive nature of the tumor microenvironment (104). In patients with recurrent glioblastoma treated with an oral CSF-1R inhibitor PLX3397, the percentage of non-classical monocytes ($CD14^{lo}CD16^+$) declined after treatment but microglia in the tumor microenvironment were only modestly reduced (105). However, while taking this into account, it may be that different glioblastoma subtypes (i.e., pro-neural glioblastoma) may be more susceptible to the reprogramming of monocytes/macrophages from CSF-1R inhibition (106). Finally, $CSF\text{-}1R^+$ myeloid cells are associated with negative outcomes in neuroblastoma patients (107). In monocytes co-cultured in the presence of neuroblastoma cells, the CSF-1R inhibitor BLZ945 partially restored HLA-DR and CD86 expression and reduced the immunosuppressive capacities of the monocytes on T cell proliferation.

GM-CSF has been used to support myelopoiesis and promote anti-tumor immunity as a stand-alone monotherapy and also to complement various immunotherapeutic approaches (108). Although GM-CSF has been shown to overcome monocyte deactivation in sepsis by inducing HLA-DR expression, the utility for use in cancer patients remains to be determined. In some cases, GM-CSF has been shown to provide a clinical benefit (109–112). But in several instances, GM-CSF has demonstrated neutral or negative results (113, 114). While it is clear that GM-CSF can act via pro- and anti- inflammatory pathways, emerging data, more prevalent in mouse models (115–119) but also from human studies (80, 120), indicate that GM-CSF strongly promotes the development of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes. The data from Ribechini et al. suggest that the timing of GM-CSF administration may be critical in the transitioning of pro-inflammatory to anti-inflammatory pathways (54). Therefore, in order to optimize GM-CSF therapy, it is critical to further understand and define how dosing, timing, and other mechanisms contribute to $CD14^+HLA\text{-}DR^{lo/neg}$ monocyte accumulation.

A common theme emerges from all studies cited. Therapies targeting immunosuppressive monocytes have a wide variety of effects on these cells, and underlying mechanisms are still not well understood. While many of these studies remain relatively limited in scope, and much work remains to better identify the optimal strategies and indications, these promising preliminary results clearly warrant further investigation into developing methods to target monocytes in cancer patients. The negative effect of immunosuppressive monocyte levels, particularly of the $CD14^+HLA\text{-}DR^{lo/neg}$ phenotype, is clear. Therefore, rigorous and well-defined immune monitoring and phenotyping of patient myeloid cells in clinical trials is justified, as their measurement is critical for understanding the mechanism(s) of action of such therapies.

THE POTENTIAL FOR UTILIZING IMMUNOSUPPRESSIVE MONOCYTES AS A PREDICTIVE BIOMARKER

The clinical significance of the broad class of MDSCs has been well documented and the pathway to utilizing these cells as biomarkers has recently been proposed (121). In many studies, the presence of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in circulation has been shown to be a systemic marker of immune suppression,

TABLE 1 | Therapeutic approaches targeting immunosuppressive monocytes.

Agent	Mode of action	Observed effects on human MDSCs
Gemcitabine	Inhibition of the expansion of MDSCs	• Preferentially decreases PMN-MDSCs and total monocytes but not monocytic MDSCs in pancreatic cancer patients
TRAIL-R2 antibody	Apoptotic programmed cell death	• Declines in different MDSC populations while not affecting other myeloid populations
Tyrosine kinase inhibitor (TKI) imatinib	Interference with signal transduction, suppressing cell proliferation, differentiation, migration, metabolism and programmed cell death	• Decreases PMN-MDSCs
Tyrosine kinase inhibitor (TKI) nilotinib	Interference with signal transduction, suppressing cell proliferation, differentiation, migration, metabolism and programmed cell death	• Decreases PMN-MDSCs
Tyrosine kinase inhibitor (TKI) dasatinib	Interference with signal transduction, suppressing cell proliferation, differentiation, migration, metabolism and programmed cell death	• Decreases PMN-MDSCs • Decreases CD14+HLA-DR$^{lo/neg}$ monocytes, which correlates with positive patient molecular responses.
Tyrosine kinase inhibitor (TKI) sunitinib	Interference with signal transduction, suppressing cell proliferation, differentiation, migration, metabolism and programmed cell death	• Preferentially inhibits suppressive activity of CD14+CD16+ monocytes, which associates with sunitinib responders
Small molecule CCR2 inhibitor PF-04136309	Prevention of monocyte trafficking to tumors (likely including immunosuppressive monocytes)	• Improves anti-tumor immunity
CSF-1R inhibitor PLX3397	Myeloid differentiation, monocytic commitment, trafficking, survival, and proliferation of monocytes and macrophages	• In recurrent glioblastoma, percentage of non-classical monocytes (CD14^{lo}CD16^{+}) declines but microglia in the tumor is only modestly reduced. (However other glioblastoma subtypes may respond differently)
CSF-1R inhibitor BLZ945	Myeloid differentiation, monocytic commitment, trafficking, survival, and proliferation of monocytes and macrophages	• In co-culture with neuroblastoma, monocytes partially recover HLA-DR and CD86 expression, reducing immunosuppression of T-Cell proliferation
GM-CSF	Stimulation of myelopoiesis and promotion of anti-tumor immunity	• Reverses monocyte deactivation in sepsis by inducing HLA-DR expression • Utility in cancer patients remains to be determined. • May also promote the development of CD14^{+}HLA-DR$^{lo/neg}$ monocytes, dependent on dosing, timing, and other mechanisms.

References provided in the text.

and has been associated with the accumulation of these cells in tumors (50). Both their ability to impair anti-tumor immune responses and that they may be a promising therapeutic target make a compelling case for the development of standardized tools and/or assays to measure CD14^{+}HLA-DR$^{lo/neg}$ monocytes in a manner that is useful for guiding therapeutic decisions for patients receiving immunotherapy.

Perhaps the simplest and most efficient way to measure CD14^{+}HLA-DR$^{lo/neg}$ monocytes is by flow cytometry of peripheral blood; therefore, acquisition of tumor biopsies which may not always be available from patients is unnecessary. The small sample of blood that is required to measure these cells justifies further investigation into monitoring them as an informative biomarker. Significant variability currently exists in the way these cells have been measured and reported in the literature. We outline these variables in **Table 2** and highlight areas of concern, including differences in flow cytometry gating strategies, cell enumeration methods, timing of sample procurement, and processing procedures. These differences in methodology have been problematic, creating variability in actual, and reported results. Mandruzzato et al. have shown that the lack of standardized gating strategies was one of the largest factors of variation when measuring the total group of MDSCs (122). Nonetheless, standardized gating strategies have been used to gain meaningful correlations to clinical outcomes. We and others (124) have shown that standardization of the measurement of these cells can result in consistent and robust assays.

Typically, CD14^{+}HLA-DR$^{lo/neg}$ monocytes are measured by flow cytometry in blood samples collected from patients. These monocytes are phenotypically positive for CD14, CD33, and CD11b (125). The source of inconsistencies is often in the measurement of monocyte HLA-DR loss, as HLA-DR expression exhibits considerable variation and is not uniform across all subtypes of monocytes. **Figure 1** displays a diagram of surface marker commonalities within the myeloid compartment. CD33 expression on human myeloid cells appears to be bi-modal as granulocytes and immature myeloid cells express moderate amounts of CD33 whereas monocytes exhibit strong CD33 expression (**Figure 1A**). MDSCs of both granulocytic and monocytic lineage reside within the CD33^{+} population of cells. CD33^{-} cells comprise cells from the lymphoid lineage (**Figure 1B**). Often CD33 and HLA-DR are used to measure MDSCs but these two markers are not solely sufficient to distinguish the three types of MDSCs. To distinguish monocytic MDSCs by flow cytometry, CD33 positive cells are gated from total leukocytes and then monocytes are further gated based on CD14 expression. The combination of CD33^{++} and CD14^{+} distinguishes monocytes from all other myeloid cells (**Figure 1D**). CD33^{+}

TABLE 2 | Recommendations for consistent and reproducible reporting for immunosuppressive monocytes by flow cytometry.

Processing steps that contribute to variation in reporting	Examples observed in the literature*	Recommendations for best practices
Phenotypes: combinations reported (2, 122, 123)	$CD14^+HLA\text{-}DR^{lo/neg}$ $CD33^+HLA\text{-}DR^-$ LIN^-, $CD14^+$, $HLA\text{-}DR^-$ $CD33^+$, $CD11b^+$, $CD14^+$	CD33 (bright): confirm myeloid origin CD14 (+): parent population HLA-DR (lo/neg): distinguish between "normal" and immunosuppressive populations CD16: identify subpopulations
Processing of blood samples	Purification of mononuclear cells/Ficoll separation Effects of cryopreservation/thawing Timing of sample collection and storage	Directly stain whole blood Blood draws taken at approximately same time of day (i.e., mornings) Samples processed within 4–6 h; held at room temperature
Quantification/ enumeration of $CD14^+HLA\text{-}DR^{lo/neg}$ cells	As % of PBMCs As % of Total Leukocytes Cells/μl HLA-DR MFI	Cells/μl % of CD14 Molecules per cell
Gating strategies	Monocyte measurement by Forward and Side scatter $CD14^+$ from Mononuclear gate Histogram or Quadrant of HLA-DR expression	$CD33^{br}$ from Mononuclear gate, then $CD14^+$ cells Fluorescence minus one (FMO) staining to determine HLA-DR negative cells

* *Not all inclusive.*

cells not expressing CD14 comprise a separate pool of $CD33^+$ subtypes (**Figure 1C**) including $CD15^{(+)}/SSC^{(hi)}/HLA\text{-}DR^{(-)}$ granulocytes and granulocytic MDSCs (currently very few reproducible markers distinguish g-MDSCs by flow cytometry) (**Figure 1E**), $CD15^{(-)}/SSC^{(lo)}/HLA\text{-}DR^{(-)}$ immature MDSCs (**Figure 1F**), and $LIN^{(-)}$ $HLA\text{-}DR^{(+++)}$ dendritic cells or other myeloid cells (**Figure 1G**). Although the illustration is meant to visualize the hierarchy of myeloid cell populations, it is likely that some myeloid progenitors may become $CD14^+$ and hence join the pool of monocytic cells.

The immunosuppressive monocyte of phenotype $CD14^+HLA\text{-}DR^{lo/neg}$ resides within the $CD33^{(++)}/CD14^{(+)}$ population of mononuclear cells (**Figure 1D**). This population may be further sub-divided based on CD16 expression into classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and non-classical ($CD14^{lo}CD16^+$) monocytes [**Figures 1H–J** and Ziegler-Heitbrock et al.(127)]. Interestingly, HLA-DR expression varies between these subgroups in that intermediate monocytes express the highest amount of HLA-DR and classical monocytes expressing the least (125). Note that the HLA-DR expression level noted as $HLA\text{-}DR^{(+)}$, is not low enough to classify as the immunosuppressive $HLA\text{-}DR^{(lo/neg)}$ phenotype. From the combined pool of the three monocyte subtypes (**Figure 1K**), the immunosuppressive phenotype may arise by loss of HLA-DR expression (**Figure 1L**). The $CD14^+HLA\text{-}DR^{lo/neg}$ cell most typically arises from the classical monocyte pool, but may also be derived from the intermediate and non-classical monocyte pools. For setting the $HLA\text{-}DR^{(+)}$ vs. $HLA\text{-}DR^{(lo/neg)}$ threshold in flow cytometry, a convenient internal negative control is available in the $CD33^-CD14^-HLA\text{-}DR^-$ mononuclear cell population. The threshold is set at the upper limit of HLA-DR in that population, thereby delineating the boundary to distinguish low or negative from high HLA-DR expression in the $CD33^+CD14^+$ monocyte population.

Further complicating the comparison of $CD14^+HLA\text{-}DR^{lo/neg}$ monocyte levels between different studies is the output of how the cells are enumerated. Examples from the literature include reported cells as a percent of peripheral blood mononuclear cells (PBMCs), a percent of total leukocytes, a percent of monocytes, total cells per volume of blood (i.e., cells/μl), mean fluorescence intensity (MFI), molecules per cell, and based on other non-flow cytometry methods such as polymerase chain reaction assays. For flow cytometry based assays, reporting the abundance of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes as a percent of PBMCs or total leukocytes is the least informative, particularly when measuring these cells from cancer patients where many patients exhibit severe leukopenia and/or lymphopenia. This phenomenon leads to artificially high percentages because the comparative denominator of total PBMCs or leukocytes can be much lower than in the control or healthy volunteer subject groups. Since it remains to be determined how much HLA-DR expression must be diminished before the monocyte becomes deactivated or immunosuppressive, it may be more appropriate to measure surface expression of HLA-DR on monocytes rather than measure cell abundance. While MFI is commonly used for measuring surface expression, it is difficult to standardize MFI values between different instruments within the same laboratory let alone between different laboratories. As such, we recommend the use of fluorescent beads such as Anti–HLA-DR/Anti-Monocyte Quantibrite™ (BD Biosciences) to better assess the quantity of surface protein expression on cells. Finally, computational approaches for reducing the effect of procedural and inter-user variability on assay results have been developed which use coefficient of variation to quantify the HLA-DR spread on monocytes in healthy subjects and patients with melanoma (57).

Another source of variation that contributes to inconsistent results is the method by which blood samples are processed. The most common processing steps for the isolation of peripheral blood mononuclear cells (PBMCs) include sucrose gradient centrifugation followed by subsequent cryopreservation of the purified cells. In direct comparisons of processing steps in samples from patients with gastrointestinal cancer, Duffy et al. found that although the processing steps yielded relatively consistent results when comparing cancer patients to healthy subjects, the absolute numbers of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were significantly different when comparing whole blood staining to freshly isolated PBMCs in the cancer patient cohort (128). Several groups have found that cryopreservation can negatively affect the immunosuppressive functions, enzymatic activity, and/or the abundance/distribution of MDSC subsets (61, 129, 130). Monneret et al. found that

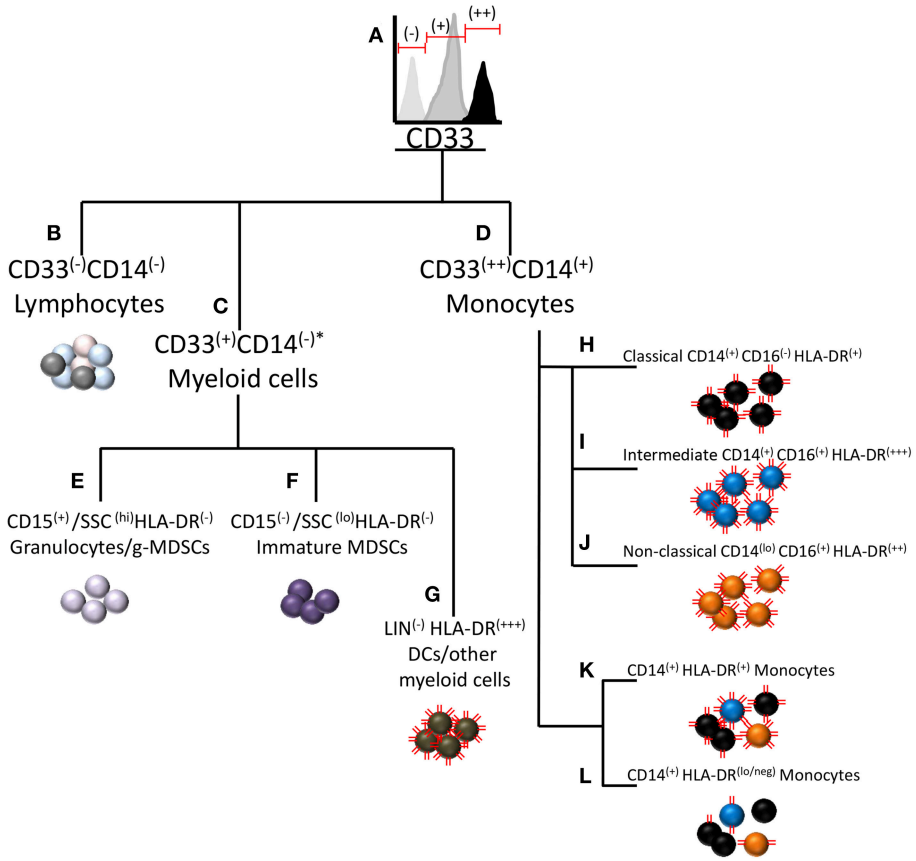


FIGURE 1 | Relationship of immunosuppressive CD14$^+$HLA-DR$^{lo/neg}$ monocytes to other myeloid cells and myeloid derived suppressor cells. A diagram of CD marker commonalities between cell types within the myeloid subclass including MDSCs and other cells with similar CD marker expression. **(A)** MDSCs of both granulocytic and monocytic lineage reside within the CD33$^+$ population of cells. **(B)** Cells not expressing CD33 are considered to be of lymphoid lineage. **(C)** Cells not expressing CD14 comprise a separate pool of CD33$^+$ subtypes. **(D)** CD14$^+$ monocytes typically strongly express CD33, hereafter noted as CD33$^{(++)}$. **(E)** CD15$^{(+)}$/SSC$^{(hi)}$/HLA-DR$^{(-)}$ normal granulocytes and granulocytic MDSCs. **(F)** CD15$^{(-)}$/SSC$^{(lo)}$/HLA-DR$^{(-)}$ immature MDSCs. **(G)** LIN$^{(-)}$ HLA-DR$^{(+++)}$ dendritic cells or other myeloid cells. Three sub-populations of monocytes are **(H)** classical monocytes (CD14$^+$CD16-) **(I)** intermediate monocytes (CD14$^+$CD16$^+$) **(J)** Non-classical monocytes (CD14^{lo}CD16$^+$) **(K)** Representation of the combined pool of the three monocyte subtypes **(L)** The CD14$^+$HLA-DR$^{lo/neg}$ cell most typically arises from the classical monocyte pool, but may also be derived from the intermediate and non-classical monocyte pools. The red lines on the cells represent relative HLA-DR expression on the cells. *Under some conditions, granulocytes can express CD14 (126).

in blood samples collected in EDTA anti-coagulant tubes, HLA-DR expression was influenced both by storage time and temperature in their study of patients with sepsis and in control subjects (131). After sample collection, increased storage time at room temperature led to dramatically increased HLA-DR expression both in terms of percent positive monocytes and MFI. Higher storage temperatures also appeared to increase HLA-DR levels as well. Docke et al. also found that processing and transport steps can influence HLA-DR and thus recommended staining unprocessed blood within 4 h of the blood draw (132). Additionally, they found that the HLA-DR values for samples that were lysed/washed vs. lysed/no wash strongly correlated despite the slightly higher overall HLA-DR values reported in the lyse/no wash samples. In summary, there are many processing steps that affect the accurate measurement of CD14$^+$HLA-DR$^{lo/neg}$ monocytes. Results from minimally processed samples appear to yield the most reliable results for HLA-DR quantification. Therefore, as whole blood staining of fresh blood is becoming more standard practice, this will no doubt improve the prospect of using CD14$^+$HLADR$^{lo/neg}$ monocytes as a biomarker for understanding responses to cancer immunotherapy.

CONCLUSIONS

There is now a large body of evidence linking CD14$^+$HLADR$^{lo/neg}$ monocytes to systemic immune suppression and paralysis and their negative affect on cancer immunotherapy. As new evidence suggests that systemic immunity plays an important role in optimal responses to cancer immunotherapy (51, 133), circulating monocytes likely contribute significantly to this phenomenon. From studies published to date, it appears that the various immunotherapeutic

approaches do not drastically change the abundance of $CD14^{+}HLADR^{lo/neg}$ monocytes but their pre-treatment levels correlate with poorer or more favorable outcomes in most settings. While deciphering the precise mechanisms of $CD14^{+}HLADR^{lo/neg}$ monocyte-mediated suppression in humans will remain difficult, the established data warrant further efforts to investigate novel ways to counteract these cells. Finally, the immunosuppressive $CD14^{+}HLA\text{-}DR^{lo/neg}$ monocyte not only may be a very good therapeutic target, but also may be a very good candidate for biomarker development. They are easy to quantify, likely to reflect general systemic immunosuppression, and may even reflect what is happening in the tumor microenvironment.

AUTHOR CONTRIBUTIONS

AM contributed to the development, writing, and illustrations of the article. DG contributed to the study concept, development, and writing of the article. MG contributed to the study concept, development, writing, and illustrations of the article. All authors read and approved the final manuscript.

FUNDING

The study was supported by internal funds from the Mayo Clinic Department of Laboratory Medicine and Pathology and Center for Regenerative Medicine.

REFERENCES

1. VanGundy ZC, Guerau-de-Arellano M, Baker JD, Strange HR, Olivo-Marston S, Muth DC, et al. Continuous retinoic acid induces the differentiation of mature regulatory monocytes but fails to induce regulatory dendritic cells. *BMC immunol.* (2014) 15:8. doi: 10.1186/1471-2172-15-8
2. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun.* (2016) 7:12150. doi: 10.1038/ncomms12150
3. Manjili MH, Wang XY, Abrams S. Evolution of Our Understanding of Myeloid Regulatory Cells: From MDSCs to Mregs. *Front Immunol.* (2014) 5:303. doi: 10.3389/fimmu.2014.00303
4. Poschke I, Kiessling R. On the armament and appearances of human myeloid-derived suppressor cells. *Clin Immunol.* (2012) 144:250–68. doi: 10.1016/j.clim.2012.06.003
5. Veglia F, Perego M, Gabrilovich D. Myeloid-derived suppressor cells coming of age. *Nat Immunol.* (2018) 19:108–19. doi: 10.1038/s41590-017-0022-x
6. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends Immunol.* (2011) 32:19–25. doi: 10.1016/j.it.2010.10.002
7. Laborde RR, Lin Y, Gustafson MP, Bulur PA, Dietz AB. Cancer vaccines in the world of immune suppressive monocytes (CD14+HLA-DRlo/neg cells): the gateway to improved responses. *Front Immunol.* (2014) 5:147. doi: 10.3389/fimmu.2014.00147
8. Millrud CR, Bergenfelz C, Leandersson K. On the origin of myeloid-derived suppressor cells. *Oncotarget.* (2017) 8:3649–65. doi: 10.18632/oncotarget.12278
9. Werfel T, Witter W, Gotze O. CD11b and CD11c antigens are rapidly increased on human natural killer cells upon activation. *J Immunol.* (1991) 147:2423–7.
10. Bakdash G, Buschow SI, Gorris MA, Halilovic A, Hato SV, Skold AE, et al. Expansion of a BDCA1+CD14+ myeloid cell population in melanoma patients may attenuate the efficacy of dendritic cell vaccines. *Cancer Res.* (2016) 76:4332–46. doi: 10.1158/0008-5472.CAN-15-1695
11. Schroder M, Melum GR, Landsverk OJ, Bujko A, Yaqub S, Gran E, et al. CD1c-expression by monocytes - implications for the use of commercial CD1c+ dendritic cell isolation kits. *PLoS ONE.* (2016) 11:e0157387. doi: 10.1371/journal.pone.0157387
12. Balk RA. Systemic inflammatory response syndrome (SIRS): where did it come from and is it still relevant today? *Virulence.* (2014) 5:20–6. doi: 10.4161/viru.27135
13. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM consensus conference committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest.* (1992) 101:1644–55. doi: 10.1378/chest.101.6.1644
14. Volk HD, Reinke P, Krausch D, Zuckermann H, Asadullah K, Muller JM, et al. Monocyte deactivation–rationale for a new therapeutic strategy in sepsis. *Intensive Care Med.* (1996) 22 (Suppl. 4):S474–81. doi: 10.1007/BF01743727
15. Volk HD, Thieme M, Heym S, Docke WD, Ruppe U, Tausch W, et al. Alterations in function and phenotype of monocytes from patients with septic disease–predictive value and new therapeutic strategies. *Behring Inst Mitt.* (1991) 88:208–15.
16. Kox WJ, Bone RC, Krausch D, Docke WD, Kox SN, Wauer H, et al. Interferon gamma-1b in the treatment of compensatory anti-inflammatory response syndrome. A new approach: proof of principle. *Arch Inter Med.* (1997) 157:389–93. doi: 10.1001/archinte.157.4.389
17. Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. *Crit Care Med.* (1996) 24:1125–8. doi: 10.1097/00003246-199607000-00010
18. Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, et al. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. *Nat Med.* (1997) 3:678–81. doi: 10.1038/nm0697-678
19. Ditschkowski M, Kreuzfelder E, Rebmann V, Ferencik S, Majetschak M, Schmid EN, et al. HLA-DR expression and soluble HLA-DR levels in septic patients after trauma. *Ann Surg.* (1999) 229:246–54. doi: 10.1097/00000658-199902000-00013
20. Monneret G, Lepape A, Voirin N, Bohe J, Venet F, Debard AL, et al. Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock. *Intens Care Med.* (2006) 32:1175–83. doi: 10.1007/s00134-006-0204-8
21. Oczenski W, Krenn H, Jilch R, Watzka H, Waldenberger F, Koller U, et al. HLA-DR as a marker for increased risk for systemic inflammation and septic complications after cardiac surgery. *Intens Care Med.* (2003) 29:1253–7. doi: 10.1007/s00134-003-1826-8
22. Perry SE, Mostafa SM, Wenstone R, Shenkin A, McLaughlin PJ. Is low monocyte HLA-DR expression helpful to predict outcome in severe sepsis? *Intens Care Med.* (2003) 29:1245–52. doi: 10.1007/s00134-003-1686-2
23. Dorhoi A, Du Plessis N. Monocytic myeloid-derived suppressor cells in chronic infections. *Front Immunol.* (2017) 8:1895. doi: 10.3389/fimmu.2017.01895
24. Yang H-M, Yu Y, Chai J-K, Hu S, Sheng Z-Y, Yao Y-M. Low HLA-DR expression on CD14+ monocytes of burn victims with sepsis, and the effect of carbachol *in vitro*. *Burns.* (2008) 34:1158–62. doi: 10.1016/j.burns.2008.01.026
25. Venet F, Tissot S, Debard AL, Faudot C, Crampe C, Pachot A, et al. Decreased monocyte human leukocyte antigen-DR expression after severe burn injury: correlation with severity and secondary septic shock. *Crit Care Med.* (2007) 35:1910–7. doi: 10.1097/01.CCM.0000275271.77350.B6
26. Antoniades CG, Berry PA, Davies ET, Hussain M, Bernal W, Vergani D, et al. Reduced monocyte HLA-DR expression: a novel biomarker of disease severity and outcome in acetaminophen-induced acute liver failure. *Hepatology.* (2006) 44:34–43. doi: 10.1002/hep.21240
27. Wasmuth HE, Kunz D, Yagmur E, Timmer-Stranghoner A, Vidacek D, Siewert E, et al. Patients with acute on chronic liver failure display "sepsis-like" immune paralysis.[see comment]. *J Hepatol.* (2005) 42:195–201. doi: 10.1016/j.jhep.2004.10.019

28. Berres ML, Schnyder B, Yagmur E, Inglis B, Stanzel S, Tischendorf JJ, et al. Longitudinal monocyte human leukocyte antigen-DR expression is a prognostic marker in critically ill patients with decompensated liver cirrhosis. *Liver Int*. (2009) 29:536–43. doi: 10.1111/j.1478-3231.2008.01870.x
29. Kylanpaa M-L, Mentula P, Kemppainen E, Puolakkainen P, Aittomaki S, Silvennoinen O, et al. Monocyte anergy is present in patients with severe acute pancreatitis and is significantly alleviated by granulocyte-macrophage colony-stimulating factor and interferon-gamma *in vitro*. *Pancreas*. (2005) 31:23–7. doi: 10.1097/01.mpa.0000164449.23524.94
30. Richter A, Nebe T, Wendl K, Schuster K, Klaebisch G, Quintel M, et al. HLA-DR expression in acute pancreatitis. *Eur J Surg Acta Chirurg*. (1999) 165:947–51. doi: 10.1080/110241599750008053
31. Gotzinger P, Sautner T, Spittler A, Barlan M, Wamser P, Roth E, et al. Severe acute pancreatitis causes alterations in HLA-DR and CD14 expression on peripheral blood monocytes independently of surgical treatment. *Eur J Surg Acta Chirurg*. (2000) 166:628–32. doi: 10.1080/11024150075000808286
32. Gustafson MP, Staff NP, Bornschlegl S, Butler GW, Maas ML, Kazamel M, et al. Comprehensive immune profiling reveals substantial immune system alterations in a subset of patients with amyotrophic lateral sclerosis. *PLoS ONE*. (2017) 12:e0182002. doi: 10.1371/journal.pone.0182002
33. Torrance HDT, Longbottom ER, Vivian ME, Lalabekyan B, Abbott TEF, Ackland GL, et al. Post-operative immune suppression is mediated via reversible, Interleukin-10 dependent pathways in circulating monocytes following major abdominal surgery. *PLoS ONE*. (2018) 13:e0203795. doi: 10.1371/journal.pone.0203795
34. Masternak K, Muhlethaler-Mottet A, Villard J, Zufferey M, Steimle V, Reith W. CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhanceosome complex. *Genes Dev*. (2000) 14:1156–66. doi: 10.1011/gad.14.9.1156
35. LeibundGut-Landmann S, Waldburger JM, Krawczyk M, Otten LA, Suter T, Fontana A, et al. Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *Eur J Immunol*. (2004) 34:1513–25. doi: 10.1002/eji.200424964
36. Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science*. (1994) 265:106–9. doi: 10.1126/science.8016643
37. Gonzalez-Junca A, Driscoll KE, Pellicciotta I, Du S, Lo CH, Roy R, et al. Autocrine TGFbeta is a survival factor for monocytes and drives immunosuppressive lineage commitment. *Cancer Immunol Res*. (2019) 7:306–20. doi: 10.1158/2326-6066.CIR-18-0310
38. Rohn W, Tang LP, Dong Y, Benveniste EN. IL-1 beta inhibits IFN-gamma-induced class II MHC expression by suppressing transcription of the class II transactivator gene. *J Immunol*. (1999) 162:886–96.
39. Koppelman B, Neefjes JJ, de Vries JE, de Waal Malefyt R. Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. *Immunity*. (1997) 7:861–71. doi: 10.1016/S1074-7613(00)80404-5
40. Thibodeau J, Bourgeois-Daigneault MC, Huppe G, Tremblay J, Aumont A, Houde M, et al. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *Eur J Immunol*. (2008) 38:1225–30. doi: 10.1002/eji.200737902
41. Le Tulzo Y, Pangault C, Amiot L, Guilloux V, Tribut O, Arvieux C, et al. Monocyte human leukocyte antigen-DR transcriptional downregulation by cortisol during septic shock. *Am J Respir Crit Care Med*. (2004) 169:1144–51. doi: 10.1164/rccm.200309-1329OC
42. Haveman JW, van den Berg AP, van den Berk JM, Mesander G, Slooff MJ, de Leij LH, et al. Low HLA-DR expression on peripheral blood monocytes predicts bacterial sepsis after liver transplantation: relation with prednisolone intake. *Trans Infect Dis*. (1999) 1:146–52. doi: 10.1034/j.1399-3062.1999.010302.x
43. Gustafson MP, Lin Y, New KC, Bulur PA, O'Neill BP, Dietz AB. Systemic immune suppression in glioblastoma: the interplay between CD14+HLA-DRlo/neg monocytes, tumor factors, and dexamethasone. *Neuro Oncol*. (2010) 12:631–44. doi: 10.1093/neuonc/noq001
44. Schwiebert LM, Schleimer RP, Radka SF, Ono SJ. Modulation of MHC class II expression in human cells by dexamethasone. *Cell Immunol*. (1995) 165:12–9. doi: 10.1006/cimm.1995.1181
45. Woiciechowsky C, Asadullah K, Nestler D, Schoning B, Glockner F, Docke WD, et al. Diminished monocytic HLA-DR expression and ex vivo cytokine secretion capacity in patients with glioblastoma: effect of tumor extirpation. *J Neuroimmunol*. (1998) 84:164–71. doi: 10.1016/S0165-5728(97)00236-1
46. Loercher AE, Nash MA, Kavanagh JJ, Platsoucas CD, Freedman RS. Identification of an IL-10-producing HLA-DR-negative monocyte subset in the malignant ascites of patients with ovarian cancer that inhibits cytokine protien expression and proliferation of autologous T cells. *J Immunology*. (1999) 163:6251–60.
47. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res*. (2006) 66:9290–8. doi: 10.1158/0008-5472.CAN-06-1819
48. Lin Y, Gustafson MP, Bulur PA, Gastineau DA, Witzig TE, Dietz AB. Immunosuppressive CD14+HLA-DR(low)/- monocytes in B-cell non-Hodgkin lymphoma. *Blood*. (2011) 117:872–81. doi: 10.1182/blood-2010-05-283820
49. Gustafson MP, Abraham RS, Lin Y, Wu W, Gastineau DA, Zent CS, et al. Association of an increased frequency of CD14(+) HLA-DR(lo/neg) monocytes with decreased time to progression in chronic lymphocytic leukaemia (CLL). *Br J Haematol*. (2012) 156:674–6. doi: 10.1111/j.1365-2141.2011.08902.x
50. Gustafson MP, Lin Y, Bleeker JS, Warad D, Tollefson MK, Crispen PL, et al. Intratumoral CD14+ cells and circulating CD14+HLA-DRlo/neg monocytes correlate with decreased survival in patients with clear cell renal cell carcinoma. *Clin Cancer Res*. (2015) 21:4224–33. doi: 10.1158/1078-0432.CCR-15-0260
51. Gustafson MP, Lin Y, LaPlant B, Liwski CJ, Maas ML, League SC, et al. Immune monitoring using the predictive power of immune profiles. *J Immunother Cancer*. (2013) 1:7. doi: 10.1186/2051-1426-1-7
52. Rodrigues JC, Gonzalez GC, Zhang L, Ibrahim G, Kelly JJ, Gustafson MP, et al. Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. *Neuro-Oncology*. (2010) 12:351–65. doi: 10.1093/neuonc/nop023
53. Okada SL, Simmons RM, Franke-Welch S, Nguyen TH, Korman AJ, Dillon SR, et al. Conditioned media from the renal cell carcinoma cell line 786.O drives human blood monocytes to a monocytic myeloid-derived suppressor cell phenotype. *Cell Immunol*. (2018) 323:49–58. doi: 10.1016/j.cellimm.2017.10.014
54. Ribechini E, Hutchinson JA, Hergovits S, Heuer M, Lucas J, Schleicher U, et al. Novel GM-CSF signals via IFN-gammaR/IRF-1 and AKT/mTOR license monocytes for suppressor function. *Blood Adv*. (2017) 1:947–60. doi: 10.1182/bloodadvances.2017006858
55. Bergenfelz C, Larsson AM, von Stedingk K, Gruvberger-Saal S, Aaltonen K, Jansson S, et al. Systemic monocytic-MDSCs are generated from monocytes and correlate with disease progression in breast cancer patients. *PLoS ONE*. (2015) 10:e0127028. doi: 10.1371/journal.pone.0127028
56. Meyer C, Cagnon L, Costa-Nunes CM, Baumgaertner P, Montandon N, Leyvraz L, et al. Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. *Cancer Immunol Immunother*. (2014) 63:247–57. doi: 10.1007/s00262-013-1508-5
57. Kitano S, Postow MA, Ziegler CG, Kuk D, Panageas KS, Cortez C, et al. Computational algorithm-driven evaluation of monocytic myeloid-derived suppressor cell frequency for prediction of clinical outcomes. *Cancer Immunol Res*. (2014) 2:812–21. doi: 10.1158/2326-6066.CIR-14-0013
58. Tarhini AA, Edington H, Butterfield LH, Lin Y, Shuai Y, Tawbi H, et al. Immune monitoring of the circulation and the tumor microenvironment in patients with regionally advanced melanoma receiving neoadjuvant ipilimumab. *PLoS ONE*. (2014) 9:e87705. doi: 10.1371/journal.pone.0087705
59. Martens A, Wistuba-Hamprecht K, Geukes Foppen M, Yuan J, Postow MA, Wong P, et al. Baseline peripheral blood biomarkers associated with clinical outcome of advanced melanoma patients treated with ipilimumab. *Clin Cancer Res*. (2016) 22:2908–18. doi: 10.1158/1078-0432.CCR-15-2412

60. Gebhardt C, Sevko A, Jiang H, Lichtenberger R, Reith M, Tarnanidis K, et al. Myeloid cells and related chronic inflammatory factors as novel predictive markers in melanoma treatment with ipilimumab. *Clin Cancer Res*. (2015) 21:5453–9. doi: 10.1158/1078-0432.CCR-15-0676
61. de Coana YP, Wolodarski M, Poschke I, Yoshimoto Y, Yang Y, Nystrom M, et al. Ipilimumab treatment decreases monocytic MDSCs and increases CD8 effector memory T cells in long-term survivors with advanced melanoma. *Oncotarget*. (2017) 8:21539–53. doi: 10.18632/oncotarget.15368
62. Krieg C, Nowicka M, Guglietta S, Schindler S, Hartmann FJ, Weber LM, et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat Med*. (2018) 24:144–53. doi: 10.1038/nm.4466
63. Eissler N, Mao Y, Brodin D, Reutersward P, Andersson Svahn H, Johnsen JI, et al. Regulation of myeloid cells by activated T cells determines the efficacy of PD-1 blockade. *Oncoimmunology*. (2016) 5:e1232222. doi: 10.1080/2162402X.2016.1232222
64. Antonios JP, Soto H, Everson RG, Moughon D, Orpilla JR, Shin NP, et al. Immunosuppressive tumor-infiltrating myeloid cells mediate adaptive immune resistance via a PD-1/PD-L1 mechanism in glioblastoma. *Neuro-Oncology*. (2017) 19:796–807. doi: 10.1093/neuonc/now287
65. Weber J, Gibney G, Kudchadkar R, Yu B, Cheng P, Martinez AJ, et al. Phase I/II study of metastatic melanoma patients treated with nivolumab who had progressed after ipilimumab. *Cancer Immunol Res*. (2016) 4:345–53. doi: 10.1158/2326-6066.CIR-15-0193
66. Chavan R, Salvador D, Gustafson MP, Dietz AB, Nevala W, Markovic SN. Untreated stage IV melanoma patients exhibit abnormal monocyte phenotypes and decreased functional capacity. . *Cancer Immunol Res*. (2014) 2:241–8. doi: 10.1158/2326-6066.CIR-13-0094
67. Campbell DE, Tustin NB, Riedel E, Tustin R III, Taylor J, Murray J, et al. Cryopreservation decreases receptor PD-1 and ligand PD-L1 coinhibitory expression on peripheral blood mononuclear cell-derived T cells and monocytes. *Clin Vaccine Immunol*. (2009) 16:1648–53. doi: 10.1128/CVI.00259-09
68. Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, et al. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. *Nat Med*. (2010) 16:452–9. doi: 10.1038/nm.2106
69. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med*. (2012) 18:1254–61. doi: 10.1038/nm.2883
70. Hansen GL, Gaudernack G, Brunsvig PF, Cvancarova M, Kyte JA. Immunological factors influencing clinical outcome in lung cancer patients after telomerase peptide vaccination. *Cancer Immunol Immunother*. (2015) 64:1609–21. doi: 10.1007/s00262-015-1766-5
71. Santegoets SJ, Stam AG, Lougheed SM, Gall H, Jooss K, Sacks N, et al. Myeloid derived suppressor and dendritic cell subsets are related to clinical outcome in prostate cancer patients treated with prostate GVAX and ipilimumab. *J Immunother Cancer*. (2014) 2:31. doi: 10.1186/s40425-014-0031-3
72. Olin MR, Low W, McKenna DH, Haines SJ, Dahlheimer T, Nascene D, et al. Vaccination with dendritic cells loaded with allogeneic brain tumor cells for recurrent malignant brain tumors induces a CD4(+)IL17(+) response. *J Immunother Cancer*. (2014) 2:4. doi: 10.1186/2051-1426-2-4
73. Poschke I, Mao Y, Adamson L, Salazar-Onfray F, Masucci G, Kiessling R. Myeloid-derived suppressor cells impair the quality of dendritic cell vaccines. *Cancer Immunol Immunother*. (2012) 61:827–38. doi: 10.1007/s00262-011-1143-y
74. Kansagra A, Inwards DJ, Ansell SM, Micallef IN, Johnston PB, Hogan WJ, et al. Infusion of autograft natural killer cell/CD14(+)HLA-DR(DIM) cell ratio predicts survival in lymphoma post autologous stem cell transplantation. *Bone Marrow Transplant*. (2018) 53:146–54. doi: 10.1038/bmt.2017.225
75. Guan Q, Blankstein AR, Anjos K, Synova O, Tulloch M, Giftakis A, et al. Functional myeloid-derived suppressor cell subsets recover rapidly after allogeneic hematopoietic stem/progenitor cell transplantation. *Biol Blood Marrow Transplant*. (2015) 21:1205–14. doi: 10.1016/j.bbmt.2015.04.015
76. Storek J, Geddes M, Khan F, Huard B, Helg C, Chalandon Y, et al. Reconstitution of the immune system after hematopoietic stem cell transplantation in humans. *Sem Immunopathol*. (2008) 30:425–37. doi: 10.1007/s00281-008-0132-5
77. Mougiakakos D, Jitschin R, von Bahr L, Poschke I, Gary R, Sundberg B, et al. Immunosuppressive CD14+HLA-DRlow/neg IDO+ myeloid cells in patients following allogeneic hematopoietic stem cell transplantation. *Leukemia*. (2013) 27:377–88. doi: 10.1038/leu.2012.215
78. Luyckx A, Schouppe E, Rutgeerts O, Lenaerts C, Fevery S, Devos T, et al. G-CSF stem cell mobilization in human donors induces polymorphonuclear and mononuclear myeloid-derived suppressor cells. *Clin Immunol*. (2012) 143:83–7. doi: 10.1016/j.clim.2012.01.011
79. Vendramin A, Gimondi S, Bermema A, Longoni P, Rizzitano S, Corradini P, et al. Graft monocytic myeloid-derived suppressor cell content predicts the risk of acute graft-versus-host disease after allogeneic transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood stem cells. *Biol Blood Marrow Transplant*. (2014) 20:2049–55. doi: 10.1016/j.bbmt.2014.09.011
80. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, et al. Identification of a new subset of myeloid suppressor cells in pheripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol*. (2007) 25:2546–53. doi: 10.1200/JCO.2006.08.5829
81. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP, et al. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4+CD25+Foxp3+ T cells. *Gastroenterology*. (2008) 135:234–43. doi: 10.1053/j.gastro.2008.03.020
82. Jitschin R, Braun M, Buttner M, Dettmer-Wilde K, Bricks J, Berger J, et al. CLL-cells induce IDOhi CD14+HLA-DRlo myeloid-derived suppressor cells that inhibit T-cell responses and promote TRegs. *Blood*. (2014) 124:750–60. doi: 10.1182/blood-2013-12-546416
83. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. *Cancer Res*. (2010) 70:4335–45. doi: 10.1158/0008-5472.CAN-09-3767
84. Weide B, Martens A, Zelba H, Stutz C, Derhovanessian E, Di Giacomo AM, et al. Myeloid-derived suppressor cells predict survival of patients with advanced melanoma: comparison with regulatory T cells and NY-ESO-1- or melan-A-specific T cells. *Clin Cancer Res*. (2014) 20:1601–9. doi: 10.1158/1078-0432.CCR-13-2508
85. Stroncek DF, Ren J, Lee DW, Tran M, Frodigh SE, Sabatino M, et al. Myeloid cells in peripheral blood mononuclear cell concentrates inhibit the expansion of chimeric antigen receptor T cells. *Cytotherapy*. (2016) 18:893–901. doi: 10.1016/j.jcyt.2016.04.003
86. Burga RA, Thorn M, Point GR, Guha P, Nguyen CT, Licata LA, et al. Liver myeloid-derived suppressor cells expand in response to liver metastases in mice and inhibit the anti-tumor efficacy of anti-CEA CAR-T. *Cancer Immunol Immunother*. (2015) 64:817–29. doi: 10.1007/s00262-015-1692-6
87. Hosoi A, Matsushita H, Shimizu K, Fujii S, Ueha S, Abe J, et al. Adoptive cytotoxic T lymphocyte therapy triggers a counter-regulatory immunosuppressive mechanism via recruitment of myeloid-derived suppressor cells. *Int J Cancer*. (2014) 134:1810–22. doi: 10.1002/ijc.28506
88. Arina A. Rethinking the role of myeloid-derived suppressor cells in adoptive T-cell therapy for cancer. *Oncoimmunology*. (2014) 3:e28464. doi: 10.4161/onci.28464
89. Liu Y, Wei G, Cheng WA, Dong Z, Sun H, Lee VY, et al. Targeting myeloid-derived suppressor cells for cancer immunotherapy. *Cancer Immunol Immunother*. (2018) 67:1181–95. doi: 10.1007/s00262-018-2175-3
90. Anani W, Shurin MR. Targeting myeloid-derived suppressor cells in cancer. *Adv Exp Med Biol*. (2017) 1036:105–28. doi: 10.1007/978-3-319-67577-0_8
91. Draghiciu O, Lubbers J, Nijman HW, Daemen T. Myeloid derived suppressor cells-An overview of combat strategies to increase immunotherapy efficacy. *Oncoimmunology*. (2015) 4:e954829. doi: 10.4161/21624011.2014.954829
92. Najjar YG, Finke JH. Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer. *Front Oncol*. (2013) 3:49. doi: 10.3389/fonc.2013.00049
93. Weber R, Fleming V, Hu X, Nagibin V, Groth C, Altevogt P, et al. Myeloid-derived suppressor cells hinder the anti-cancer activity of immune checkpoint inhibitors. *Front Immunol*. (2018) 9:1310. doi: 10.3389/fimmu.2018.01310

94. Le HK, Graham L, Cha E, Morales JK, Manjili MH, Bear HD. Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *Int Immunopharmacol.* (2009) 9:900–9. doi: 10.1016/j.intimp.2009.03.015
95. Eriksson E, Wenthe J, Irenaeus S, Loskog A, Ullenhag G. Gemcitabine reduces MDSCs, tregs and TGFbeta-1 while restoring the teff/treg ratio in patients with pancreatic cancer. *J Trans Med*. (2016) 14:282. doi: 10.1186/s12967-016-1037-z
96. Dominguez GA, Condamine T, Mony S, Hashimoto A, Wang F, Liu Q, et al. Selective targeting of myeloid-derived suppressor cells in cancer patients using DS-8273a, an agonistic TRAIL-R2 antibody. *Clin Cancer Res*. (2017) 23:2942–50. doi: 10.1158/1078-0432.CCR-16-1784
97. Giallongo C, Parrinello NL, La Cava P, Camiolo G, Romano A, Scalia M, et al. Monocytic myeloid-derived suppressor cells as prognostic factor in chronic myeloid leukaemia patients treated with dasatinib. *J Cell Mol Med*. (2018) 22:1070–80. doi: 10.1111/jcmm.13326
98. Chen HM, Ma G, Gildener-Leapman N, Eisenstein S, Coakley BA, Ozao J, et al. Myeloid-derived suppressor cells as an immune parameter in patients with concurrent sunitinib and stereotactic body radiotherapy. *Clin Cancer Res*. (2015) 21:4073–85. doi: 10.1158/1078-0432.CCR-14-2742
99. Nywening TM, Wang-Gillam A, Sanford DE, Belt BA, Panni RZ, Cusworth BM, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. *Lancet Oncol*. (2016) 17:651–62. doi: 10.1016/S1470-2045(16)00078-4
100. Nywening TM, Belt BA, Cullinan DR, Panni RZ, Han BJ, Sanford DE, et al. Targeting both tumour-associated CXCR2(+) neutrophils and CCR2(+) macrophages disrupts myeloid recruitment and improves chemotherapeutic responses in pancreatic ductal adenocarcinoma. *Gut*. (2018) 67:1112–23. doi: 10.1136/gutjnl-2017-313738
101. Chang AL, Miska J, Wainwright DA, Dey M, Rivetta CV, Yu D, et al. CCL2 Produced by the glioma microenvironment is essential for the recruitment of regulatory T cells and myeloid-derived suppressor cells. *Cancer Res*. (2016) 76:5671–82. doi: 10.1158/0008-5472.CAN-16-0144
102. Eruslanov E, Neuberger M, Daurkin I, Perrin GQ, Algood C, Dahm P, et al. Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer. *Int J Cancer*. (2012) 130:1109–19. doi: 10.1002/ijc.26123
103. Stanley ER, Chitu V. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb Perspect Biol.* (2014) 6:a021857. doi: 10.1101/cshperspect.a021857
104. Chitu V, Stanley ER. Colony-stimulating factor-1 in immunity and inflammation. *Curr Opin Immunol.* (2006) 18:39–48. doi: 10.1016/j.coi.2005.11.006
105. Butowski N, Colman H, De Groot JF, Omuro AM, Nayak L, Wen PY, et al. Orally administered colony stimulating factor 1 receptor inhibitor PLX3397 in recurrent glioblastoma: an Ivy foundation early phase clinical trials consortium phase II study. *Neuro-Oncology*. (2016) 18:557–64. doi: 10.1093/neuonc/nov245
106. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med.* (2013) 19:1264–72. doi: 10.1038/nm.3337
107. Mao Y, Eissler N, Blanc KL, Johnsen JI, Kogner P, Kiessling R. Targeting Suppressive myeloid cells potentiates checkpoint inhibitors to control spontaneous neuroblastoma. *Clin Cancer Res*. (2016) 22:3849–59. doi: 10.1158/1078-0432.CCR-15-1912
108. Kaufman HL, Ruby CE, Hughes T, Slingluff CL, Jr. Current status of granulocyte-macrophage colony-stimulating factor in the immunotherapy of melanoma. *J Immunother Cancer*. (2014) 2:11. doi: 10.1186/2051-1426-2-11
109. Lipson EJ, Sharfman WH, Chen S, McMiller TL, Pritchard TS, Salas JT, et al. Safety and immunologic correlates of Melanoma GVAX, a GM-CSF secreting allogeneic melanoma cell vaccine administered in the adjuvant setting. *J Trans Med.* (2015) 13:214. doi: 10.1186/s12967-015-0572-3
110. Lacher MD, Bauer G, Fury B, Graeve S, Fledderman EL, Petrie TD, et al. SV-BR-1-GM, a clinically effective GM-CSF-secreting breast cancer cell line, expresses an immune signature and directly activates CD4(+) T lymphocytes. *Front Immunol.* (2018) 9:776. doi: 10.3389/fimmu.2018.00776
111. Lawson DH, Lee S, Zhao F, Tarhini AA, Margolin KA, Ernstoff MS, et al. Randomized, placebo-controlled, phase III trial of yeast-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) versus peptide vaccination versus GM-CSF plus peptide vaccination versus placebo in patients with no evidence of disease after complete surgical resection of locally advanced and/or stage iv melanoma: a trial of the eastern cooperative oncology group-american college of radiology imaging network cancer research group (e4697). *J Clin Oncol*. (2015) 33:4066–76. doi: 10.1200/JCO.2015.62.0500
112. Le DT, Wang-Gillam A, Picozzi V, Greten TF, Crocenzi T, Springett G, et al. Safety and survival with GVAX pancreas prime and Listeria Monocytogenes-expressing mesothelin (CRS-207) boost vaccines for metastatic pancreatic cancer. *J Clin Oncol.* (2015) 33:1325–33. doi: 10.1200/JCO.2014.57.4244
113. Faries MB, Hsueh EC, Ye X, Hoban M, Morton DL. Effect of granulocyte/macrophage colony-stimulating factor on vaccination with an allogeneic whole-cell melanoma vaccine. *Clin Cancer Res*. (2009) 15:7029–35. doi: 10.1158/1078-0432.CCR-09-1540
114. Slingluff CL Jr, Petroni GR, Olson WC, Smolkin ME, Ross MI, Haas NB, et al. Effect of granulocyte/macrophage colony-stimulating factor on circulating CD8+ and CD4+ T-cell responses to a multipeptide melanoma vaccine: outcome of a multicenter randomized trial. *Clin Cancer Res*. (2009) 15:7036–44. doi: 10.1158/1078-0432.CCR-09-1544
115. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res.* (2004) 64:6337–43. doi: 10.1158/0008-5472.CAN-04-0757
116. Morales JK, Kmieciak M, Knutson KL, Bear HD, Manjili MH. GM-CSF is one of the main breast tumor-derived soluble factors involved in the differentiation of CD11b-Gr1- bone marrow progenitor cells into myeloid-derived suppressor cells. *Breast Cancer Res Treatment*. (2010) 123:39–49. doi: 10.1007/s10549-009-0622-8
117. Thorn M, Guha P, Cunetta M, Espat NJ, Miller G, Junghans RP, et al. Tumor-associated GM-CSF overexpression induces immunoinhibitory molecules via STAT3 in myeloid-suppressor cells infiltrating liver metastases. *Cancer Gene Therapy*. (2016) 23:188–98. doi: 10.1038/cgt.2016.19
118. Strauss L, Sangaletti S, Consonni FM, Szebeni G, Morlacchi S, Totaro MG, et al. RORC1 regulates tumor-promoting "Emergency" granulo-monocytopoiesis. *Cancer Cell*. (2015) 28:253–69. doi: 10.1016/j.ccell.2015.07.006
119. Lesokhin AM, Hohl TM, Kitano S, Cortez C, Hirschhorn-Cymerman D, Avogadri F, et al. Monocytic CCR2(+) myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment. *Cancer Res.* (2012) 72:876–86. doi: 10.1158/0008-5472.CAN-11-1792
120. Kohanbash G, McKaveney K, Sakaki M, Ueda R, Mintz AH, Amankulor N, et al. GM-CSF promotes the immunosuppressive activity of glioma-infiltrating myeloid cells through interleukin-4 receptor-alpha. *Cancer Res.* (2013) 73:6413–23. doi: 10.1158/0008-5472.CAN-12-4124
121. Okla K, Wertel I, Wawruszak A, Bobinski M, Kotarski J. Blood-based analyses of cancer: Circulating myeloid-derived suppressor cells - is a new era coming? *Crit Rev Clin Lab Sci.* (2018) 55:376–407. doi: 10.1080/10408363.2018.1477729
122. Mandruzzato S, Brandau S, Britten CM, Bronte V, Damuzzo V, Gouttefangeas C, et al. Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study. *Cancer Immunol Immunother.* (2016) 65:161–9. doi: 10.1007/s00262-015-1782-5
123. Solito S, Marigo I, Pinton L, Damuzzo V, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity in human cancers. *Ann N Y Acad Sci.* (2014) 1319:47–65. doi: 10.1111/nyas.12469
124. Demaret J, Walencik A, Jacob MC, Timsit JF, Venet F, Lepape A, et al. Inter-laboratory assessment of flow cytometric monocyte HLA-DR expression in clinical samples. *Cytomet Part B Clin Cytomet.* (2013) 84:59–62. doi: 10.1002/cyto.b.21043

125. Gustafson MP, Lin Y, Maas ML, Van Keulen VP, Johnston PB, Peikert T, et al. A method for identification and analysis of non-overlapping myeloid immunophenotypes in humans. *PLoS ONE*. (2015) 10:e0121546. doi: 10.1371/journal.pone.0121546
126. Wagner C, Deppisch R, Denefleh B, Hug F, Andrassay K, Hansch GM. Expression patterns of the lipopolysaccharide receptor CD14, and the FCg receptors CD16 and CD64 on polymorphonuclear neutrophils: data from patients with severe bacterial infections and lipopolysaccharide-exposed cells. *Shock*. (2003) 19:5–12. doi: 10.1097/00024382-200301000-00002
127. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. (2010) 116:e74–80. doi: 10.1182/blood-2010-02-258558
128. Duffy A, Zhao F, Haile L, Gamrekelashvili J, Fioravanti S, Ma C, et al. Comparative analysis of monocytic and granulocytic myeloid-derived suppessor cell subsets in patients with gastrointestinal malignancies. *Cancer Immunol Immunother*. (2013) 62:299–307. doi: 10.1007/s00262-012-1332-3
129. Kotsakis A, Harasymczuk M, Schilling B, Georgoulias V, Argiris A, Whiteside TL. Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples. *J Immunol Methods*. (2012) 381:14–22. doi: 10.1016/j.jim.2012.04.004
130. Idorn M, Kollgaard T, Kongsted P, Sengelov L, Thor Straten P. Correlation between frequencies of blood monocytic myeloid-derived suppressor cells, regulatory T cells and negative prognostic markers in patients with castration-resistant metastatic prostate cancer. *Cancer Immunol Immunother*. (2014) 63:1177–87. doi: 10.1007/s00262-014-1591-2
131. Monneret G, Elmenkouri N, Bohe J, Debard AL, Gutowski MC, Bienvenu J, et al. Analytical requirements for measuring monocytic human lymphocyte antigen DR by flow cytometry: application to the monitoring of patients with septic shock. *Clin Chem*. (2002) 48:1589–92.
132. Docke WD, Hoflich C, Davis KA, Rottgers K, Meisel C, Kiefer P, et al. Monitoring temporary immunodepression by flow cytometric measurement of monocytic HLA-DR expression: a multicenter standardized study. *Clinical Chem*. (2005) 51:2341–7. doi: 10.1373/clinchem.2005.052639
133. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, et al. Systemic immunity is required for effective cancer immunotherapy. *Cell*. (2017) 168:487–502.e15. doi: 10.1016/j.cell.2016.12.022

Conflict of Interest Statement: MG has intellectual property and two patents or pending patents associated with the enumeration of immune system profiles.

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

frontiers in Immunology

PERSPECTIVE
published: 31 May 2019
doi: 10.3389/fimmu.2019.01201

Tracking Monocytes and Macrophages in Tumors With Live Imaging

*Marie Laviron, Christophe Combadière and Alexandre Boissonnas**

Centre d'Immunologie et des Maladies Infectieuses CIMI, CNRS, Sorbonne Université, Inserm, Paris, France

In most cancers, myeloid cells represent the major component of the immune microenvironment. Deciphering the impact of these cells on tumor growth and in response to various anti-tumor therapies is a key issue. Many studies have elucidated the role of tumor-associated monocytes and tumor-associated macrophages (TAM) in tumor development, angiogenesis, and therapeutic failure. In contrast, tumor dendritic cells (DC) are associated with tumor antigen uptake and T-cell priming. Myeloid subpopulations display differences in ontogeny, state of differentiation and distribution within the neoplastic tissue, making them difficult to study. The development of high-dimensional genomic and cytometric analyses has unveiled the large functional diversity of myeloid cells. Important fundamental insights on the biology of myeloid cells have also been provided by a boom in functional fluorescent imaging techniques, in particular for TAM. These approaches allow the tracking of cell behavior in native physiological environments, incorporating spatio-temporal dimensions in the study of their functional activity. Nevertheless, tracking myeloid cells within the TME remains a challenging process as many markers overlap between monocytes, macrophages, DC, and neutrophils. Therefore, perfect discrimination between myeloid subsets remains impossible to date. Herein we review the specific functions of myeloid cells in tumor development unveiled by image-based tracking, the limits of fluorescent reporters commonly used to accurately track specific myeloid cells, and novel combinations of myeloid-associated fluorescent reporters that better discriminate the relative contributions of these cells to tumor biology according to their origin and tissue localization.

Keywords: tumor-associated macrophages, live imaging, fluorescence reporters, immuno oncology, multiphoton imaging, two-photon microscopy

OPEN ACCESS

Edited by:
Wei Zheng,
Albert Einstein College of Medicine, United States

Reviewed by:
Jose Javier Bravo-Cordero, Icahn School of Medicine at Mount Sinai, United States
Keehoon Jung, Seoul National University, South Korea
Leo Marc Carlin, University of Glasgow, United Kingdom

****Correspondence:***
Alexandre Boissonnas
alexandre.boissonnas@upmc.fr

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *22 March 2019*
Accepted: *13 May 2019*
Published: *31 May 2019*

Citation:
Laviron M, Combadière C and Boissonnas A (2019) Tracking Monocytes and Macrophages in Tumors With Live Imaging. Front. Immunol. 10:1201. doi: 10.3389/fimmu.2019.01201

INTRODUCTION

Myeloid cells form a vast and heterogeneous group of cells that play a major role in shaping the tumor microenvironment (TME). Tumor associated macrophages (TAM) represent the most abundant myeloid subset across multiple cancer types, and they generally correlate with poor outcomes. Dendritic cells (DC) in tumors represent a less abundant subset, and contradictory results surround their association with tumor prognosis. DC are classified into subpopulations exhibiting different specificity for priming T-cells (1, 2). Macrophages and DC subsets display a strong overlap of phenotypic markers, adding a high level of complexity to accurately identify

them. So far, TAM have been considered to arise primarily from monocyte cells. Recent discoveries regarding tissue macrophage ontogeny challenge this assumption (3). Different tissues of origin are likely to contribute in part to how TAM heterogeneity arises (4–6). Flow cytometry allows qualitative and quantitative characterization of these cells but does not preserve their *in situ* localization to study native cell-cell interactions. In contrast, intra-vital imaging at cellular-scale resolution offers the ability to study cell migration and interactions in living tissue in real-time. It is tempting to consider "truth" what is visible to the eye, and thus direct visualization of cell interactions tends to provide more confidence in the interpretation of a biological process. A main hurdle of this approach rests on the accurate tracking of these cells since the number of available markers are more limited than for flow cytometry and many markers overlap between monocytes, macrophages, DC and even neutrophils, potentially leading to misinterpretations. Moreover, one must keep in mind that imaging experiments usually focus on specific cell subsets, avoiding the potential contribution of the "unseen." Herein, we review how fluorescent imaging, and more specifically *in situ* live imaging, has contributed to the characterization of TAM and tumor-DC. We discuss limitations of the most common models used for the discrimination and tracking of these different subsets, and we present some perspectives derived from the combination of different fluorescent reporter mouse strains used to unveil microanatomical niches of myeloid subsets in tumors.

FUNCTIONAL IMAGING OF TUMOR-ASSOCIATED MYELOID CELLS

Microscopy studies represent a necessary approach to truly comprehend the relationship between cells in their physiological environment (7). Beyond the simple identification of cell distribution across the tissue provided by histological analysis, the development of live imaging *in situ* has generated fundamental insights in cellular functions and is termed "functional imaging." Here, we mention studies based on monocyte and TAM imaging to highlight how this approach has contributed to our knowledge of their function within tumors.

Functional Imaging of Tumor-Associated Myeloid Cell Dynamics and Interactions With Tumor Cells

Intra-vital imaging of TAM has helped to identify their role in tumor invasiveness and metastasis (8, 9). Direct visualization of fluorescent macrophages and tumor cell lines has revealed CSF1 and EGF-dependent chemotaxis, respectively, (10) and has led to the elaboration of a tumor cell/macrophage cross-talk model (7, 11). *In vitro* imaging is an important complementary approach to study the molecular pathways involved in this model. Beyond paracrine loops, the combination of *in vitro* and *in vivo* imaging has provided evidence that physical contacts between macrophages and tumor cells correlate with invadopodium formation through the induction of RhoA activity on tumor cells (12). The strength of real time imaging is elegantly illustrated by the work of Harney et al showing that the role of Tie2+ perivascular macrophages in this intravasation process is transient and mainly occurs in highly defined microanatomical niches termed "Tumor Microenvironment of Metastasis" (TMEM) (13). Another study has found that macrophages orchestrating early dissemination in breast cancer are $CD206^{Hi}$ and $Tie2^{+}$ and migrate toward tumor cells through CCL2 production by the latter (14).

Macrophages have also been involved in the "streaming cell movement" of tumor cells, defined as the migration of multiple cells in a single file pattern (15). Directional streaming toward the endothelium results from CXCR4 upregulation on TAM and CXCL12 secretion by peripheral fibroblasts (16). Cocultures in 3D-matrices have provided the subcellular resolution to identify a macrophage/tumor cell communication mechanism involving the formation of tunneling nanotubes between the two cell types that is required to induce this directional cell streaming (17). This heterotypic interaction might favor the switch from a mesenchymal migration mode of tumor cells toward an MMP-independent ameboid-like migration as observed in spheroid culture (18). Cytoplasmic exchange between macrophages and tumor cells has been confirmed *in vivo* in zebrafish (19). *In vivo* visualization of migratory activity of TAM, tumor-DC and neutrophils has been observed using differentially ingested dextran particles or differential staining by intravascular injection of fluorescent antibodies in MMTV-PyMT/cfms-$EGFP^{+}$ mice. Sessile cells exhibited strong endocytosis and MMP activity, however TAM and tumor-DC could not be disciminated based on the tested labeling combination (20). Similar labeling approaches have unveiled that migratory capacities of myeloid cells in mammary cancer were less sensitive to hypoxia than regulatory T-cells (21).

Considering macrophage ontogeny and tissue specification has raised the question of their differential function in pathological contexts, particularly in cancer development. Although microglial cells have been considered as the primary TAM subset in brain tumors, it is commonly held that the majority of TAM among many other tumors are monocyte-derived (MoD-TAM) (22). Evidence is recently accumulating that tissue-resident macrophages represent a distinct functional subset from MoD-TAM in other cancer types (16, 23, 24). While resident macrophages were associated with ECM production, recruited macrophages were more involved in the modulation of the adaptive immune response (24), in addition to matrix remodeling and tumor cell clearance following chemotherapeutic treatment (16).

So far, very little information on the role of tissue-resident macrophages in solid tumors is available from imaging studies. The reporter model used in our recent study has been an interesting option for simultaneous tracking of macrophages of different origins in lung tumors (16). MoD-TAM and monocytes tended to accumulate in the periphery of advanced lung tumor nodules and displayed higher displacements than their resident counterparts (16). Their increased migratory behavior also fits with the observation of streaming TAM recruited in a CCR2-dependent manner (25). Accordingly, CCR2-dependent recruited TAM in lung tumors have been associated with remodeling activity and higher tumor cell dissemination (16).

So far, modulating the CCL2/CCR2 axis appears useful in identifying the monocytic origin of TAM. Nevertheless, while the accumulation of tissue resident macrophages has been shown to be CCR2-independent in lung tumors (16), this subset binds CCL2, suggesting that they might respond to a local CCL2 gradient. One should consider that targeting the CCR2 axis may directly or indirectly affect recruited as well as resident TAM. Resident TAM do not necessarily have an embryonic origin but could also arise from local proliferation of MoD-resident macrophages that have progressively colonized the tissue at steady state as observed in several tissues (26). Fate mapping models to track embryonic-derived macrophages by imaging are necessary to determine whether resident TAM are of embryonic origin.

Functional Imaging of TAM Role in Metastatic Seeding

With the opportunity to track single cells in real time, live imaging has greatly improved our knowledge on the early events of metastatic seeding, in particular through the development of *in vivo* lung imaging (27). Patrolling monocytes have been reported to rapidly engulf tumor material in lung capillaries reducing metastasis development (28). This patrolling activity has also been efficiently monitored using a peritoneal window in colorectal tumors treated with anti-VEGFR2 therapy, highlighting a protumoral activity through neutrophil recruitment (29). Patrolling monocytes do not appear to be the only myeloid cells involved in this process. Rather, a series of sequential waves involving different myeloid subsets are able to uptake tumor material in the lung (30). CCL2-dependent monocyte recruitment has been strongly implicated in metastatic seeding by experiments utilizing CCL2 blockade or global macrophage depletion (31, 32). The relative roles of interstitial lung macrophages and monocyte-derived cells on this early process remain unclear.

Functional Imaging of TAM and Tumor-DC Interactions With Lymphocytes

Live imaging has also contributed to identifying direct interactions of myeloid cells with T-cells in the TME. Trapping of antigen specific T-cells by myeloid cells in sustained and non-productive interactions has been proposed to favor immunosuppression (33, 34). Macrophage depletion has been associated with increased CD8 T-cell infiltration and improved response to anti-PD-1 "checkpoint" immunotherapy (35). Macrophage/Treg interactions after radiotherapy have also been visualized in a model of head and neck cancer. TNF-mediated cross talk between the two subsets is a proposed mechanism responsible for how an immunosuppressive environment dampens therapeutic efficacy (36). While the vast majority of tumor-infiltrating T-cells seem to be in contact with TAM correlating with poor ability to induce effector functions, Broz et al. have identified a sparse subset of tumor-DC with strong immunostimulatory capacities (2). Recruitment of this subset via NK cell crosstalk mediated by FLT3 ligand and resulting physical interactions defines a positive prognostic factor for anti-PD-1 therapy in melanoma patients (37). Overall, this supports the idea that TAM are usually associated with immune suppressive activity while tumor-DC are more immunostimulatory (38).

Overall, monitoring myeloid cell dynamics, morphology, local distribution in specific TMEM, and interactions with other partners of the TME has unveiled many of their key biological mechanisms. However, the capacity to accurately identify specific myeloid subsets by imaging can be limiting.

TRACKING MYELOID CELLS IN TUMORS

Specific identification of myeloid cells by imaging is challenging because of their heterogeneity, plasticity, and overlapping markers.

In vivo antibody injection represents an interesting alternative for cell identification, but there are multiple limitations of this approach. Efficient cell staining is limited by tissue penetration of antibodies, and the persistence of the staining is low due to degradation and recycling activities in living tissues. Finally, the impact of multiple *in vivo* antibody staining on cell dynamics and function cannot be neglected, and findings regarding cell behavior should be interpreted with caution. Fluorescent reporter mice are, thus far, the best option to overcome these limitations. However, the lack of cell-specifc labeling ability still presents a challenge. Promoter-driven fluorescent protein (FP) production is never restricted to a specific subset. Moreover, it is not recommended to associate reporter expression with endogenous protein expression. Therefore, a careful phenotypic characterization of each model using flow cytometry is required to adequately define the imaged cell populations.

Many transgenic mice (listed below) have been developed with various fluorescent reporters to attempt to discriminate specific myeloid populations.

The development of a Csf1r-EGFP transgene (MacGreen) has confirmed that this receptor is expressed in monocytes, tissue-resident macrophages and some populations of DC, such as the Langerhans cells; yet is also present in trophoblasts and granulocytes (39, 40). The deletion of a conserved distal element from the Csf1r promoter on the ΔCSF1R-ECFP reporter (MacBlue) mouse ablated expression in trophoblasts and reduced expression in granulocytes (41). Reporter gene expression is maintained in alveolar macrophages, microglia, and Langerhans cells, however it is ablated in most resident macrophage populations including osteoclasts (42), Kupffer cells (43), and lung interstital macrophages (44). Hawley et al. created a Csf1r-mApple mouse (MacApple) with the same pattern of expression as MacGreen mice (45). Crossing MacApple with MacBlue mice results in specific patterns of fluorescent expression among monocytes and macrophages as observed in the lung and the brain. The authors propose that ECFP expression may be present in cells relying more on IL-34 or CSF2 while $ECFP^{-}$ $mApple^{+}$ macrophages would depend more on CSF1 for their

homeostasis (45, 46). The regulation of CSF1R expression requires further investigation.

The *Cx3cr1*^{EGFP} reporter mouse (47) is commonly used to monitor patrolling monocytes (29, 48–50) and tissue macrophages (51), but this reporter is also expressed by subsets of NK cells and dendritic cells as well as epidermal T-cells harboring a dendritic-like morphology. EGFP upregulation on subsets of T-cells has been also reported during viral infection (52). Whether tumor-infiltrating T-cells upregulate CX3CR1 must be investigated when using this strain as they can represent an important confounding subset when imaging the TME. We have developed an additional dimension of resolution using the combination of MacBlue x *Cx3cr1*^{EGFP} x MacApple reporter mice. This strain provides an improved display of the myeloid compartement heterogeneity in lung tumors, allowing the visualization of recruited, resident interstitial, and alveolar macrophages as well as neutrophils based on differential expression of the fluorescent reporters (**Figure 1A**). This further highlights microanatomical niches with specific myeloid subset distributions (**Figure 1B**). Although EGFP expression is lower in classical compared to non-classical monocytes (and has thus been used to track the latter), the discrimination between both subsets by imaging is imprecise. The high expression of ECFP in the MacBlue mouse improves the detection of both subsets, but their discrimination is still not possible (53, 54).

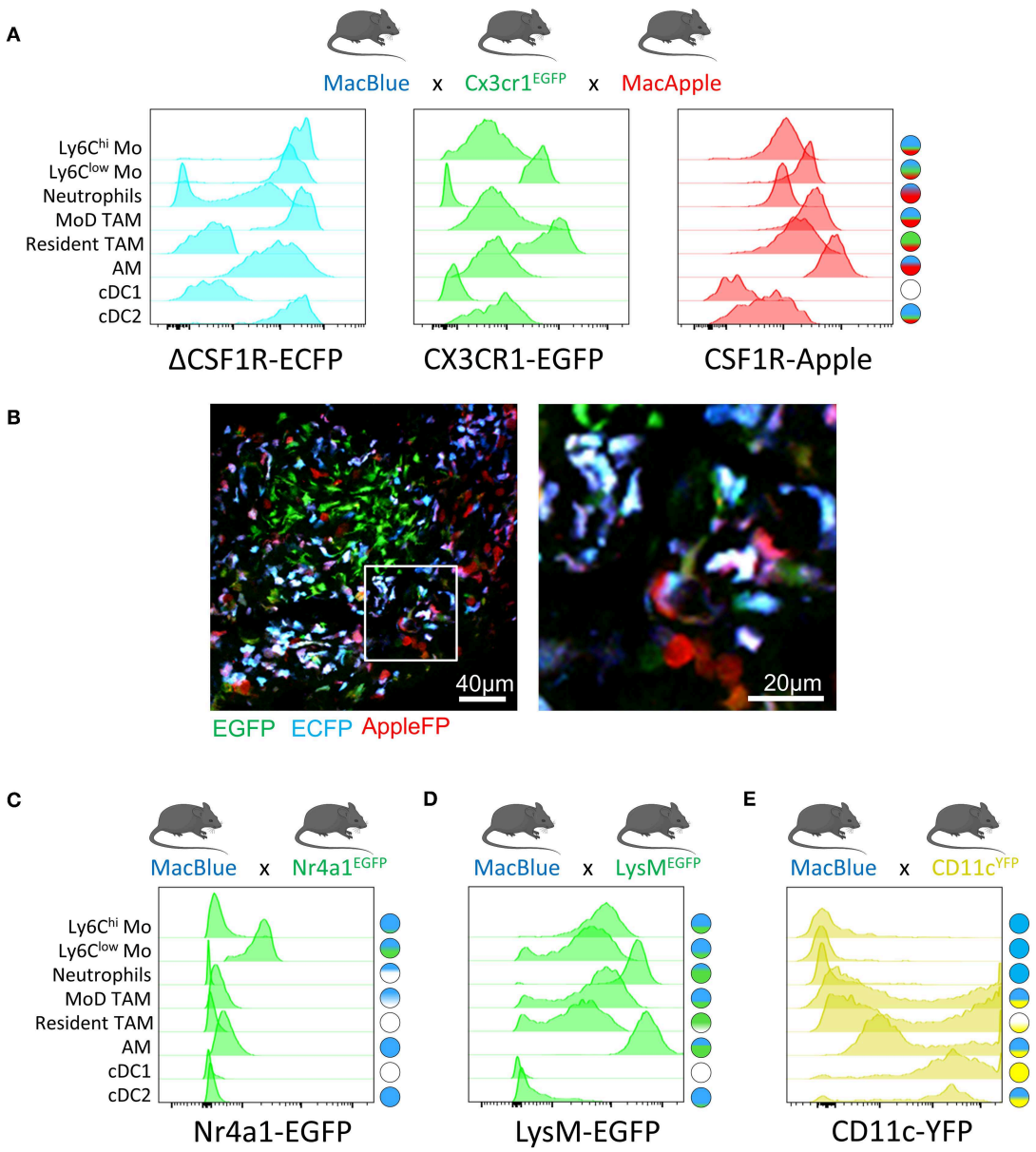
The *Nr4a1*^{gfp} fluorescent reporter mouse provides a good marker to monitor non-classical monocytes in the lungs (28). Combination between MacBlue and *Nr4a1*^{gfp} might offer an opportunity to simultaneously track both subsets (**Figure 1C**).

FP expression guided by the *Ccr2* promoter would be expected to preferentially label classical monocytes, but this fluorescent reporter is also highly expressed on NK cells [(55) and personal observation]. NK cells are often abundant in the TME and can lead to misinterpretation of imaging studies using this reporter. Combination with other reporters may therefore improve specificity. For instance, combining *Ccr2*^{RFP} and *Cx3cr1*^{EGFP} reporters allows tracking of the relative accumulation of CCR2^{hi}CX3CR1^{low} and CCR2^{low}CX3CR1^{hi} cells in glioblastoma, arguing for distinct origins of TAM in this model (56). As NK subsets also express EGFP in the *Cx3cr1*^{EGFP}, the risk of NK contamination when imaging and identifyinng myeloid cells in the TME using this mouse strain must be considered.

LysM^{EGFP} reporter mice display bright expression of GFP based on the lysozyme M locus and are widely used to visualize monocytes and macrophages. However, this marker is also strongly expressed in neutrophils (50, 57). Using this reporter for live imaging is challenging as monocytes, macrophages, and neutrophils are closely related in the TME and the discrimination of these populations requires additional markers. The combination of *LysM*^{EGFP} with MacBlue might be considered, but the strong overlap of expression of these two reporters between granulocytes, monocytes and macrophages limits their accurate identification by imaging (**Figure 1D**).

Mouse strains expressing FP driven by the *Itgax* promoter (CD11c) typically provide very bright fluorescent signal and are available in different colors (58). Although Itgax-based reporters are routinely associated with DC, it is clear that numerous TAM will express the FP and thus prevent the exclusive visualization of DC using this unique reporter (**Figure 1E**). The combination of *CD11c*^{RFP} with *Cx3cr1*^{EGFP} in the study by Broz et al has provided an additional dimension to better discriminate DC and TAM in breast tumors (2). The combination of *CD11*^{RFP} and *Xcr1*^{venus} reporters provides also an alternative to more accurately identify DC by imaging (59).

Altogether, these transgenic models have demonstrated utility in providing new insights on the dynamics of different myeloid populations (**Figure 1F**). Furthermore, the combination of different fluorescent reporters appears to be a valid and worthwhile approach to target the cells more accurately. We have already demonstrated that the relative expression of the fluorescent reporter in MacBlue x *Cx3cr1*^{EGFP} mice identifies TAM subsets of distinct origins with specific anatomic distribution (16). TAM microanatomical niches are even more marked in the spontaneous mammary tumor model PyMT-ChOVA combined with the MacBlue x *Cx3cr1*^{EGFP} x MacApple reporters. Subsets with relative dominant expression of the three FP have been identified (**Figure 2A**). EGFP^{+} cells are mainly localized to the neoplasic mammary epithelium basal membrane and ECFP^{+} are more clustered in the stroma. In addition to genetic fluorescent reporters, two-photon imaging can be used to generate fluorescence from specific cellular structures without the need of an exogenous fluorescent probe. Coherent anti-Stokes Raman scattering (CARS) imaging, for example, allows imaging of lipid deposits showing that a MacApple^{+} subset is enriched in the adipose tissue of the PyMT tumors (**Figure 2B**) and favoring the notion of spatial diversity of TAM (60). Whether or not these subsets originate from resident macrophages of the mammary epithelium and surrounding adipose tissue needs further investigation. Second harmonic generation (SHG) is another label-free approach based on the intrinsic optical properties of extracellular structures that has been used to highlight T-cell trafficking in the collagen matrix of the TME (61, 62). Tracking the evolution of collagen density according to tumor stage can be correlated with the functional characterization of TAM, as they are major actors in ECM remodeling. Szulczewski et al. have reported a label-free metabolic imaging protocol allowing for the visualization of NADH and FAD based on their autofluorescent properties. This technique has identified that macrophages express high levels of FAD and are mainly glycolytic, enabling their discrimination from tumor cells without adding any exogenous staining molecule (63). Label-free sensing of biomolecules typically does not result in photobleaching and reflects physiological content and distribution when compared with exogenous fluorescent probes. This label-free imaging also provides an opportunity to obtain information from human samples. As these methods lack specificity, complementary markers are necessary to study myeloid function.



F

	MacBlue	MacGreen	Cx3cr1-EGFP	LyzM-GFP	Nr4a1-GFP	CD11c-YFP	MacApple	CCR2-RFP
Ly6CHi Mo	+++	++	+	++	+	-	++	++
Ly6Clow Mo	+++	+++	+++	++/-	+++	-	+++	-
Neutrophils	+/-	++	-	+++	-	-	++	?
AM	+++	+++	-	+++	-	+/-	+++	-
cDC1	-	+/-	-	-	-	+++	+/-	?
cDC2	+++	+/-	+/-	+/-	-	+++	+/-	?
Other reported subsets	Microglia, Langherans, some B cells	All tissue mac, eosino, some B cells	Microglia, Osteoclast, Langherans, some NK, T cells	Some tissue mac	Activated T cells	Langherans some T, B cells	All tissue mac, eosino, some B cells	NK cells, some T cells

FIGURE 1 | Combination of distinct fluorescent reporter mice identify myeloid cell diversity in the tumor, MacBlue, *Cx3cr1*^{EGFP}, and MacApple mice were intercrossed to generate a combined fluorescent mouse strain. TC-1 lung carcinoma cell line was inoculated and different myeloid subsets in the lung tumor were analyzed for their

(Continued)

FIGURE 1 | relative expression of the fluorescent reporters by flow cytometry **(A)** and tissue distribution by multiphoton microscopy **(B)**. At a single reporter level, the overlap is major between different subsets but the resulting combination of fluorescent reporters for each cell highlights a more specific signature for each subset population (see schematic cell fluorescent signature on the right). **(B)** Left image shows the distribution of distinct myeloid cells in a lung tumor nodule. Right image represents magnification of left image. Discrimination of the distinct subsets is based on the known expression of each reporter seen in **(A)**. Mostly Monocytes and MoD-TAM (Blue/Green/ Red) are distinct from resident TAM (Green), neutrophils (Red) and Alveolar macrophages (Blue/Red). The image was acquired using a Zeiss 7MP multiphoton microscope coupled with a Chameleon Visio II (at 840 nm) and an OPO Mpx (at 1104 nm). **(C)** Combination of Macblue and Nr4a1^{GFP} reporter mice allows the distinction of Ly6Clow from classical monocytes and MoD-TAM. Cell fluorescent signature on the right is generated according to the relative expression of each reporter for all subsets. EGFP is exclusively found in Ly6C^{low} monocytes, ECFP expression is presented in **(A)**. **(D)** Combination of Macblue and LysM^{EGFP} reporter mice allows the distinction of Neutrophils from monocytes and macrophages. EGFP Expression is brighter in neutrophils than in resident interstitial macrophages but similar to alveolar macrophages. Due to spectral overlap between ECFP and EGFP, the accurate discrimination between these subsets can be limited. **(E)** In the *Itgax^{YFP}* reporter mice (CD11c^{YFP}), the YFP is strongly expressed by classical DC (cDC1) and CD11b+ DC (cDC2) but is also in found in a fraction of Resident and MoD-TAM discriminated by the MacBlue reporter. Therefore, CD11c should not be used as an exclusive marker of DC. For all histogram plots, subsets are defined as: CD11b^{+} Ly6C^{hi} Ly6G^{-} SiglecF^{-} CD64^{low} for Ly6C^{hi} Mo; CD11b^{+} Ly6C$^{low/-}$ Ly6G^{-} SiglecF^{-} CD64^{low} for Ly6C^{low} Mo; CD11b^{+} Ly6G^{+} SiglecF^{-} for Neutrophils; CD11b^{+} Ly6C^{-} CD64^{+} ECFP^{+} EGFP^{+} for MoD TAM; CD11b^{+} Ly6C^{-} CD64^{+} ECFP^{-} EGFP^{+} for Resident TAM; CD11b^{+} Ly6C^{-} CD64^{+} CD11c^{+} SiglecF^{+} for alveolar mac (AM); CD11b^{-} CD11c^{+} CD64^{-} MHC-II^{+} for cDC1; CD11b^{+} CD11c^{+} CD64^{-} MHC-II^{+} for cDC2. **(F)** Table summarizing the relative expression of the different reporters across the indicated immune subsets according to + and-signs. +/– stands for differential expression among one given population.

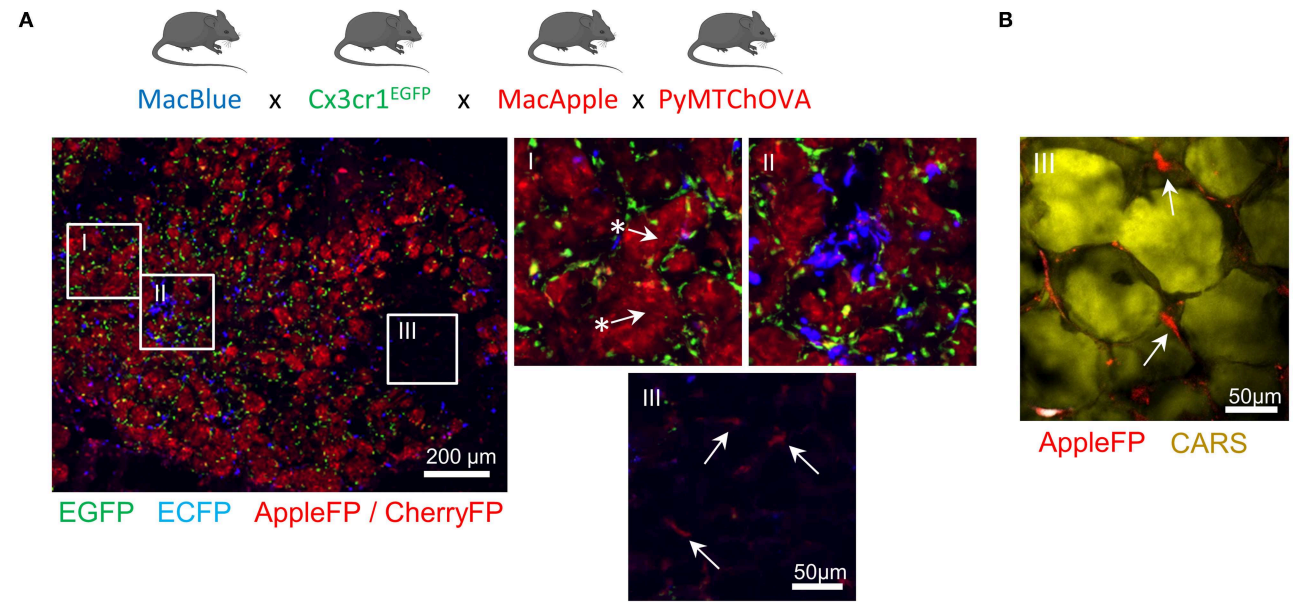


FIGURE 2 | Identification of specific myeloid cell distribution in spontaneous mammary carcinoma. MacBlue x *Cx3cr1^{EGFP}* x MacApple mice were crossed with PyMT-ChOVA mice from Engelhardt et al. (33). Briefly this mouse develops spontaneous multifocal mammary tumors expressing CherryFP and Ovalbumin. **(A)** Whole mammary tumors cryo-section shows microanatomical niches of the PyMT tumor with specific enrichment of myeloid cells with distinct fluorescent signatures. EGFP^{+} cells (green) localize at the basal membrane of the mammary carcinomas (region I) and are homogeneously distributed across the neoplasic tissue, ECFP^{+} cells (blue) accumulate in sparse clusters (region II). AppleFP cells cannot be discriminated from CherryFP using these settings but Apple^{+} cells (red) are visualized in the tumor-associated mammary fat pad (region III) confirming the existence of another subset of myeloid cell. Arrows with * highlight CherryFP^{+} tumor nodules and arrows indicate AppleFP^{+} cells. Images were acquired using a Zeiss epifluorescent microscope (Axio Observer Z1). **(B)** Mammary fat pad-associated AppleFP^{+} myeloid cells were confirmed by CARS imaging (2,846 cm^{-1}) allowing the visualization of lipid deposits of adipocytes (in yellow). Image was acquired using a Zeiss 7MP multiphoton microscope coupled with a Chameleon Visio II (at 840 nm) and an OPO Mpx (at 1,104 nm) synchronized by a delay line (Coherent).

CONCLUDING REMARKS

The delineation of myeloid heterogenity relies on our ability to multiply the number of simultaneously imaged parameters. Although high-dimensional analysis by flow/mass cytometry and single cell transcriptomics is now accessible, accomplishing this characterization with spatiotemporal resolution using optical imaging remains challenging. Because of the strong overlap of commonly used fluorescent reporters between several myeloid subsets, mouse models must be carefully chosen based on the population of interest. The development of spectral unmixing (64) may offer a promising alternative technique to multiply the number of fluorescent parameters recorded simultaneously, but so far has been restricted to analysis of fixed tissue. The use of imaging windows allows longer-term tracking of cellular behavior (65). This approach may also contribute to better understand myeloid functions over time and in response to therapy. Tracking myeloid cell subsets using combinations of complementary approaches, such as *in vivo* fluorescent antibody labeling, dextran uptake, endogenous fluorescent reporters, and

label-free optical imaging processes, is likely to yield a full appreciation of the phenotypic and functional diversity of TAM and DC. Fate mapping models to label embryonically derived macrophages might additionally identify tumor myeloid cell origin and will certainly be the goal of imaging studies in the near future. Despite some complexity that can dampen the accurate identification of myeloid subsets in the TME, previous studies have been extraordinarily rewarding in our understanding of tumor-associated myeloid cell biology.

DATA AVAILABILITY

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

ETHICS STATEMENT

Mouse experimentation were approved by the French animal experimentation and ethics committee and validated by "Service Protection et Santé Animales, Environnement" with the number A-75-2065.

AUTHOR CONTRIBUTIONS

ML performed the experiments. All authors wrote the manuscript.

FUNDING

This work was supported by Fondation ARC pour la recherche sur le cancer, l'INCa (2018-1-PLBIO-06-1) and DIM Malinf. ML is supported by Sorbonne University program Idex SUPER.

ACKNOWLEDGMENTS

The authors wish to thank T. Hammoudi for manuscript editing, D.A. Hume for the generous gift of the MacBlue and MacApple mice and M.F. Krummel for the PyMT-ChOVA.

REFERENCES

1. Laoui D, Keirsse J, Morias Y, Van Overmeire E, Geeraerts X, Elkrim Y, et al. The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nat Commun*. (2016) 7:13720. doi: 10.1038/ncomms13720
2. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, et al. Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell*. (2014) 26:638–52. doi: 10.1016/j.ccell.2014.09.007
3. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*. (2014) 14:392–404. doi: 10.1038/nri3671
4. Kiss M, Van Gassen S, Movahedi K, Saeys Y, Laoui D. Myeloid cell heterogeneity in cancer: not a single cell alike. *Cell Immunol*. (2018) 330:188–201. doi: 10.1016/j.cellimm.2018.02.008
5. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol*. (2014) 14:571–8. doi: 10.1038/nri3712
6. Guilliams M, van de Laar, L. A Hitchhiker's guide to myeloid cell subsets: practical implementation of a novel mononuclear phagocyte classification system. *Front Immunol*. (2015) 6:406. doi: 10.3389/fimmu.2015.00406
7. Sidani M, Wyckoff J, Xue C, Segall JE, Condeelis J. Probing the microenvironment of mammary tumors using multiphoton microscopy. *J Mammary Gland Biol Neoplasia*. (2006) 11:151–63. doi: 10.1007/s10911-006-9021-5
8. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell*. (2006) 124:263–6. doi: 10.1016/j.cell.2006.01.007
9. Dovas A, Patsialou A, Harney AS, Condeelis J, Cox D. Imaging interactions between macrophages and tumour cells that are involved in metastasis *in vivo* and *in vitro*. *J Microscopy*. (2013) 251:261–9. doi: 10.1111/j.1365-2818.2012.03667.x
10. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. (2004) 64:7022–9. doi: 10.1158/0008-5472.CAN-04-1449
11. Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res*. (2007) 67:2649–56. doi: 10.1158/0008-5472.CAN-06-1823
12. Roh-Johnson M, Bravo-Cordero JJ, Patsialou A, Sharma VP, Guo P, Liu H, et al. Macrophage contact induces RhoA GTPase signaling to trigger tumor cell intravasation. *Oncogene*. (2014) 33:4203–12. doi: 10.1038/onc.2013.377
13. Harney AS, Arwert EN, Entenberg D, Wang Y, Guo P, Qian BZ, et al. Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage-derived VEGFA. *Cancer Discov*. (2015) 5:932–43. doi: 10.1158/2159-8290.CD-15-0012
14. Linde N, Casanova-Acebes M, Sosa MS, Mortha A, Rahman A, Farias E, et al. Macrophages orchestrate breast cancer early dissemination and metastasis. *Nat Commun*. (2018) 9:21. doi: 10.1038/s41467-017-02481-5
15. Roussos ET, Balsamo M, Alford SK, Wyckoff JB, Gligorijevic B, Wang Y, et al. Mena invasive (MenaINV) promotes multicellular streaming motility and transendothelial migration in a mouse model of breast cancer. *J Cell Sci*. (2011) 124:2120–31. doi: 10.1242/jcs.086231
16. Loyher PL, Hamon P, Laviron M, Meghraoui-Kheddar A, Goncalves E, Deng Z, et al. Macrophages of distinct origins contribute to tumor development in the lung. *J Exp Med*. (2018) 215:2536–53. doi: 10.1084/jem.20180534
17. Hanna SJ, McCoy-Simandle K, Leung E, Genna A, Condeelis J, Cox D. Tunneling nanotubes, a novel mode of tumor cell-macrophage communication in tumor cell invasion. *J Cell Sci*. (2019) 132:819–23. doi: 10.1242/jcs.223321
18. Guiet R, Van Goethem E, Cougoule C, Balor S, Valette A, Al Saati T, et al. The process of macrophage migration promotes matrix metalloproteinase-independent invasion by tumor cells. *J Immunol*. (2011) 187:3806–14. doi: 10.4049/jimmunol.1101245
19. Roh-Johnson M, Shah AN, Stonick JA, Poudel KR, Kargl J, Yang GH, et al. Macrophage-dependent cytoplasmic transfer during melanoma invasion *in vivo*. *Dev Cell*. (2017) 43:549–62.e6. doi: 10.1016/j.devcel.2017.11.003
20. Lohela M, Casbon AJ, Olow A, Bonham L, Branstetter D, Weng N, et al. Intravital imaging reveals distinct responses of depleting dynamic tumor-associated macrophage and dendritic cell subpopulations. *Proc Natl Acad Sci USA*. (2014) 111:E5086–95. doi: 10.1073/pnas.1419899111
21. Egeblad M, Ewald AJ, Askautrud HA, Truitt ML, Welm BE, Bainbridge E, et al. Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Dis Model Mech*. (2008) 1:155–67; discussion: 165. doi: 10.1242/dmm.000596
22. Franklin RA, Li MO. Ontogeny of tumor-associated macrophages and its implication in cancer regulation. *Trends Cancer*. (2016) 2:20–34. doi: 10.1016/j.trecan.2015.11.004
23. Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, et al. Macrophage ontogeny underlies differences in

tumor-specific education in brain malignancies. *Cell Rep*. (2016) 17:2445–59. doi: 10.1016/j.celrep.2016.10.052
24. Zhu Y, Herndon JM, Sojka DK, Kim KW, Knolhoff BL, Zuo C, et al. Tissue-resident macrophages in pancreatic ductal adenocarcinoma originate from embryonic hematopoiesis and promote tumor progression. *Immunity*. (2017) 47:323–8.e6. doi: 10.1016/j.immuni.2017.07.014
25. Arwert EN, Harney AS, Entenberg D, Wang Y, Sahai E, Pollard JW, et al. A unidirectional transition from migratory to perivascular macrophage is required for tumor cell intravasation. *Cell Rep*. (2018) 23:1239–48. doi: 10.1016/j.celrep.2018.04.007
26. Guilliams M, Scott CL. Does niche competition determine the origin of tissue-resident macrophages? *Nat Rev Immunol*. (2017) 17:451–60. doi: 10.1038/nri.2017.42
27. Thornton EE, Krummel MF, Looney MR. Live imaging of the lung. *Curr Protocols Cytometry Chapter*. (2012) 12:Unit12.28. doi: 10.1002/0471142956.cy1228s60
28. Hanna RN, Cekic C, Sag D, Tacke R, Thomas GD, Nowyhed H, et al. Patrolling monocytes control tumor metastasis to the lung. *Science*. (2015) 350:985–90. doi: 10.1126/science.aac9407
29. Jung K, Heishi T, Khan OF, Kowalski PS, Incio J, Rahbari NN, et al. Ly6Clo monocytes drive immunosuppression and confer resistance to anti-VEGFR2 cancer therapy. *J Clin Invest*. (2017) 127:3039–51. doi: 10.1172/JCI93182
30. Headley MB, Bins A, Nip A, Roberts EW, Looney MR, Gerard A, et al. Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature*. (2016) 531:513–7. doi: 10.1038/nature16985
31. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*. (2011) 475:222–5. doi: 10.1038/nature10138
32. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS ONE*. (2009) 4:e6562. doi: 10.1371/journal.pone.0006562
33. Engelhardt JJ, Boldajipour B, Beemiller P, Pandurangi P, Sorensen C, Werb Z, et al. Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. *Cancer Cell*. (2012) 21:402–17. doi: 10.1016/j.ccr.2012.01.008
34. Boissonnas A, Licata F, Poupel L, Jacquelin S, Fetler L, Krumeich S, et al. CD8+ tumor-infiltrating T cells are trapped in the tumor-dendritic cell network. *Neoplasia*. (2013) 15:85–94. doi: 10.1593/neo.121572
35. Peranzoni E, Lemoine J, Vimeux L, Feuillet V, Barrin S, Kantari-Mimoun C, et al. Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. *Proc Natl Acad Sci USA*. (2018) 115:E4041–50. doi: 10.1073/pnas.1720948115
36. Mondini M, Loyher PL, Hamon P, M. Gerbe De Thore, Laviron M, Berthelot K, et al. CCR2-dependent recruitment of Tregs and monocytes following radiotherapy is associated with TNFalpha-mediated resistance. *Cancer Immunol Res*. (2019). doi: 10.1158/2326-6066.CIR-18-0633
37. Barry KC, Hsu J, Broz ML, Cueto FJ, Binnewies M, Combes AJ, et al. A natural killer-dendritic cell axis defines checkpoint therapy-responsive tumor microenvironments. *Nat Med*. (2018) 24:1178–91. doi: 10.1038/s41591-018-0085-8
38. Broz ML, Krummel MF. The emerging understanding of myeloid cells as partners and targets in tumor rejection. *Cancer Immunol Res*. (2015) 3:313–9. doi: 10.1158/2326-6066.CIR-15-0041
39. Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, et al. Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages *in vitro* in response to CSF-1. *J Leukoc Biol*. (2007) 82:111–23. doi: 10.1189/jlb.1206713
40. Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, et al. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood*. (2003) 101:1155–63. doi: 10.1182/blood-2002-02-0569
41. Ovchinnikov DA, van Zuylen WJ, DeBats CE, Alexander KA, Kellie S, Hume DA. Expression of Gal4-dependent transgenes in cells of the mononuclear phagocyte system labeled with enhanced cyan fluorescent protein using Csf1r-Gal4VP16/UAS-ECFP double-transgenic mice. *J Leukoc Biol*. (2008) 83:430–3. doi: 10.1189/jlb.0807585
42. Ovchinnikov DA, DeBats CE, Sester DP, Sweet MJ, Hume DA. A conserved distal segment of the mouse CSF-1 receptor promoter is required for maximal expression of a reporter gene in macrophages and osteoclasts of transgenic mice. *J Leukoc Biol*. (2010) 87:815–22. doi: 10.1189/jlb.0809557
43. Sauter KA, Pridans C, Sehgal A, Bain CC, Scott C, Moffat L, et al. The MacBlue binary transgene (csf1r-gal4VP16/UAS-ECFP) provides a novel marker for visualisation of subsets of monocytes, macrophages and dendritic cells and responsiveness to CSF1 administration. *PLoS ONE*. (2014) 9:e105429. doi: 10.1371/journal.pone.0105429
44. Rodero MP, Poupel L, Loyher PL, Hamon P, Licata F, Pessel C, et al. Immune surveillance of the lung by migrating tissue monocytes. *Elife*. (2015) 4. doi: 10.7554/eLife.07847
45. Hawley CA, Rojo R, Raper A, Sauter KA, Lisowski ZM, Grabert K, et al. Csf1r-mApple transgene expression and ligand binding *in vivo* reveal dynamics of CSF1R expression within the mononuclear phagocyte system. *J Immunol*. (2018) 200:2209–23. doi: 10.4049/jimmunol.1701488
46. Jenkins SJ, Hume DA. Homeostasis in the mononuclear phagocyte system. *Trends Immunol*. (2014) 35:358–67. doi: 10.1016/j.it.2014.06.006
47. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol*. (2000) 20:4106–14. doi: 10.1128/MCB.20.11.4106-4114.2000
48. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. (2007) 317:666–70. doi: 10.1126/science.1142883
49. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. (2009) 325:612–6. doi: 10.1126/science.1175202
50. Jung K, Kim P, Leuschner F, Gorbatov R, Kim JK, Ueno T, et al. Endoscopic time-lapse imaging of immune cells in infarcted mouse hearts. *Circ Res*. (2013) 112:891–9. doi: 10.1161/CIRCRESAHA.111.300484
51. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science*. (2010) 327:656–61. doi: 10.1126/science.1178331
52. Gordon CL, Lee LN, Swadling L, Hutchings C, Zinser M, Highton AJ, et al. Induction and maintenance of CX3CR1-intermediate peripheral memory CD8(+) T cells by persistent viruses and vaccines. *Cell Rep*. (2018) 23:768–82. doi: 10.1016/j.celrep.2018.03.074
53. Jacquelin S, Licata F, Dorgham K, Hermand P, Poupel L, Guyon E, et al. CX3CR1 reduces Ly6Chigh-monocyte motility within, and release from the bone marrow after chemotherapy in mice. *Blood*. (2013) 122:674–83. doi: 10.1182/blood-2013-01-480749
54. Hamon P, Loyher PL, Baudesson de Chanville C, Licata F, Combadiere C, Boissonnas A. CX3CR1-dependent endothelial margination modulates Ly6Chigh monocyte systemic deployment upon inflammation in mice. *Blood*. (2017) 129:1296–307. doi: 10.1182/blood-2016-08-732164
55. Saederup N, Cardona AE, Croft K, Mizutani M, Cotleur AC, Tsou CL, et al. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS ONE*. (2010) 5:e13693. doi: 10.1371/journal.pone.0013693
56. Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al. Cellular and molecular identity of tumor-associated macrophages in glioblastoma. *Cancer Res*. (2017) 77:2266–78. doi: 10.1158/0008-5472.CAN-16-2310
57. Faust N, Varas F, Kelly LM, Heck S, Graf T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood*. (2000) 96:719–26.
58. Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks *in vivo*. *Nat Immunol*. (2004) 5:1243–50. doi: 10.1038/ni1139
59. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, et al. Critical role for $CD103^{+}$/$CD141^{+}$ dendritic cells bearing CCR7 for tumor antigen trafficking and priming of T cell immunity in melanoma. *Cancer Cell*. (2016) 30:324–36. doi: 10.1016/j.ccell.2016.06.003
60. Yang M, McKay D, Pollard JW, Lewis CE. Diverse functions of macrophages in different tumor microenvironments. *Cancer Res*. (2018) 78:5492–503. doi: 10.1158/0008-5472.CAN-18-1367

61. Boissonnas A, Fetler L, Zeelenberg IS, Hugues S, Amigorena S. *In vivo* imaging of cytotoxic T cell infiltration and elimination of a solid tumor. *J Exp Med*. (2007) 204:345–56. doi: 10.1084/jem.20061890
62. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean MC, Validire P, Trautmann A, et al. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. *J Clin Invest*. (2012) 122:899–910. doi: 10.1172/JCI45817
63. Szulczewski JM, Inman DR, Entenberg D, Ponik SM, Aguirre-Ghiso J, Castracane J, et al. *In vivo* visualization of stromal macrophages via label-free FLIM-based metabolite imaging. *Sci Rep*. (2016) 6:25086. doi: 10.1038/srep25086
64. Wickenhauser C, Bethmann D, Feng Z, Jensen SM, Ballesteros-Merino C, Massa C, et al. Multispectral fluorescence imaging allows for distinctive topographic assessment and subclassification of tumor-infiltrating and surrounding immune cells. *Methods Mol Biol*. (2019) 1913:13–31. doi: 10.1007/978-1-4939-8979-9_2
65. Alieva M, Ritsma L, Giedt RJ, Weissleder R, van Rheenen J. Imaging windows for long-term intravital imaging: general overview and technical insights. *Intravital*. (2014) 3:e29917. doi: 10.4161/intv.29917

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 04 June 2019
doi: 10.3389/fimmu.2019.01141

High Salt Inhibits Tumor Growth by Enhancing Anti-tumor Immunity

*Ralf Willebrand[1], Ibrahim Hamad[1], Lauren Van Zeebroeck[1], Máté Kiss[2,3], Kirsten Bruderek[4], Anneleen Geuzens[1], Dries Swinnen[1], Beatriz Fernandes Côrte-Real[1], Lajos Markó[5], Els Lebegge[2,3], Damya Laoui[2,3], Josephine Kemna[6,7], Thomas Kammertoens[6,7], Sven Brandau[4], Jo A. Van Ginderachter[2,3] and Markus Kleinewietfeld[1]**

[1] VIB Laboratory of Translational Immunomodulation, VIB Center for Inflammation Research, University of Hasselt, Campus Diepenbeek, Hasselt, Belgium, [2] Cellular and Molecular Immunology Lab, Vrije Universiteit Brussel, Brussels, Belgium, [3] Myeloid Cell Immunology Lab, VIB Center for Inflammation Research, Brussels, Belgium, [4] Research Division, Department of Otorhinolaryngology, West German Cancer Center, University Hospital Essen, Essen, Germany, [5] Experimental and Clinical Research Center, A Joint Cooperation of Max Delbrück Center for Molecular Medicine and Charité University Medicine Berlin, Berlin, Germany, [6] Institute of Immunology, Charité - Universitätsmedizin Berlin, Berlin, Germany, [7] Max Delbrück Center for Molecular Medicine, Berlin, Germany*

OPEN ACCESS

Edited by:
Maija Hollmén,
University of Turku, Finland

Reviewed by:
Frederique Vegran,
INSERM U1231 Lipides, Nutrition, Cancer (LNC), France
Sirpa Jalkanen,
University of Turku, Finland

***Correspondence:**
Markus Kleinewietfeld
markus.kleinewietfeld@uhasselt.vib.be

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *05 February 2019*
Accepted: *07 May 2019*
Published: *04 June 2019*

Citation:
Willebrand R, Hamad I, Van Zeebroeck L, Kiss M, Bruderek K, Geuzens A, Swinnen D, Côrte-Real BF, Markó L, Lebegge E, Laoui D, Kemna J, Kammertoens T, Brandau S, Van Ginderachter JA and Kleinewietfeld M (2019) High Salt Inhibits Tumor Growth by Enhancing Anti-tumor Immunity. Front. Immunol. 10:1141. doi: 10.3389/fimmu.2019.01141

Excess salt intake could affect the immune system by shifting the immune cell balance toward a pro-inflammatory state. Since this shift of the immune balance is thought to be beneficial in anti-cancer immunity, we tested the impact of high salt diets on tumor growth in mice. Here we show that high salt significantly inhibited tumor growth in two independent murine tumor transplantation models. Although high salt fed tumor-bearing mice showed alterations in T cell populations, the effect seemed to be largely independent of adaptive immune cells. In contrast, depletion of myeloid-derived suppressor cells (MDSCs) significantly reverted the inhibitory effect on tumor growth. In line with this, high salt conditions almost completely blocked murine MDSC function *in vitro*. Importantly, similar effects were observed in human MDSCs isolated from cancer patients. Thus, high salt conditions seem to inhibit tumor growth by enabling more pronounced anti-tumor immunity through the functional modulation of MDSCs. Our findings might have critical relevance for cancer immunotherapy.

Keywords: cancer, dietary factor, MDSC, cancer immunotherapy, sodium chloride (dietary)

INTRODUCTION

The balance between pro- and anti-inflammatory cells and signals is critical for preserving immune homeostasis and a disturbed immune cell balance is believed to contribute to autoimmunity and cancer. Recent data have demonstrated that a high salt diet (HSD) could influence the immune cell balance toward a pro-inflammatory state, where the induction of pro-inflammatory cells, such as T helper 17 cells (T_H17) and M1-like macrophages is promoted and the function of anti-inflammatory cells, such as M2-like macrophages and regulatory T cells (T_{regs}) is impaired (1–4). High salt intake is a ubiquitous phenomenon of Western diets and is indeed implicated in a plethora of diseases like cardiovascular and metabolic as well as autoimmune diseases (5, 6). Particularly the pro-inflammatory effects of a HSD are believed to be associated with autoimmune diseases like e.g., multiple sclerosis (MS) and inflammatory bowel diseases (IBD) (7). These pro-inflammatory effects of high salt on the immune cell balance raise the question if high salt conditions could also affect anti-tumor immunity and cancer.

The immune system is able to recognize neoplasms and after therapeutic intervention it can also attack and eradicate tumors as recent advances in the field of cancer immunotherapy (e.g., immune checkpoint inhibition) have shown. Indeed, immunotherapy is one of the most promising approaches to treat cancer (8, 9). However, a major obstacle for successful cancer immunotherapy is the highly immuno-suppressive environment induced by many tumors. It is well-documented that the tumor microenvironment frequently induces an immune protective and tolerogenic environment by e.g., promoting the induction of various immune suppressive cell types or by inducing the expression of immune suppressive cytokines (10–14).

Important players in the tumor microenvironment are myeloid cells, which can support tumor growth by providing growth factors, such as vascular endothelial growth factor (VEGF) (15) and additionally can be strongly immunosuppressive. One of these tumor promoting myeloid cell types are myeloid-derived suppressor cells (MDSCs) (16–18). MDSCs are a classically defined as a heterogeneous population of immature myeloid cells that fail to terminally differentiate and exert a strong immune suppressive potential in mice and humans. The induction of MDSCs from myeloid progenitors occurs in the bone marrow (BM) and spleen usually in the context of chronic inflammation and often leads to an accumulation of these cells in the periphery in cancerous conditions. Here they can inhibit various other immune cells like T and B cells, dendritic cells (DC) and natural killer cells (NK) and thereby contribute to an immune suppressive state using different molecular mechanisms of suppression. However, also in the absence of T cells, GR1 positive myeloid cells can support tumor growth (19) e.g., by the promotion of tumor angiogenesis (15). The presence of MDSCs in cancer patients is associated with a poor disease prognosis and tumor recurrence (17, 20). MDSCs can be further subdivided based on their origin and phenotype into granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and monocytic (M-MDSCs) (17, 21). Both MDSC subsets can be frequently detected in BM, spleen, blood and tumor tissues of cancer patients. In mice PMN-MDSCs are defined as $CD11b^{+}Ly6G^{high}Ly6C^{low}$, whereas M-MDSCs are defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells, although both marker combinations are not entirely specific and can also include neutrophils or classical monocyte populations (17). In humans PMN-MDSCs are characterized by $CD11b^{+}CD14^{-}CD15^{+}HLA\text{-}DR^{-}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ expression and M-MDSCs are $CD11b^{+}CD14^{+}CD15^{-}HLA\text{-}DR^{low/-}$ cells (17). MDSCs further show a high grade of plasticity. They can react to environmental triggers like altered cytokine milieus or hypoxia and adapt their phenotype and function accordingly or can even differentiate into cells with pro-inflammatory potential (18, 22). However, how and if MDSCs react to changes in the ionic microenvironment as e.g., found in tumor tissues through increased necrosis (23) or through induction by HSD (24, 25) is unknown.

Since high salt affects various adaptive and innate immune cells it is plausible that MDSCs may also react to elevated Na^{+} concentrations. Considering the immune stimulatory effects of high salt, these conditions may be favorable for enhanced tumor immunology by boosting pro-inflammatory effector cells and blocking anti-inflammatory cells. We therefore sought to analyze the impact of HSD on tumor growth in murine tumor transplantation models. Here we show that a HSD significantly reduces tumor growth in two independent tumor transplantation models. The effect seems to be largely dependent on myeloid cells by impacting MDSC function and thereby leading to enhanced anti-tumor immunity. Thus, our study identified a novel effect of how high dietary salt intake could modify innate immune reactions by modulating murine and human MDSC function. These data may offer novel strategies for improving cancer immunotherapies.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles River and housed in the facility of the University of Hasselt under standardized conditions. Further, C57BL/6 mice were purchased from Janvier and housed in the facility of Vrije Universiteit Brussel (VUB) under standardized conditions. $RAG2^{-/-}$ mice were kindly provided by Thomas Blankenstein. Animal studies were approved by the ethics committees of animal studies at the University of Hasselt (201738) and VUB (14-220-26).

Diet and Tumor Inoculation

Mice were either fed a normal diet (Control group) containing 0.5% NaCl or sodium enriched diet (HSD group) containing 4% NaCl as well as 1% NaCl enriched tap water for 2 weeks before tumor inoculation. In some experiments, diet switch was started directly before tumor inoculation. Both diets were purchased from SSNIFF (Ctrl: E15430-04, HSD: E15431-34; Soest, Germany). Mice were maintained on the respective diet during the course of the experiment. B16F10 melanoma cells (ATCC) were cultured in DMEM (Sigma Aldrich) supplemented with 10% FCS (Gibco) and Penicillin/Streptomycin (Gibco) and Lewis Lung carcinoma (LLC) cells (ATCC) were cultured in RPMI (Lonza) supplemented with 10% FCS and Penicillin/Streptomycin. Cells were maintained mycoplasma free, tested continuously by HEK-blue mycoplasma detection (Invivogen). Tumor cells were subcutaneously injected in the left abdominal flank (LLC at $1 \times 10e6$ expose 6 and B16F10 at $2 \times 10e5$ expose 5 cells/mouse). Tumor growth was monitored three times per week by using a caliper. Tumor volume was calculated with the ellipsoid formula ($\pi/6$*a*b*c) as described before (26).

Flow Cytometry and Preparation of Single Cell Suspensions

Blood was taken by tail vein puncture. Spleens and lymph node(s) were mashed through a 70 μm cell strainer. Tumor tissue was minced and subjected to digestion cocktail containing collagenase at 500 μg/ml and DNAse at 40 units/ml for 30 min at 37°C and passed through a 70 μm cell strainer. Single cell suspensions were subjected to red blood cell lysis (eBioscience). Cells were washed with MACS buffer (0.5% BSA 2 mM EDTA) and subjected to flow cytometry (further named FACS) staining protocol or resuspended in complete RPMI medium and

restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 250 ng/ml Ionomycin (Sigma) in the presence of GolgiPlug (BD) for 5 h to detect cytokines by FACS. Single cell suspensions were firstly stained with fixable Live/Dead cell kit (Thermo Fisher Scientific) for 10 min at room temperature. Cells were then incubated with antibody cocktails for 30 min at 4°C in MACS buffer. Intracellular staining was performed using the FoxP3 staining kit (eBioscience) according to the manufacturers protocol. The following antibodies were used: CD3 (Biolegend or eBioscience, 17A2), CD4 (Biolegend or BD Pharmingen, RM4-5), CD8 (eBioscience, 53-6.7), CD11b (eBioscience, M1/70), CD25 (BD Pharmingen, PC61), CD44 (eBioscience, IM7), CD45.2 (BD, 104), CD62L (eBioscience, MEL-14), CD183 (Biolegend, CXCR3-173), CD192 (Biolegend, SA203G11), CD196 (Biolegend, 29-2LI7), CD274 (Biolegend, 10F.9G2), CD279 (BD, J43), FoxP3 (eBioscience, FJK-16s), F4/80 (eBioscience, BM8), I-A/I-E (BD, M5/114.15.2), IFNγ (eBioscience, XMG1.2), IL-9 (Biolegend, RM9A4), IL-10 (eBioscience, JES5-16E3), IL-17 (eBioscience, eBio17B7), Ly6C (Biolegend, HK1.4), Ly6G (Biolegend, 1A8), Siglec-F (BD, E50-2440) and TNFα (Biolegend, MP6-XT22), Eomes (eBioscience, Dan11mag), T-bet (Biolegend, 4B10), CD19 (Biolegend, MB19-1), CD5 (Biolegend, 53-7.3). MDSCs were defined as $CD11b^{+}Ly6C^{high}Ly6G^{-}$ (M-MDSC) and as $CD11b^{+}Ly6C^{med}Ly6G^{high}$ (PMN-MDSC) pre gated on $CD45.2^{+}$ live cells excluding doublets and dead cells. Cells were acquired on a BD FACS-Fortessa instrument (BD) and analyzed using FlowJo V.10.1 software (FlowJo LLC) and by using FlowSOM.

FlowSOM Analysis

FACS data was manually gated on single live and/or $CD11b^{+}$ or $CD4^{+}$ T cells and later exported as FCS files in FlowJo V.10.1 (FlowJo, LLC). The automated analysis of exported FCS files was done by using FlowSOM algorithm, a R bioconductor package that uses self-organizing maps for dimensional reduction visualization of flow cytometry data (27). All data was concatenated scaled and logical transformed on import. Cells were assigned to a Self-Organizing Map (SOM) with a 10 × 10 grid, grouping similar cells into 100 nodes. Each node in the FlowSOM tree gets a score indicating its correspondence with this requested cell profile. To visualize similar nodes in branches, a minimal spanning tree (MST) was constructed and cell counts were log scaled and nodes with similar expression markers were clustered within metaclusters. The FlowSOM algorithm was run three times to ensure reproducibility of the results. Comparisons between groups (HSD and Ctrl) were performed using a Mann-Whitney test by computing the mean percentage per sample group in each cluster and by testing statistical significance on every node within metaclusters. *P*-values were two-sided and analysis was performed using RStudio (version 3.4.4).

Apoptosis Assay

B16F10 and LLC cells were cultured under 40 mM NaCl concentration or 80 mM Mannitol (Sigma Aldrich) as an osmolyte control and additional control cells were cultured in medium only. Cells were harvested after 48 h by trypsinization and resuspended in AnnexinV binding buffer (0.01 M Hepes, 0.14 M NaCl, 2.5 mM $CaCl_2$). AnnexinV-FITC (BD) was added for 30 min. Propidium iodide (PI) was added at 1 μg/ml shortly before acquisition on a FACS Calibur instrument (BD).

ELISA

Serum samples from tumor-bearing control and HSD fed mice were subjected to TNFα-, IL17A, IL-10-, and IFNγ- specific ELISA. All ELISA-kits were purchased from RD-Systems and performed according to the manufacturers protocol. Finally, wells were incubated with the horseradish peroxidase substrate o-phenylenediamine dihydrochloride (OPD) (Thermo Scientific) and optical density measurement was done on the iMark microplate reader (Biorad) with a 450 nm wavelength filter.

Antibody Depletion

Anti-Gr-1 (clone RB6-8C5) depletion antibody was purchased from BioXCell (West Lebanon, NH). 200 μg were i.p. injected 4 days after tumor injection and later on every second day. Control mice received PBS injection at the same time. Depletion efficiency was monitored by FACS analysis of blood samples using antibodies against CD11b and Ly6-C.

Quantitative Real-Time PCR

Tumor or spleen tissue was placed in RLT buffer containing b-mercaptoethanol and shredded in a Tissue-lyser (Qiagen). RNA was isolated from the lysates using the RNeasy Kit (Qiagen). RNA was reversely transcribed using the Quanta cDNA Kit (Quanta Biosciences). Quantitative real-time PCR was performed with the Power up SYBR Green Master Mix (Applied Bioscience). Samples were measured on the Step ONE Plus RT-PCR machine (Applied Biosciences). The following primers were used: *Tnfα* forward: 5′-GAGCAATGACTCCAAAGTAG-3′, *Tnfα* reverse: 5′-CGTAGCAAACCACCAAGTGG-3′, *Ifnγ* forward: 5′-AAAGAGATAATCTGGCTCTGC-3′, *Ifnγ* reverse: 5′-GCTCTGAGACAATGAACGCT-3′, *Nos2* forward: 5′-CCCTTCAATGGTTGGTACATGG-3′, *Nos2* reverse: 5′-ACATTGATCTCCGTGACAGCC-3′, *IL-10* forward: 5′-ATAACTGCACCCACTTCCCA-3′, *IL-10* reverse: 5′-GGGCATCACTTCTACCAGGT-3′, *Csf2* forward: 5′-TTTACTTTTCCTGGGCATTG-3′, *Csf2* reverse: 5′-TAGCTGGCTGTCATGTTCAA-3′, *Sgk1* forward: 5′-CCAAACCCTCCGACTTTCAC-3′, *Sgk1* reverse: 5′-CCTTGTGCCTAGCCAGAAGAA-3′, *Il17a* forward: 5′-ATCCCTCAAAGCTCAGCGTGTC-3′, *Il17a* reverse: 5′-GGGTCTTCATTGCGGTGGAGAG-3′, *Pbgd* forward: 5′ TGGTTGTTCACTCCCTGAAGG-3′ and *Pbgd* reverse: 5′-AAAGACAACAGCATCACAAGGGT-3′, *Hprt* forward: 5′-GTTGGATACAGGCCAGACTTTGTT-3′ and *Hprt* reverse: 5′-GAGGGTAGGCTGGCCTATAGGCT-3′. Data was analyzed by $2^{-\Delta\Delta Ct}$ method.

Murine MDSC Isolation and Suppression Assay

Subcutaneous LLC tumors were excised and treated with 10 U/ml collagenase I, 400 U/ml collagenase IV and 30 U/m1 DNase I (Worthington) for 30 min at 37°C. Tumors and spleens were squashed and filtered. Red blood cells in spleen and tumor cell suspensions were removed using erythrocyte lysis

buffer. To purify MDSCs, $CD11b^+$ cells were enriched by using anti-CD11b microbeads (Miltenyi Biotec). MDSCs were sorted from $CD11b^+$ cells using FACS Aria II (BD Biosciences) (**Supplementary Figure 10**). Post sort analysis revealed on average cell purity above 90%. For suppression assays, sorted MDSCs were added at different ratios to splenocytes (2×10^5 splenocytes/well) stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) in flat-bottom 96-well plates in RPMI medium supplemented with 10% FCS, 300 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 0.02 mM 2-mercaptoethanol in the presence or absence of additional 40 mM NaCl or 80 mM Mannitol solution in the cultures. After 24 h, ^{3}H-thymidine was added and T-cell proliferation was measured after another 18 h of culture as counts per minute (cpm) on a Wallac 1450 Liquid Scintillation Counter. Suppressive capacity of MDSCs isolated from HSD or control diet receiving animals was measured in a similar manner, without adding additional NaCl.

Human MDSC Isolation and Suppression Assay

PMN-MDSCs and autologous $CD3^+$ responder T cells from cancer patients were isolated and tested in suppression assays as described before (28). In brief, MDSCs were isolated from CD3-depleted PBMC by FACS using anti-human CD66b-FITC, anti-human CD33PE, anti-human HLA-DR-APC, and anti-human lineage cocktail (CD3, CD20, CD19, CD56, all BV421). Post sort analysis by FACS revealed a purity of at least 90%. T lymphocytes were labeled with 10 μM Cell Proliferation Dye eFluor® 450 (CPDye405) according to manufacturer instructions (eBioscience, Frankfurt am Main, Germany). For induction of T cell proliferation cells were stimulated in L-arginine free RPMI 1640 medium (Thermo Fisher scientific, Karlsruhe, Germany) supplemented with 10% (v/v) heat-inactivated FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin (Thermo Fisher scientific), and 150 μM L-Arginine (both Sigma-Aldrich) in 96 well round bottom plates coated with CD3 (1 μg/ml, clone OKT-3, eBioscience) and CD28 (2 μg/ml, clone 28.2, Beckman coulter). Autologous PMN-MDSC subsets were added in a T-cell: MDSC ratio of 2.5:1. To study the effect of high salt conditions additional 40 mM NaCl solution (Sigma-Aldrich) were added to the medium. CPDye405 intensity was analyzed by flow cytometry after 4 days of co-culture and proliferation. Proliferation index calculation is based on dye dilution and was calculated with ModFit LT3.3 (Verity Software, Topsham, US) according to an algorithm provided by the software. Written informed consent was obtained from all human subjects prior to inclusion in this project in accordance with the ethical standards of the institutional review board, ethical approval was granted by University of Essen, Germany (07/3500 and 16/7135).

Immunohistochemistry

Immunohistochemistry on tumor sections was done as described before (26). In brief, 5 μm sections of OCT-tissue tech (Sakura) embedded LLC tumor tissues were mounted on slides air-dried overnight and fixed in acetone for 10 min and air-dried for another 20 min. Slides were treated with 0.2% galantine (Sigma Aldrich) and 0.2% Triton X-100 in PBS and additionally blocked with antibody diluent (Dako) for 1 h at RT. All antibody stainings were performed in Dako antibody diluent solution. Primary antibodies were incubated overnight at 4°C. After 3 times washing with PBS, second antibodies were added for 1 h together with Hoechst 33342 (Sigma Aldrich) at room temperature. Negative controls were generated by staining with secondary antibodies and Hoechst 33342 only. After staining, the slides were covered with slowfade (Life Technologies) and analyzed with ObserverD.1 or LSM710 confocal microscopes (Zeiss). The following anti-mouse antibodies were used for confocal and fluorescence microscopy: CD31 (clone MEC13.3, BD # 550274, isotype control rat IgG2a), cleaved caspase 3 (cell signaling # 9604S), CD146 (clone ME9-F1, BD # 562230, isotype control rat IgG2a). As secondary antibodies Alexa 488-, Alexa 568-, and Alexa 647- labeled: anti-rat IgG and anti-rabbit IgG (Life Technologies) were used. Hoechst 33342 was used for staining nuclei.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data were analyzed by unpaired *t*-test. Data tested against a specified value were analyzed by one-sample *t*-test. Repeated measurement two-way ANOVA using Sidak's multiple comparison tests was applied on tumor growth data. Data were presented, if not indicated elsewhere, as mean ± S.E.M. $P < 0.05$ was considered to be statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

High Salt Intake Inhibits Tumor Growth in Mice

To examine the effects of HSD on cancer development we used the B16F10 syngeneic melanoma transplantation model. This poorly immunogenic tumor model (29, 30) was chosen to analyze possible immune activating effects of a HSD in mice. We first applied a protocol previously used in models of hypertension and autoimmunity by pre-feeding mice with a 4% NaCl containing chow and 1% NaCl in the drinking water compared to a control diet before tumor inoculation (**Figure 1A**) (1, 4). Of note, HSD fed mice showed a significantly inhibited tumor growth in the B16 tumor model (**Figure 1B**). Delayed tumor outgrowth was evident as early as day 11 post-injection (p.i.), leading to significant differences in tumor size between both groups at day 13 p.i. and at the day of sacrifice (day 15–17 p.i.) (**Figures 1B,C**). This effect seemed to be specific for the dietary regimens, since besides water intake no other confounders like e.g., general appearance, weight gain, and food intake were different (**Supplementary Figure 1** and data not shown) nor was there a direct effect of high sodium concentrations (additional 40 mM NaCl) on tumor cell viability during *in vitro* culture nor any effect of mannitol as an osmolyte control (**Supplementary Figure 2**). The NaCl concentrations used *in vitro* are comparable to the *in vivo* situation in high salt fed animals (24, 25). Only at concentrations

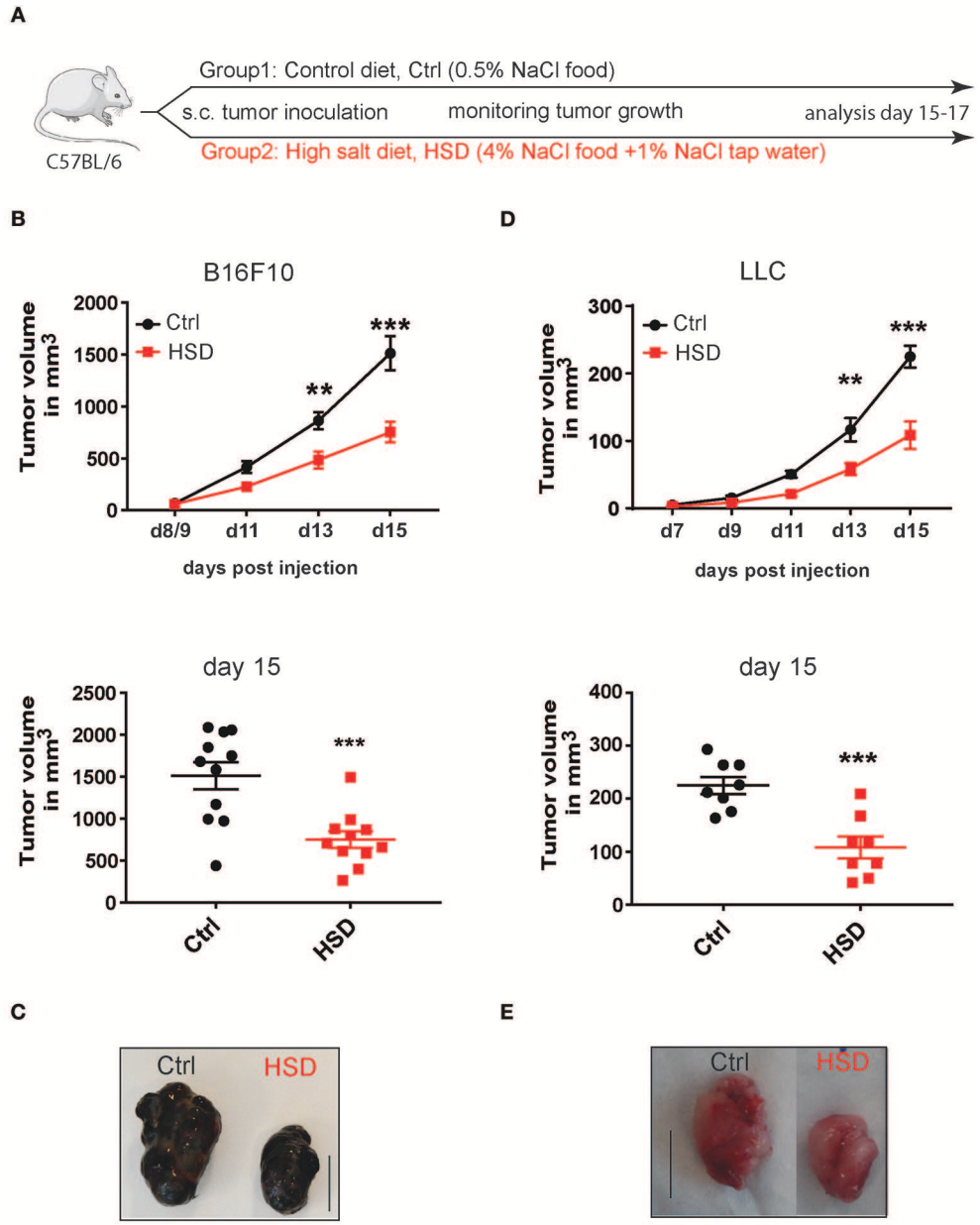


FIGURE 1 | High salt diet inhibits tumor growth in mice. **(A)** Experimental design. C57BL/6 mice were kept on control diet (Ctrl) or were fed a high salt diet (HSD) before tumor inoculation. After tumor challenge the mice were further kept on the same diets until sacrifice. **(B)** Mice pre-fed on the respective diets were challenged with B16F10 melanoma cells by subcutaneous (s.c.) injection. Growth curve and dotplot shows tumor volume as mean ± S.E.M. ($n = 11$) at the indicated time points pooled from two of three independent experiments with similar results. **(C)** Representative pictures were taken from tumors of control and HSD fed mice at day 15 post-induction (p.i.) (scale bar = 1 cm). **(D)** Mice that were fed a control diet or HSD for 2 weeks were subcutaneously injected with Lewis lung carcinoma cells (LLC) and tumor growth was monitored over time. Growth curve and dotplots shows tumor volume as mean ± S.E.M. ($n = 8$) at the indicated time points from one representative of three independent experiments with at least 5 mice per group. **(E)** Representative pictures were taken from LLC tumors of control and HSD fed mice at day 20 p.i. (scale bar = 1 cm). Statistical analysis was performed by Two-way repeated-measure Anova test (**$p < 0.01$, ***$p < 0.001$).

much higher than 40 mM NaCl high salt conditions were toxic to tumor cells as reported for other tested cell types before (1) (**Supplementary Figure 2**). To examine if the results were reproducible also in other transplanted tumor models, we tested the HSD regimen in the Lewis lung carcinoma model (LLC) (31). Similar to the B16 model, HSD also significantly delayed LLC tumor growth (**Figures 1D,E**). Thus, HSD was able to significantly inhibit tumor growth in two independent tumor transplantation models.

Salt-Induced Changes of the Immune System in Tumor-Bearing Mice

Since it is well-known that a HSD could have a profound impact on the host immune system by several mechanisms (7)

we first analyzed general immune parameters in tumor-bearing mice receiving a HSD compared to controls. Transcriptional analysis by quantitative real-time PCR with reverse transcription (qRT-PCR) of tumor tissue from mice at day 15–17 after tumor cell inoculation revealed a significant increase of tumor necrosis factor alpha (*Tnfα*), interferon-γ (*Ifnγ*) and a tendency of increased nitric oxide synthase 2 (*Nos2*) expression ($p = 0.0549$), whereas transcripts for interleukin (IL) 10 (*Il10*) and granulocyte macrophage-colony stimulating factor 2 (*Csf2*) remained unchanged (**Figure 2A**). The expression of serum and glucocorticoid-regulated kinase (*Sgk1)*, as a prominent salt signature gene (32) was similarly not changed (**Figure 2A**). When analyzing spleen cells from tumor-bearing mice at day 15–17 p.i., we detected similarly significant changes in *Tnfα*, *Ifnγ*, and *Nos2* expression and a significant increase in *Sgk1* expression (**Figure 2B**). Similar to the LLC model, we found increases in *Tnfα*, *Ifnγ*, and *Nos2* expression in the HSD B16 model (**Supplementary Figure 3A**). However, in both tissues we were unable to detect changes in *Il17a* expression. Moreover, ELISA-mediated analysis of serum cytokines of tumor-bearing animals on day 15–17 p.i. did not show any differences for TNFα, IFNγ, IL-10, or IL-17A between both groups (data not shown). However, by intracellular FACS analysis we detected increases of TNFα and IFNγ expression in tumor infiltrating cells (**Supplementary Figure 3B**).

We next analyzed by FACS the abundance of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in different tissues of tumor-bearing mice. FACS analysis of tumors, spleens, peripheral blood, tumor draining lymph nodes (dLN) and mesenteric lymph nodes (mLN) revealed no significant changes between control and HSD groups (**Figure 2C**; **Supplementary Figure 3C**). Although there were no obvious changes in T cell populations, particularly the higher expression of *Tnfα* and *Ifnγ* indicated a more pro-inflammatory environment in HSD fed mice compared to controls and suggests that the observed effect of delayed tumor growth is potentially related to changes in the host immune system.

High Salt Mediated Effect on Tumor Growth Is Largely Independent of T Cells

Since it is known that a HSD can profoundly affect the phenotype and function of $CD4^+$ T cells, particularly T_H17 cells and Tregs (1, 4, 6, 33, 34), we further examined these subsets in more detail in tumor-bearing animals receiving either a HSD or control diet by multicolor FACS analysis of different tissues as shown in **Figure 2C**. In line with the increased cytokine expression (**Figures 2A,B**; **Supplementary Figure 3A**), we detected a significantly higher number of effector-memory $CD4^+$ T cells (T_{EM}) and T_H1-like cells in the mLN of HSD fed tumor-bearing mice based on an antibody panel containing CD3, CD4, CD44, CD62L, CCR6, and CXCR3 specific antibodies by FlowSOM analysis at day 15–17 p.i. (**Figure 3A**). However, cells isolated from other tissues, including tumor-infiltrating cells, did not show any significant differences (**Supplementary Figure 4** and data not shown). The higher percentage of T_{EM} cells was confirmed by a manual gating strategy for CD44 and CD62L expression in $CD4^+$ T cells of mLN (**Figure 3B**). HSD fed animals further displayed a higher percentage of $CXCR3^+$ $CD4^+$ T cells in mLN cells, indicative of an increase in T_H1 cells in HSD fed tumor-bearing animals (**Figure 3C**). Of note, an increase of T_H1 cells was reported before in a model of lupus nephritis in an SGK1 dependent manner (35) and possibly could explain the HSD mediated effect on tumor growth. However, we only detected subtle changes of *Sgk1* expression (**Figures 2A,B**) that makes this scenario unlikely. Moreover, there seemed to be no changes in T_H17-like cells based on CCR6 chemokine receptor expression (**Figure 3C**).

We next extended the analysis by intracellular FACS after PMA/ionomycin restimulation *in vitro* for intracellular cytokine detection. Again, we were not able to detect significant changes for IL-17A (**Figure 3D**), indicating that T_H17 cells may not play a significant role in the delayed tumor growth of animals receiving a HSD. In contrast to the observed increases of T_H1-like cells, we were also not able to detect more IFNγ expressing cells in HSD fed mice, indicating that the observed increase of T_H1-like cells based on CXCR3 expression in mLN is of rather minor relevance (**Figure 3D**). This was similar in all tissues analyzed (data not shown).

Since we and others demonstrated before that a HSD could also impact $Foxp3^+$ Tregs (7, 34), for instance by inducing a T_H1-like effector phenotype, we carefully analyzed the frequency and cytokine expression of Tregs in different tissues of both groups. However, the detailed analysis of $Foxp3^+$ Tregs did not show any significantly altered frequency or phenotype (**Figures 3E,F**; **Supplementary Figure 5**) indicating that Tregs may not play a critical role in the HSD induced delayed tumor growth in this model. Besides $CD4^+$ T cells, we were not able to detect any significant changes in $CD8^+$ T cells nor NK cells in the tissues and at time points analyzed (**Supplementary Figures 6, 7**).

Having analyzed changes in the phenotype and function of T cells in high salt fed tumor-bearing mice, we next wanted to directly evaluate the contribution of T cells to tumor growth reduction mediated by HSD. To this end, we applied a similar tumor transplantation model using LLC and B16 tumor cells in *RAG2* deficient animals, completely lacking mature T and B cells (36). Surprisingly, while tumor growth reduction was slightly less efficient, the HSD effect was still detectable in *RAG2* deficient mice (**Figure 3G**; **Supplementary Figure 8**), indicating that T cells rather play a minor role in this model. Thus, although we were able to detect differences in T cell populations between both groups, the HSD mediated effect on tumor growth seemed to be mostly independent of alterations in T cells as well as B cells.

High Salt Intake Modulates Myeloid-Derived Suppressor Cells

Since T cells were not the major driving force behind the HSD mediated effect on tumor growth, we further analyzed innate immune cells in more detail. We and others have shown before that particularly M1-type- and M2-type macrophages were sensitive to HSD conditions (2, 3, 37). Thus, we hypothesized that innate immune cells may be key for the observed effect

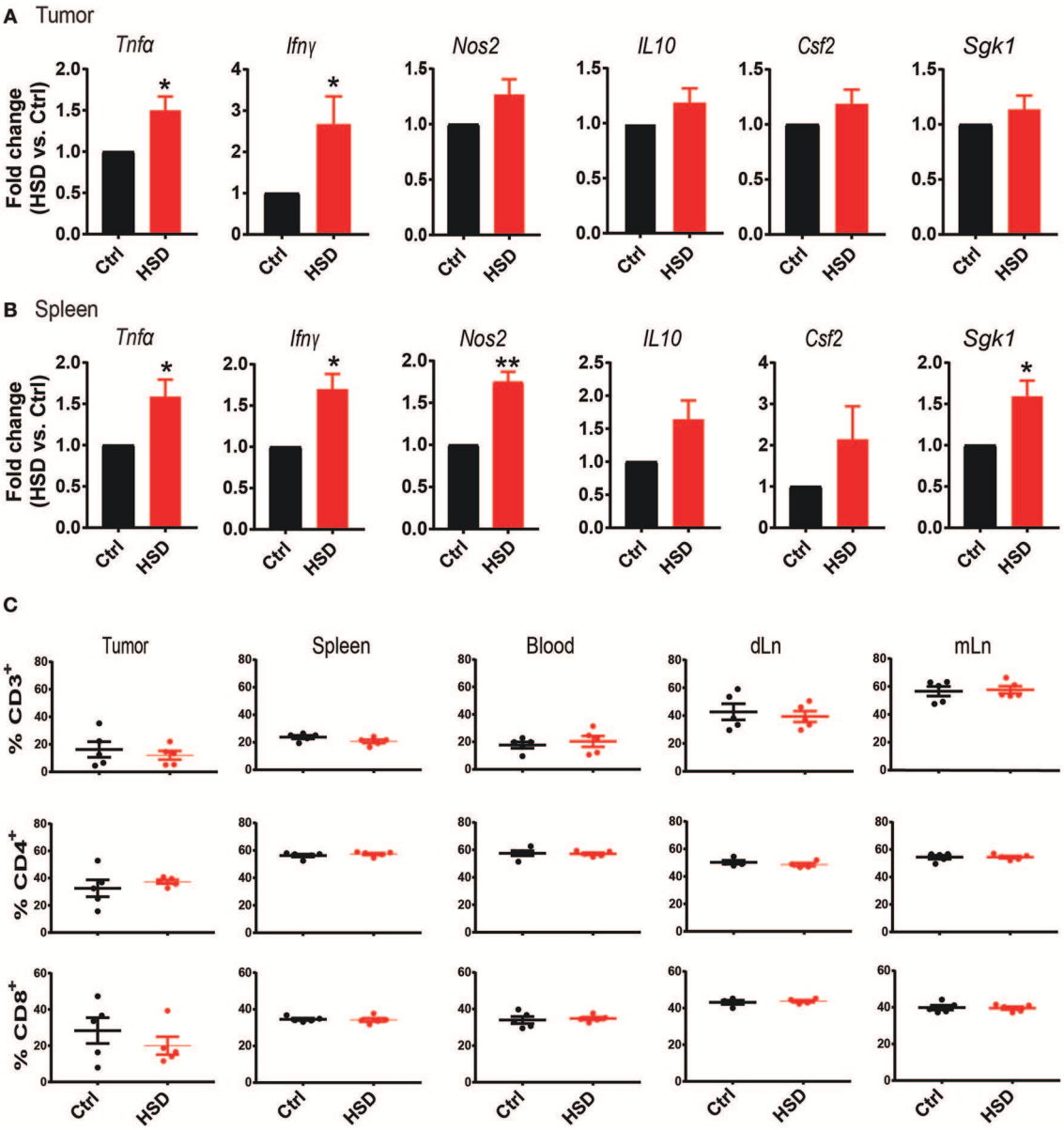


FIGURE 2 | High salt diet creates a pro-inflammatory environment in tumor-bearing mice. **(A)** Quantitative RT-PCR analysis of LLC tumor tissue. Bar graphs show fold change as mean ± S.E.M. from HSD samples normalized to control samples. Data are pooled from two independent experiments ($n = 8$–10). **(B)** Quantitative RT-PCR analysis of spleen samples from LLC tumor-bearing mice. Bar graphs show fold change as mean ± S.E.M. from HSD samples normalized to control samples ($n = 5$). **(C)** Tumors as well as the indicated organs from tumor-bearing mice were subjected to FACS analysis of T cell subsets. Cellular events were defined according to an extended lymphocyte gate, excluding doublets and dead cells. T cells were defined as $CD3^+$ and further gated according to CD4 and CD8 expression. Bar graphs show mean ± S.E.M. of $CD3^+$ cells (upper row), $CD4^+$ T cells (center row, gated on $CD3^+$), and $CD8^+$ T cells (lower row, gated on $CD3^+$). Samples were analyzed on day 15–17 p.i. statistical significance was determined by *t*-test (*$p < 0.05$, **$p < 0.01$).

of inhibited tumor growth in HSD receiving animals. In this respect, particularly myeloid-derived suppressor cells are known to have a critical impact on tumor growth (17, 28). MDSCs are classified as myeloid cells with suppressive function and can be identified in mice using the markers CD11b, Ly6C, and Ly6G for PMN-MDSC ($CD11b^+Ly6G^{high}Ly6C^{low}$) and M-MDSCs ($CD11b^+Ly6G^-Ly6C^{high}$) (17, 28, 38–40).

We examined subsets of myeloid cells including potential MDSC populations in tumor-bearing mice receiving either HSD or control diet by FACS in different tissues at different time points (**Figure 4**). Of note, FlowSOM analysis of samples from peripheral blood at day 14 p.i. showed significant changes in myeloid cell populations (**Figure 4A**). We observed a similar trend in $CD11b^+Ly6G^{high}Ly6C^{low}$ PMN-MDSCs by manual

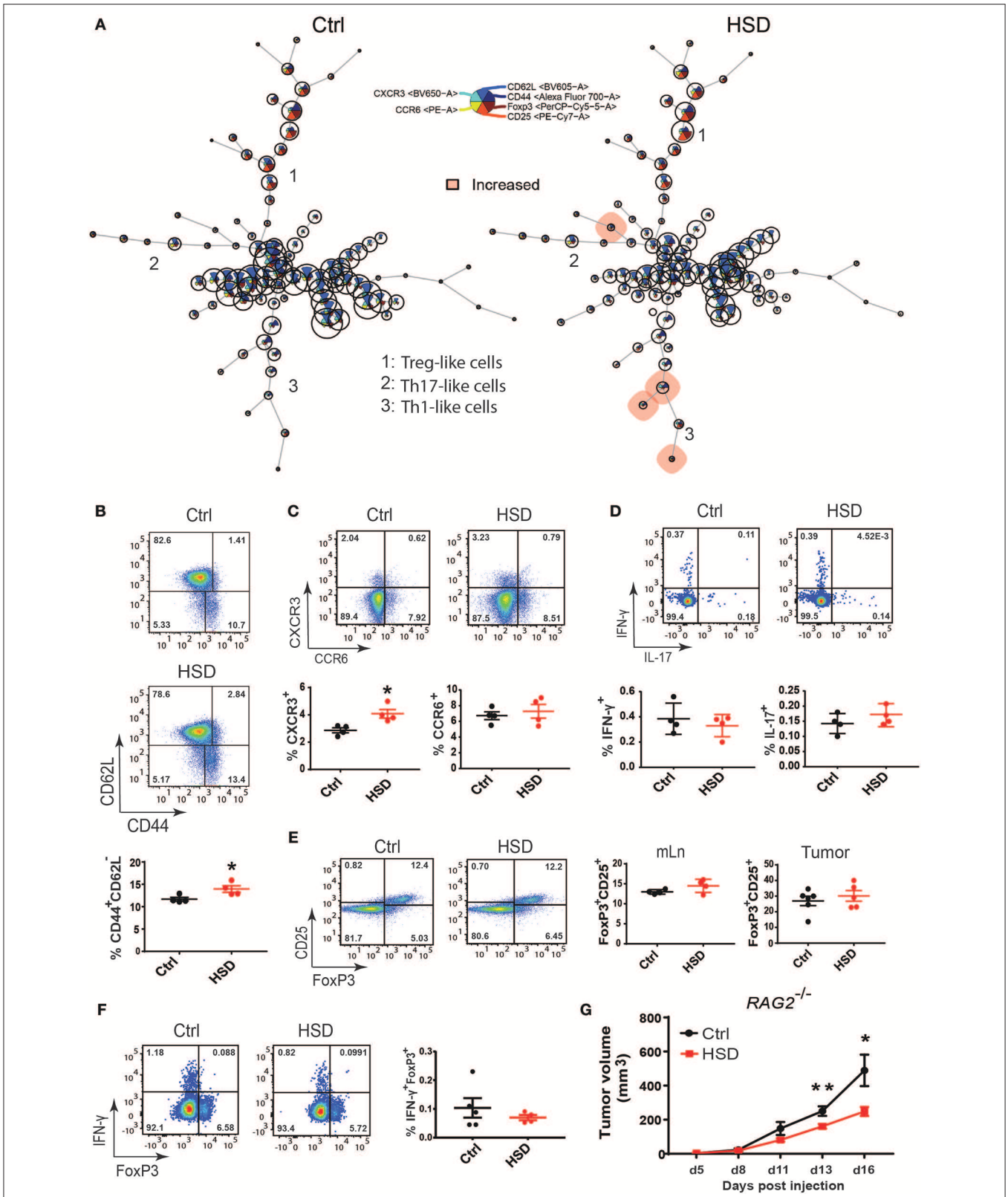


FIGURE 3 | The impact of T cells on high salt mediated reduced tumor growth. **(A)** FlowSOM visualization of flow cytometry data across mesenteric lymph nodes (mLN). Single live $CD4^+$ cells for each sample (Ctrl, $n = 4$; HSD, $n = 4$) were exported and concatenated then analyzed using FlowSOM, which arranges the cells into

(Continued)

FIGURE 3 | clusters (represented by circles) according to similarities in their expression profiles. Each node represents one cluster (total = 100 nodes). Colored nodes highlight statistically significant changes ($p < 0.05$) in cell population between two groups (HSD and Ctrl). **(B)** The same mLN samples as in **(A)** were analyzed by manual gating for **(B)** $CD4^+$ effector-memory T cells ($CD44^+CD62L^-$). **(C)** Th1-like cells ($CD4^+CXCR3^+$) and Th17-like cells ($CD4^+CCR6^+$). **(D)** IL-17 and IFNγ producing $CD4^+$ T cells after PMA/Ionomycin restimulation and intracellular staining. Statistical significance was determined by unpaired *t*-test (*$p < 0.05$). **(E)** $FoxP3^+CD25^+$ regulatory T cells in tumor-bearing mice. FACS plots show the indicated parameters after pre-gating on $CD3^+CD4^+$ T cells. Dotplots show frequency of the indicated populations as mean ± S.E.M. ($n = 4$/group) representative of three independent experiments. **(F)** IFNγ-producing regulatory T cells after restimulation of mLN single cell suspensions from LLC tumor-bearing mice. Representative FACS plots show FoxP3 against IFNγ after pre-gating on $CD3^+CD4^+$ T cells. Dotplots show frequency of $FoxP3^+IFN\gamma^+$ cells as mean ± S.E.M. from 4 to 5 mice in each group. Similar results were obtained from B16 tumor-bearing mice. **(G)** *RAG2*$^{-/-}$ mice were fed a high salt diet (HSD) or control diet (Ctrl) and challenged with LLC tumor cells. Growth curve shows tumor volume as mean ± S.E.M. for 7 mice in each group. Statistical analysis was performed by Two-way repeated-measure Anova test (*$p < 0.05$, **$p < 0.01$).

gating (**Figure 4B**; **Supplementary Figure 9**) and confirmed an increase of PMN-MDSCs at day 14 p.i. ($p = 0.0559$) as well as day 9 ($p = 0.1336$), although not reaching statistical significance. When analyzing MDSC subsets in tumors and spleens at day 15–17 p.i., however, we could not detect any significant differences in frequencies of MDSCs (**Figure 4C**; **Supplementary Figure 10**). In addition, we could not reveal any significant changes in monocyte/macrophage populations (**Figure 4C**; **Supplementary Figure 10**), indicating that by HSD the composition of myeloid cell populations was changed systemically (in peripheral blood) but not locally in the tumor. Since MDSCs could also affect tumor angiogenesis (15), we analyzed abundance of endothelial cells in tumor sections by CD31 immunohistology. Specificity for blood vessel endothelial cells was confirmed by CD146 co-staining (41) (data not shown). However, we could not detect major differences in CD31 staining indicative for alterations in the degree of tumor angiogenesis between the two groups at time points analyzed (**Supplementary Figure 11**).

The above data suggested that HSD induced changes on the myeloid compartment and that particularly MDSCs might be key to HSD induced inhibited tumor growth. To directly test the impact of MDSCs in the HSD tumor model, we applied an antibody-mediated depletion of MDSCs by using anti-GR1 antibodies, known to be very efficient in depleting MDSCs in mice (42). In line with previous studies (43), this protocol efficiently depleted MDSCs and neutrophils in the model system as monitored by FACS analysis in blood and tumors (**Supplementary Figure 12**). Importantly, by depleting MDSCs using anti-GR1 antibodies, the inhibitory effect of HSD on tumor growth was completely abolished (**Figure 4D**). Antibody treated HSD fed animals displayed a similar tumor growth as animals receiving the control diet. These data indicate that the population of MDSCs are essential players in the HSD mediated inhibitory effect on tumor growth.

High Salt Blocks Suppressive Function of Murine and Human MDSCs

The above data clearly pointed toward phenotypic and functional changes of MDSCs upon HSD and indicated that these changes in MDSC populations significantly contributed to the inhibitory effect of high salt on tumor growth. Although the number of MDSCs in spleen and tumor was not altered upon HSD, it is possible that HSD alters the function of these cells. To further test if high salt may directly affect MDSCs, we analyzed the impact of increased sodium concentrations on MDSC phenotype and function *in vitro* (**Figure 5**). To mimic *in vitro* the high salt conditions in the interstitial tissues of animals receiving a HSD diet (24, 25), we used an established protocol by increasing the sodium concentration in cell cultures by adding an additional 40 mM of NaCl (1, 3, 4, 33, 34). To test the effects of high salt on MDSC function we isolated MDSCs from spleens and tumors of LLC tumor-bearing mice and examined these cells *in vitro* for their capacity to suppress effector T cells under control or high salt conditions (+40 mM NaCl). It has been shown that immature myeloid cells can affect tumor growth also in the absence of T cells (19) and although the *in vivo* experiments in *RAG2* deficient mice clearly pointed to a T cell independent mechanism for myeloid cell mediated tumor growth reduction, we still resorted to the *in vitro* T cell suppression assay, as an established method to analyze changes in immunoregulatory phenotype after salt exposition.

MDSCs were isolated based on the expression of specific markers as described before (16, 21) (**Supplementary Figures 13A,B**). Of note, high salt conditions blocked MDSC suppressive capacity *in vitro* almost completely (**Figure 5A**; **Supplementary Figure 14A**). Particularly, the effect was observed in M-MDSCs isolated from tumors and spleens indicating that this subset was highly affected by high sodium conditions *in vitro*. A similar tendency, although to a lower extent, was also observed for PMN-MDSCs (data not shown). As an osmolyte control, we tested the effects of 80 mM mannitol in cultures but did not observe a similar effect, indicating that the effect was rather specific to Na^+ ions and was not simply due to changes in osmotic pressure (**Supplementary Figure 13C**). Of note, a similar effect of functional inactivation was also observed, when testing the suppressive capacity of MDSCs isolated from HSD and control fed mice. MDSCs isolated from tumors of HSD fed mice showed lower suppressive function compared to MDSCs isolated from tumors of control fed mice, indicating that the functional changes occur also under high salt conditions *in vivo* (**Supplementary Figure 14B**).

To examine if this effect may also apply to human MDSCs, we isolated MDSCs from peripheral blood of cancer patients (**Supplementary Table 1**). Since in humans the subset of PMN-MDSCs have been demonstrated to be the subset with highest suppressive activity and clinical relevance in cancer patients (16, 28), we isolated MDSCs as $HLA\text{-}DR^-CD33^{dim}CD66b^+Lin^-CD14^{dim}$ cells from five independent cancer patients (**Figure 5B**). We then tested MDSC functionality in suppression assays under control or high salt (+40 mM NaCl) conditions as measured by fluorescent dye dilution of labeled

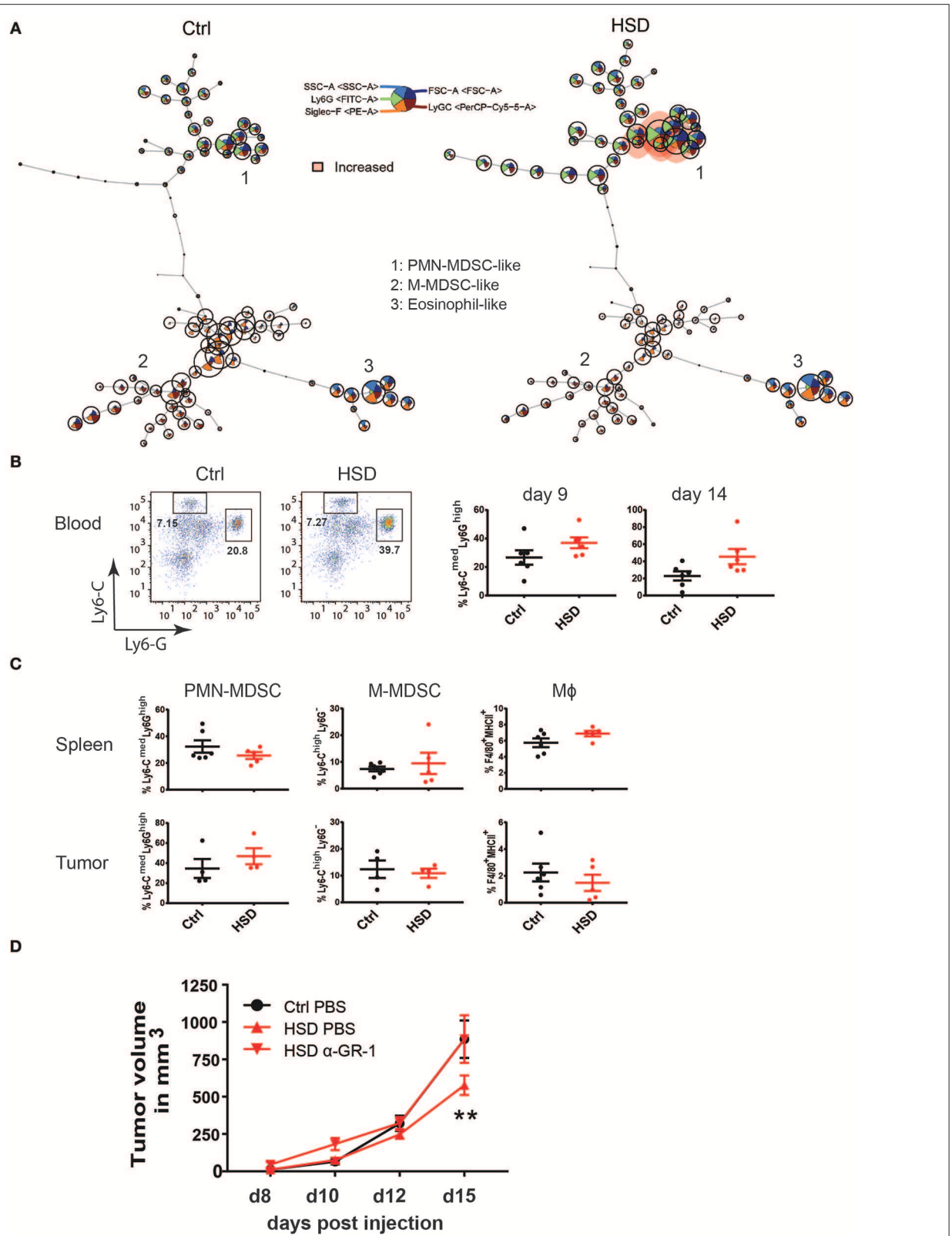


FIGURE 4 | High salt diet mediates changes in myeloid cells in tumor-bearing mice. **(A)** FlowSOM visualization of flow cytometry data in blood. Single live $CD45^{+}CD11b^{+}$ cells for each sample (Ctrl and HSD, $n = 6$/group) were exported and concatenated then analyzed using FlowSOM, which arranges the cells into

(Continued)

FIGURE 4 | clusters (represented by circles) according to similarities in their expression profiles. Each node represents one cluster (total = 100 nodes). Colored nodes highlight statistically significant changes ($p < 0.05$) in cell population between two groups (HSD and Ctrl). **(B)** Blood samples from B16 tumor-bearing mice were analyzed for MDSC populations at day 9 and 14 p.i. FACS plots show representative distribution of M-MDSC-like cells (Ly6-C^{high}LyG^{-} and PMN-MDSC-like cells (Ly6C^{med}Ly6-G^{high}) after gating on CD11b^{+} cells. Bar graphs show the frequency of each population in high salt diet fed mice (HSD) compared to control mice (Ctrl) as mean ± S.E.M. from 6 mice in each group. Statistical significance was determined by unpaired *t*-test. **(C)** Single cell suspensions from spleens and tumors were analyzed as in **(B)** at day 16. Dotplots show the frequency of each population in high salt diet fed mice (HSD) compared to control mice (Ctrl) as mean ± S.E.M. from 5 to 6 mice in each group. **(D)** High salt diet (HSD) fed mice were treated with an anti-GR-1 antibody or PBS as control from day 4 after B16 melanoma cell inoculation on consecutively every second day. Growth curve shows tumor volume as mean ± S.E.M. of 5 mice in each group. Statistical analysis was performed by Two-way repeated-measure Anova (**$p < 0.01$).

T cells as described before (28) (**Figure 5C**). Of note, high salt conditions blocked the suppressive function of MDSCs in T cell suppression assays of all five patients (**Figure 5D**). This data clearly show that increased sodium concentrations, mimicking high salt conditions in tissues of HSD fed animals, could indeed markedly change the inflammatory phenotype of MDSCs and alter their function (here shown for immunosuppression) in mice and humans. While it remains to be shown how exactly myeloid cells reduce tumor growth, the animal experiments using HSD show that such changes can directly affect tumor growth *in vivo*.

DISCUSSION

High dietary salt intake is believed to be associated with various diseases (5). Besides implications in cardiovascular pathologies, recent data have clearly shown that high salt intake could profoundly modulate the immune system through direct and indirect mechanisms-mainly leading to shifts toward a pro-inflammatory milieu (6, 7). However, the majority of current *in vivo* data is based on studies in rodents, using protocols of extremes of high salt intake that likely cannot extrapolate to humans and therefore findings have to be analyzed carefully if they can apply to the human situation (7, 44). Nevertheless, few available studies in humans indicate that even moderate changes in salt intake could impact host immunity and clinical parameters in a similar manner compared to experimental animal studies. For instance, a daily increase of 6 g NaCl for 14 days seemed to affect already T_H17 cell frequency and blood pressure as reported for a small human pilot study (4, 45). The data presented here indicates that a high salt diet could also strongly affect tumor growth by enhancing anti-tumor immunity through the modulation of MDSC function. Thus, in the context of an immune response to cancer, a high salt diet may positively affect anti-tumor immunity, similar to enhanced immune responses toward certain pathogens as shown before for *Leishmania* infection in skin (2).

In humans, high sodium intake might be a risk factor for the development of gastric cancer (46, 47). However, animal studies on gastric cancer development show contradictory results (48–51) indicating that the exact role of sodium intake in cancer development is still not well-defined and the stomach represents a very peculiar milieu because of the acidic nature and the ionic composition in the gastric mucosa.

When analyzing major immune parameters in tumor-bearing mice between control and high salt fed mice, we could detect increases in *Tnfa*, *Ifng*, and *Nos2* expression in spleen and tumor tissues, indicating a more pro-inflammatory environment in HSD mice. Although we couldn't measure any significant changes in T cell frequencies between the two groups, HSD impacted adaptive immune cells in our model, as measured by increased effector-memory and T_H1-like cells in tumor-bearing mice. However, this was only evident in mLN of tumor-bearing mice, indicating that these changes may not play a relevant role for the observed effects. A possible explanation for this observation might be the known impact of HSD on the gut microbiota and T cells (4). We neither did detect changes in T_H17 cells, a T cell subpopulation usually enhanced under high salt conditions particularly in experimental settings of neuro- and gut-inflammation. However, this can be due to the nature of the inflammatory conditions of the tumor models used or due to different time points and tissues analyzed compared to other experimental models of inflammation under HSD (45). In contrast to CD4^{+} T effector cells, we were not able to detect any significant differences in the CD8^{+} T cell compartment at the examined time points in different tissues. Since regulatory T cells can also critically be affected by high salt (34) and they greatly impact anti-tumor immunity (52), we also examined this suppressive CD4^{+} T cell subset thoroughly in our model. However, Foxp3^{+} Tregs didn't show any differences in frequency or altered subpopulations [e.g., IFNγ^{+} T_H1-like Tregs (34)] between both groups in all tissues analyzed. Nevertheless, we cannot exclude functional changes, since we did not test the *in vitro* suppressive capacity of isolated Tregs from tumor-bearing mice that was shown to be impaired under high salt conditions *in vitro* and in humanized mouse models in settings of a xenogeneic graft-vs.-host disease model (x-GvHD) *in vivo* (34). However, importantly, since we observed almost similar effects of delayed tumor growth under HSD in *RAG2*$^{-/-}$ animals, the effect seems not to be critically dependent on T cells in both tumor models tested, suggesting that the changes in phenotype seen in the T cell compartment are a reflection of the changes in the pro-inflammatory milieu but do not significantly contribute to tumor control.

Therefore, we further examined innate immune cells in tumor-bearing mice. Monocyte/macrophages have been shown before to be sensitive to high salt (2, 3, 37) and particularly M1-type macrophages were induced to become more pro-inflammatory in an p38MAPK/NFAT5 dependent manner and to express higher levels of a pro-inflammatory gene signature,

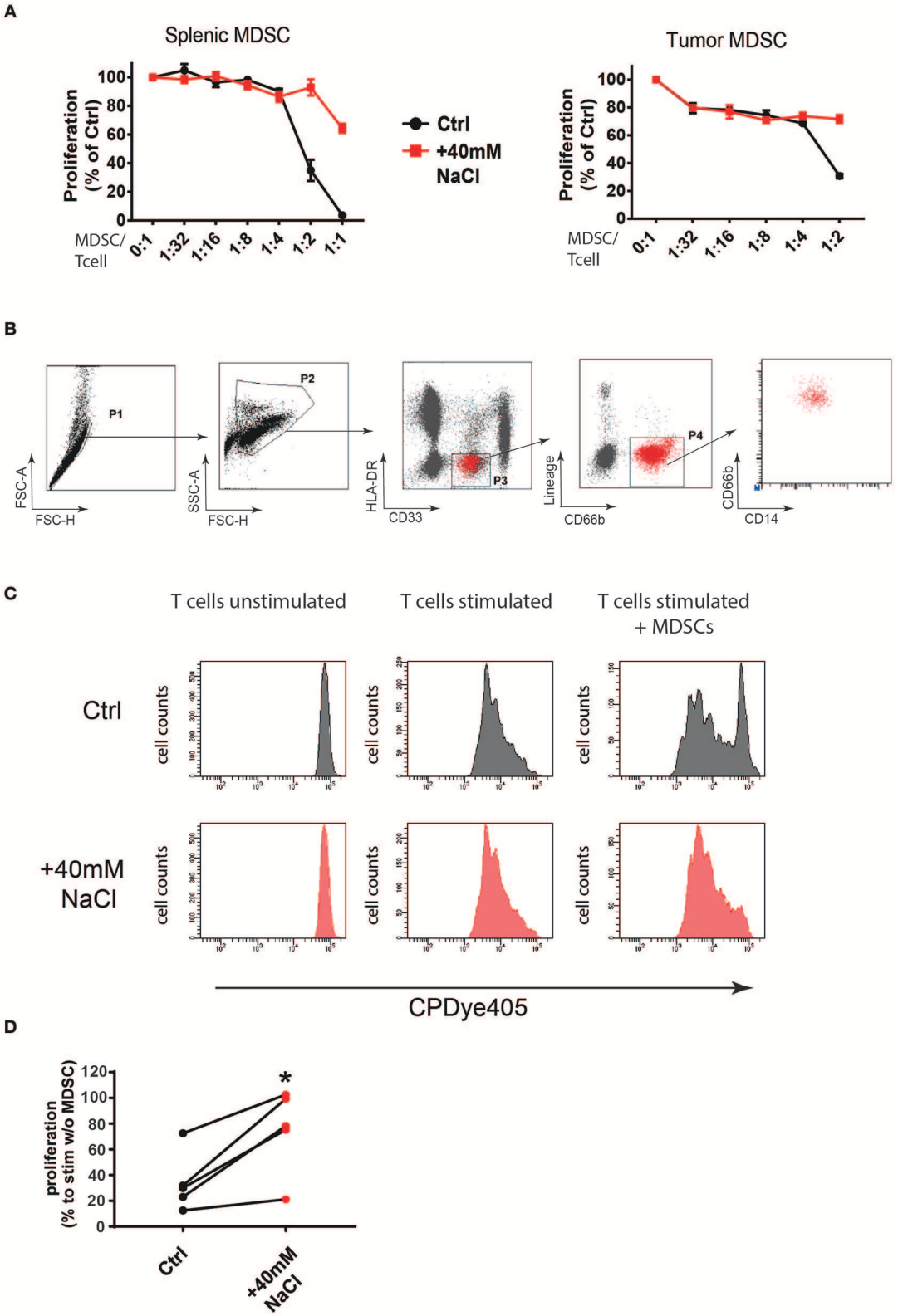


FIGURE 5 | High salt conditions block murine and human MDSC function *in vitro*. **(A)** MDSCs isolated from LLC tumors and spleens of tumor-bearing mice were cultured with splenocytes at the indicated ratios in the presence of anti-CD3/CD28 stimulating antibodies. Cells were either cultured under high salt (+40 mM NaCl) or

(Continued)

FIGURE 5 | control conditions (Ctrl). Proliferation of responder splenocytes was measured by ^{3}H-thymidin incorporation. Curves show proliferation normalized to controls (stimulated splenocytes without addition of MDSCs) as mean ± S.E.M from triplicates representative for three independent experiments with similar results. **(B)** PMN-MDSCs were sorted from PBMCs of patients with oropharynx or bladder cancer. FACS plots show the gating strategy. **(C)** FACS purified human PMN-MDSC were co-cultured with CPDye405-labeled autologous CD3^{+} T cells under control (Ctrl) or high salt conditions (+40 mM NaCl) for 4 days at a 1:2.5 ratio. FACS histograms from a representative patient are shown. **(D)** Graph shows proliferation index from five independent patients under control or high salt (+40 mM NaCl) conditions. Statistical significance was determined by unpaired *t*-test (*$p < 0.05$).

including *Tnfα* and *Nos2* (2, 37) two genes that were also highly induced in HSD fed animals. However, we could not detect any significant changes in frequencies of monocyte/macrophage populations locally in the tumor milieu, although we cannot exclude functional changes on these cells upon HSD. In addition, we could not detect any changes in NK cell frequencies. Nevertheless, when analyzing CD11b^{+}Ly6G^{high}Ly6C^{low} and CD11b^{+}Ly6G^{-}Ly6C^{high} myeloid cell populations in tumor-bearing animals that include MDSCs, we observed systemically increased numbers of CD11b^{+}Ly6G^{high}Ly6C^{low} MDSC-like cells in high salt fed mice. Particularly in blood at early time points after tumor inoculation, these PMN-MDSCs seemed to be increased in cell numbers under HSD compared to controls, indicating an effect of HSD in tumor-bearing mice in this myeloid cell population. Since MDSCs are critical modulators of anti-tumor immunity (16–18) we thus analyzed this subset in more detail.

Interestingly, the depletion of GR1^{+} cells annihilated the HSD effect on tumor growth, clearly pointing toward a functional role of MDSCs. We thus tested the impact of high sodium concentrations on the suppressive capacity of MDSCs *in vitro*, as a surrogate marker for their suppressive vs. pro-inflammatory activity. Of note, high salt concentrations *in vitro*, mimicking sodium content in tissues of HSD fed animals, significantly blocked the function of MDSCs isolated from tumor-bearing mice in suppression assays. Moreover, MDSCs isolated directly from tumors of HSD fed mice showed similarly a lack of suppression compared to cells isolated from tumors of control fed mice. In this respect, it is of interest that a previous study found accumulation of functional MDSCs in different models of hypertension, including a salt sensitive setting, implicating a functional role in blood pressure regulation. However, how the specific salt sensitive L-N^{G}-Nitroarginine Methyl Ester (L-NAME) model for hypertension compares to the tumor models is currently unclear and would be of interest to be investigated in further studies (53). Our data indicate that high sodium content may directly affect particularly MDSC function in tumor-bearing mice, consequently leading to a shift in the immune balance toward a pro-inflammatory environment. However, since T cells do not seem to be the major force driving the anti-tumoral effect, as demonstrated by the *RAG2*$^{-/-}$ experiments, the key executing cell type seems to be rather of innate origin. Possibly, as observed by increases of *Tnfa*, *Ifng*, and *Nos2* expression, it could well be that in HSD fed animals besides other innate immune cells like macrophages, tumor-infiltrating MDSCs themselves became more pro-inflammatory and anti-tumoral. In this respect it is of interest that MDSCs were shown to be highly plastic cell types with the ability to convert toward proinflammatory effector cells (22).

Importantly, a clear modulation of suppressive activity under high salt conditions was also observed in circulating human MDSCs from cancer patients. This indicates that high salt could similarly affect MDSC function in humans. Thus, potential molecular changes induced by high salt conditions may offer novel therapeutic targets to possibly assist cancer immunotherapy. MDSCs are considered to be a major hurdle in cancer immunotherapy, preventing efficient immune attack against tumors e.g., when using checkpoint inhibitors (20, 54). Therefore, the further exploration of this effect may have potential in immune therapies for targeting MDSC function. However, the detailed analysis of the molecular effect has to be addressed in future studies and is out of the scope of this study. It would be of interest for future studies to analyze the role of known molecular high salt targets like p38/MAPK, SGK1 and NFAT5 and if they are also involved in salt exposed MDSCs, as it has been shown for T cell and monocyte/macrophage populations. Of note, p38/MAPK signaling is also of crucial importance for the pro-inflammatory and pro-tumoral activity of tumor-associated PMN in humans (55). In addition, it would be of interest to test in future studies if the functional changes in MDSCs under high salt exposure are also related to epigenetic remodeling and changes in immuno-metabolism as observed for M2-type macrophages (3).

In summary, we show that a high salt diet significantly delays tumor growth in two independent murine tumor transplantation models. This effect seems to be mediated through enhanced anti-tumor immunity by a functional inactivation of MDSCs. Since high salt conditions also affected human MDSCs in a similar manner, our data suggest that the targeting of this mechanism could potentially be a novel beneficial strategy to block MDSC function in settings of cancer immunotherapy.

ETHICS STATEMENT

Animal studies:

The protocol was approved by the ethics committees of animal studies at the University of Hasselt (201738) and Vrije Universiteit Brussel (14-220-26).

Human specimen:

Written informed consent was obtained from all human subjects prior to inclusion in this project in accordance with the ethical standards of the institutional review board and with the Declaration of Helsinki, ethical approval was granted by University of Duisburg-Essen, Germany (07/3500 and 16/7135).

AUTHOR CONTRIBUTIONS

RW and IH designed and performed experiments and analyzed data. LV, MKi, KB, AG, DS, BC-R, LM, EL, DL, and JK performed experiments and analyzed data. TK, SB, and JV supervised experiments and analysis. MKl conceived the project, designed, and supervised experiments and analysis and wrote the manuscript with further input from all authors.

FUNDING

MKi was supported by a Ph.D. grant from the Research Foundation Flanders (FWO), Belgium. MKl was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (640116), by a SALK-grant from the government of Flanders, Belgium and by an Odysseus-grant of the FWO, Belgium.

ACKNOWLEDGMENTS

We thank Yvan Saeys and Sofie van Gassen for help with FlowSOM analysis, we further thank Christian Friese for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01141/full#supplementary-material

REFERENCES

1. Kleinewietfeld M, Manzel A, Titze J, Kvakan H, Yosef N, Linker RA, et al. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature*. (2013) 496:518–22. doi: 10.1038/nature11868
2. Jantsch J, Schatz V, Friedrich D, Schroder A, Kopp C, Siegert I, et al. Cutaneous Na+ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. *Cell Metab*. (2015) 21:493–501. doi: 10.1016/j.cmet.2015.02.003
3. Binger KJ, Gebhardt M, Heinig M, Rintisch C, Schroeder A, Neuhofer W, et al. High salt reduces the activation of IL-4- and IL-13-stimulated macrophages. *J Clin Invest*. (2015) 125:4223–38. doi: 10.1172/JCI80919
4. Wilck N, Matus MG, Kearney SM, Olesen SW, Forslund K, Bartolomaeus H, et al. Salt-responsive gut commensal modulates TH17 axis and disease. *Nature*. (2017) 551:585–9. doi: 10.1038/nature24628
5. Brown IJ, Tzoulaki I, Candeias V, Elliott P. Salt intakes around the world: implications for public health. *Int J Epidemiol*. (2009) 38:791–813. doi: 10.1093/ije/dyp139
6. Manzel A, Muller DN, Hafler DA, Erdman SE, Linker RA, Kleinewietfeld M. Role of "Western diet" in inflammatory autoimmune diseases. *Curr Allergy Asthma Rep*. (2014) 14:404. doi: 10.1007/s11882-013-0404-6
7. Willebrand R, Kleinewietfeld M. The role of salt for immune cell function and disease. *Immunology*. (2018) 154:346–53. doi: 10.1111/imm.12915
8. Farkona S, Diamandis EP, Blasutig IM. Cancer immunotherapy: the beginning of the end of cancer? *BMC Med*. (2016) 14:73. doi: 10.1186/s12916-016-0623-5
9. Gibney GT, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. *Lancet Oncol*. (2016) 17:e542–51. doi: 10.1016/S1470-2045(16)30406-5
10. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev*. (2008) 18:11–8. doi: 10.1016/j.gde.2007.12.007
11. Cavallo F, De Giovanni C, Nanni P, Forni G, Lollini PL. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother*. (2011) 60:319–26. doi: 10.1007/s00262-010-0968-0
12. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity*. (2013) 39:1–10. doi: 10.1016/j.immuni.2013.07.012
13. Hernandez C, Huebener P, Schwabe RF. Damage-associated molecular patterns in cancer: a double-edged sword. *Oncogene*. (2016) 35:5931–41. doi: 10.1038/onc.2016.104
14. Kammertoens T, Schuler T, Blankenstein T. Immunotherapy: target the stroma to hit the tumor. *Trends Mol Med*. (2005) 11:225–31. doi: 10.1016/j.molmed.2005.03.002
15. Shojaei F, Zhong C, Wu X, Yu L, Ferrara N. Role of myeloid cells in tumor angiogenesis and growth. *Trends Cell Biol*. (2008) 18:372–8. doi: 10.1016/j.tcb.2008.06.003
16. Bruger AM, Dorhoi A, Esendagli G, Barczyk-Kahlert K, van der Bruggen P, Lipoldova M, et al. How to measure the immunosuppressive activity of MDSC: assays, problems and potential solutions. *Cancer Immunol Immunother*. (2018) 68:631–44. doi: 10.1007/s00262-018-2170-8
17. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun*. (2016) 7:12150. doi: 10.1038/ncomms12150
18. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. (2012) 12:253–68. doi: 10.1038/nri3175
19. Pekarek LA, Starr BA, Toledano AY, Schreiber H. Inhibition of tumor growth by elimination of granulocytes. *J Exp Med*. (1995) 181:435–40.
20. Liu Y, Wei G, Cheng WA, Dong Z, Sun H, Lee VY, et al. Targeting myeloid-derived suppressor cells for cancer immunotherapy. *Cancer Immunol Immunother*. (2018) 67:1181–95. doi: 10.1007/s00262-018-2175-3
21. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*. (2008) 111:4233–44. doi: 10.1182/blood-2007-07-099226
22. Ben-Meir K, Twaik N, Baniyash M. Plasticity and biological diversity of myeloid derived suppressor cells. *Curr Opin Immunol*. (2018) 51:154–61. doi: 10.1016/j.coi.2018.03.015
23. Eil R, Vodnala SK, Clever D, Klebanoff CA, Sukumar M, Pan JH, et al. Ionic immune suppression within the tumour microenvironment limits T cell effector function. *Nature*. (2016) 537:539–43. doi: 10.1038/nature19364
24. Machnik A, Neuhofer W, Jantsch J, Dahlmann A, Tammela T, Machura K, et al. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. *Nat Med*. (2009) 15:545–52. doi: 10.1038/nm.1960
25. Wiig H, Schroder A, Neuhofer W, Jantsch J, Kopp C, Karlsen TV, et al. Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. *J Clin Invest*. (2013) 123:2803–15. doi: 10.1172/JCI60113
26. Kammertoens T, Friese C, Arina A, Idel C, Briesemeister D, Rothe M, et al. Tumour ischaemia by interferon-gamma resembles physiological blood vessel regression. *Nature*. (2017) 545:98–102. doi: 10.1038/nature22311
27. Van Gassen S, Callebaut B, Van Helden MJ, Lambrecht BN, Demeester P, Dhaene T, et al. FlowSOM: using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry A*. (2015) 87:636–45. doi: 10.1002/cyto.a.22625
28. Lang S, Bruderek K, Kaspar C, Hoing B, Kanaan O, Dominas N, et al. Clinical relevance and suppressive capacity of human myeloid-derived suppressor cell subsets. *Clin Cancer Res*. (2018) 24:4834–44. doi: 10.1158/1078-0432.CCR-17-3726
29. Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. *Curr Protoc Immunol*. (2001). 39:20.1.1–29 doi: 10.1002/0471142735.im2001s39

30. Fidler IJ, Darnell JH, Budmen MB. Tumoricidal properties of mouse macrophages activated with mediators from rat lymphocytes stimulated with concanavalin A. *Cancer Res*. (1976) 36:3608–15.
31. Colegio OR, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. (2014) 513:559–63. doi: 10.1038/nature13490
32. Binger KJ, Linker RA, Muller DN, Kleinewietfeld M. Sodium chloride, SGK1, and Th17 activation. *Pflugers Arch*. (2015) 467:543–50. doi: 10.1007/s00424-014-1659-z
33. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature*. (2013) 496:513–7. doi: 10.1038/nature11984
34. Hernandez AL, Kitz A, Wu C, Lowther DE, Rodriguez DM, Vudattu N, et al. Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. *J Clin Invest*. (2015) 125:4212–22. doi: 10.1172/JCI81151
35. Yang X, Yao G, Chen W, Tang X, Feng X, Sun L. Exacerbation of lupus nephritis by high sodium chloride related to activation of SGK1 pathway. *Int Immunopharmacol*. (2015) 29:568–73. doi: 10.1016/j.intimp.2015.09.027
36. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. (1992) 68:855–67
37. Zhang WC, Zheng XJ, Du LJ, Sun JY, Shen ZX, Shi C, et al. High salt primes a specific activation state of macrophages, M(Na). *Cell Res*. (2015) 25:893–910. doi: 10.1038/cr.2015.87
38. Moses K, Brandau S. Human neutrophils: their role in cancer and relation to myeloid-derived suppressor cells. *Semin Immunol*. (2016) 28:187–96. doi: 10.1016/j.smim.2016.03.018
39. Mandruzzato S, Brandau S, Britten CM, Bronte V, Damuzzo V, Gouttefangeas C, et al. Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study. *Cancer Immunol Immunother*. (2016) 65:161–9. doi: 10.1007/s00262-015-1782-5
40. Dumitru CA, Moses K, Trellakis S, Lang S, Brandau S. Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology. *Cancer Immunol Immunother*. (2012) 61:1155–67. doi: 10.1007/s00262-012-1294-5
41. Schrage A, Loddenkemper C, Erben U, Lauer U, Hausdorf G, Jungblut PR, et al. Murine CD146 is widely expressed on endothelial cells and is recognized by the monoclonal antibody ME-9F1. *Histochem Cell Biol*. (2008) 129:441–51. doi: 10.1007/s00418-008-0379-x
42. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. (2008) 83:64–70. doi: 10.1189/jlb.0407247
43. Moses K, Klein JC, Mann L, Klingberg A, Gunzer M, Brandau S. Survival of residual neutrophils and accelerated myelopoiesis limit the efficacy of antibody-mediated depletion of Ly-6G$^+$ cells in tumor-bearing mice. *J Leukoc Biol*. (2016) 99:811–23. doi: 10.1189/jlb.1HI0715-289R
44. Haase S, Wilck N, Kleinewietfeld M, Muller DN, Linker RA. Sodium chloride triggers Th17 mediated autoimmunity. *J Neuroimmunol*. (2019) 329:9–13. doi: 10.1016/j.jneuroim.2018.06.016
45. Muller DN, Wilck N, Haase S, Kleinewietfeld M, Linker RA. Sodium in the microenvironment regulates immune responses and tissue homeostasis. *Nat Rev Immunol*. (2019) 19:243–54. doi: 10.1038/s41577-018-0113-4
46. Dungal N, Sigurjonsson J. Gastric cancer and diet. A pilot study on dietary habits in two districts differing markedly in respect of mortality from gastric cancer. *Br J Cancer*. (1967) 21:270–6. doi: 10.1038/bjc.1967.29
47. Kelley JR, Duggan JM. Gastric cancer epidemiology and risk factors. *J Clin Epidemiol*. (2003) 56:1–9. doi: 10.1016/S0895-4356(02)00534-6
48. Gaddy JA, Radin JN, Loh JT, Zhang F, Washington MK, Peek RM Jr, et al. High dietary salt intake exacerbates *Helicobacter pylori*-induced gastric carcinogenesis. *Infect Immun*. (2013) 81:2258–67. doi: 10.1128/IAI.01271-12
49. Fox JG, Dangler CA, Taylor NS, King A, Koh TJ, Wang TC. High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances *Helicobacter pylori* colonization in C57BL/6 mice. *Cancer Res*. (1999) 59:4823–8.
50. Kato S, Tsukamoto T, Mizoshita T, Tanaka H, Kumagai T, Ota H, et al. High salt diets dose-dependently promote gastric chemical carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils associated with a shift in mucin production from glandular to surface mucous cells. *Int J Cancer*. (2006) 119:1558–66. doi: 10.1002/ijc.21810
51. Rogers AB, Taylor NS, Whary MT, Stefanich ED, Wang TC, Fox JG. *Helicobacter pylori* but not high salt induces gastric intraepithelial neoplasia in B6129 mice. *Cancer Res*. (2005) 65:10709–15. doi: 10.1158/0008-5472.CAN-05-1846
52. Takeuchi Y, Nishikawa H. Roles of regulatory T cells in cancer immunity. *Int Immunol*. (2016) 28:401–9. doi: 10.1093/intimm/dxw025
53. Shah KH, Shi P, Giani JF, Janjulia T, Bernstein EA, Li Y, et al. Myeloid suppressor cells accumulate and regulate blood pressure in hypertension. *Circ Res*. (2015) 117:858–69. doi: 10.1161/CIRCRESAHA.115.306539
54. Weber R, Fleming V, Hu X, Nagibin V, Groth C, Altevogt P, et al. Myeloid-derived suppressor cells hinder the anti-cancer activity of immune checkpoint inhibitors. *Front Immunol*. (2018) 9:1310. doi: 10.3389/fimmu.2018.01310
55. Dumitru CA, Fechner MK, Hoffmann TK, Lang S, Brandau S. A novel p38-MAPK signaling axis modulates neutrophil biology in head and neck cancer. *J Leukoc Biol*. (2012) 91:591–8. doi: 10.1189/jlb.0411193

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

frontiers in Immunology

MINI REVIEW
published: 12 July 2019
doi: 10.3389/fimmu.2019.01563

The Remarkable Plasticity of Macrophages: A Chance to Fight Cancer

Nadège Bercovici[1,2,3], *Marion V. Guérin*[1,2,3], *Alain Trautmann*[1,2,3] *and Emmanuel Donnadieu*[1,2,3]*

[1] INSERM, U1016, Institut Cochin, Paris, France, [2] CNRS, UMR8104, Paris, France, [3] Université Paris Descartes, Sorbonne Paris Cité, Paris, France

It is well established that tumor-associated macrophages (TAM) found in most advanced tumors have a pro-tumoral role. In this context, TAM limit the activity of tumor-infiltrating lymphocytes (TIL), and a number of mechanisms have been described including a trapping in the stroma, impeding TIL to reach malignant cells. Based on these results, a number of therapeutic approaches have been designed to deplete TAM. However, during tumor regression induced by immunotherapeutic treatments, recent studies revealed that TAM can switch from pro-tumoral to anti-tumoral and actively cooperate with TIL. Here, we will review the two faces of TAM in their interaction with TIL. We will summarize how they can inhibit T cell activities in growing tumors, and how they may also, together with T cells, successfully contribute to tumor eradication after an appropriate stimulation. Finally, we will discuss current promising therapies combining TAM reprogramming with T cell-based immunotherapy.

Keywords: cancer, macrophages, T cells, immunotherapy, migration, inflammation

OPEN ACCESS

Edited by:
Maija Hollmén, University of Turku, Finland

Reviewed by:
Paola Allavena, Milan University, Italy
Reno Debets, Erasmus University Rotterdam, Netherlands

***Correspondence:**
Emmanuel Donnadieu
emmanuel.donnadieu@inserm.fr

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *18 March 2019*
Accepted: *24 June 2019*
Published: *12 July 2019*

Citation:
Bercovici N, Guérin MV, Trautmann A and Donnadieu E (2019) The Remarkable Plasticity of Macrophages: A Chance to Fight Cancer. Front. Immunol. 10:1563. doi: 10.3389/fimmu.2019.01563

INTRODUCTION

Macrophages are amongst the most versatile cells in the body. Resident macrophages are abundant in all tissues where, like microglia in the brain or Kuppfer cells in the liver, these "*pro-tissular macrophages*" contribute to optimize the functioning of the tissue in which they are, by maintaining it clean and preventing an unnecessary inflammation (1). Besides, following an appropriate stimulation, e.g., following an infection, macrophages may be key contributors to immune responses (2). They participate to a variety of functions, primarily as effector cells to eliminate the invading bodies but also to drive an acute inflammation, to promote the recruitment of other immune cells as well as to present antigens to T cells. The switch from the pro-tissular, anti-inflammatory state to the pro-immune, inflammatory one, may take place within a few minutes. This is what happens to subcapsular macrophages when they detect the arrival of pathogens in the lymph node subcapsular sinus (3). This switch may take hours or days, when it involves the recruitment of blood monocytes, followed by their appropriate differentiation in the tissue. Even though the distinction between *pro-tissular* and *pro-immune* macrophages shares similarities with the M2/M1 distinction, we favor the idea that the most important difference between these two macrophage subtypes is functional rather than phenotypic.

In advanced tumors, macrophages favor tumor growth and are associated with a bad outcome in most cancers. Therefore, tumor-associated macrophages (TAM) are usually considered as simply "pro-tumoral". This has not always been the case. In the 1990s, a potential role of macrophages

for cancer treatment has been a popular idea and this concept has begun to emerge. Indeed, in sensitized tumors, macrophages may be anti-tumoral, with the modulation of some gene expression (4).

We will summarize here some specific consequences of the functioning of macrophages in progressing tumors, in which their dominant role is pro-tumoral and immunosuppressive. In particular, we will focus on the mechanisms by which TAM limit TIL from reaching tumor cells. We will continue by considering how one can favor the switch of TAM to pro-immune cells exerting an anti-tumoral action. For these two TAM faces, our focus will be on positive or negative interactions between TAM and TIL, as summarized in the **Figure 1**.

TAM INHIBIT T CELL ACTIVITIES IN PROGRESSING TUMORS

TAM can promote tumor growth by a variety of mechanisms that include tumor cell proliferation, metastasis, angiogenesis and inhibition of T cell anti-tumoral activities. A considerable number of excellent reviews have been published on the various ways in which TAM contribute to tumor growth [for instance see (5)]. Yet, the mechanisms by which TAM negatively control T cells are not completely understood and we would like to focus on those related to intratumoral T cell migration.

TAM Impair T Cell Migration Within Tumors

Our team has recently shown that, in untreated progressing tumors, TAM have a detrimental impact on TIL ability to migrate within tumors and contact malignant cells (6). By using an experimental system based on thick slices made from fresh tumor biopsies combined with fluorescent imaging microscopy, we evidenced the presence of stable conjugates formed between TAM and CD3 T cells in the stroma of human lung tumors as illustrated in the **Figure 2A**. If such interactions do not result in T cell activation, macrophages could contribute to sequestering lymphocytes away from tumor cells (6). Remarkably, in mouse mammary tumor models we found that the depletion of TAM with pexidartinib, an inhibitor of the colony stimulating factor 1 receptor (CSF1R), increased the motility of TIL and their ability to reach tumor cells. This is consistent with data obtained in a mouse model of pancreatic carcinoma but with a CD8 T cell-macrophage trapping process that occurs outside the tumor (7). Whether a similar mechanism also affects CD4 T cells is unknown for the moment. In murine lymph nodes, macrophages were shown to sequester γδT cells unable to recirculate in the blood (8).

Altogether, these data suggest that TAM participate to the exclusion of TIL from the vicinity of cancer cells which is considered to be a major hurdle for T cell-based anti-tumor immunotherapy.

Mechanisms Underlying Blockade of T Cell Migration by TAM

The mechanisms by which TAM prevent CD8 T cells from reaching tumor cells is not known at the moment. We favor an adhesion process between both cell types triggered by an antigen recognition which by itself is insufficient to trigger full T cell activation. This would be in line with data showing antigen-dependent interactions between CD8 T cells and myeloid cells in a spontaneous mammary carcinoma murine model (9). However, the nature of the adhesion molecules involved in such cell-cell conjugates needs to be further investigated.

An effect of TAM on environmental factors controlling the motility of T cells cannot be ruled out. Studies performed over the last few years have provided evidence for a role of the structure of the tissue and the presence of chemokines in regulating the migration of T cells (10). By tracking T cells in fresh human lung tumor slices, we reported an important role of chemokines produced by tumor cells in the ability of T cells to infiltrate tumor islets (11). Such chemokines contribute to a low grade chronic inflammation. In mice harboring mammary tumors, we found that the depletion of TAM resulted in more inflammatory chemokines, such as CCL2 and CXCL10, which are likely to enhance the entry of T cells into the tumor and their intratumoral migration (6). The reason of an enhanced production of chemokines upon TAM depletion is not known for the moment. One possibility is that TAM could participate to the degradation and/or inactivation (e.g., nitration) of inflammatory chemokines, a process reported to occur in murine tumors (12).

A hallmark of advanced tumors is the development of a fibrosis characterized by an excessive accumulation of collagen I, likely to favor tumor progression and prevent antitumor T cell functions by limiting lymphocytes from migrating and contacting tumor cells, as we have previously demonstrated (13). Thus, a dense extracellular matrix (ECM) made by activated carcinoma-associated fibroblasts (CAF) might be responsible for the excluded T cell profile observed in various human carcinomas. The cells and elements that are susceptible to enhance collagen I production by CAF include macrophages. In many physiological situations like breast development, macrophages actively participate to the construction of the tissue (14, 15). In addition, the number of *pro-tissular* macrophages parallels the amount of tissue fibrosis in many human tumors. For example in colorectal tumors and in melanoma, a mesenchymal signature, associated with a bad outcomes and resistance of PD-1 therapy, are characterized by genes involved in extracellular matrix remodeling, angiogenesis, wound healing and TAM suggesting that pro-tissular macrophages and CAF are part of a similar environment (16–18). Evidence obtained in mouse models of colon cancer and pancreatic ductal adenocarcinoma indicates a role of TAM in ECM production within the tumor suggesting that TAM could indirectly inhibit

Abbreviations: CAF, carcinoma-associated fibroblasts; CAR, chimeric antigen receptor; CSF1R, colony stimulating factor 1 receptor; ECM, extracellular matrix; HDAC, histone deacetylase; IFN, interferon; PI3K, phosphatidylinositol 3-kinase; STING, stimulator of interferon genes; TAM, tumor-associated macrophages; TIL, tumor-infiltrating lymphocytes; TGFβ, transforming growth factor beta.

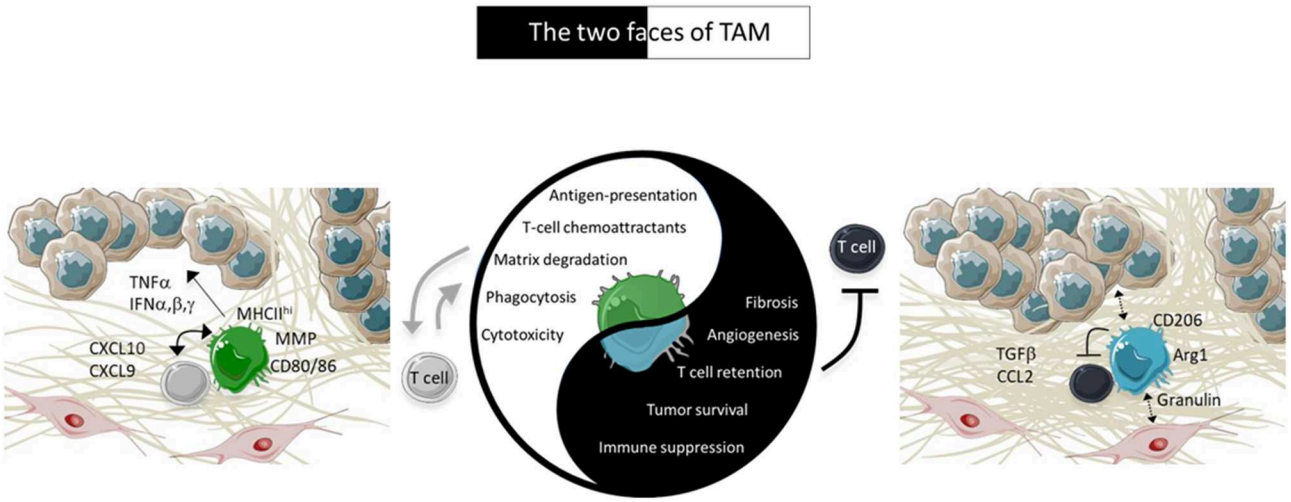


FIGURE 1 | Characteristics of TAM in tumors. In advanced tumors, TAM are pro-tissular and promote tumor growth by several ways including by trapping TIL in the stroma. However, under appropriate activation, TAM positively cooperate with TIL to induce tumor regression. The pro-tissular and pro-immune TAM subtypes are present in two different environments depicted in the figure. Pro-tissular TAM reside in a mesenchymal environment enriched in a dense ECM network and TGFβ. Conversely, pro-immune TAM are distributed in an inflammatory milieu enriched in type I IFN and T-cell chemoattractants.

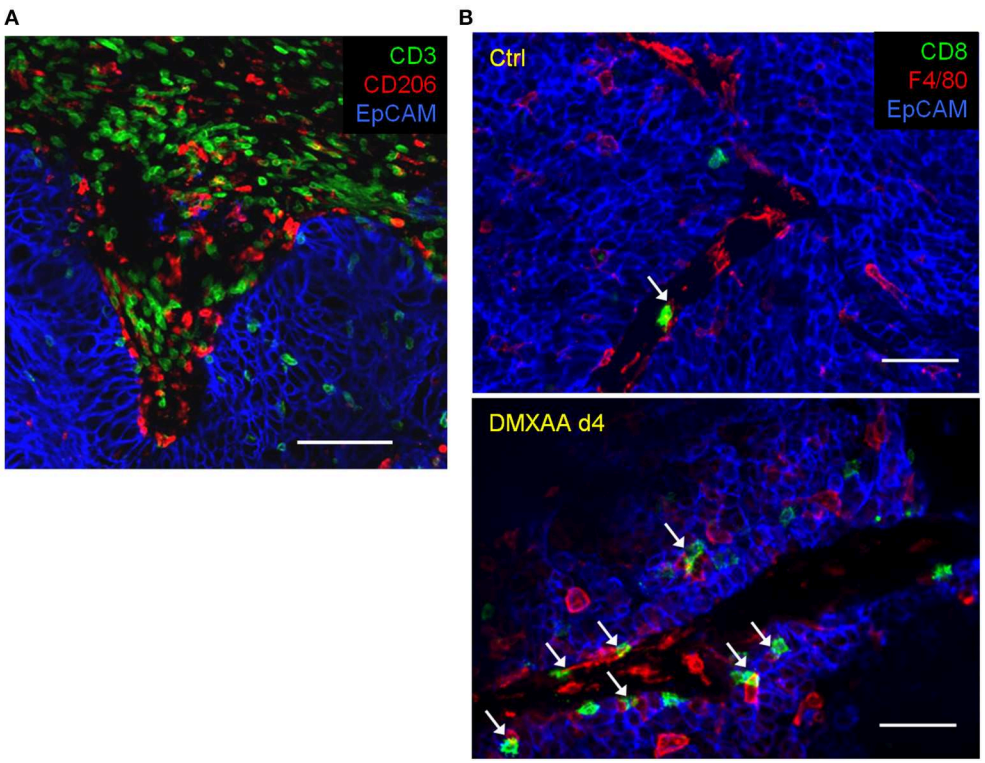


FIGURE 2 | Within tumors, T cells and macrophages are often in contact. **(A)** In a human lung tumor, numerous CD3 T cells are potentially interacting with macrophages (stained by CD206). Bar, 100 μm. **(B)** Interactions between TIL and TAM in mammary carcinoma mouse tumors (MMTV-PyMT) before and after 4 days (4d) of treatment with the STING agonist DMXAA. Of note, the immunotherapeutic agent induces a massive recruitment of CD8 T cells and macrophages (stained by F4/80) with many contacts between both cell types. White arrows indicate TAM-T cells contacts. Bar 50 μm.

T cell migration through the construction of a dense stroma (19, 20). TAM can fine-tune fibrosis by depositing and/or remodeling the ECM (20) but indirect effects through cross-talks with CAF are also envisioned (**Figure 1**). In that context, a recent study demonstrates that TAM activate CAF to produce excessive amount of the ECM, excluding T cells from tumor cells, through

the secretion of granulin, a growth factor belonging to the epithelin family (21).

Macrophage-Depletion Strategies May Potentiate Anti-tumor T-Cell Therapies

The aforementioned studies demonstrating a negative impact of TAM on T cells fostered the development of strategies combining pro-tissular macrophage-depletion with approaches that boost T cells. In preclinical mouse tumor models, the depletion of TAM has been combined with T cell-based therapies, both anti-PD-1 and adoptive T cell transfer, which results in enhanced efficacy of the immunotherapy treatment (22–26). For example, we have shown that a macrophage-depletion strategy through CSF1R inhibition, which by itself has a minor effect on the tumor growth, also improved the efficacy of an anti-PD-1 treatment (6).

Based on these results, several therapeutic applications to impair TAM recruitment or survival are either entering or have entered clinical trials (27). CSF1R inhibitors are currently being tested, the most advanced being the small-molecule Pexidartinib (28). However, CSF1R inhibitors have shown very limited antitumor effects in patients as single agents, suggesting the need to combine these inhibitors with other approaches, including immune checkpoint inhibitors. Such combination strategies are ongoing in a number of solid malignancies (NCT02452424, NCT02713529). Macrophages also use the CCL2/CCR2 axis to enter into tumors. Thus, anti-CCR2 approaches are being developed to reduce the number of immunosuppressive macrophages into solid malignancies (29). In addition, chemotherapeutic agents (e.g., gemcitabine, cyclophosphamide, trabectedin), although not specific to TAM, have been shown to deplete myeloid cells (30–32).

TOWARD STRATEGIES FAVORING AN ANTI-TUMORAL ROLE OF TAM

There is increasing evidence that an appropriate activation of macrophages, rather than their depletion, would drastically potentiate an anti-tumor immune response. Macrophages appropriately stimulated by TLR ligands or after abundant cell death, be it induced by radiotherapy, chemotherapy or other means, are key players of an acute inflammation, with numerous consequences. First, inflammatory macrophages release chemokines, leading to the recruitment of innate immune cells and T cells (33). Another major feature of acute inflammation is the activation of the tumor vasculature which controls T cell extravasation (34, 35). In addition, activated macrophages can attack and reduce the density of the intratumoral ECM (36), thus facilitating TIL mobility in the tumor microenvironment. Finally, macrophages are the most abundant cells in tumors, after tumor cells themselves, which constitute a major asset to propagate de novo inflammatory process in the tumor microenvironment. A careful analysis of the various TAM subsets infiltrating human tumors revealed in addition that a high density of anti-tumoral TAM correlated with a favorable prognosis (37).

Historically, various clinical trials were initiated, for instance with the injection of activated macrophages (38) or cytokines and microbial derived molecules (39–43) aiming at activating macrophages directly *in vivo*. Anti-tumoral effects of such macrophage activators have been reported, but such approaches have been globally disappointing. The conclusion to be drawn from this scientific period is that targeting only macrophages cannot induce a systematic tumor regression.

These provisional failures and the very fast increase of T cell knowledge led, in the following years, to an almost complete oversight of anti-tumoral capabilities of macrophages. However, recent advances in myeloid cell biology are putting macrophages back into play, as we will discuss.

Inhibition of Pro-tumoral TAM Orientation

The inhibition of molecules that contribute to a pro-tumoral TAM orientation represents an interesting strategy. Here we will focus on TGFβ, PI3Kγ, and some HDAC (histone deacetylases) which will nicely illustrate our point.

TGFβ Inhibition

The major effect of TGFβ, in large tumors in particular, is to resolve inflammation, to facilitate wound healing and to contribute to immunosuppression. TGFβ does it in various ways. In tumors, TAM are both a source and a target of TGFβ, which is involved in a positive feed-back loop for stabilizing the pro-tumoral TAM phenotype. In parallel, TGFβ promotes the activation of fibroblasts while it inhibits the expression of several molecules necessary for the cytotoxic activity of T cells. Therefore, this molecule appeared to be a central mediator in the TAM/CAF/TIL axis. In the literature on erythropoiesis or on fibrotic diseases, one can find that an anti-TGFβ treatment could be done, in principle, by reducing the concentration of circulating TGFβ with anti-TGFβ antibodies or with TGFβ ligand traps (44), or with pharmacological blockers of TGFβ signaling, such as SB431542 (45). Importantly for the activity of anti-tumor T cells, the combination of TGFβ blockade and anti-PDL1 has been shown to decrease the activity of fibroblasts and the density of ECM relieving the exclusion of T cells from malignant cells (46, 47). Another major effect of TGFβ is the inhibition of IFNβ signaling as we have recently shown in murine spontaneous tumors (48). Type I IFN are central in the initiation of T-cell responses and this finding could be of major importance for combined anti-tumor treatments, in which an anti-TGFβ would not be active on its own, but only for amplifying the potency of T cells. An anti-TGFβ treatment could modify the TAM phenotype and sensitivity to STING agonist within 3 days in this spontaneous tumor model (48).

Class II HDAC Inhibition

Class IIa HDAC, which includes HDAC4, act quite differently from other HDAC. A specific Class IIa HDAC inhibitor, which appears to have no effect on T cells, is able to induce an inflammatory state by promoting the infiltration of phagocytic and immunostimulatory $CD40^{+}$ TAM, resulting in an anti-tumoral action (49). Thus, specific inhibitors of class IIa HDAC might prove to be of great interest in combined anti-tumoral

therapies. Note that, in different instances, TGFβ effects appear to be mediated by HDAC4 (48, 50, 51). Thus, the two molecules may use common pathways to maintain an immunosuppressive, anti-inflammatory macrophage activity.

PI3Kγ Inhibition

PI3Kγ is a PI3K activated by chemokine receptors (52). In myeloid cells, PI3Kγ is important both for the recruitment of monocytes/macrophages and neutrophils (53), and for the resolution of inflammation, in particular with macrophages phagocyting apoptotic neutrophils. In macrophages, the dominant role of PI3Kγ is the resolution of inflammation and immunosuppression (54). Under these conditions, specific inhibitors of PI3Kγ (55, 56) could obviously be of major interest in combined anti-tumoral therapies.

Note that a recent study has evidenced the role of the scavenger receptor clever-1 in TAM pro-tumoral activities. In mouse tumor models, clever-1 blockade leads to macrophage repolarization that become immunostimulatory enhancing T cell responses against tumors (57). A phase I clinical trial with a blocking anti-clever-1 antibody is currently ongoing in various solid tumors (NCT03733990).

Activation of Anti-tumoral TAM Activities

We would like to shed light on two main pathways promoting an anti-tumoral TAM activity. One involves the CD40 pathway, the other one the induction of type I IFN.

The CD40L-CD40 pathway may be activated by CD40 agonists or with cells that express CD40L as it is the case with activated TIL or NKT (58). In a mouse model of pancreatic cancer, the density of the ECM was shown to be reduced after an anti-CD40 agonist treatment, through the activation of matrix metalloproteinases production by TAM, which may facilitate the motility of T cells (59). Further work from the same group indicates that a CD40 agonist triggers the release of IFNγ and CCL2 responsible for both the recruitment of monocytes/macrophages into the tumor and their polarization toward ECM-degrading cells (60).

Even if anti-CD40 antibodies may not only target TAM and DCs, but also other CD40-expressing cells such as endothelial cells, in combination with gemcitabine, CD40 agonists have already been shown to induce clinical responses in patients with surgically incurable pancreatic cancer (59). This CD40-dependent TAM activation is more efficient when combined with T cell activation (61), or with TLR9 stimulation (62).

Type I IFN has also been shown to enhance anti-tumor activities of myeloid cells. The release of IFNα/β in tumors can be achieved by irradiation (63), some chemotherapeutic agents (64) but more efficiently by a direct activation of the STING (Stimulator of Interferon Genes) molecule. TLR ligands, such as CpG, may also result in the production of IFNα/β by TAM. We have recently shown that this type I IFN contribution to anti-tumoral treatments may be strongly inhibited by TGFβ that accumulates abundantly in spontaneous tumors (48). TGFβ inhibition may therefore be an important element of an efficient combined treatment stimulating anti-tumor activity of TAM.

Overall, the balance toward anti-tumor activity of TAM maybe switched ON if one aims at inhibiting their pro-tissular activity while favoring their pro-immune activity. Various clinical trials are ongoing with such macrophages targeting agents (65).

The duration of an increased anti-tumoral activity of TAM is a question that warrants further investigations. Recruited macrophages with high cytotoxic and phagocytic activities were found to accumulate between 4 and 8 days following treatments (30, 33, 66). Thereafter, factors of the tumor microenvironment, such as VEGF, have been shown to influence the reconstitution of the TAM compartment (30), and to promote tumor outgrowth. The persistence of anti-tumor activity of macrophages may also depend on their interactions with anti-tumor T cells as will be discussed below.

ACTIVATED TAM COOPERATE WITH TIL FOR A GLOBAL ANTI-TUMORAL ACTIVITY

An increasing number of reports lead to the conclusion that T cells and TAM can cooperate to fight tumors. We have shown that activated TAM were necessary to reject transplanted tumors after therapeutic vaccination (66) or STING /type I IFN activation (33). Importantly, STING exerts an anti-tumoral activity involving both TAM and T cells (33, 48) with a key role exerted by IFNα/β production by TAM. In such an acute inflammatory context, the depletion of TAM drastically reduced the production of T-cell chemoattractants and the accumulation of CD8 T cells in tumors. Thus, TAM can either favor or prevent intra-tumoral T cell infiltration, depending on whether an inflammatory/immune response has been triggered or not. In addition, a positive feed-back may be observed, with T cells amplifying the activity of immunostimulatory TAM (66). This demonstrates that TAM and anti-tumoral CD8 TIL can work in synergy to reject tumors following an appropriate stimulation.

As a matter of fact, TAM-T cells positive interactions have been observed in various settings, but were rarely put at the forefront. Such positive interactions have been reported after intratumoral injection of TLR3 or TLR9 agonists (67, 68), after the use of checkpoints blockers anti-PD1/anti-CTLA4 (69), or after the adoptive transfer of tumor-infiltrating T cells (70) as well as chimeric antigen receptor (CAR) T cells (71). **Table 1** summarizes clinical trials in which a combination of drugs targeting TAM and TIL has been evaluated.

Consequences of TAM-T Cell Cooperation

After facilitating the entry of T cells in sensitized tumors, TAM can interact closely with T cells as illustrated in **Figure 2B** and present tumor antigens, and thus reactivate them (72). The importance of such a reactivation may be illustrated by the fact that MHC class I expression on tumor infiltrating myeloid cells is strikingly crucial for the rejection of B16 tumor cells by adoptively transferred tumor-specific CD8 T cells (73).

Thus, TAM may help T cells, but reciprocally, T cells can contribute to macrophage activation, and the release of IFNγ

TABLE 1 | Ongoing clinical trials targeting TAM and TIL in solid tumors.

	Macrophage/TIL targets	Clinical trial number	Investigators	Indications	Study design	Immune response evaluation	Phase
Depletion of pro-tumoral TAM	Anti-CCR2/CCR5/anti-PD1	NCT03184870	Bristol-Myers Squibb	Solid tumors	aCCR2/CCR5 vs. aCCR2/CCR5 + aPD1 vs. aCCR2/CCR5+ chemotherapies	Decrease in regulatory T cells & tumor-associated macrophages	I
	Anti-CFS1R/anti-PD1	NCT02526017	Five Prime Therapeutics, Inc.	Solid tumors	aCSF1R + aPD1 vs. aCSF1R alone	Changes in macrophage and T-cell levels/Changes in gene expression in peripheral T-cell and other leukocyte phenotypes, and levels of peripheral myeloid-derived suppressor cells	I
	Anti-CFS1R/anti-PDL1	NCT03238027	Syndax Pharmaceuticals, Inc.	Solid tumors	aCSF1R alone vs. aCSF1R + aPDL1	Inflammatory cytokines/TIL expansion	I
	Anti-CSF1R/anti-PDL1	NCT02323191	Hoffmann-La Roche	Solid tumors	aCSF1R + aPDL1	TAM depletion	I + II
Inhibition of pro-tumoral TAM activity	Anti-CTLA-4, Anti-PDL1/OX40L Ig	NTC02705482	MedImmune LLC	Advanced solid tumors	OX40L Ig + aPDL1 vs. OX40L Ig + aCTLA4	TIL expansion	I
	Anti-PDL1/OX40L Ig	NTC02221960	MedImmune LLC	Recurrent or Metastatic Solid Tumors	OX40LIg alone vs. OX40L Ig + aPDL1	Biomarkers activity on TIL	I
	PD1-Fc-OX40L	NTC03894618	Shattuck Labs	Solid tumors and lymphomas	1 or 2 inejctions i.t		I
	TGFbRI inhibitor/anti-PDL1	NCT02937272	Eli Lilly and Company	Solid tumors	TGFbRI inh orally alone vs. TGFbRI inh orally + anti-PDL1 i.v		I
	TGFb inhibitor/anti-PD1	NCT02423343	Eli Lilly and Company	Solid tumors (NLSC/HCC)	TGFB inh orally + anti-PD1 i.v		I + II
Activation of anti-tumoral TAM activity	TLR7, 8 agonist/anti-PDL1	NTC02556463	MedImmune LLC	Solid tumors	aTLR7/8 alone vs. aTLR7/8 + aPDL1	TIL expansion/Inflammatory cytokine levels	I
	TRL9 agonist/OX40 agonist	NCT03831295	Stanford Cancer Institute Palo Alto	Solid neoplasms	TLR9 agonist x3 i.t + OX40 agonist x2 i.v and x3 i.t vs. TLR9 agonist x3 i.t + OX40 agonist x3 i.v and x3 i.t		I
	TLR4 agonist/anti-PD1, ICOS agonist, OX40 agonist	NCT03447314	GlaxoSmithKline	Neoplasms	OX40 + TLR4 agonists vs. ICOS + TLR4 agonists vs. aPD1 + TRL4 agonists vs. OX40 + ICOS + TLR4 agonists		I
	STING agonist/anti-PD1	NCT03172936	Novartis Pharmaceuticals	Solid tumors and lymphomas	One vs. 3 doses of STING agonist (i.t) + 1 injection of anti-PD1 (i.v)	Cytokines, TIL expansion in targeted and non-targeted lesions	I
	STING agonist/anti-CTLA4	NCT02675439	Novartis Pharmaceuticals	Solid tumors and lymphomas	3 injections of STING agonist (i.t) vs. 2 injections of STING agonist (i.t) + 1 injection of aCTLA4	Measurement of CD8-TIL counts/RNA expression analysis of IFN gamma and immunomodulatory genes	I
	CD40 agonist/anti-PDL1	NCT02304393	Hoffmann-La Roche	Advanced/ metastatic solid tumors	1 dose of CD40 agonist i.v + aPDL1 vs. 1 dose of CD40 agonist s.c + aPDL1	TIL expansion, PDL1 expression on tumor and immune infiltrating cells	I
	anti-CD47, IFN-α2/anti-PD1, anti-PDL1	NCT02890368	Trillium Therapeutics Inc.	Solid tumors	aCD47 Monotherapy/aCD47 + PD-1/PD-L1 Inhibitor/aCD47 + pegylated IFN-α2/aCD47 + T-Vec/aCD47 + radiation	Anti-tumor activity	I
	GMCSF/iNeo-Vac-P01 (peptides)	NCT03662815	Sir Run Run Shaw Hospital	Solid tumors	iNeo-Vac-P01 (peptides)+ GM-CSF x7 doses	IFN-gamma measurement/CD4 and CD8 T cells subsets	I
	Ad-IFNγ/TIL adoptive transfer	NCT01082887	Nantes University Hospital	Metastatic melanoma	2 injections of Ad-IFNγ (i.t) +2 injections of TIL (i.v)		I+II

seems determinant in this process (66). For instance, the anti-tumor potency of some adoptively transferred T cells was shown to rely on the IFNγ-dependent activation of TAM (74).

Another consequence of such a TAM repolarization is the acquisition of effector functions by these activated cells. Indeed, appropriately activated TAM can phagocyte and engulf tumor cells (75, 76). Additionally, they can kill tumor cells, as shown by several groups (67, 68), including ours (66). TAM, endowed with cytotoxic and cytostatic activities, can kill malignant cells by TNFα secretion (66, 77), NO (74) and sometimes on TRAIL (78).

CONCLUSION

The common point of view that TAM are pro-tumoral cells is only correct in one specific situation: that of growing tumors. We have recalled that TAM could play such a role in different ways, including by trapping T cells in the tumor stroma and by reducing their mobility and therefore their capacity to reach cancer cells.

However, we have also discussed that TAM, when appropriately stimulated, have the capacity to cooperate with T cells for an anti-tumoral action. Despite the well-established importance of such a cooperation in anti-infectious immune responses, its importance in anti-tumoral responses has been too often neglected. First, 30 years ago, it has been neglected by those who attempted to treat cancer by only stimulating the innate immune system. More recently, the importance of the TAM-T cell cooperation has again been ignored by those who considered that, for anti-tumoral immune responses, T cells were the good guys and macrophages the bad ones.

We have shown here that an efficient strategy should aim at stimulating both T cells and TAM so as to promote their cooperation. This cooperation is not just about a help provided by TAM to T cells: *the two cell types may be both helpers for the other, and final effectors against the tumor*. Treatments aiming at stimulating only one cell type (only T cells or only macrophages) should be systematically replaced by well-thought combined treatments for stimulating both of them. In particular, *T cell-focused treatments* with checkpoint inhibitors or CAR T cells would greatly benefit being combined with *activators of pro-immune TAM* activities such as CD40 agonists, STING activators or otherof IFNα inducers, and with *inhibitors of pro-tissular TAM functions*, i.e., inhibitors ofTGFβ or class IIa HDAC. The only caveat we would put on it is the potential immune-related adverse effects that may follow efficient tumor rejection. But taking into account that such triple combinations have the greatest chances to promote efficient anti-tumoral therapies would be a fundamental step forward.

AUTHOR CONTRIBUTIONS

ED, AT, and NB conceived the article, wrote the first version of the manuscript, and integrated inputs from MG. MG designed the **Table 1** with the contribution of NB and the **Figure 1** with the contribution of AT. ED and AT designed the **Figure 2**. All authors approved the final version of the article.

ACKNOWLEDGMENTS

This work was granted by Worldwide Cancer Research, the Plan Cancer (Tumor Heterogeneity and Ecosystem Program), the Comité de Paris de La Ligue Contre le Cancer, the Cancer Research for Personalized Medicine (CARPEM), CNRS, INSERM, and University Paris-Descartes. MG was granted by the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche. We are grateful to E. Peranzoni and IMAG'IC facility of the Cochin Institute for the **Figure 2**.

REFERENCES

1. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. (2013) 496:445–55. doi: 10.1038/nature12034
2. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*. (2010) 11:889–96. doi: 10.1038/ni.1937
3. Sagoo P, Garcia Z, Breart B, Lemaitre F, Michonneau D, Albert ML, et al. *In vivo* imaging of inflammasome activation reveals a subcapsular macrophage burst response that mobilizes innate and adaptive immunity. *Nat Med*. (2016) 22:64–71. doi: 10.1038/nm.4016
4. Guerriero JL. Macrophages: the road less traveled, changing anticancer therapy. *Trends Mol Med*. (2018) 24:472–89. doi: 10.1016/j.molmed.2018.03.006
5. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol*. (2019) 19:369–82. doi: 10.1038/s41577-019-0127-6
6. Peranzoni E, Lemoine J, Vimeux L, Feuillet V, Barrin S, Kantari-Mimoun C, et al. Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. *Proc Natl Acad Sci USA*. (2018) 115:E4041–E50. doi: 10.1073/pnas.1720948115
7. Beatty GL, Winograd R, Evans RA, Long KB, Luque SL, Lee JW, et al. Exclusion of T cells from pancreatic carcinomas in mice is regulated by Ly6C(low) F4/80(+) extratumoral macrophages. *Gastroenterology*. (2015) 149:201–10. doi: 10.1053/j.gastro.2015.04.010
8. Audemard-Verger A, Riviere M, Durand A, Peranzoni E, Guichard V, Hamon P, et al. Macrophages induce long-term trapping of gammadelta T cells with innate-like properties within secondary lymphoid organs in the steady state. *J Immunol*. (2017) 199:1998–2007. doi: 10.4049/jimmunol.1700430
9. Engelhardt JJ, Boldajipour B, Beemiller P, Pandurangi P, Sorensen C, Werb Z, et al. Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. *Cancer Cell*. (2012) 21:402–17. doi: 10.1016/j.ccr.2012.01.008
10. Krummel MF, Bartumeus F, Gerard A. T cell migration, search strategies and mechanisms. *Nat Rev Immunol*. (2016) 16:193–201. doi: 10.1038/nri.2015.16
11. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean MC, Validire P, Trautmann A, et al. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. *J Clin Investigat*. (2012) 122:899–910. doi: 10.1172/JCI45817
12. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, et al. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med*. (2011) 208:1949–62. doi: 10.1084/jem.20101956
13. Peranzoni E, Rivas-Caicedo A, Bougherara H, Salmon H, Donnadieu E. Positive and negative influence of the matrix architecture on antitumor immune surveillance. *Cell Mol Life Sci*. (2013) 70:4431–48. doi: 10.1007/s00018-013-1339-8

14. Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn.* (2006) 235:3222–9. doi: 10.1002/dvdy.20972
15. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity*. (2016) 44:450–62. doi: 10.1016/j.immuni.2016.02.015
16. Becht E, de Reynies A, Giraldo NA, Pilati C, Buttard B, Lacroix L, et al. Immune and stromal classification of colorectal cancer is associated with molecular subtypes and relevant for precision immunotherapy. *Clin Cancer Res.* (2016) 22:4057–66. doi: 10.1158/1078-0432.CCR-15-2879
17. Calon A, Lonardo E, Berenguer-Llergo A, Espinet E, Hernando-Momblona X, Iglesias M, et al. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet.* (2015) 47:320–9. doi: 10.1038/ng.3225
18. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. *Cell.* (2016) 165:35–44. doi: 10.1016/j.cell.2016.02.065
19. Afik R, Zigmond E, Vugman M, Klepfish M, Shimshoni E, Pasmanik-Chor M, et al. Tumor macrophages are pivotal constructors of tumor collagenous matrix. *J Exp Med.* (2016) 213:2315–31. doi: 10.1084/jem.20151193
20. Zhu Y, Herndon JM, Sojka DK, Kim KW, Knolhoff BL, Zuo C, et al. Tissue-resident macrophages in pancreatic ductal adenocarcinoma originate from embryonic hematopoiesis and promote tumor progression. *Immunity*. (2017) 47:323–38 e6. doi: 10.1016/j.immuni.2017.07.014
21. Quaranta V, Rainer C, Nielsen SR, Raymant ML, Ahmed MS, Engle DD, et al. Macrophage-derived granulin drives resistance to immune checkpoint inhibition in metastatic pancreatic cancer. *Cancer Res.* (2018) 78:4253–69. doi: 10.1158/0008-5472.CAN-17-3876
22. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* (2011) 1:54–67. doi: 10.1158/2159-8274.CD-10-0028
23. Mok S, Koya RC, Tsui C, Xu J, Robert L, Wu L, et al. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. *Cancer Res.* (2014) 74:153–61. doi: 10.1158/0008-5472.CAN-13-1816
24. Neubert NJ, Schmittnaegel M, Bordry N, Nassiri S, Wald N, Martignier C, et al. T cell-induced CSF1 promotes melanoma resistance to PD1 blockade. *Sci Transl Med.* (2018) 10:436. doi: 10.1126/scitranslmed.aan3311
25. Strachan DC, Ruffell B, Oei Y, Bissell MJ, Coussens LM, Pryer N, et al. CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by CD8+ T cells. *Oncoimmunology.* (2013) 2:e26968. doi: 10.4161/onci.26968
26. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* (2014) 74:5057–69. doi: 10.1158/0008-5472.CAN-13-3723
27. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol.* (2017) 14:399–416. doi: 10.1038/nrclinonc.2016.217
28. Cannarile MA, Weisser M, Jacob W, Jegg AM, Ries CH, Ruttinger D. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J Immunother Cancer.* (2017) 5:53. doi: 10.1186/s40425-017-0257-y
29. Nywening TM, Wang-Gillam A, Sanford DE, Belt BA, Panni RZ, Cusworth BM, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. *Lancet Oncol.* (2016) 17:651–62. doi: 10.1016/S1470-2045(16)00078-4
30. Loyher PL, Hamon P, Laviron M, Meghraoui-Kheddar A, Goncalves E, Deng Z, et al. Macrophages of distinct origins contribute to tumor development in the lung. *J Exp Med.* (2018) 215:2536–53. doi: 10.1084/jem.20180534
31. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res.* (2005) 11:6713–21. doi: 10.1158/1078-0432.CCR-05-0883
32. Allavena P, Signorelli M, Chieppa M, Erba E, Bianchi G, Marchesi F, et al. Anti-inflammatory properties of the novel antitumor agent yondelis (trabectedin): inhibition of macrophage differentiation and cytokine production. *Cancer Res.* (2005) 65:2964–71. doi: 10.1158/0008-5472.CAN-04-4037
33. Weiss JM, Guerin MV, Regnier F, Renault G, Galy-Fauroux I, Vimeux L, et al. The STING agonist DMXAA triggers a cooperation between T lymphocytes and myeloid cells that leads to tumor regression. *Oncoimmunology.* (2017) 6:e1346765. doi: 10.1080/2162402X.2017.1346765
34. Ganss R, Ryschich E, Klar E, Arnold B, Hammerling GJ. Combination of T-cell therapy and trigger of inflammation induces remodeling of the vasculature and tumor eradication. *Cancer Res.* (2002) 62:1462–70.
35. Sektioglu IM, Carretero R, Bender N, Bogdan C, Garbi N, Umansky V, et al. Macrophage-derived nitric oxide initiates T-cell diapedesis and tumor rejection. *Oncoimmunology.* (2016) 5:e1204506. doi: 10.1080/2162402X.2016.1204506
36. Long KB, Beatty GL. Harnessing the antitumor potential of macrophages for cancer immunotherapy. *Oncoimmunology.* (2013) 2:e26860. doi: 10.4161/onci.26860
37. Fridman WH, Zitvogel L, Sautes-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol.* (2017) 14:717–34. doi: 10.1038/nrclinonc.2017.101
38. Pages F, Lebel-Binay S, Vieillefond A, Deneux L, Cambillau M, Soubrane O, et al. Local immunostimulation induced by intravesical administration of autologous interferon-gamma-activated macrophages in patients with superficial bladder cancer. *Clin Exp Immunol.* (2002) 127:303–9. doi: 10.1046/j.1365-2249.2002.01766.x
39. Axelrod RS, Havas HF, Murasko DM, Bushnell B, Guan CF. Effect of the mixed bacterial vaccine on the immune response of patients with non-small cell lung cancer and refractory malignancies. *Cancer.* (1988) 61:2219–30.
40. Engelhardt R, Mackensen A, Galanos C. Phase I trial of intravenously administered endotoxin (Salmonella abortus equi) in cancer patients. *Cancer Res.* (1991) 51:2524–30.
41. Klimp AH, de Vries EG, Scherphof GL, Daemen T. A potential role of macrophage activation in the treatment of cancer. *Crit Rev Oncol.* (2002) 44:143–61. doi: 10.1016/S1040-8428(01)00203-7
42. Nardin A, Lefebvre ML, Labroquere K, Faure O, Abastado JP. Liposomal muramyl tripeptide phosphatidylethanolamine: targeting and activating macrophages for adjuvant treatment of osteosarcoma. *Curr Cancer Drug Targets.* (2006) 6:123–33. doi: 10.2174/156800906776056473
43. Tang ZY, Zhou HY, Zhao G, Chai LM, Zhou M, Lu JZ, et al. Preliminary result of mixed bacterial vaccine as adjuvant treatment of hepatocellular carcinoma. *Med Oncol Tumor Pharmacother.* (1991) 8:23–8.
44. Suragani RN, Cadena SM, Cawley SM, Sako D, Mitchell D, Li R, et al. Transforming growth factor-beta superfamily ligand trap ACE-536 corrects anemia by promoting late-stage erythropoiesis. *Nat Med.* (2014) 20:408–14. doi: 10.1038/nm.3512
45. Farzaneh M, Derakhshan Z, Hallajzadeh J, Sarani NH, Nejabatdoust A, Khoshnam SE. Suppression of TGF-beta and ERK signaling pathways as a new strategy to provide rodent and non-rodent pluripotent stem cells. *Curr Stem Cell Res Ther.* (2019) 14:466–73. doi: 10.2174/1871527318666190314110529
46. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFbeta attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature.* (2018) 554:544–8. doi: 10.1038/nature25501
47. Tauriello DVF, Palomo-Ponce S, Stork D, Berenguer-Llergo A, Badia-Ramentol J, Iglesias M, et al. TGFbeta drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature.* (2018) 554:538–43. doi: 10.1038/nature25492
48. Guerin M, Regnier F, Feuillet V, Vimeux L, Weiss JM, Guilbert T, et al. TGFbeta blocks STING-induced IFN release and tumor rejection in spontaneous mammary tumors. *BioRxiv.* (2018). doi: 10.1101/336990
49. Guerriero JL, Sotayo A, Ponichtera HE, Castrillon JA, Pourzia AL, Schad S, et al. Class IIa HDAC inhibition reduces breast tumours and metastases through anti-tumour macrophages. *Nature.* (2017) 543:428–32. doi: 10.1038/nature21409
50. Glenisson W, Castronovo V, Waltregny D. Histone deacetylase 4 is required for TGFbeta1-induced myofibroblastic differentiation. *Biochim Biophys Acta.* (2007) 1773:1572–82. doi: 10.1016/j.bbamcr.2007.05.016

51. Guo W, Saito S, Sanchez CG, Zhuang Y, Gongora Rosero RE, Shan B, et al. TGF-beta1 stimulates HDAC4 nucleus-to-cytoplasm translocation and NADPH oxidase 4-derived reactive oxygen species in normal human lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol*. (2017) 312:L936–L44. doi: 10.1152/ajplung.00256.2016
52. Solinas G, Becattini B. The role of PI3Kgamma in metabolism and macrophage activation. *Oncotarget*. (2017) 8:106145–6. doi: 10.18632/oncotarget.22068
53. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, et al. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. (2000) 287:1049–53. doi: 10.1126/science.287.5455.1049
54. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kgamma is a molecular switch that controls immune suppression. *Nature*. (2016) 539:437–42. doi: 10.1038/nature19834
55. Evans CA, Liu T, Lescarbeau A, Nair SJ, Grenier L, Pradeilles JA, et al. Discovery of a selective phosphoinositide-3-kinase (PI3K)-gamma inhibitor (IPI-549) as an immuno-oncology clinical candidate. *ACS Med Chem Lett*. (2016) 7:862–7. doi: 10.1021/acsmedchemlett.6b00238
56. Pemberton N, Mogemark M, Arlbrandt S, Bold P, Cox RJ, Gardelli C, et al. Discovery of highly isoform selective orally bioavailable phosphoinositide 3-kinase (PI3K)-gamma inhibitors. *J Med Chem*. (2018) 61:5435–41. doi: 10.1021/acs.jmedchem.8b00447
57. Viitala M, Virtakoivu R, Tadayon S, Rannikko J, Jalkanen S, Hollmen M. Immunotherapeutic blockade of macrophage clever-1 reactivates the CD8(+) T-cell response against immunosuppressive tumors. *Clin Cancer Res*. (2019) 25:3289–303. doi: 10.1158/1078-0432.CCR-18-3016
58. Cortesi F, Delfanti G, Grilli A, Calcinotto A, Gorini F, Pucci F, et al. Bimodal CD40/Fas-dependent crosstalk between iNKT cells and tumor-associated macrophages impairs prostate cancer progression. *Cell Rep*. (2018) 22:3006–20. doi: 10.1016/j.celrep.2018.02.058
59. Beatty GL, Chiorean EG, Fishman MP, Saboury B, Teitelbaum UR, Sun W, et al. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science*. (2011) 331:1612–6. doi: 10.1126/science.1198443
60. Long KB, Gladney WL, Tooker GM, Graham K, Fraietta JA, Beatty GL. IFNgamma and CCL2 cooperate to redirect tumor-infiltrating monocytes to degrade fibrosis and enhance chemotherapy efficacy in pancreatic carcinoma. *Cancer Discov*. (2016) 6:400–13. doi: 10.1158/2159-8290.CD-15-1032
61. Jackaman C, Lew AM, Zhan Y, Allan JE, Koloska B, Graham PT, et al. Deliberately provoking local inflammation drives tumors to become their own protective vaccine site. *Int Immunol*. (2008) 20:1467–79. doi: 10.1093/intimm/dxn104
62. Jensen JL, Rakhmilevich A, Heninger E, Broman AT, Hope C, Phan F, et al. Tumoricidal effects of macrophage-activating immunotherapy in a murine model of relapsed/refractory multiple myeloma. *Cancer Immunol Res*. (2015) 3:881–90. doi: 10.1158/2326-6066.CIR-15-0025-T
63. Vanpouille-Box C, Demaria S, Formenti SC, Galluzzi L. Cytosolic DNA sensing in organismal tumor control. *Cancer Cell*. (2018) 34:361–78. doi: 10.1016/j.ccell.2018.05.013
64. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, et al. Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat Med*. (2014) 20:1301–9. doi: 10.1038/nm.3708
65. Pathria P, Louis TL, Varner JA. Targeting tumor-associated macrophages in cancer. *Trends Immunol*. (2019) 40:310–27. doi: 10.1016/j.it.2019.02.003
66. Thoreau M, Penny HL, Tan K, Regnier F, Weiss JM, Lee B, et al. Vaccine-induced tumor regression requires a dynamic cooperation between T cells and myeloid cells at the tumor site. *Oncotarget*. (2015) 6:27832–46. doi: 10.18632/oncotarget.4940
67. Shime H, Matsumoto M, Oshiumi H, Tanaka S, Nakane A, Iwakura Y, et al. Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors. *Proc Natl Acad Sci USA*. (2012) 109:2066–71. doi: 10.1073/pnas.1113099109
68. Shirota Y, Shirota H, Klinman DM. Intratumoral injection of CpG oligonucleotides induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells. *J Immunol*. (2012) 188:1592–9. doi: 10.4049/jimmunol.1101304
69. Gubin MM, Esaulova E, Ward JP, Malkova ON, Runci D, Wong P, et al. High-dimensional analysis delineates myeloid and lymphoid compartment remodeling during successful immune-checkpoint cancer therapy. *Cell*. (2018) 175:1443. doi: 10.1016/j.cell.2018.11.003
70. Hollenbaugh JA, Dutton RW. IFN-gamma regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. *J Immunol*. (2006) 177:3004–11. doi: 10.4049/jimmunol.177.5.3004
71. Textor A, Listopad JJ, Wuhrmann LL, Perez C, Kruschinski A, Chmielewski M, et al. Efficacy of CAR T-cell therapy in large tumors relies upon stromal targeting by IFNgamma. *Cancer Res*. (2014) 74:6796–805. doi: 10.1158/0008-5472.CAN-14-0079
72. Ma Y, Mattarollo SR, Adjemian S, Yang H, Aymeric L, Hannani D, et al. CCL2/CCR2-dependent recruitment of functional antigen-presenting cells into tumors upon chemotherapy. *Cancer Res*. (2014) 74:436–45. doi: 10.1158/0008-5472.CAN-13-1265
73. Kerkar SP, Goldszmid RS, Muranski P, Chinnasamy D, Yu Z, Reger RN, et al. IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors. *J Clin Investigat*. (2011) 121:4746–57. doi: 10.1172/JCI58814
74. Vicetti Miguel RD, Cherpes TL, Watson LJ, McKenna KC. CTL induction of tumoricidal nitric oxide production by intratumoral macrophages is critical for tumor elimination. *J Immunol*. (2010) 185:6706–18. doi: 10.4049/jimmunol.0903411
75. Bougherara H, Nemati F, Nicolas A, Massonnet G, Pugniere M, Ngo C, et al. The humanized anti-human AMHRII mAb 3C23K exerts an anti-tumor activity against human ovarian cancer through tumor-associated macrophages. *Oncotarget*. (2017) 8:99950–65. doi: 10.18632/oncotarget.21556
76. Montalvao F, Garcia Z, Celli S, Breart B, Deguine J, Van Rooijen N, et al. The mechanism of anti-CD20-mediated B cell depletion revealed by intravital imaging. *J Clin Investigat*. (2013) 123:5098–103. doi: 10.1172/JCI 70972
77. Zhang B, Karrison T, Rowley DA, Schreiber H. IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. *J Clin Investigat*. (2008) 118:1398–404. doi: 10.1172/JCI 33522
78. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, Fanger NA. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. *J Exp Med*. (1999) 189:1343–54. doi: 10.1084/jem.189.8.1343

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

frontiers in Immunology

MINI REVIEW
published: 19 July 2019
doi: 10.3389/fimmu.2019.01710

Neutrophils as a Therapeutic Target in Cancer

*Zvi Granot**

Department of Developmental Biology and Cancer Research, The Institute for Medical Research Israel-Canada, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Neutrophils are the most abundant population of white blood cells in the human circulation. They are terminally differentiated myeloid cells which were traditionally associated with fighting infections and inflammatory processes. While this perception of neutrophils is still widely prevalent, in the past decade it has become clear that neutrophils also play a critical role in tumor growth and progression. The unique tumor microenvironment, consisting of the non-malignant stroma that surrounds tumor cells, is shaped by numerous cues emanating from both tumor cells and stromal cells which support the growing tumor. Various immune cells, including neutrophils, make up a significant proportion of the tumor stroma. Immune cells exist for the protection of the host against various threats including the detection and elimination of cancerous cells. However, in the context of cancer immune cells are often coerced into a tumor supportive phenotype. This is also the case for neutrophils, which are often described to possess tumor promoting properties and to associate with poorer prognosis. The fact that neutrophils may contribute to tumor growth and progression suggests they may be targets for anti-cancer therapies. This review discusses the various functions neutrophils may play in cancer and the possibility of targeting these functions as a novel mode of immunotherapy.

Keywords: neutrophils, cancer, metastasis, tumor microenvironment, therapy

OPEN ACCESS

Edited by:
Jeffrey W. Pollard,
University of Edinburgh,
United Kingdom

Reviewed by:
Kawaljit Kaur,
University of California, Los Angeles,
United States
Sanjivan Gautam,
National Institutes of Health (NIH),
United States

***Correspondence:**
Zvi Granot
zvikag@ekmd.huji.ac.il

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *30 April 2019*
Accepted: *08 July 2019*
Published: *19 July 2019*

Citation:
Granot Z (2019) Neutrophils as a Therapeutic Target in Cancer. Front. Immunol. 10:1710. doi: 10.3389/fimmu.2019.01710

DISTINCT NEUTROPHIL SUBSETS OR A FUNCTIONAL SPECTRUM?

Neutrophils are phagocytes which play a key role in protection of the host against microbial infections as well as taking a critical part in inflammatory processes. In the context of cancer, neutrophils were also shown to play other, non-conventional roles, and may either promote (1, 2) or limit tumor growth (3–5). The conflicting reports regarding neutrophil function in cancer suggest that like other cells of the immune system, neutrophils may be divided into distinct subsets. However, until recently neutrophils were viewed as a homogeneous population of terminally differentiated cells. Still, in a recent study we were able to show that neutrophils in the context of cancer may be divided into 3 subsets—Normal Density Neutrophils (NDN), mature and immature Low Density Neutrophils (LDN) (6). We were able to associate cytotoxic anti-tumor properties with NDN and immunosuppressive pro-tumor properties with LDN (6). In fact, neutrophils subsets distinguishable by their density were found in a wide range of clinical scenarios and are not only associated with cancer (7). Unlike cells of the adaptive immune system, which can be easily defined based on surface expression of unique markers, such surface markers are not well-characterized for neutrophils. In fact, several studies suggested possible markers but these still need to be better

validated (8, 9). Still, neutrophil subsets may be distinguished according to their different physical properties (6) and there is increasing evidence for the existence of various neutrophil subsets which may be defined by their functionality. The lack of validated surface markers, together with their short half-life, makes neutrophils very difficult to study. Further, although specific functionally distinct subsets may be identified, it is still not clear whether these are truly specific subsets or are they simply found on extreme ends of a functional spectrum. That said, the accumulating data regarding neutrophil function in cancer highlights various functional aspects that may be targeted or modified to benefit patients. Following is an account of neutrophil functions and characteristics in the context of cancer and a discussion of how and whether targeting these aspects is feasible or beneficial for cancer therapy.

NEUTROPHIL TO LYMPHOCYTE RATIO

Neutrophils are notorious for their tumor promoting properties (1, 2, 10). First and foremost, high neutrophil numbers, otherwise manifested as the Neutrophil to Lymphocyte Ratio (NLR), represent a poor prognostic factor. This was found to be applicable to breast, colon, liver, and many other types of cancer (10). The reasons for the increase in neutrophil numbers are not always fully understood. Some tumors express high levels of colony stimulating factors (i.e., G-CSF and GM-CSF) which may account for the increase in mobilized neutrophils. Other tumors are in a state of smoldering inflammation which may also drive the increase in neutrophil numbers.

NLR relates to the numbers of circulating neutrophils, however, the extent of neutrophil infiltration of tumors also appears to have an adverse prognostic value (11). High neutrophil infiltration is associated with poor prognosis, advanced stage cancer and lower recurrence free survival (12–16). Some evidence suggest that high NLR may correlate with the number of tumor associated neutrophils (17). However, this needs to be further evaluated.

These observations raise a question regarding the possible targeting of neutrophils as a means for better patient outcome. Neutrophils are critical for anti-microbial protection, the option of eliminating neutrophils as a therapeutic strategy cannot be seriously considered since neutropenia is a life threatening condition. A possible alternative would be the depletion of specific neutrophil subpopulations while sparing those subpopulations essential for anti-microbial protection (see above). Unfortunately, although the existence of distinct neutrophil subsets in cancer has been convincingly demonstrated, our understanding of neutrophil subsets and the features making them distinct is still lacking. Specifically, as long as there are no clear markers to distinguish specific subsets, eliminating specific subsets for therapeutic purposes is impossible.

PRO-TUMOR NEUTROPHIL FUNCTIONS

Angiogenesis

The angiogenic switch that characterizes a transition toward a more aggressive tumor phenotype is regulated by the expression of angiogenic factors such as VEGF (18). As such, targeting angiogenesis should serve to limit tumor growth. This indeed turned out to be the case to a limited extent and in certain types of cancer (19). When looking for the source of angiogenic factors in the tumor microenvironment, neutrophils, together with other stromal cells, were shown to provide proangiogenic factors and actively promote tumor angiogenesis. Specifically, neutrophils were shown to provide MMP9, VEGF and HGF (**Figure 1**). Furthermore, neutrophils were shown to provide factors that circumvent common anti-angiogenic therapies targeting VEGF (20). Taken together, these observations highlight a key role for neutrophils in propagating tumor angiogenesis and suggest that targeting of neutrophil mediated angiogenesis, or targeting of the angiogenic neutrophil subpopulation (if such subpopulation indeed exists), may be used as an anti-angiogenic therapeutic approach.

Tumor Cell Dissemination

Metastasis is the final and lethal stage in cancer progression. For tumor cells to metastasize they need to acquire unique features that support the transition from the primary site, their survival in the circulation and the successful metastatic seeding in a distant organ. In this context neutrophils were shown to play various roles to promote the intravasation of tumor cells (MMPs and neutrophil elastase, **Figure 1**), their survival in the circulation (21), their adherence to the endothelium at the future site of metastasis (priming of the premetastatic niche and NETs) and the process of extravasation (**Figure 1**). Recently neutrophils were also shown to play a critical role in the awakening of dormant tumor cells and the initiation of metastases growth (22). Targeting of neutrophil function in each of these stages of metastatic dissemination may have significant implications on metastatic progression. This is elegantly demonstrated in a recent study by Albrengues et al. (22) who show that NETs are required for promoting the exit from dormancy and the establishment of marcometastases. This finding is noteworthy since it proposes a clinical scenario where intervention is still possible, i.e., administration of DNAse to eliminate NETs to maintain tumor cells dormant and prolong distant metastasis free survival.

Immune Suppression

The term Myeloid Derived Suppressor Cells (MDSC) encompasses a wide range of myeloid cells which possess immunosuppressive properties. In the context of cancer, these cells have the capacity to suppress cytotoxic T cells and promote immune evasion. The broadness of the MDSC umbrella also covers neutrophils but since it is a relatively well-defined population the term suppressive neutrophils is more accurate. We have previously shown that immunosuppressive neutrophils are propagated to promote the resolution of an inflammatory process. It seems that a similar rationale is employed in the context of cancer—the propagation of immunosuppressive neutrophils serves the resolution of tumor associated inflammation. However, since the tumor is in a continuous state of inflammation that does not resolve, suppressive neutrophils are mobilized excessively to the point where they become the dominant subpopulation of neutrophils. Under these conditions

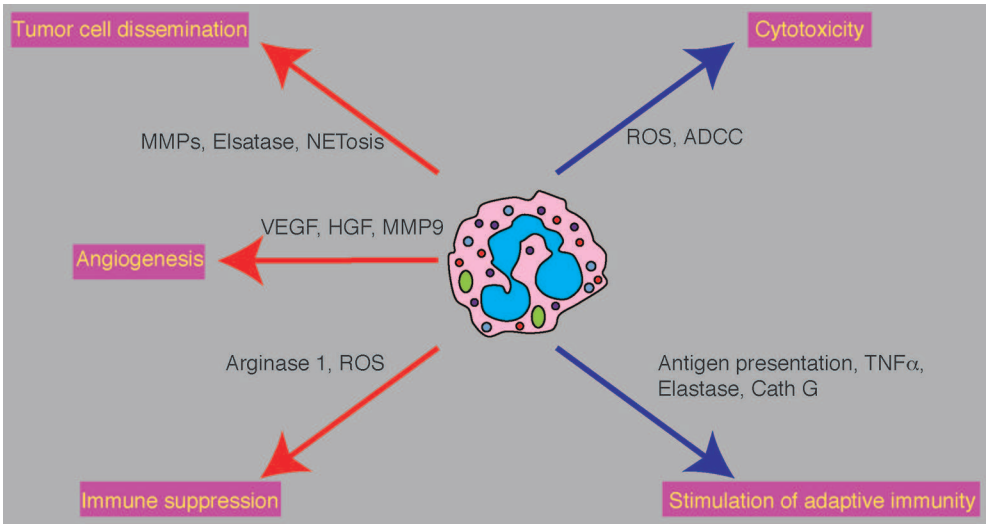


FIGURE 1 | Neutrophil functions in cancer and potential therapeutic targets. Neutrophils play various and conflicting roles in cancer. Tumor promoting functions (red arrows) and anti tumor functions (blue arrows) are executed by specific molecular mediators. Tumor promoting propeties: Neutrophils promote tumor cell dissemination by degradation of the ECM at the primary and premetastatic sites and promote tumor cell seeding by deploying NETs. Promotion of angiogenesis is mediated by secretion of VEGF and HGF and the release of angiogenic factors from the ECM by neutrophil derived MMP9. Neutrophil mediate immune supprssion via the secretion of ROS and Arginase 1 to limit T cell dependent anti-tumor immunty. Anti-tumor properties: Neutrophils limit tumor growth and metastatic progression by eliminating tumor cells either directly or via antibody dependent mechanisms. Neutrophils can stimulate anti-tumor adaptive immune by acting as antigen presenting cells, secretion of TNFα, secretion of Elastase and secretion of Cathepsin G (Cath G).

the overall neutrophil contribution is pro-tumorigenic. Immunosuppressive neutrophils (often referred to as G-MDSC) contain large amounts of arginase I (**Figure 1**) which suppresses T cell proliferation through deprivation of L-arginine (23, 24). Immunosuppressive neutrophils were also shown to generate high levels of hydrogen peroxide (**Figure 1**) and thus block T cell proliferation (25, 26). These observations provide insight into the role played by neutrophils that are maintaining an immunosuppressive tumor microenvironment and highlight their role in facilitating metastatic spread through suppression of adaptive immune components (6, 25, 27). These observations suggest that administration of immunotherapies concomitant with blocking of neutrophil-mediated immunosuppression may further potentiate anti-tumor adaptive immunity. This notion was in fact demonstrated in two separate studies; the first showing that blocking of c-MET in neutrophils improves the efficacy of immunotherapy by limiting the recruitment of immunosuppressive neutrophils (28). The second study, recently published by Veglia et al. (29) shows that FATP2 deficient neutrophils lose their immunosuppressive properties leading to a significant delay in tumor progression.

ANTI TUMOR NEUTROPHIL FUNCTIONS

Neutrophil Cytotoxicity

While most of the data regarding neutrophil function in cancer supports a pro-tumorigenic role, neutrophil may also eliminate cancerous cells and limit metastatic seeding. Unlike other neutrophil properties discussed above, neutrophil cytotoxicity requires a high level of specificity. Neutrophils need to be activated, they need to be attracted to tumor cells, they must identify tumor cells as a target, they must form physical contact with tumor cells and must secrete cytotoxic mediators (H_2O_2) to induce tumor cell apoptosis (**Figure 1**). Neutrophil recognition of tumor cells may be mediated either directly [RAGE-Cathepsin G (30)] or in an antibody dependent fashion [ADCC (31)]. In addition, tumor cells must be susceptible to neutrophil cytotoxicity (i.e., express the H_2O_2-dependent TRPM2 Ca^{2+} channel) for neutrophils to exert this favorable function (32). It seems that although cytotoxic neutrophils may be detected throughout the course of the disease, neutrophil cytotoxicity is mostly evident in early stages of tumor progression. This is most likely due to suppressive conditions that govern the tumor microenvironment. Since TRPM2 expression in tumor cells varies, not all tumor cells are equally susceptible to neutrophil cytotoxicity. Neutrophil resistant tumor cells should be targeted by other means. However, preventing the transition form HDN to LDN (perhaps by blocking TGFβ activity) should enhance the proportion of anti-tumor neutrophils and may be considered as a possible anti-cancer therapy. Further, the transfusion of cytotoxic neutrophils, although somewhat challenging, is actively being evaluated (*Lift BioSciences*).

Stimulation of Adaptive Immune Responses

The notion that adaptive immunity is the major effector in anti-tumor immune responses is well-accepted. However, there is evidence supporting a role for neutrophils in this respect too. For example, neutrophils were shown to interact with T cells

and are required for proper anti-tumor $CD4^+$ and $CD8^+$ T-cell responses (33–36). In fact, neutrophils were shown to present antigens and provide accessory signals for T cell activation (37, 38). In addition, N1 tumor associated neutrophils were shown to require T-cells for their anti-tumor activity at the primary site, which may indicate possible stimulation of T cells by neutrophils (33). Finally, neutrophils are able to recruit and activate T-cells via secretion of cytokines, including TNFα, Cathepsin G and neutrophil elastase (27) (**Figure 1**).

CONTEXT DEPENDENT NEUTROPHIL FUNCTION

Neutrophils may present with either tumor promoting or tumor limiting properties. It is not yet clear whether this is a manifestation of distinct subsets or the extreme ends of a wide functional spectrum. Regardless, neutrophils are the first responders of the immune system and as such are equipped with a wide variety of receptors. This makes neutrophils highly responsive to cues in their microenvironment and may explain why neutrophils function one way at the primary tumor and in a completely different way in the pre-metastatic niche. Indeed, neutrophil function was found to be dramatically modified by factors such as TGFβ and type I interferons.

TGFβ

TGFβ is a highly versatile molecule which may act as both tumor suppressor and oncogene. However, when examining the effect it exerts on neutrophil function in cancer it is regarded as pro tumoral. Fridlender et al. (33) were the first to show that TGFβ in the tumor microenvironment acts to block neutrophil cytotoxicity. In this study they also coined the "N1" anti-tumor and "N2" pro-tumor terminology to describe neutrophil function in cancer. Their study showed that TGFβ both blocked the anti-tumor function of neutrophils and restricted their entry into the tumor (33). Later studies provided better insight into the effect of TGFβ on neutrophil function in cancer. First, TGFβ directly blocks the production of H_2O_2, a key mediator of neutrophil cytotoxicity, by activated neutrophils. Second, TGFβ was found to block the migration of tumor neutrophils toward tumor cells. And third, TGFβ was found to change the ratio between HDN and LDN (see above). Together, these observations demonstrate that TGFβ not only blocks the favorable anti-tumor functions of neutrophils, it also increases the proportion of tumor promoting neutrophils thereby supporting tumor growth. Since TGFβ is abundant at the primary and metastatic tumors, neutrophil cytotoxicity is not evident in these sites but rather the pro-tumor functions are manifested. In contrast, during the early stages of metastatic dissemination, circulating tumor cells arriving to the future site of metastasis are not protected by high levels of TGFβ and are susceptible to neutrophil cytotoxicity. Hence neutrophil cytotoxicity is evident at the time of metastatic seeding and possibly at early stages of tumorigenesis but not in the microenvironment of an established tumor.

IFNs

Type I interferons have an effect on neutrophil function that opposes that of TGFβ. IFNs were first identified as having anti-viral functions and later on were also found to play an anti-tumorigenic role. IFNs mediate an anti-tumor immune response by activating various immune cells (39). On top of modulating the function of lymphocytes and macrophages, IFN-β was found to suppress the expression of proangiogenic factors, such as VEGF and MMP9, thereby limiting tumor growth (40). In addition to modifying the expression of protumorigenic factors, IFN-β enhances the recruitment of neutrophils and their life span in primary tumors (41, 42). Finally, type I IFN activity was found to inhibit neutrophil-mediated priming of a receptive premetastatic niche (43).

Together, these observations support the notion that neutrophil function in cancer is heavily dictated by the specific microenvironment. More importantly, these data suggest that rather than modifying the function of neutrophils or depleting specific subsets, one may achieve a therapeutic benefit mediated by neutrophils via modulation of the tumor microenvironment. Essentially, blocking TGFβ activity or enhancing IFNs activity at the tumor microenvironment should facilitate neutrophil anti-tumor cytotoxicity and may be considered as a mode of anti-tumor immunotherapy.

CONCLUDING REMARKS

Neutrophils are essential for host protection against microbial infections and as such cannot be eliminated as a mode of therapy. However, the progress made in recent years highlighting the fact that neutrophils are not a homogeneous population of cells, opens new opportunities for targeting neutrophils as a mode of cancer therapy. Better characterization of neutrophils, their different subsets and distinct functions may serve to specifically deplete harmful populations and enhance neutrophils' favorable functions. However, taking into account the fast rate of neutrophil replenishment, this strategy will require continuous administration of antibodies. This therapeutic approach is not without risk and previous studies using antibodies to deplete neutrophils show that ultimately the depleting antibodies lose their efficacy.

A different strategy for the manipulation of neutrophil function in cancer is via the modulation of the tumor microenvironment in a fashion that would permit neutrophil anti-tumor functions. Indeed, using small molecules to block TGFβ showed a dramatic effect on tumor growth that was dependent on neutrophils. Furthermore, Novitskiet al. demonstrated that tumor growth and metastatic spread are blocked when using a mouse model of myeloid-specific deletion of TGFβR2 (44). Together, these observations suggest that modifying TGFβ activity in neutrophils *in vivo* may be sufficient for stimulating a robust anti-tumor response. That said, current therapies targeting TGFβ signaling prove to be toxic and are not tolerated well. A possible alternative for circumventing the toxicity of systemic administration of small molecule

TGFβ blockers is a more targeted approach. Future therapies using neutrophil specific drug delivery may serve to harness neutrophils toward fighting cancer. Such technology is yet to be developed.

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer*. (2016) 16:431–46. doi: 10.1038/nrc.2016.52
2. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature*. (2015) 528:413–7. doi: 10.1038/nature16140
3. Granot Z, Jablonska J. Distinct functions of neutrophil in cancer and its regulation. *Mediators Inflamm*. (2015) 2015:701067. doi: 10.1155/2015/701067
4. Singhal S, Bhojnagarwala PS, O'Brien S, Moon EK, Garfall AL, Rao AS, et al. Origin and role of a subset of tumor-associated neutrophils with antigen-presenting cell features in early-stage human lung cancer. *Cancer Cell*. (2016) 30:120–35. doi: 10.1016/j.ccell.2016.06.001
5. Finisguerra V, Di Conza G, Di Matteo M, Serneels J, Costa S, Thompson AA, et al. MET is required for the recruitment of anti-tumoural neutrophils. *Nature*. (2015) 522:349–53. doi: 10.1038/nature14407
6. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep*. (2015) 10:562–73. doi: 10.1016/j.celrep.2014.12.039
7. Jablonska J, Granot Z. Neutrophil, quo vadis? *J Leukoc Biol*. (2017) 102:685–8. doi: 10.1189/jlb.3MR0117-015R
8. Condamine T, Dominguez GA, Youn JI, Kossenkov AV, Mony S, Alicea-Torres K, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. *Sci Immunol*. (2016) 1:aaf8943. doi: 10.1126/sciimmunol.aaf8943
9. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature $CD10^{+}$ and immature $CD10^{-}$ neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood*. (2017) 129:1343–56. doi: 10.1182/blood-2016-04-713206
10. Sionov RV, Fridlender ZG, Granot Z. The multifaceted roles neutrophils play in the tumor microenvironment. *Cancer Microenviron*. (2015) 8:125–58. doi: 10.1007/s12307-014-0147-5
11. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. (2015) 21:938–45. doi: 10.1038/nm.3909
12. Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C. Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in northern Italy. *Mod Pathol*. (2002) 15:831–7. doi: 10.1097/01.MP.0000020391.98998.6B
13. Zhao JJ, Pan K, Wang W, Chen JG, Wu YH, Lv L, et al. The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. *PLoS ONE*. (2012) 7:e33655. doi: 10.1371/journal.pone.0033655
14. Reid MD, Basturk O, Thirabanjasak D, Hruban RH, Klimstra DS, Bagci P, et al. Tumor-infiltrating neutrophils in pancreatic neoplasia. *Mod Pathol*. (2011) 24:1612–9. doi: 10.1038/modpathol.2011.113
15. Ino Y, Yamazaki-Itoh R, Shimada K, Iwasaki M, Kosuge T, Kanai Y, et al. Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. *Br J Cancer*. (2013) 108:914–23. doi: 10.1038/bjc.2013.32
16. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol*. (2009) 27:4709–17. doi: 10.1200/JCO.2008.18.9498
17. Takakura K, Ito Z, Suka M, Kanai T, Matsumoto Y, Odahara S, et al. Comprehensive assessment of the prognosis of pancreatic cancer: peripheral blood neutrophil-lymphocyte ratio and immunohistochemical analyses of the tumour site. *Scand J Gastroenterol*. (2016) 51:610–7. doi: 10.3109/00365521.2015.1121515
18. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. (1996) 86:353–64. doi: 10.1016/S0092-8674(00)80108-7
19. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer Cell*. (2014) 26:605–22. doi: 10.1016/j.ccell.2014.10.006
20. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc Natl Acad Sci USA*. (2008) 105:2640–5. doi: 10.1073/pnas.0712185105
21. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature*. (2019) 566:553–7. doi: 10.1038/s41586-019-0915-y
22. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:eaao4227. doi: 10.1126/science.aao4227
23. Raber P, Ochoa AC, Rodriguez PC. Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T cell suppression and therapeutic perspectives. *Immunol Invest*. (2012) 41:614–34. doi: 10.3109/08820139.2012.680634
24. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunol Rev*. (2008) 222:180–91. doi: 10.1111/j.1600-065X.2008.00608.x
25. Corzo CA, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Sotomayor E, et al. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol*. (2009) 182:5693–701. doi: 10.4049/jimmunol.0900092
26. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. (2009) 9:162–74. doi: 10.1038/nri2506
27. Mishalian I, Bayuh R, Eruslanov E, Michaeli J, Levy L, Zolotarov L, et al. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17–a new mechanism of impaired antitumor immunity. *Int J Cancer*. (2014) 135:1178–86. doi: 10.1002/ijc.28770
28. Glodde N, Bald T, van den Boorn-Konijnenberg D, Nakamura K, O'Donnell JS, Szczepanski S, et al. Reactive neutrophil responses dependent on the receptor tyrosine kinase c-MET limit cancer immunotherapy. *Immunity*. (2017) 47:789–802 e9. doi: 10.1016/j.immuni.2017.09.012
29. Veglia F, Tyurin VA, Blasi M, De Leo A, Kossenkov AV, Donthireddy L, et al. Fatty acid transport protein 2 reprograms neutrophils in cancer. *Nature*. (2019) 569:73–78 doi: 10.1038/s41586-019-1118-2
30. Sionov RV, Fainsod-Levi T, Zelter T, Polyansky L, Pham CT, Z. G. Neutrophil cathepsin G and tumor cell RAGE facilitate neutrophil anti-tumor cytotoxicity. *Oncoimmunology*. (2019). doi: 10.1080/2162402X.2019.1624129
31. Sionov RV, Assi S, Gershkovitz M, Sagiv JY, Polyansky L, Mishalian I, et al. Isolation and characterization of neutrophils with anti-tumor properties. *J Vis Exp*. (2015) 2015:e52933. doi: 10.3791/52933
32. Gershkovitz M, Caspi Y, Fainsod-Levi T, Katz B, Michaeli J, Khawaled S, et al. TRPM2 mediates neutrophil killing of disseminated tumor cells. *Cancer Res*. (2018) 78:2680–90. doi: 10.1158/0008-5472.CAN-17-3614
33. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
34. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *J Clin Invest*. (2014) 124:5466–80. doi: 10.1172/JCI77053
35. Stoppacciaro A, Melani C, Parenza M, Mastracchio A, Bassi C, Baroni C, et al. Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation

and T cell-produced interferon gamma. *J Exp Med*. (1993) 178:151–61. doi: 10.1084/jem.178.1.151

36. Potter NS, Harding CV. Neutrophils process exogenous bacteria via an alternate class I MHC processing pathway for presentation of peptides to T lymphocytes. *J Immunol.* (2001) 167:2538–46. doi: 10.4049/jimmunol.167.5.2538
37. Radsak M, Iking-Konert C, Stegmaier S, Andrassy K, Hansch GM. Polymorphonuclear neutrophils as accessory cells for T-cell activation: major histocompatibility complex class II restricted antigen-dependent induction of T-cell proliferation. *Immunology*. (2000) 101:521–30. doi: 10.1046/j.1365-2567.2000.00140.x
38. Reali E, Guerrini R, Moretti S, Spisani S, Lanza F, Tomatis R, et al. Polymorphonuclear neutrophils pulsed with synthetic peptides efficiently activate memory cytotoxic T lymphocytes. *J Leukoc Biol*. (1996) 60:207–13. doi: 10.1002/jlb.60.2.207
39. Chervenick PA, Boggs DR, Marsh JC, Cartwright GE, Wintrobe MM. Quantitative studies of blood and bone marrow neutrophils in normal mice. *Am J Physiol*. (1968) 215:353–60. doi: 10.1152/ajplegacy.1968.215.2.353
40. Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev*. (2007) 219:88–102. doi: 10.1111/j.1600-065X.2007.00550.x
41. de la Luz Sierra M, Sakakibara S, Gasperini P, Salvucci O, Jiang K, McCormick PJ, et al. The transcription factor Gfi1 regulates G-CSF signaling and neutrophil development through the Ras activator RasGRP1. *Blood*. (2010) 115:3970–9. doi: 10.1182/blood-2009-10-246967
42. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest.* (2010) 120:2423–31. doi: 10.1172/JCI41649
43. Weisel KC, Bautz F, Seitz G, Yildirim S, Kanz L, Mohle R. Modulation of CXC chemokine receptor expression and function in human neutrophils during aging *in vitro* suggests a role in their clearance from circulation. *Mediators Inflamm*. (2009) 2009:790174. doi: 10.1155/2009/790174
44. Novitskiy SV, Pickup MW, Chytil A, Polosukhina D, Owens P, Moses HL. Deletion of TGF-beta signaling in myeloid cells enhances their anti-tumorigenic properties. *J Leukoc Biol*. (2012) 92:641–51. doi: 10.1189/jlb.1211639

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 25 July 2019
doi: 10.3389/fimmu.2019.01611

Tuning the Tumor Myeloid Microenvironment to Fight Cancer

Nadine S. Jahchan[1], Adriana M. Mujal[2†], Joshua L. Pollack[1], Mikhail Binnewies[1], Venkataraman Sriram[1], Leonard Reyno[1] and Matthew F. Krummel[2,3]**

[1] Pionyr Immunotherapeutics, South San Francisco, CA, United States, [2] Department of Pathology, University of California, San Francisco, San Francisco, CA, United States, [3] ImmunoX Initiative, University of California, San Francisco, San Francisco, CA, United States

The tumor microenvironment (TME) of diverse cancer types is often characterized by high levels of infiltrating myeloid cells including monocytes, macrophages, dendritic cells, and granulocytes. These cells perform a variety of functions in the TME, varying from immune suppressive to immune stimulatory roles. In this review, we summarize the different myeloid cell populations in the TME and the intratumoral myeloid targeting approaches that are being clinically investigated, and discuss strategies that identify new myeloid subpopulations within the TME. The TME therapies include agents that modulate the functional activities of myeloid populations, that impact recruitment and survival of myeloid subpopulations, and that functionally reprogram or activate myeloid populations. We discuss the benefits, limitations and potential side effects of these therapeutic approaches.

Keywords: tumor micoenvironment, macrophage, tumor associated macrophage (TAM), immune checkpoint blockade (ICB), dendritic cell (DC), myeloid cells, myeloid tuning, monocytes

OPEN ACCESS

Edited by:
Jeffrey W. Pollard, University of Edinburgh, United Kingdom

Reviewed by:
Luca Cassetta, University of Edinburgh, United Kingdom
Jo A. Van Ginderachter, Vrije University Brussel, Belgium

****Correspondence:***
Nadine S. Jahchan
nadine.jahchan@pionyrtx.com
Matthew F. Krummel
matthew.krummel@ucsf.edu

†Present address:
Adriana M. Mujal, Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY, United States

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *01 May 2019*
Accepted: *27 June 2019*
Published: *25 July 2019*

Citation:
Jahchan NS, Mujal AM, Pollack JL, Binnewies M, Sriram V, Reyno L and Krummel MF (2019) Tuning the Tumor Myeloid Microenvironment to Fight Cancer. Front. Immunol. 10:1611. doi: 10.3389/fimmu.2019.01611

INTRODUCTION

The tumor microenvironment (TME) consists of a cellular multitude including fibroblasts, endothelial cells, and immune cells from the lymphoid and myeloid lineage (1–3). The TME shapes the growth of tumor cells and influences responses to therapies (4). In cancer patients, the immune system fails to suppress tumor growth in part due to the presence of active immune checkpoints or "brakes," that usually result in the suppression of T-cell function (5, 6). CTLA-4 was the first immune checkpoint identified on T-cells in 1996 (7) and led to the development of the anti-CTLA-4 antibody Ipilimumab that is now approved in the clinic (8). PD-1 (9) was the second immune checkpoint identified (10) and led to the development of multiple anti-PD-1 and anti-PD-L1 monoclonal antibodies that are now approved therapies (11, 12). These Immune Checkpoint Blockade (ICB) therapies mainly function by re-engaging the immune system to promote anti-tumor activity. In the clinic, ICB therapies have shown profound clinical benefits and durable responses in a subset of patients in multiple tumor indications, including metastatic melanoma, NSCLC, and renal cell carcinoma (13). Only about 25% of patients across all indications respond to ICB therapies, highlighting the importance for additional therapies to treat the remaining non-responsive patients (14). There is currently a major effort to develop therapies that block additional immune inhibitory pathways (e.g., TIM3, LAG3, IDO, VISTA, and KIR) or that activate immune co-stimulatory receptors (e.g., CD40, GITR, ICOS, CD137, and OX40) (15). To date, these second generation immune-therapies have yet to yield significant clinical efficacy beyond anti-PD-1, anti-PD-L1, or anti-CTLA-4 therapies. For instance, IDO-1 inhibitors failed to provide benefit

as monotherapy in Phase I/II clinical trials (16, 17) and in combination with anti-PD-1 therapy in a Phase III clinical trial in advanced melanoma patients (18), highlighting the challenges to understand the biology of this drug target and to explore further combination therapies in the clinic.

Both the lack of progress in next-generation ICB agents targeting the T cells as well as identification of resistance and regulatory pathways beyond T cells in the TME has renewed interest in identification of novel targets in the TME. Profiling the immune cells in the TME of patients with advanced techniques demonstrated significant differences in the immune infiltrates and composition of the TME within patients from the same tumor types, especially in cells from the myeloid lineage (19–23). The intratumoral myeloid cells in the TME are heterogeneous in nature and include mononuclear cells (monocytes, macrophages, dendritic cells), and polymorphonuclear granulocytes (3, 19, 24, 25). In normal tissues, these myeloid cells assist in damage repair and provide a first line of defense against dangers such as pathogens and viruses. They are not uniform, either in form or function, presumably to ensure versatile responses to the diverse challenges faced in normal and disease physiology. In the TME, they can either suppress or promote anti-tumor immunity and play an important role in phagocytosis and antigen presentation to T-cells (24, 26, 27). For instance, myeloid inhibitory cells such as tumor-associated macrophages (TAMs) can limit responses to chemotherapy, irradiation, and angiogenic inhibitors (28–30). In contrast, stimulatory myeloid cells such as migratory dendritic cells (DCs) are critical for eliciting potent anti-tumor T-cell responses, and patients with higher migratory DCs have significantly increased overall survival (19, 20, 24, 26, 27, 31). Despite the potential to mediate antitumor effector T cell immunity, however, steady-state DC populations also maintain peripheral T cell tolerance (32, 33) and these baseline homeostatic processes may compromise their stimulatory capacity in some patients (34, 35). Therefore, strategies to target specific myeloid populations and cellular programs in the clinic have attracted considerable attention from many companies, and multiple drug agents are currently being evaluated in the clinic. In this review, we describe the myeloid subpopulations in the TME and summarize the different myeloid tuning strategies to target these cells.

MYELOID SUBPOPULATIONS IN THE TUMOR MICROENVIRONMENT

The myeloid cell populations within the TME, their ontogeny and development, the key chemokines required for their trafficking and survival, as well as the gene products that are used by many researchers to define the various myeloid populations in humans and mice are outlined in **Figure 1** and discussed in more detail below.

Macrophages

The most abundant myeloid population in tumors are generally TAMs (27, 36). Though an inclusive term, TAMs have heterogeneous ontogeny and can broadly be classified on this basis as monocyte-derived macrophages "mo-Macs" or yolk-sac derived tissue-resident macrophages (29, 30, 36, 37). While high frequencies of TAMs are generally associated with poor prognosis in a wide variety of cancer indications, correlations between high TAM density and improved survival have also emerged (30, 38, 39). These discordant observations raise questions about whether there are qualities of TAMs that can make them beneficial to an adaptive response, and also reinforce the need for markers to rigorously distinguish TAMs from distinct origins, distinct phenotypes, as well as from other myeloid populations (**Figure 1**). Like some of their other myeloid counterparts, TAMs take on distinct activation states. Although it trivializes the diversity *in vivo*, TAMs are often reduced to existing in binary states of classical ("M1") or alternative ("M2") activation based on *in vitro* studies that skewed macrophage differentiation with the single chemokines IFN-γ vs. IL-4, respectively. Although more reductionist than what occurs *in vivo,* these two cellular profiles exemplify the possibility of polarized transcriptional and secretory programs, and those in turn may explain conflicting correlations in patient outcome (40). For instance, "M2" signatures, which sometimes correlate with poor prognosis, tend to be anti-inflammatory and associated with tissue remodeling and wound-healing processes (30, 38). However, it is now clear that activation and polarization of macrophages consist of a range of non-terminal phenotypes rather than two binary states and there are multiple factors contributing to their intratumoral and intertumoral heterogeneity, such as the anatomical location, cancer subtype, and exposure to a myriad of environmental factors corrupting the TAMs to exist in a katzenjammer state (30, 38, 41, 42).

The heterogeneity of macrophages may be due to lineage-imprinted differences between mo-Macs and tissue-resident macrophages (30, 37, 43, 44). While some tissue-resident macrophages express tissue-specific markers (45), recent advances have improved separation of mo-Macs and tissue-resident macrophages in the TME (36, 37). In these studies, tissue-resident macrophages exhibited a stronger "M2" profile (36) and took on a wound repair program (37) while mo-Macs exhibited the ability to prime CD8+ T cells, although these experiments were performed *in vitro* with pulsed antigen, bypassing normal cross-presentation machinery (37). RNA-sequencing (RNA-seq) analyses of breast and endometrial cancer TAMs in comparison with FACS sorted tissue-resident macrophages from normal tissues confirmed the existence of tissue-specific niches that influence macrophage and TAM profiles irrespective of their common precursor cells (45). A better understanding of macrophage origin and heterogeneity is vital when exploring the effects of targeting the macrophage population within the TME. Recent studies using single-cell profiling by RNA-seq suggest a more complex heterogeneity and plasticity of macrophages that could further affect tumor development and responsiveness to immunotherapy (21–23).

Dendritic Cells

Conventional DCs (cDCs) similarly exhibit diversity, broadly delimited as cDC1 and cDC2, with commitment to each occurring early in specific precursor populations, called

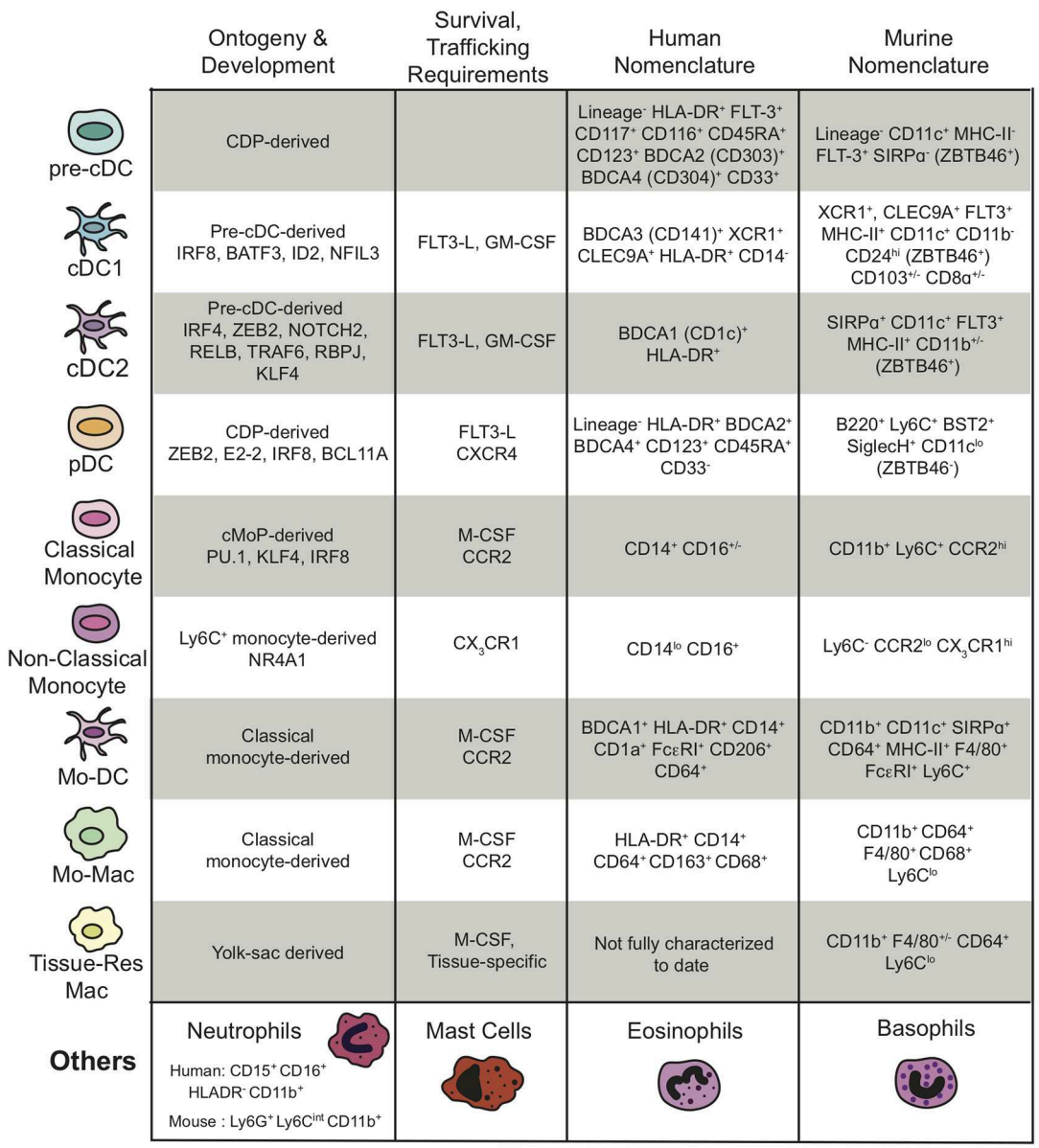


FIGURE 1 | Summary of the different subtypes of myeloid cells present in the TME, their ontogeny and development, their survival and trafficking requirements, and their human and mouse nomenclatures including transcription factors and cell surface markers. The "Others" myeloid cells in the last row represent the granulocytes. "Lineage$^-$" is defined as CD3$^-$CD14$^-$CD16$^-$CD19$^-$ for the human nomenclature and CD3$^-$NKp46$^-$B220$^-$ for the murine nomenclature.

pre-cDCs (46) and the two mature classes corresponding to differential transcription factor requirements and having functional specialization (47–49). Pre-cDCs are detectable in the blood, lymphoid, and non-lymphoid tissue, and can also be found in the TME (50). Although cellularity may vary, both cDC1s and cDC2s can be found in mouse and human tumors (21, 27, 51) and take on distinct roles in the priming of anti-tumor T cells. cDCs, particularly cDC1s, require FLT3-ligand (FLT3-L) for development and *in situ* proliferation, as well as GM-CSF for survival in peripheral tissue (52). Although there have been reports of some cancers producing GM-CSF (53), the origin of these cytokines in the TME is largely uncharacterized. Notably, recent data suggests that natural killer cells act as a rich source of FLT3-L in the TME (20).

cDC1s excel at antigen cross-presentation and are critical for initiating CD8$^+$ T cell responses across a number of immunological settings, including tumor models (27, 51, 54). In mice, cDC1s have two major subclasses: lymphoid tissue resident CD8a$^+$ DCs and non-lymphoid tissue (NLT) migratory CD103$^+$ DCs, which are strikingly similar to one another transcriptionally and share expression of the chemokine receptor XCR1 (49, 51, 55). Together cDC1s depend on transcription factors IRF8 (49)

and BATF3 (54) for development, although strict requirements between the subsets may differ (48). Although genetic models eliminating these genes are useful for broad depletion of cDC1s (54), more recent use of mixed bone marrow chimeras demonstrated a specific and critical role for $CCR7^+$ $CD103^+$ DCs in migration and initiation of $CD8^+$ T-cells responses in tumor-draining lymph nodes (LNs) (26, 51). In addition to outperforming the other DC subset at cross-presentation, tumor cDC1s are a primary producer of IL-12 (27), which contributes to $CD8^+$ T-cell proliferation and effector function and is associated with higher rates of responsiveness to chemotherapy (56). Furthermore, cDC1s exert potent anti-tumor activity in the TME despite being an extremely rare population (27). Tumor cDC1 production of CXCL9 and CXCL10 can recruit activated T-cells to the TME (57) where local cDC1 re-stimulation of T-cells support anti-tumor activity (27). Although the mechanistic requirements and consequences of DC re-activation are still not well-understood, tumor cDC1s may promote higher T-cell motility and contact with cancer cells (20, 57, 58).

In contrast to cDC1s, cDC2s typically preferentially activate $CD4^+$ T-cells through presentation of peptides on MHC-II, express SIRPα, and are dependent on the transcription factor IRF4 (49, 52). Despite this overarching classification, cDC2s encapsulate a great degree of heterogeneity (55). While historically cDC2s have largely been identified as $CD11b^+$ DCs (47), dermal cDC2s do include a $CD11b^{hi}$, and $CD11b^{lo}$ KLF4-dependent population (59), highlighting the advantage of using SIRPα as a defining marker. Another complicating feature of cDC2s is that they share many surface markers with monocytes and macrophages (e.g., CD11b, CD11c, SIRPα, CX3CR1, CCR2, CD14). While this overlap has made it difficult to precisely define and isolate cDC2s, additional markers including CD64, MERTK, and Ly6C have been proposed to selectively identify macrophages and monocytes (60). ZBTB46 has also emerged as a cDC lineage-restricted transcription factor and may help to clarify ontogeny (61). In humans, cDC2s are best aligned with the $CD1c^+$ ($BDCA1^+$) subset found in the blood and various tissues including tumor (35, 62, 63) and comprise at least two subset populations as revealed by recent single-cell RNA-sequencing analysis (35, 64).

Inflammatory DCs

Although cDCs are tautologically pre-cDC-derived, monocytes can be recruited to sites of inflammation and differentiate into mo-DCs, also called inflammatory or iDCs, in response to a number of infectious or adjuvant agents (65). Monocyte ontogeny is primarily demarcated by CCR2-dependency and surface markers, and transcriptional profiling of skin cell populations revealed that mo-DCs exhibit a similar gene signature to $CD11b^+$ cDCs (60). In some cases, mo-DCs may substitute for cDCs functionally or shape T-cell differentiation (65). As with cDCs, mo-DCs have been identified in the TME of mice (66) and human tumor ascites (65), and may also contribute to anti-tumor immunity as they were suggested to actively suppress T-cell responses (66). Indeed, anthracycline chemotherapy can prompt massive recruitment and differentiation of monocytes. In this model the therapeutic benefit of chemotherapy relied on $CD11b^+$ cells (67), suggesting that these mo-DCs may exhibit anti-tumor activity. Many questions remain as to how mo-DCs develop, if mo-DC populations from these studies share common transcriptional programs, and how they are functionally distinct from their peer cDCs. While seemingly semantic, clarity on origin, and functional specification will allow for more consistent comparisons across models and shed light on the myeloid populations that contribute to anti-tumor responses.

Plasmacytoid DCs

Plasmacytoid DCs (pDCs) develop from the common DC progenitor (CDP), but are independent of the cDC lineage (47). While they can promote antiviral responses through type-I interferon, pDCs can also induce tolerance and have been associated with poor outcome in breast and ovarian cancer (68). Despite their proposed tolerogenic properties, however, some studies have found potent anti-tumor activity in pDCs upon therapeutic stimulation (68). It is important to note, however, that a recent study identified human $CD123^+$ $CD33^+$ pre-cDCs to exhibit substantial surface marker overlap with pDCs (69). Although CD33 and several other markers can separate pre-cDCs from pDCs (69), the cells used in older studies of pDCs may be contaminated with pre-cDCs, and conclusions drawn may warrant reevaluation.

Monocytes

In both mouse and human, monocytes develop in a colony-stimulating factor 1 (CSF-1) dependent manner primarily in the bone marrow, through differentiation of monocyte-committed common monocyte progenitor (cMoP) population (70, 71). Although single-cell sequencing approaches are rapidly identifying subsets of these cells in bone-marrow, blood, and tissue, two primary subtypes, classical and non-classical or "patrolling" monocytes, clearly exist within the blood (72, 73).

In human, classical monocytes are characterized by their expression of both CD14 and CD16, while in mouse they are described as being $Ly6C^{hi}CCR2^+$. Classical monocytes, hereby referred to as $Ly6C^{hi}$ monocytes, are thought to persist in circulation for 1–2 days, at which point they have either entered a tissue site in response to a stimulus, differentiated into a non-classical monocyte, or died (74). Studies on a population of cells known as monocytic myeloid-derived suppressor cells (mMDSC) (75), which includes monocytes, have been shown to promote tumor growth through the production of various immunosuppressive cytokines and factors (76–78) and the suppression T-cell proliferation and function (79), suggesting that perhaps, even as an undifferentiated precursor monocytes may contain functional capacity of consequence. Furthermore, a recent study using multiphoton intravital imaging of the lung pre-metastatic site in mice revealed that as pioneering metastatic tumor cells arrived and died, distinct waves of myeloid cells ingested tumor material, supplying antigen to both pro- and anti-tumor immune compartments (80). Monocytes were found to engulf the majority of tumor material, potentially sequestering valuable tumor antigen from stimulatory DC populations and

genetic ablation of monocytes resulted in higher antigen loads in those DC.

Non-classical monocytes, hereby referred to as Ly6C^{low} monocytes, are described in human as being CD14^{low} CD16^{+} and in mouse as Ly6C^{low}CCR2^{-}Nr4a1^{+} (81). Unlike their Ly6C^{hi} monocyte counterparts, the function and critical features of Ly6C^{low} monocytes are poorly understood, particularly in the TME. Ly6C^{low} monocytes are generally characterized as being blood-resident, which helps explain data suggesting a role for them in the surveying of endothelial integrity (82, 83). While the role and even presence of Ly6C^{low} monocytes in the TME is debatable, Ly6C^{low} monocyte involvement in the metastatic site is clearer. A recent study (84) using Nr4a1-deficient mice, which lack Ly6C^{low} monocytes, demonstrated that in the absence of Ly6C^{low} monocytes tumor metastatic burden significantly increased but could be reduced by adoptive transfer of *Nr4a1*-proficient LyC6^{low} monocytes. It was shown that infiltrating Ly6C^{low} monocytes detect tumor through CX3CR1 and were capable of phagocytosing tumor material.

Granulocytes

These cells include tumor associated **neutrophils** (TANs), which are distinct from circulating neutrophils in phenotype, cell surface markers, and chemokine activity. These neutrophils are recruited to the tumor site through various chemokine and receptor systems and their accumulation in the tumor is influenced by multiple factors and interactions with other cells types and environmental cues in the TME (85). Although neutrophils can inhibit or promote tumor progression based on their active role as regulators of the immune system and their impact on the TME, clinical evidence show their correlation with poor prognosis in multiple tumor indications such as melanoma, lung, melanoma, and renal carcinomas (86–90). Various reviews have highlighted the paradoxical role of neutrophils and provided insights on the mechanisms for their recruitment to the tumor site, their functional plasticity and polarization, and their activation to support tumor progression or enhance their antitumor functions (91–97). At present, numerous laboratories are engaged in single-cell sequencing efforts focusing on neutrophil heterogeneity, polarization, and lineage determination.

Beyond neutrophils, inflamed tissue can contain **mast cells, eosinophils, or basophils,** but little is known of the possible role(s) for these cells in cancer progression and the surrounding microenvironment. Similar to monocytes and macrophages, these cells can produce various angiogenic and lymphangiogenic factors important for tumor development and metastasis, chronic inflammation, and tissue injury and remodeling (98, 99). Mast cells are the stromal components of the inflammatory microenvironment and secrete a myriad of protumorigenic and antitumorigenic molecules depending on the environment, the tumor type, or their peritumoral or intratumoral localization (100). Eosinophils and basophils can also have protumorigenic or antitumorigenic roles, depending on their modulatory and regulatory functions to other immune cells in the TME or to their cytotoxic effects against tumor cells (101–103). Increasing evidence suggests that neutrophils, mast cells, eosinophils, or basophils can be potential therapeutic targets in different types of tumors (3, 100). However, there are still many unanswered questions that should be addressed before we understand their exact function in tumor progression and design accurate strategies for targeting them.

Both monocytes and neutrophils are often referred to as "myeloid-derived suppressor cells" or MDSC, a name given based on data suggesting a pro-tumoral, immune suppressive function when cultured with T-cells. For clarity purposes, we will refer to all myeloid cells in this review based on their individual population name.

THERAPEUTIC TARGETING OF MYELOID POPULATIONS IN THE TME

Given that there are populations of myeloid cells within the TME that impede productive anti-tumor immunity, it is of great interest to target myeloid populations that block anti-tumor immunity antagonistically, or to activate stimulatory cells that can help promote anti-tumor immunity. In this review we discuss the notion of "myeloid tuning," which broadly involves the use of therapeutic compounds to change the **composition of myeloid cells** in the TME or to alter **their functional attributes**. **Figure 2** describes the six myeloid tuning strategies and highlights the myeloid targets known to inhibit recruitment, block survival, affect proliferation, induce immune activation, alter differentiation, and stimulate reprogramming of myeloid cells in the TME (**Figure 2** and **Table 1**). Multiple recent reviews have described various strategies to target the myeloid cells in the TME (3, 25, 29, 30, 38, 104–106). Here we aim to focus on the ongoing clinical trials of agents targeting the TME myeloid cell populations that are showing early therapeutic promise, placing them within the "myeloid tuning" mechanisms-of-action framework.

Targets and Therapies That Alter TME Myeloid Population Composition by Altering Cell Recruitment, Proliferation, and Survival

Altering the recruitment of specific subsets of myeloid cells to the tumor, or modulating their proliferation or survival is viewed as a promising approach to promote durable anti-tumor responses either as single agent therapies or in combination with currently available cancer therapies. Many of the myeloid protein targets that are being pursued therapeutically to alter TME myeloid composition (**Figure 2**) vary in their specificity or lack thereof for specific subsets, and are discussed below.

CCL2-CCR2 Axis

The chemokine CCL2 and its receptor CCR2 are critical for attracting monocytes into tissues. CCR2 inhibition retains monocytes in the bone marrow and reduces the number of TAMs in tumors, leading to decreased tumor burden and metastasis in different tumor indications (107–114). Previous reviews have described different strategies to prevent CCL2-mediated recruitment of myeloid cells and elucidated the

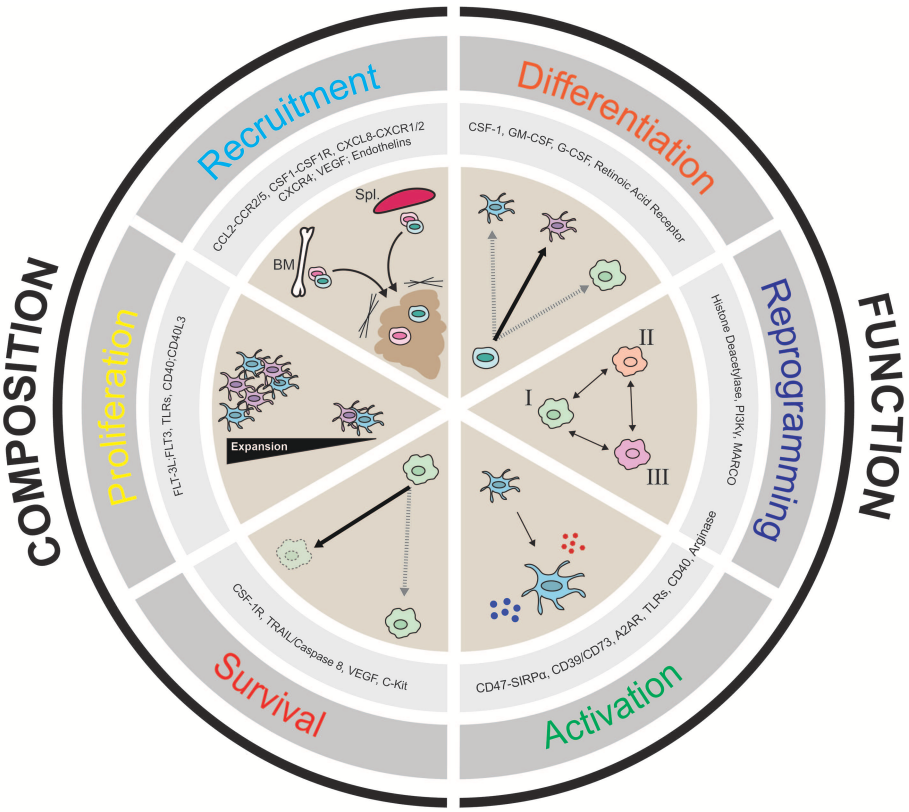


FIGURE 2 | Cartoon depicting the six **"Pillars of Myeloid Tuning"** and the myeloid targets within each category. The myeloid tuning strategies affecting the **Composition** of the TME include targets modulating Recruitment (CCL2-CCR2/5, CSF1-CSF1R, CXCL8-CXCR1/2; CXCL12-CXCR4, VEGF-VEGFR, Endothelins), Proliferation (FLT3L-FLT3, TLRs, CD40-CD40L), and Survival (CSF1R, TRAIL/Caspase 8, VEGF, c-kit). The myeloid tuning strategies altering the **Function** of the TME include targets inducing Differentiation (CSF1, GM-CSF, G-CSF, Retinoic Acid Receptor ATRA), Reprogramming (Histone Deacetylase, CSF1R, MARCO, Arginase, PI3Kγ), and Activation (CD47-SIRPα, A2AR, CD73/CD39, STING, TLRs, CD40, Arginase). The indicated myeloid targets in each category are not comprehensive.

pharmacological difficulties in safely and efficiently blocking this CCL2/CCR2 axis (29, 30, 115–118). Multiple experimental agents targeting the CCL2/CCR2 axis also showed limited efficacy in the clinic, and the clinical testing of some of these agents were recently discontinued (e.g., Carlumab from Centocor/J&J, Plozalizumab from Millenium Pharamceuticals, and PF-04136309 from Pfizer). The limited therapeutic efficacy of the Carlumab was attributed to the profound accumulation of total CCL2 in the periphery due to high chemokine synthesis rate and the significant discrepancy between the *in vitro* and *in vivo* K_D values (119, 120). The limited efficacy and lack of durable responses of these agents could in part be linked to the rapid compensation by granulocytes, the lack of effect on tissue resident macrophages, and the rebound in monocyte recruitment after treatment cessation as seen in pre-clinical models (37, 121, 122). The anti-CCR2 mAb Plozalizumab was terminated in a Phase I trial in malignant melanoma (NCT02723006) due to a classified business decision in May 2018. PF-04136309, a small molecule antagonizing CCR2, was used in combination with FOLFIRINOX in a Phase Ib study in resectable pancreatic ductal carcinoma (NCT01413022). Treatment related toxicities of grade ≥3 adverse events were seen in ≥10% of patients treated with both therapies, which included neutropenia, lymphopenia, hypokalemia, and diarrhea (123). Another clinical trial in metastatic pancreatic patients using PF-04136309 in combination with nab-paclitaxel and gemcitabine was terminated in May 2017 (NCT02732938) reported by the sponsor as due to a change in portfolio strategy without commenting on either safety or efficacy signals. Previously, it had been reported that in 21 enrolled patients, the drug had encouraging safety, PK, and efficacy profiles (124).

NOX-E36, an Emapticap pegol RNA Aptamer that targets CCL2 showed an acceptable clinical safety profile in type II diabetes patients and decreased the CCR2+ monocytes blood count as expected [NCT01547897; (125)]. NOX-E36 therapy in a mouse tumor model inhibited the infiltration of tumor-associated macrophages leading to significant changes of the TME and a reduction in liver tumor burden (126). The small molecule inhibitor CCX-872, which targets CCR2, is currently in the clinic for the treatment of patients with advanced and metastatic pancreatic cancer (NCT02345408), and data from the ongoing Phase Ib trial demonstrated promising safety and overall survival with the CCX872 and FOLFIRINOX combination therapy compared to FOLFIRINOX alone (127, 128).

TABLE 1 | Summary of ongoing clinical trials with agents that target myeloid cells.

Target	Myeloid target cells	Drug name	Drug class	Sponsor	Indications	Clinical trials
CCL2	Chemokine for Monocytes and other immune cells	NOX-E36	PEG-Aptamer	Noxxon Pharma AG	NSCLC and PDAC	Phase Ib/II Planned
CCR2 CCR5	Monocytes Macrophages, DCs, T-cells	BMS-813160	SM	Bristol-Myers Squibb Co	Advanced Solid Tumors	Phase Ib/II, NCT03184870
CCR2	Monocytes Macrophages, DCs, T-cells	CCX872-B	SM	ChemoCentryx Inc	Metastatic Adenocarcinoma of the Pancreas	Phase Ib; NCT02345408
CXCR2	Neutrophils, Mast cells, Macrophages, Monocytes	SX-682	SM	Synthrix Biosystems Inc	Metastatic Melanoma	Phase I; NCT03161431
CXCL8	Chemokine for neutrophils and monocytes	BMS-986253	SM	Bristol-Myers Squibb Co	Hormone sensitive prostate cancer	Phase Ib/II; NCT03689699
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	PLX-3397	SM	Plexxikonn Inc	Tenosynovial Giant Cell Tumor	Phase III NCT02371369
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	PLX-3397	SM	Plexxikonn Inc	Solid Tumors GBM and Gliosarcoma Refractory Leukemias and Refractory Solid Tumors KIT-mutated Melanoma Metastatic Breast Cancer	Phase I; NCT01004861 Phase I/II; NCT02777710 Phase I/II; NCT01790503 Phase I/II; NCT02390752 Phase I/II; NCT02975700 Phase Ib/II; NCT01596751
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	ARRY-382	SM	Array BioPharma Inc	Solid Tumors	Phase Ib/II NCT02880371
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	Cabiralizumab	mAb	Bristol-Myers Squibb Co	Advanced Pancreatic Cancer Stage IV Pancreatic Cancer Resectable Biliary Tract Cancer	Phase II; NCT03336216 Phase II; NCT03697564 Phase II; NCT03768531
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	Cabiralizumab	mAb	Bristol-Myers Squibb Co	Advanced Melanoma, NSCLC, and RCC Tenosynovial Giant Cell Tumor Selected advanced cancers	Phase I; NCT03502330 Phase I/II; NCT02471716 Phase I; NCT02526017
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	BLZ-945	SM	Novartis AG	Advanced Solid Tumors	Phase I/II; NCT02829723
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	LY-3022855	mAb	Eli Lilly and Co	Melanoma Pancreatic Cancer	Phase I/II; NCT03101254 Phase I; NCT03153410
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, epithelial cells)	Emactuzumab	mAb	F. Hoffmann-La Roche Ltd	Advanced Solid Tumors Platinum-Resistant Ovarian Cancer	Phase I; NCT02323191 Phase II; NCT02923739
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	AMG-820	mAb	Amgen	Pancreatic Cancer, Colorectal Cancer, NSCLC	Phase Ib/II; NCT02713529
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	DCC-3014	SM	Deciphera Pharmaceuticals LLC	Hematological Tumors; Solid Tumors	Phase I; NCT03069469
CSF1R	Monocytes, Macrophages, DCs (also on microglia, Paneth cells, Ostecolasts, Epithelial cells)	SNDX-6352	SM	Syndax Pharmaceuticals Inc	Solid Tumors	Phase I; NCT03238027
M-CSF	Growth factor for monocytes, macrophages, and other cells	Lacnotuzumab	mAb	Novartis AG	Advanced Malignancies	Phase Ib/II; NCT02807844

(Continued)

TABLE 1 | Continued

Target	Myeloid target cells	Drug name	Drug class	Sponsor	Indications	Clinical trials
M-CSF	Growth factor for monocytes, macrophages, and other cells	PD-0360324	mAb	Pfizer Inc	Platinum-Resistant Epithelial Ovarian Cancer	Phase II; NCT02948101
CD47	Tumor Cells, Red Blood Cells	Hu-5F9G4	mAb	Forty-Seven Inc	Hematological Malignancies Relapsed/Refractory B-cell Non-Hodgkin's Lymphoma Ovarian Cancer Solid Tumors and Advanced Colorectal Cancer	Phase I; NCT03248479 Phase Ib/II; NCT02953509 Phase I; NCT03558139 Phase Ib/II; NCT02953782
CD47	Tumor Cells, Red Blood Cells	IBI-188	mAb	Innovent Biologics Inc	Advanced Malignant Tumors and Lymphoma Advanced Malignancies	Phase I: NCT03763149 Phase I: NCT03717103
CD47	Tumor Cells, Red Blood Cells	CC-90002	mAb	Celgene Corp	Advanced Solid and Hematologic Cancers	Phase I; NCT02367196
CD47	Tumor Cells, Red Blood Cells	SRF-231	mAb	Surface Oncology Inc	Advanced Solid and Hematologic Cancers	Phase I; NCT03512340
SIRPα	Macrophages, DCs	ALX-148	Fusion protein	ALX Oncology Inc	Advanced Solid Tumors and Lymphoma	Phase I; NCT03013218
SIRPα	Macrophages, DCs	TTI-621	Fusion protein	Trillium Therapeutics Inc	Hematologic Malignancies and Selected Solid Tumors Relapsed and Refractory Solid Tumors	Phase I; NCT02663518 Phase I; NCT02890368
SIRPα	Macrophages, DCs	TTI-622	Fusion protein	Trillium Therapeutics Inc	Relapsed or Refractory Lymphoma or Myeloma	Phase I; NCT03530683
PI3Kγ	Macrophages, neutrophils, eosinophils. Mast cells	IPI-549	SM	Infinity Pharmaceuticals Inc	Advanced Solid Tumors Advanced HPV+ and HPV- HNSCC	Phase I; NCT02637531 Phase II; NCT03795610
A2AR	T-cells, monocytes, macrophages, DCs, NKs	CPI-444	SM	Corvus Pharma	Advanced Cancers	Phase I; NCT02655822
A2AR	T-cells, monocytes, macrophages, DCs, NKs	PBF-509	SM	Novartis AG	Advanced NSCLC	Phase I; NCT02403193
A2AR	T-cells, monocytes, macrophages, DCs, NKs	AB-928	SM	Arcus Biosciences Inc	Advanced Malignancies Gastrointestinal Malignancies TNBC and Gynecologic Malignancies Lung Cancer	Phase I; NCT03629756 Phase I; NCT03720678 Phase I; NCT03719326 Phase I; NCT03846310
CD73	Ectonucleotidase in the TME	MEDI-9447	mAb	MedImmune LLC	Advanced EGFRm NSCLC Relapsed Ovarian Cancer Metastatic Triple-Negative Breast Cancer Metastatic Pancreatic Cancer	Phase Ib/II; NCT03381274 Phase II; NCT03267589 Phase I/II; NCT03616886 Phase Ib/II; NCT03611556
CD73	Ectonucleotidase in the TME	CPI-006	mAb	Corvus Pharma	Advanced Cancers	Phase I; NCT03454451
CD73	Ectonucleotidase in the TME	BMS-986179	mAb	Bristol-Myers Squibb Co	Advanced Solid Tumors	Phase I/IIa; NCT02754141
CD73	Ectonucleotidase in the TME	AB-680	SM	Arcus Biosciences Inc	Healthy Volunteers	Phase I; NCT03677973
CD73	Ectonucleotidase in the TME	NZV-930	mAb	Novartis AG	Advanced Malignancies	Phase I; NCT03549000
Arginase	Macrophages, Neutrophils	CB-1158	SM	Calithera/Incyte Corp	Advanced and Metastatic Solid Tumors Relapsed or Refractory Multiple Myeloma	Phase I/II; NCT02903914, NCT03314935 Phase I/II; NCT003837509
Arginase	Macrophages, Neutrophils	AEB-1102	Rec Enzyme	Aeglea Biotherapeutics	Advanced Solid Tumors Extensive Disease SCLC	Phase I; NCT02561234 Phase I/II; NCT03371979
Arginase	Macrophages, Neutrophils	ARG1-18	Vaccine	Herlev Hospital	Metastatic Solid Tumors	Phase I; NCT03689192
TLR3	DCs, Macrophages, T-cells	Rintatolimod	Oligonucleotide	Hemispherx Biopharma Inc	Recurrent Ovarian Cancer Metastatic Colorectal Cancer Peritoneal Surface Malignancies Metastatic TNBC	Phase II; NCT03734692 Phase I; NCT03403634 Phase I/II; NCT02151448 Phase I; NCT03599453
TLR4	Macrophages, Monocytes, Granulocytes, DCs	G100	Rec Adenovirus	Immune Design Corp	Follicular Non-Hodgkin's Lymphoma Cutaneous T-cell Lymphoma	Phase I/II; NCT02501473 Phase II; NCT03742804

(Continued)

TABLE 1 | Continued

Target	Myeloid target cells	Drug name	Drug class	Sponsor	Indications	Clinical trials
TLR4	Macrophages, Monocytes, Granulocytes, DCs	GSK-091	SM	GlaxoSmithKline Plc	Advanced Solid Tumors	Phase I; NCT03447314
TLR4	Macrophages, Monocytes, Granulocytes, DCs	ECI-006	Oligonucleotide	eTheRNA Immunotherapies	Metastatic Melanoma	Phase I; NCT03394937
TLR5	Macrophages, Monocytes, DCs, T-cells, Intestinal Epithelial cells	M-VM3	Vaccine	Panacela Labs Inc	Prostate Cancer	Phase Ib; NCT02844699
TLR7	B-cells, DCs, Monocytes, Macrophages, Neutrophils	Imiquimod UGN-102	SM	UroGen Pharmaceuticals Ltd	Non-muscle Invasive Bladder Cancer (NMIBC)	Phase II; NCT03558503
TLR7 TLR8	B-cells, DCs, Monocytes, Macrophages, Neutrophils	NKTR-262	SM	Nektar Therapeutics	Advanced or Metastatic Solid Tumor Malignancies	Phase I/II; NCT03435640
TLR7 TLR8	B-cells, DCs, Monocytes, Macrophages, Neutrophils	Resiquimod R848	SM	Galderma SA	Metastatic Melanoma	Phase II; NCT00960752
TLR8	DCs, Monocytes, Macrophages, Neutrophils	Motolimod VTX-2337	SM	Celgene Corp	Recurrent, Platinum-Resistant Ovarian Cancer	Phase I/II; NCT02431559
TLR9	B-cells, T-cells, Macrophages, Monocytes, Neutrophils	Lefitolimod MGN1703	Oligonucleotide	Mologen AG	Metastatic Colorectal Cancer Advanced Solid Tumors	Phase III; NCT02077868 Phase I; NCT02668770
TLR9	B-cells, T-cells, Macrophages, Monocytes, Neutrophils	Tilsotolimod	Oligonucleotide	Idera Pharmaceuticals Inc	Solid Tumors	Phase II; NCT03865082
TLR9	B-cells, T-cells, Macrophages, Monocytes, Neutrophils	AST-008	Oligonucleotide	Exicure Inc	Advanced Solid Tumors	Phase Ib/II; NCT03684785
TLR9	B-cells, T-cells, Macrophages, Monocytes, Neutrophils	CMP-001	Oligonucleotide	Checkmate Pharmaceuticals Inc	Metastatic Colorectal Cancer Non-small Cell Lung Cancer Advanced Melanoma Melanoma with LN disease	Phase I; NCT03507699 Phase I; NCT03438318 Phase I; NCT02680184 Phase II; NCT03618641
TLR9	B-cells, T-cells, Macrophages, Monocytes, Neutrophils	SD-101	Oligonucleotide	Dynavax Technologies Corp	Relapsed or Refractory Follicular Lymphoma B-Cell Non-Hodgkin Lymphoma Advanced or Metastatic Solid Malignancies	Phase Ib/II; NCT02927964 Phase I; NCT03410901 Phase I; NCT03831295
TLR9	B-cells, T-cells, Macrophages, Monocytes, Neutrophils	DV-281	Oligonucleotide	Dynavax Technologies Corp	Non-small Cell Lung Carcinoma	Phase I; NCT03326752
DC	DCs	Poly-ICLC (Hiltonol)	Vaccine	Oncovir Inc	MRP Colon Cancer Unresectable Solid Cancers Recurrent Pediatric Gliomas Solid Cancer Prostate Cancer	Phase I/II; NCT02834052 Phase I/II; NCT03721679 Phase II; NCT01188096 Phase II; NCT02423863 Phase I; NCT0362103
FLT3L	DC Progenitors, pDCs, cDCs	rhuFlt3L/(CDX-301)	Rec protein	Celldex Therapeutics	Low Grade B-Cell Lymphomas Advanced NSCLC	Phase I/I; NCT01976585 Phase II; NCT02839265
STING	T-cells, NK cells, DCs, Monocytes, Macrophages	MK-1454	SM	Merck & Co Inc	Advanced/Metastatic Solid Tumors and Lymphomas	Phase I; NCT03010176
STING	T-cells, NK cells, DCs, Monocytes, Macrophages	ADU-S100 (MIW815)	SM	Aduro BioTech Inc	Advanced/Metastatic Solid Tumors and Lymphomas	Phase I; NCT02675439 NCT03172936
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	APX-005M	mAb	Apexigen Inc	Solid Tumors Advanced Sarcomas Metastatic Melanoma Pediatric CNS Tumors Metastatic Pancreatic Cancer	Phase I; NCT02482168 Phase II; NCT03719430 Phase I/II; NCT02706353 Phase I; NCT03389802 Phase Ib/II; NCT03214250
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	Selicrelumab	mAb	F. Hoffmann-La Roche Ltd	Advanced/Metastatic Solid Tumors	Phase I; NCT02665416, NCT02304393
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	ABBV-927	mAb	AbbVie Inc	Advanced Solid Tumors Advanced Head and Neck Cancer	Phase I; NCT02988960 Phase I; NCT03818542
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	MEDI-5083	Fusion protein	MedImmune LLC	Advanced Solid Tumors	Phase I; NCT03089645

(Continued)

TABLE 1 | Continued

Target	Myeloid target cells	Drug name	Drug class	Sponsor	Indications	Clinical trials
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	SEA-CD40	mAb	Seattle Genetics Inc	Advanced Malignancies	Phase I; NCT02376699
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	JNJ-7107 (ADC-1013)	mAb	Johnson & Johnson	Advanced Stage Solid Tumors	Phase I; NCT02829099
CD40	DCs, Macrophages, Monocytes, B-cells, Endothelial Cells, Tumor Cells	CDX-1140	mAb	Celldex Therapeutics Inc	Advanced Malignancies	Phase I; NCT03329950

CCL5-CCR5 Axis

Notably, alternative recruitment of monocytes can be achieved via the CCL5-CCR5 axis (129) and inhibiting that axis also restricted cancer growth in colorectal cancer (130) and blocked metastasis of basal breast cancer cells. A dual small molecule inhibitor, BMS-813160, that targets both CCR2 and CCR5, is being tested in patients with advanced pancreatic cancer in combination with Nivolumab and Gemcitabine and Nab-paclitaxel (NCT03184870).

Emerging data suggest that tumor-produced **IL-8** (CXCL8) plays an important role in recruiting neutrophils and monocytes into the TME of many cancer types (131). Neutralization of IL-8 by the mAb HuMax-IL8 in TNBC decreases the recruitment of neutrophils (also referred to as PMN-MDSCs) to the tumor site and facilitates immune-mediated killing (132). The IL-8 inhibitor BMS-986253 is being tested in a Phase Ib/II trial in combination with Nivolumab in hormone-sensitive prostate cancer [NCT03689699; (133)].

CSF1-CSF1R Axis

The CSF1/CSF1R axis plays a key role in the differentiation, recruitment, proliferation, and survival of both monocytes and macrophages (134). Multiple inhibitors of the CSF1/CSF1R axis are being clinically developed, and these inhibitors have been extensively reviewed (29, 135–137). The most advanced agent in clinical testing is the small molecule selective kinase inhibitor Pexidartinib (PLX-3397), which is being tested in a Phase III trial in Tenosynovial Giant Cell Tumors (TGCT; NCT02371369). Pexidartinib demonstrated efficacy in TGCT (136, 138, 139). TGCT is driven by the translocation of chromosome 1 and 2 (1p13 to 2q35), which leads to the overexpression of CSF1 caused by the fusion of *CSF1* to *COL6A3* (140). Pexidartinib is also being investigated for the treatment of various solid tumors, such as metastatic breast cancer, advanced ovarian cancer, colorectal cancer, and pancreatic cancer, in combination with chemotherapy or ICBs (**Table 1**). In pre-clinical models, PLX-3397 increased the efficacy of anti-PD-1 or chemotherapy treatments (141, 142). While PLX-3397 is a CSF1R inhibitor, it also targets the c-kit and FLT3 receptor tyrosine kinases (RTKs), which are expressed on other myeloid populations including mast cells and dendritic cells. Two other small molecules CSF1R inhibitors are in development, BLZ-945 and ARRY-382. BLZ-945 is currently in Phase I/II trails for patients with advanced solid tumors (NCT02829723). In preclinical studies, BLZ-945 was shown to repolarize TAMs to become antitumoral in mouse models of glioblastoma by downregulating genes that have been associated with an M2-like macrophage polarization phenotype (143), and to decrease tumor progression as monotherapy and in combination with ICBs in a mouse model of neuroblastoma (144). ARRY-382 is also being tested in Phase I/II in patients with advanced solid tumors (NCT02880371). Preliminary clinical data demonstrated partial responses with a manageable tolerability profile (145).

The anti-CSF1R mAb, Cabiralizumab blocks the activation and survival of monocytes and macrophages by inhibiting the binding of the two ligands CSF1 and IL-34 to CSF1R (146, 147). Cabiralizumab is being tested in a Phase I clinical trial in advanced solid tumors (NCT02526017), in a Phase II trial in advanced pancreatic cancer (NCT03336216), and in TGCT (NCT02471716). Preliminary data suggests tolerable safety profiles in combination with Nivolumab and durable clinical benefits in heavily pretreated patients with pancreatic cancer (148). Recent data showed that treatment with Cabiralizumab and Nivolumab depletes immunosuppressive TAMs and promotes a pro-inflammatory TME (149). For instance, tumors from treated patients had a decrease in CSF1R+ macrophages, an increase in CD8+ T cells, and an increase in pro-inflammatory genes. Moreover, these patients had increased levels of CSF1/IL-34 and decreased levels non-classical monocytes in the periphery (149). In addition, Cabiralizumab demonstrated initial clinical benefits in patients with Pigmented Villonodular Synovitis (150). In addition to Cabiralizumab, several other antagonistic anti-CSF1R mAbs are in clinical development (see **Table 1**). AMG-820 (a fully human IgG2 targeting CSF1R) resulted in a 32% stable disease in a Phase II study (NCT01444404) in patients with relapsed or refractory advanced solid tumors and induced adverse effects including periorbital edema, increased aspartate aminotransferase, fatigue, nausea, blurred vision, and deafness (151). AMG-820 is also being tested in combination with pembrolizumab in patients with pancreatic, NSCLC, and colorectal cancer (NCT02713529). LY3022855 (a humanized IgG1 targeting CSF1R) is being tested in a Phase I/II trial in patients with metastatic melanoma in combination with BRAF/MEK inhibitors (NCT03101254). Emactuzumab is a mAb that blocks CSF1R dimerization, and demonstrated a 7% complete response rate in a Phase I

trial with TCGT patients and had no reported dose toxicity (152). Targeting the CSF1R ligand CSF1 has also proven to be a promising strategy. Two mAbs developed by Novartis (Lacnotuzumab) and Pfizer (PD-0360324) are currently in the clinic. Recent data from Lacnotuzumab (MCS110)'s Phase Ib clinical trial in advanced malignancies showed it is tolerated and has preliminary antitumor activity, especially in the pancreatic cancer cohort. However, grade 3 suspected drug-related adverse effects were observed and included periorbital edema, increased blood creatine phosphokinase, and increased aspartate aminotransferase (AST) (153).

While targeting the CSF1/CSF1R axis has shown clinical promise, novel resistance, and compensatory mechanisms could emerge. For instance, acquired and inherent resistance to CSF1R blockade has been reported in pre-clinical mouse models of glioblastoma multiforme and other cancer types harboring specific genetic alterations (105). Moreover, a recent study identified a compensation between CSF1R+ macrophages and Foxp3+ regulatory T-cells (Tregs) that can drive resistance to immunotherapy in a mouse model of colorectal cancer (154). In addition, the common side effects observed in most of the CSF1/CSF1R antagonistic small molecules and mAbs developed could be caused by the systemic depletion of tissue resident macrophages in normal tissues. In addition to targeting the CSF1/CSF1R axis to reduce tumor associated myeloid cells there are a number of additional agents, including trabectidin (Yondelis®), lurbinectedin, and the bisphosphonates clodronate and zoledronic acid (3, 29, 116). While there are multiple ongoing clinical trials to evaluate bisphosphonates, there is no available data regarding their anti-tumor activity. Therefore, finding new targets that are selectively upregulated in the TAMs and tumor-associated monocytes is crucial and might lead to more clinical benefits with fewer side effects.

Targets and Therapies That Alter TME Myeloid Population Function by Altering Cell Activation, Reprograming, and Differentiation

Altering the activation status of pro-tumorigenic myeloid cells to inhibit their immunosuppressive activity (reversal of immunosuppression) or altering pro-tumorigenic myeloid cells by differentiating or reprograming them to become anti-tumorigenic is viewed as another promising approach to promote durable anti-tumor responses either as single agent therapies or in combination with currently available cancer therapies. Another approach to alter the TME myeloid population function is to induce activation of anti-tumorigenic myeloid cells such as DCs. Many of the myeloid protein targets that are being therapeutically pursued to alter TME myeloid function are shown in **Figure 2** and listed in **Table 1** and are discussed below.

CD47-SIRPα Regulation of Phagocytosis

The CD47-SIRPα axis is a myeloid specific ICB that inhibits phagocytosis of tumor cells by macrophages and other myeloid cells (155). The "don't eat me signal" CD47 is overexpressed on the majority of hematopoietic malignancies and solid tumors and is also expressed on red blood cells (156–158). CD47 binds its ligand SIRPα, a RTK expressed on the cell surface of macrophages and dendritic cells and associates with the downstream inhibitory tyrosine phosphatases SHP-1 and SHP-2 (159). Recent review articles present the various clinical strategies to enhance phagocytosis by targeting the CD47-SIRPα axis (116, 155, 160, 161), and discuss the limitations and potential toxicities of targeting this axis (116, 160). The antagonistic anti-CD47 mAb Hu-5F9G4 induces phagocytosis of tumor cells by blocking the CD47-SIRPα interaction (162). Hu-5F9G4 was evaluated in a Phase Ib dose escalation study in patients with relapsed/refractory non-Hodgkin lymphoma, follicular lymphoma and diffuse large B-cell lymphoma (DLBCL) in combination with rituximab (NCT02953509). In this small study of 22 subjects (with 21/22 known to be refractory to single agent rituximab), anti-tumor responses were observed in 50% of subjects (including 36% with complete response). Hu-5F9G4 in combination with rituximab at standard rituximab doses was generally safe and a maximum tolerated dose of the antibody was not declared (163). The main on- target side effect was anemia, which could be mitigated and managed by initially "priming" subjects with a 1 mg/kg dose of Hu-5F9G4 to eliminate aging red cells prior to introducing therapeutic intent dosing. Dose limiting toxicities not requiring treatment discontinuation were reported in two subjects (pulmonary embolism and grade 4 neutropenia requiring G-CSF) (163). A third subject developed idiopathic thrombocytopenic purpura treated with glucocorticoids and immune globulin and required treatment discontinuation. Hu-5F9G4 is also being evaluated in patients with solid tumors (NCT02216409), and acute myeloid leukemia (AML) (NCT02678338) with and without azacitidine (NCT03248479). Recent data has shown this combination therapy leads to an increase in phagocytosis of AML blast cells by human macrophages *in vitro* and clearance of AML *in vivo*, leading to a prolonged survival compared to Hu-5F9G4 or azacitidine alone (164). Since anemia and neutropenia have been a concern for anti-CD47 therapies (165), strategies for better priming and maintenance doses are crucial. To this point, studies demonstrated that an initial priming dose of Hu-5F9G4 resulted in a near complete loss of CD47 antigen only on RBCs and not on white blood cells and AML bone marrow blasts, suggesting that CD47 pruning (loss) is protective for RBCs and could decrease the potential for toxicities (166). Hu-5F9G4 is also being evaluated in combination with Cetuximab in patients with solid tumors and advanced colorectal cancer in a Phase I/II study (NCT02953782). Clinical trials for another anti-CD47 mAb, CC90002, was recently terminated in patients with AML for unspecified hematologic toxicities described as reversible (NCT02641002), but it is still being tested in a Phase I dose escalation study in patients with other hematological cancers and advanced/refractory solid tumors followed by combination treatment with Rituximab (NCT02367196).

TTI-621, a SIRPα-Fc (human IgG1 Fc) fusion protein that blocks the CD47-SIRPα interaction, is being tested in patients with hematologic malignancies and solid tumors (NCT02663518), while TTI-622 (a SIRPα-Fc (human IgG4 Fc)

fusion protein is being evaluated in a Phase I clinical trial in relapsed or refractory lymphoma or myeloma (NCT03530683). Currently it is unknown whether the SIRPα fusion proteins will have better efficacy and/or a better tolerability profile compared to the anti-CD47 mAb therapies.

Immunosuppressive Adenosine Signaling

Other strategies to activate the myeloid cells in the TME include the inhibition of their immunosuppressive functions, such as blocking the arginase, CD39/CD73 ectonucleotidases, and the adenosine A2A receptor (A2AR) pathways. Toward the latter, the extracellular adenosine concentrations and downstream signaling via the A2AR pathway has been shown to create a highly immunosuppressive microenvironment by significantly decreasing the immune responses in inflamed tissues and tumors (167–169). Many companies are developing mAbs and small molecules against these targets and some are being evaluated in the clinic (**Table 1**), and were recently reviewed (169). CPI-444 is a small molecule inhibitor targeting A2AR and is being evaluated in a Phase I trial in patients with advanced cancers (NCT02403193). Recent data from patients with refractory renal cell carcinoma showed that CPI-444 was well tolerated and prolonged survival as monotherapy and in combination with Atezolizumab (170). In addition, the expression of a novel adenosine biomarker signature in pre-treated tumor biopsies was significantly associated with tumor response to CPI-444 (171–173).

CD73 is the ectonucleotidase that catalyzes the irreversible conversion of AMP to adenosine, leading to the high levels of adenosine observed in the TME (174, 175). The monoclonal antibody MEDI-9447 (Oleclumab) antagonizes the enzymatic activity of CD73 through two distinct conformation-mediated mechanisms, which allows it to block both soluble and cell-surface CD73 in a non-competitive manner (176). MEDI-9477 can mediate changes in the infiltrating lymphoid and myeloid populations in the TME of mouse models, such as activation of macrophages and increasing CD8+ effector cells (177). In advanced pancreatic or colorectal cancer patients treated with Oleclumab (NCT0253774), free soluble CD73 and CD73 bound on peripheral T-cells were decreased across all doses and patients, and tumoral CD73 expression was also decreased (178). Oleclumab monotherapy and in combination with durvalumab showed manageable safety profile and encouraging clinical activity in colorectal and pancreatic cancer patients [NCT0253774; (178)]. While the adenosine pathway may be a key immunoregulatory node, we have to be prudently cautious about targeting specific members of the pathway without taking into account the biochemical pathway redundancies and feedback mechanisms that counter-regulate them.

TLRs and CD40 Agonists

Toll like receptors play important roles in the activation of the innate immune response and have been pivotal targets in cancer immunotherapy. They can selectively activate a subset of DC and macrophage populations to take on stimulatory and pro-inflammatory phenotypes (179–181). TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 agonists are being clinically evaluated (**Table 1**). The TLR7 agonist Imiquimod (topical cream) was approved for the treatment of basal cell carcinoma and showed additional efficacy in breast cancer skin metastases and melanoma. Imiquimod is believed to stimulate cytokine production, increase the infiltration of macrophages, DCs, and lymphocytes, and directly induce apoptosis in the tumor cells (182). Urogen Pharmaceuticals developed imiquimod (UGN-201) in a reverse thermal hydrogel formulated with the chemotherapeutic agent Mitomycin C (MMC), which is being evaluated in a Phase II trial in patients with low grade non-muscle invasive bladder cancer (NCT03558503). G100 is an intratumoral TLR4 agonist (composed of glucopranosyl lipid A in stable emulsion) that creates a systemic immune response when injected locally as a vaccine. G100 has been evaluated in multiple clinical trials and data from Phase I (NCT02501473) showed that it is well tolerated with clinical activity as a monotherapy and in combination with the anti-PD-1 antibody Pembrolizumab (183, 184). In addition, patients with TLR4 expression at baseline had a significant improved overall response rate (185). In a proof-of-concept trial in Merkel cell carcinoma patients (NCT02035657), intratumoral G100 induced anti-tumor immune responses leading to tumor regression without systemic toxicities (186). Based on encouraging results from a small early phase data, advanced trials are ongoing with intralesional SD-101 (a class c CpG Oligonucleotide TLR9 agonist) in combination with Pembrolizumab (187). The most advanced TLR9 agonist in the clinic is Lefitolimod (MGN1703), which is a synthetic DNA-based agonist that results in an antitumor immunomodulation, including increased release of cytokines and chemokines from peripheral blood mononuclear cells (PBMCs) and an increase in expression of surface activation markers of cells on a variety of immune cells (188–190). Lefitolimod is being evaluated in a pivotal Phase III trial of first-line maintenance in 549 enrolled patients with metastatic colorectal cancer (NCT02099868), following promising data in previous Phase I and II trials, where MGN1703 showed therapeutic efficacy in multiple solid tumors and was well-tolerated in long-term treatment with high doses (191–193).

The TNFR family member CD40 is expressed on the vast majority of myeloid cells such as DCs, macrophages, monocytes, and is also expressed on B-cells, tumor cells, and endothelial cells. Signals transduced by CD40 result in upregulation of multiple proteins critical to effector T-cell priming, including immunostimulatory cytokines, major histocompatibility (MHC) molecules, and the co-stimulatory ligands CD80 and CD86 (194–196). Multiple CD40 agonists have been developed to activate innate and adaptive immunity and some are being evaluated in the clinic [(197), **Table 1**]. APX-005M is the most advanced CD40 agonist in the clinic and is being tested in a Phase II trial in patients with advanced sarcomas (NCT03719430) and in Phase I/II in patients with metastatic melanoma [NCT02706353; (198)] and metastatic pancreatic cancer (NCT03214250). Recent preliminary data from the Phase Ib clinical trial in previously untreated metastatic pancreatic cancer showed that 20 out of the 24 patients had tumor shrinkage when treated with standard of care chemotherapy with and without Nivolumab. However, toxicity was a key concern as 13 out of the 24 patients

experienced adverse effects and had to discontinue the treatment combinations (199). This trial has now progressed to the Phase II stage (NCT03214250).

Reprogramming Targets

Targets that are considered likely to induce switching in TAMs from a pro-tumoral to a tumoricidal state include class I and class II histone deacetylases (200–202), the macrophage receptor with collagenous structure MARCO (203), CD11b (204), and PI3Kγ (205, 206). Within these, PI3Kγ is a key regulator of the pro-tumoral and immunosuppressive state of TAMs and its genetic and pharmacological inhibition switches the TAMs to a pro-inflammatory state and subsequent tumor growth inhibition (205, 206). The selective small molecule PI3Kγ inhibitor IPI-549 was evaluated in a Phase I/Ib clinical trial in 220 patients with advanced solid tumors as monotherapy and in combination with Nivolumab (NCT02637531). IPI-549 was shown to be well-tolerated at all the doses tested and showed 40 percent disease control and durable partial responses in patients with indications not typically responsive to anti-PD1 therapy (207, 208). Data from peripheral blood from IPI-549 treated patients showed upregulation of IFN-gamma responsive factors and an increase in proliferation of exhausted memory T-cells (207, 208). In addition, paired tumor biopsies from monotherapy IPI-549 treated patients showed a decrease in CD163, sometimes called an "M2" macrophage marker (208), consistent with the mechanism-of-action in the pre-clinical studies of IPI-549 inducing immune activation and reducing immune suppression (205).

Many of the above discussed targets and drugs used in the clinic are not specific to specific subpopulations of myeloid cells and might be contributing to some of the side effects and toxicities discussed above. In order to identify novel targets specifically expressed on unique myeloid subsets, such as macrophages, neutrophils, and DCs, sophisticated technologies need to be employed. These include single-cell RNA-sequencing (scRNA-seq) and mass cytometry, and are discussed below.

IDENTIFYING NOVEL TME MYELOID SUBPOPULATIONS

To improve the efficacy and safety of agents that target myeloid subpopulations in the TME it will likely be necessary to have a deeper understanding of the extent of the functional diversity of intratumor myeloid subpopulations. Modern, high-throughput scRNA-seq, and cytometry by time of flight (CYTOF) technologies (209) have begun to revolutionize our understanding of the TME, both in terms of intra- and inter-tumoral variability. Historically, most efforts to understand the architecture and complexity of the TME were confined to the use of bulk RNA sequencing and microarray technologies which, while providing some sample and indication level differentiation, offer little insight into the cellular composition heterogeneity of an individual tumor. Granularity of gene expression associated with various stromal, malignant, and immune cell populations as well as any heterogeneity existing within those populations is lost upon averaging across cells to yield a single transcriptional profile. A variety of cellular deconvolution methodologies (210–214) were described to attempt to recapture this heterogeneity, but they rely on the existence of specific cellular markers that possess little or no collinearity between cell types. This approach works well for the major cellular constituents of a tissue but has limited efficacy in classifying subpopulations of cells or identifying rare, novel subsets. The capacity for new scRNA-seq methods to capture tens of thousands of unique, cellular transcriptomes in a single experimental run, particularly when combined with high-throughput flow cytometric sorting as an *a priori* enrichment strategy, offers a unique and powerful window into the TME. It enables not only the measurement of relative abundances of diverse cell types, but also the relationship, substructure and differentiation processes within those cells. Single cell methodologies now exist to profile mRNA, DNA, epitope levels, methylation, transcription factor binding, chromatin accessibility, and in some cases even preserving spatial information (215). Although insights and advances driven by single cell sequencing of the intratumoral myeloid compartment are, as of yet, limited, key lessons are beginning to emerge.

While a variety of human tumor ecosystems have been profiled at single cell resolution (21, 22, 216–223) only a few contain sufficient myeloid cells to adequately address questions of subpopulation heterogeneity, lineage dynamics, or ontogeny. To date, most studies interrogating the myeloid compartment of the TME focused specifically on macrophages, as they are, by far, the most abundant cell type in that milieu. In breast cancer, a positive correlation of M1- and M2-derived gene signatures across the aggregate of multiple subclusters of TAMs was shown (22) and identified a concomitant increase of M2-markers, MARCO, NRP2, and CD276 along with CCL3, sometimes associated with antitumoral functions, across macrophage lineages derived from trajectory-based analyses (22). These findings were corroborated in a study that performed single cell profiling of human gliomas, and correlated expression profiles of the M1-marker, TNFα, and the M2-marker, IL10, as evidence that a binary model of macrophage activation may not exist *in vivo* and instead may be better examined according to a spectrum-based model (222, 224). Similarly, the application of mass cytometry in clear cell renal cell carcinoma (220) revealed 17 separate TAM clusters, across which canonical *in vivo* differentiation markers exhibited a range of expression, not the expected binary distribution. Similar to activation status, macrophage ontogeny has been sparsely examined in the context of human single cell sequencing datasets. In IDH-mutant low-grade glioma (219) researchers found a spectrum of differentiation based on gene expression between tissue resident microglia and blood-derived macrophages whereas in late stage glioma, primarily glioblastoma, the two populations of macrophages appear quite distinct (222). Utilizing the aggregation of tumor and healthy cells to classify gene signatures or gene sets that differentiate TAMs from their tissue-resident brethren in a single lung adenocarcinoma patient, TREM2, MARCO, APOE, and CD81 were shown to be specifically upregulated in TAMs, relative to alveolar macrophages (21).

Investigation into the intratumoral complement of monocytes, dendritic cells, and granulocytes is, to this point, sorely lacking from a single cell sequencing perspective. This

will undoubtedly improve, however, as cellular encapsulation technologies yield higher throughput and researchers begin to focus specifically on individual cellular populations via flow cytometric enrichment. This approach has already begun to yield dividends in the periphery, particularly in various dendritic cell populations with respect to ontogeny (46, 69) and the discovery of novel cellular subtypes (64). To this point, most single cell tumor studies have taken a macroscopic view of the tumor microenvironment: either all cells or partially enriched subsets are submitted for encapsulation and sequencing. Typically, samples from multiple patients are aggregated to generate sufficient numbers of cells to either differentiate between cell type or to provide a more global, indication-specific view of the tumor ecosystem. In this scenario, we urge researchers to also provide patient-specific analyses as the aggregation of samples homogenizes inter-patient variability in much the same way bulk sequencing homogenizes expression profiles across cell types. This issue is particularly important for human samples which, compared to tumors from in-bred mouse strains, are marked by extremely variable microenvironment composition.

Different issues arise when attempting to understand the heterogeneity within and between closely related cell types. Nearly all single cell technologies rely on the downstream identification of discrete cellular clusters. As recently reviewed by Andrew and Hemberg (225), these cluster identification algorithms range between K-means, hierarchical, graph, and density-based methods, each implemented in a variety of different ways. For divergent cell types that possess disparate functional programs, these methods generally converge. However, in the context of cells with a shared ontogeny, it can be quite difficult to arrive at a consistent pattern of clustering, particularly in light of the fact that most algorithms require *a priori* knowledge of resulting cluster number or require upfront modulation of parameters that directly dictate cluster number. In practice, this often means setting a fold-change cutoff that is reached by a set number of markers as the defining criterion for a cluster. The identification of a robust clustering of cells does not mean that those clusters have different biological and functional status. From an analytical standpoint, genes that differ between clusters may be assessed via gene set enrichment techniques to understand functional consequence and, of course, if those genes allow flow cytometric-based sorting, those populations may be compared with relevant experimental techniques.

CONCLUSION

The different myeloid tuning strategies we discuss in this review describe the various myeloid targets and agents being investigated in the clinic. Some of these agents modulate the function of myeloid populations to inhibit their immunosuppressive activities and make them more anti-tumorigenic and some agents impact recruitment and survival of myeloid subpopulations. Few myeloid targeting strategies in the clinic have yielded promising results and many have been terminated due to toxicities related to the specificity or lack of tumor specificity of the target or to the properties of the agent being used. It is too early for us to know how these agents will play out in the clinic as many of the clinical trials are still ongoing and we have to wait for the results to determine their success or failure. However, the majority of the targets being pursued are not exclusively expressed on just one population of myeloid cells but rather they can be expressed on multiple myeloid populations, and even at times on lymphocytes and tumor cells.

While the understanding of intra- and intertumoral myeloid composition is in its nascent stages, particularly in humans, single cell sequencing technology will almost assuredly serve to identify heretofore unknown cellular subsets that may yield actionable targets in the fight against cancer. Additional pre-clinical studies are needed to determine the function of those novel targets in the TME and the pathophysiological relevance of the newly identified cellular cluster subsets. Finally, a more granular understanding of the kinetics and environmental queues that drive peripheral monocyte transition to TAM phenotypes could yield upstream targets designed to prevent the development of these type of suppressive cells.

AUTHOR CONTRIBUTIONS

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

FUNDING

The authors declare no funding received from other sources except from their current employer Pionyr Immunotherapeutics. MK is supported by funding from the NIH (R01AI114787) and NCI (R01CA197363).

ACKNOWLEDGMENTS

The authors apologize to the many authors they could not cite due to space constraints. The authors thank Drs. Michel Streuli and Kevin Baker for helpful discussions and for critically reviewing the manuscript.

REFERENCES

1. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci.* (2012) 125:5591–6. doi: 10.1242/jcs.116392
2. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci.* (2010) 123:4195–200. doi: 10.1242/jcs.023820
3. Schupp J, Krebs FK, Zimmer N, Trzeciak E, Schuppan D, Tuettenberg A. Targeting myeloid cells in the tumor sustaining microenvironment. *Cell Immunol.* (2017) 2017:13. doi: 10.1016/j.cellimm.2017.10.013
4. Kenny PA, Lee GY, Bissell MJ. Targeting the tumor microenvironment. *Front Biosci.* (2007) 12:3468–74. doi: 10.3390/ijms20040840
5. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* (2012) 12:252–64. doi: 10.1038/nrc3239

6. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell.* (2015) 27:450–61. doi: 10.1016/j.ccell.2015.03.001
7. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* (1996) 271:1734–6.
8. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* (2010) 363:711. doi: 10.1056/NEJMOA1003466
9. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* (1992) 11:3887–95.
10. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) 192:1027–34. doi: 10.1084/jem.192.7.1027
11. Iwai Y, Hamanishi J, Chamoto K, Honjo T. Cancer immunotherapies targeting the PD-1 signaling pathway. *J Biomed Sci.* (2017) 24:26. doi: 10.1186/s12929-017-0329-9
12. Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol.* (2013) 14:1212–8. doi: 10.1038/ni.2762
13. Callahan MK, Postow MA, Wolchok JD. Targeting T cell co-receptors for cancer therapy. *Immunity.* (2016) 44:1069–78. doi: 10.1016/j.immuni.2016.04.023
14. Chen DS, Mellman I. Elements of cancer immunity and the cancer–immune set point. *Nature.* (2017) 541:321–30. doi: 10.1038/nature21349
15. Dempke WCM, Fenchel K, Uciechowski P, Dale SP. Second- and third-generation drugs for immuno-oncology treatment-The more the better? *Eur J Cancer.* (2017) 74:55–72. doi: 10.1016/j.ejca.2017.01.001
16. Beatty GL, Dwyer PJ, Clark J, Shi JG, Bowman KJ, Scherle PA, et al. First-in-human phase I study of the oral inhibitor of indoleamine 2,3-dioxygenase-1 epacadostat (INCB024360) in patients with advanced solid malignancies. *Clin Cancer Res.* (2017) 23:3269–76. doi: 10.1158/1078-0432.CCR-16-2272
17. Zhu MMT, Dancsok AR, Nielsen TO. Indoleamine dioxygenase inhibitors: clinical rationale and current development. *Curr Oncol Rep.* (2019) 21:2. doi: 10.1007/s11912-019-0750-1
18. Muller AJ, Manfredi MG, Zakharia Y, Prendergast GC. Inhibiting IDO pathways to treat cancer: lessons from the ECHO-301 trial and beyond. *Semin Immunopathol.* (2019) 41:41–48. doi: 10.1007/s00281-018-0702-0
19. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med.* (2018) 24:541–50. doi: 10.1038/s41591-018-0014-x
20. Barry KC, Hsu J, Broz ML, Cueto FJ, Binnewies M, Combes AJ, et al. A natural killer-dendritic cell axis defines checkpoint therapy-responsive tumor microenvironments. *Nat Med.* (2018) 24:1178–91. doi: 10.1038/s41591-018-0085-8
21. Lavin Y, Kobayashi S, Leader A, Amir ED, Elefant N, Bigenwald C, et al. Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses. *Cell.* (2017) 169:750–65.e17. doi: 10.1016/j.cell.2017.04.014
22. Azizi E, Carr AJ, Plitas G, Cornish AE, Konopacki C, Prabhakaran S, et al. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell.* (2018) 174:1293–308.e36. doi: 10.1016/j.cell.2018.05.060
23. Gubin MM, Esaulova E, Ward JP, Malkova ON, Runci D, Wong P, et al. High-dimensional analysis delineates myeloid and lymphoid compartment remodeling during successful immune-checkpoint cancer therapy. *Cell.* (2018) 175:1014–30.e19. doi: 10.1016/j.cell.2018.09.030
24. Broz ML, Krummel MF. The emerging understanding of myeloid cells as partners and targets in tumor rejection. *Cancer Immunol Res.* (2015) 3:313. doi: 10.1158/2326-6066.CIR-15-0041
25. Awad RM, De Vlaeminck Y, Maebe J, Goyvaerts C, Breckpot K. Turn back the TIMe: targeting tumor infiltrating myeloid cells to revert cancer progression. *Front Immunol.* (2018) 9:1977. doi: 10.3389/fimmu.2018.01977
26. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, et al. Critical role for CD103 + /CD141 + dendritic cells bearing CCR7 for tumor antigen trafficking and priming of T cell immunity in melanoma article critical role for CD103 + /CD141 + dendritic cells bearing CCR7 for tumor antigen trafficking and priming of T. *Cancer Cell.* (2016) 30:324–36. doi: 10.1016/j.ccell.2016.06.003
27. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, et al. Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell.* (2014) 26:638–52. doi: 10.1016/j.ccell.2014.09.007
28. Cassetta L, Kitamura T. Macrophage targeting: opening new possibilities for cancer immunotherapy. *Immunology.* (2018) 2018:12976. doi: 10.1111/imm.12976
29. Cassetta L, Pollard JW. Targeting macrophages: therapeutic approaches in cancer. *Nat Rev Drug Discov.* (2018) 17:887–904. doi: 10.1038/nrd.2018.169
30. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol.* (2019) 2019:6. doi: 10.1038/s41577-019-0127-6
31. Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, et al. NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control. *Cell.* (2018) 172:1022–37.e14. doi: 10.1016/j.cell.2018.01.004
32. Iberg CA, Jones A, Hawiger D. Dendritic cells as inducers of peripheral tolerance. *Trends Immunol.* (2017) 38:793–804. doi: 10.1016/j.it.2017.07.007
33. McGovern N, Shin A, Low G, Low D, Duan K, Yao LJ, et al. Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. *Nature.* (2017) 546:662–6. doi: 10.1038/nature22795
34. Nirschl CJ, Suá Rez-Fariñ M, Shalek AK, Troyanskaya O. IFNg-dependent tissue-immune homeostasis is co-opted in the tumor microenvironment. *Cell.* (2017) 170:127–41. doi: 10.1016/j.cell.2017.06.016
35. Binnewies M, Mujal AM, Pollack JL, Combes AJ, Hardison EA, Barry KC, et al. Unleashing type-2 dendritic cells to drive protective antitumor CD4+ T cell immunity. *Cell.* (2019) 177:556–71.e16. doi: 10.1016/j.cell.2019.02.005
36. Franklin RA, Liao W, Sarkar A, Kim M V, Bivona MR, Liu K, et al. The cellular and molecular origin of tumor-associated macrophages. *Science.* (2014) 344:921–5. doi: 10.1126/science.1252510
37. Zhu Y, Herndon JM, Sojka DK, Kim K-W, Knolhoff BL, Zuo C, et al. Tissue-resident macrophages in pancreatic ductal adenocarcinoma originate from embryonic hematopoiesis and promote tumor progression. *Immunity.* (2017) 47:323–38.e6. doi: 10.1016/j.immuni.2017.07.014
38. Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. *Cancer Cell.* (2015) 27:462–72. doi: 10.1016/j.ccell.2015.02.015
39. Zhang Q, Liu L, Gong C, Shi H, Zeng Y, Wang X, et al. Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PLoS ONE.* (2012) 7:e50946. doi: 10.1371/journal.pone.0050946
40. Engblom C, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. *Nat Rev Cancer.* (2016) 16:447–62. doi: 10.1038/nrc.2016.54
41. Bosurgi L, Cao YG, Cabeza-Cabrerizo M, Tucci A, Hughes LD, Kong Y, et al. Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells. *Science.* (2017) 356:1072–6. doi: 10.1126/science.aai8132
42. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
43. Loyher P-L, Hamon P, Laviron M, Meghraoui-Kheddar A, Goncalves E, Deng Z, et al. Macrophages of distinct origins contribute to tumor development in the lung. *J Exp Med.* (2018) 215:2536–53. doi: 10.1084/jem.20180534
44. Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, et al. Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies. *Cell Rep.* (2016) 17:2445–59. doi: 10.1016/j.celrep.2016.10.052
45. Cassetta L, Fragkogianni S, Sims AH, Swierczak A, Forrester LM, Zhang H, et al. Human tumor-associated macrophage and monocyte transcriptional landscapes reveal cancer-specific reprogramming, biomarkers, and therapeutic targets. *Cancer Cell.* (2019) 35:588–602.e10. doi: 10.1016/j.ccell.2019.02.009
46. Schlitzer A, Sivakamasundari V, Chen J, Sumatoh HR, Schreuder J, Lum J, et al. Identification of cDC1- and cDC2-committed DC progenitors reveals early lineage priming at the common DC progenitor stage in the bone marrow. *Nat Immunol.* (2015) 16:718–28. doi: 10.1038/ni.3200
47. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature

based on ontogeny. *Nat Rev Immunol.* (2014) 14:571–8. doi: 10.1038/nri3712
48. Murphy KM. Transcriptional control of dendritic cell development. *Adv Immunol.* (2016) 34:239–67. doi: 10.1016/B978-0-12-417028-5.00009-0
49. Guilliams M, Dutertre C-A, Scott CL, McGovern N, Sichien D, Chakarov S, et al. Unsupervised high-dimensional analysis aligns dendritic cells across tissues and species. *Immunity.* (2016) 45:669–84. doi: 10.1016/j.immuni.2016.08.015
50. Diao J, Zhao J, Winter E, Cattral MS. Tumors conventional dendritic cell precursors in recruitment and differentiation of. *J Immunol Ref.* (2010) 184:1261–7. doi: 10.4049/jimmunol.0903050
51. Salmon H, Idoyaga J, Rahman A, Leboeuf M, Remark R, Jordan S, et al. Expansion and activation of CD103(+) dendritic cell progenitors at the tumor site enhances tumor responses to therapeutic PD-L1 and BRAF inhibition. *Immunity.* (2016) 44:924–38. doi: 10.1016/j.immuni.2016.03.012
52. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol.* (2013) 31:563–604. doi: 10.1146/annurev-immunol-020711-074950
53. Bayne LJ, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ, et al. Tumor-derived granulocyte-macrophage colony stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. (2012) 21:822–35. doi: 10.1016/j.ccr.2012.04.025
54. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Supporting online material for Batf3 deficiency reveals a critical role for CD8α + dendritic cells in cytotoxic T cell immunity. *Publ Sci.* (2008) 322:1164206. doi: 10.1126/science.1164206
55. Miller JC, Brown BD, Shay T, Gautier EL, Jojic V, Cohain A, et al. Deciphering the transcriptional network of the DC lineage HHS public access. *Nat Immunol.* (2012) 13:888–99. doi: 10.1038/ni.2370
56. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CMT, Pryer N, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell.* (2014) 26:623–37. doi: 10.1016/j.ccell.2014.09.006
57. Spranger S, Dai D, Horton B, Gajewski TF. Tumor-residing Batf3 dendritic cells are required for effector T cell trafficking and adoptive T cell therapy. *Cancer Cell.* (2017) 31:711–23.e4. doi: 10.1016/j.ccell.2017.04.003
58. Garris CS, Arlauckas SP, Kohler RH, Trefny MP, Garren S, Piot C, et al. Successful anti-PD-1 cancer immunotherapy requires T cell-dendritic cell crosstalk involving the cytokines IFN-γ and IL-12. *Immunity.* (2018) 49:1148–61.e7. doi: 10.1016/j.immuni.2018.09.024
59. Tussiwand R, Everts B, Pearce EJ, Murphy KM, Grajales-Reyes GE, Kretzer NM, et al. Klf4 Expression in conventional dendritic cells is required for T helper 2 cell responses article Klf4 expression in conventional dendritic cells is required for T helper 2 cell responses. *Immunity.* (2015) 42:916–28. doi: 10.1016/j.immuni.2015.04.017
60. Tamoutounour S, Guilliams M, Dé Ric F, Sanchis M, Liu H, Terhorst D, et al. Article origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity.* (2013) 39:925–38. doi: 10.1016/j.immuni.2013.10.004
61. Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseño CG, Iwata A, et al. Transcriptional control of dendritic cell development. *Annu Rev Immunol.* (2016) 34:93–119. doi: 10.1146/annurev-immunol-032713-120204
62. Schlitzer A, Mcgovern N, Teo P, Zelante T, Atarashi K, Low D, et al. Article IRF4 transcription factor-dependent CD11b + dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity.* (2013) 38:970–83. doi: 10.1016/j.immuni.2013.04.011
63. Haniffa M, Shin A, Bigley V, Mcgovern N, Teo P, See P, et al. Article human tissues contain CD141 hi cross-presenting dendritic cells with functional homology to mouse CD103 + nonlymphoid dendritic cells. *Immunity.* (2012) 37:60–73. doi: 10.1016/j.immuni.2012.04.012
64. Villani A-C, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science.* (2017) 356:4573. doi: 10.1126/science.aah4573
65. Segura E, Touzot M, Bohineust A, Cappuccio A, Chiocchia G, Hosmalin A, et al. Article human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity.* (2013) 38:336–48. doi: 10.1016/j.immuni.2012.10.018
66. Laoui D, Keirsse J, Morias Y, Van Overmeire E, Geeraerts X, Elkrim Y, et al. The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nat Commun.* (2016) 2016:13720 doi: 10.1038/ncomms13720
67. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity.* (2013) 38:729–41. doi: 10.1016/j.immuni.2013.03.003
68. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol.* (2015) 15:471–85. doi: 10.1038/nri3865
69. See P, Dutertre C-A, Chen J, Günther P, McGovern N, Irac SE, et al. Mapping the human DC lineage through the integration of high-dimensional techniques. *Science.* (2017) 356:eaag3009. doi: 10.1126/science.aag3009
70. Kawamura S, Onai N, Miya F, Sato T, Tsunoda T, Kurabayashi K, et al. Identification of a human clonogenic progenitor with strict monocyte differentiation potential: a counterpart of mouse cMoPs. *Immunity.* (2017) 46:835–48.e4. doi: 10.1016/j.immuni.2017.04.019
71. Hettinger J, Richards DM, Hansson J, Barra MM, Joschko A-C, Krijgsveld J, et al. Origin of monocytes and macrophages in a committed progenitor. *Nat Immunol.* (2013) 14:821–30. doi: 10.1038/ni.2638
72. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol.* (2011) 11:762–74. doi: 10.1038/nri3070
73. Olingy CE, Dinh HQ, Hedrick CC. Monocyte heterogeneity and functions in cancer. *J Leukoc Biol.* (2019) 2019:JLB.4RI0818-311R. doi: 10.1002/JLB.4RI0818-311R
74. Jakubzick CV, Randolph GJ, Henson PM. Monocyte differentiation and antigen-presenting functions. *Nat Rev Immunol.* (2017) 17:349–62. doi: 10.1038/nri.2017.28
75. Holmgaard RB, Zamarin D, Li Y, Gasmi B, Munn DH, Allison JP, et al. Tumor-expressed IDO recruits and activates MDSCs in a treg-dependent manner. *Cell Rep.* (2015) 13:412–24. doi: 10.1016/j.celrep.2015.08.077
76. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med.* (2005) 202:931–9. doi: 10.1084/jem.20050715
77. Donkor MK, Lahue E, Hoke TA, Shafer LR, Coskun U, Solheim JC, et al. Mammary tumor heterogeneity in the expansion of myeloid-derived suppressor cells. *Int Immunopharmacol.* (2009) 9:937–48. doi: 10.1016/j.intimp.2009.03.021
78. Mao Y, Poschke I, Wennerberg E, de Pico Coaña Y, Egyhazi Brage S, Schultz I, et al. Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. *Cancer Res.* (2013) 73:3877–87. doi: 10.1158/0008-5472.CAN-12-4115
79. Lu T, Ramakrishnan R, Altiok S, Youn J-I, Cheng P, Celis E, et al. Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. *J Clin Invest.* (2011) 121:4015–29. doi: 10.1172/JCI45862
80. Headley MB, Bins A, Nip A, Roberts EW, Looney MR, Gerard A, et al. Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature.* (2016) 531:513–7. doi: 10.1038/nature16985
81. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity.* (2013) 39:599–610. doi: 10.1016/j.immuni.2013.08.007
82. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science.* (2007) 317:666–70. doi: 10.1126/science.1142883
83. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell.* (2013) 153:362–75. doi: 10.1016/j.cell.2013.03.010
84. Hanna RN, Cekic C, Sag D, Tacke R, Thomas GD, Nowyhed H, et al. Patrolling monocytes control tumor metastasis to the lung. *Science.* (2015) 350:985–90. doi: 10.1126/science.aac9407
85. SenGupta S, Subramanian BC, Parent CA. Getting TANned: how the tumor microenvironment drives neutrophil recruitment. *J Leukoc Biol.* (2018) 105:JLB.3RI0718-282R. doi: 10.1002/JLB.3RI0718-282R

86. Wislez M, Rabbe N, Marchal J, Milleron B, Crestani B, Mayaud C, et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death 1. *Cancer Res.* (2003) 63:1405–12.
87. Schmidt H, Bastholt L, Geertsen P, Christensen IJ, Larsen S, Gehl J, et al. Elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma: a prognostic model. *Br J Cancer.* (2005) 93:273–8. doi: 10.1038/sj.bjc.6602702
88. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol.* (2009) 27:4709–17. doi: 10.1200/JCO.2008.18.9498
89. Trellakis S, Bruderek K, Dumitru CA, Gholaman H, Gu X, Bankfalvi A, et al. Polymorphonuclear granulocytes in human head and neck cancer: enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. *Int J Cancer.* (2011) 129:2183–93. doi: 10.1002/ijc.25892
90. Atzpodien J, Reitz M. Peripheral blood neutrophils as independent immunologic predictor of response and long-term survival upon immunotherapy in metastatic renal-cell carcinoma. *Cancer Biother Radiopharm.* (2008) 23:129–34. doi: 10.1089/cbr.2007.0429
91. Sionov RV, Fridlender ZG, Granot Z. The multifaceted roles neutrophils play in the tumor microenvironment. *Cancer Microenviron.* (2015) 8:125–58. doi: 10.1007/s12307-014-0147-5
92. Swierczak A, Mouchemore KA, Hamilton JA, Anderson RL. Neutrophils: important contributors to tumor progression and metastasis. *Cancer Metastasis Rev.* (2015) 34:735–51. doi: 10.1007/s10555-015-9594-9
93. Uribe-Querol E, Rosales C. Neutrophils in cancer: two sides of the same coin. *J Immunol Res.* (2015) 2015:983698. doi: 10.1155/2015/983698
94. Hurt B, Schulick R, Edil B, El Kasmi KC, Barnett C. Cancer-promoting mechanisms of tumor-associated neutrophils. *Am J Surg.* (2017) 214:938–44. doi: 10.1016/j.amjsurg.2017.08.003
95. Shaul ME, Fridlender ZG. Neutrophils as active regulators of the immune system in the tumor microenvironment. *J Leukoc Biol.* (2017) 102:343–9. doi: 10.1189/jlb.5MR1216-508R
96. Shaul ME, Fridlender ZG. Cancer-related circulating and tumor-associated neutrophils - subtypes, sources and function. *FEBS J.* (2018) 285:4316–42. doi: 10.1111/febs.14524
97. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis.* (2012) 33:949–55. doi: 10.1093/carcin/bgs123
98. Marone G, Varricchi G, Loffredo S, Granata F. Mast cells and basophils in inflammatory and tumor angiogenesis and lymphangiogenesis. *Eur J Pharmacol.* (2016) 778:146–51. doi: 10.1016/j.ejphar.2015.03.088
99. Varricchi G, Loffredo S, Galdiero MR, Marone G, Cristinziano L, Granata F, et al. Innate effector cells in angiogenesis and lymphangiogenesis. *Curr Opin Immunol.* (2018) 53:152–60. doi: 10.1016/j.coi.2018.05.002
100. Varricchi G, Galdiero MR, Loffredo S, Marone G, Iannone R, Marone G, et al. Are mast cells MASTers in cancer? *Front Immunol.* (2017) 8:424. doi: 10.3389/fimmu.2017.00424
101. Simon SCS, Utikal J, Umansky V. Opposing roles of eosinophils in cancer. *Cancer Immunol Immunother.* (2018) 2018:4. doi: 10.1007/s00262-018-2255-4
102. Reichman H, Karo-Atar D, Munitz A. Emerging roles for eosinophils in the tumor microenvironment. *Trends Cancer.* (2016) 2:664–75. doi: 10.1016/j.trecan.2016.10.002
103. Chirumbolo S, Bjørklund G, Sboarina A, Vella A. The role of basophils as innate immune regulatory cells in allergy and immunotherapy. *Hum Vaccines Immunother.* (2018) 2018:1417711. doi: 10.1080/21645515.2017.1417711
104. Fleming V, Groth C, Altevogt P, Umansky V, Nagibin V, Weber R, et al. Targeting myeloid-derived suppressor cells to bypass tumor-induced immunosuppression. *Front Immunol.* (2018) 2018:398. doi: 10.3389/fimmu.2018.00398
105. Quail DF, Joyce JA. Molecular pathways: deciphering mechanisms of resistance to macrophage-targeted therapies. *Clin Cancer Res.* (2017) 23:876–84. doi: 10.1158/1078-0432.CCR-16-0133
106. Pathria P, Louis TL, Varner JA. Targeting tumor-associated macrophages in cancer. *Trends Immunol.* (2019) 40:310–27. doi: 10.1016/J.IT.2019.02.003
107. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interf Cytokine Res.* (2009) 29:313–26. doi: 10.1089/jir.2008.0027
108. Snyder LA, Kaiser EA, Zhang J, Li J, Qian B-Z, Pollard JW, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature.* (2011) 475:222–5. doi: 10.1038/nature10138
109. Mayer A, Plambeck-Suess SM, Belt BA, Linehan DC, Carpenter D, Deshpande AD, et al. inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 Axis. *Clin Cancer Res.* (2013) 19:3404–15. doi: 10.1158/1078-0432.ccr-13-0525
110. Schmall A, Al-Tamari HM, Herold S, Kampschulte M, Weigert A, Wietelmann A, et al. Macrophage and cancer cell cross-talk via CCR2 and CX3CR1 is a fundamental mechanism driving lung cancer. *Am J Respir Crit Care Med.* (2015) 191:437–47. doi: 10.1164/rccm.201406-1137OC
111. Chun E, Lavoie S, Michaud M, Odze R, Glickman JN, Correspondence WSG. CCL2 promotes colorectal carcinogenesis by enhancing polymorphonuclear myeloid-derived suppressor cell population and function. *Cell Rep.* (2015) 12:244–57. doi: 10.1016/j.celrep.2015.06.024
112. Bin Fang W, Jokar I, Zou A, Lambert D, Dendukuri P, Cheng N. CCL2/CCR2 chemokine signaling coordinates survival and motility of breast cancer cells through Smad3 protein-and p42/44 mitogen-activated protein kinase (MAPK)-dependent mechanisms. *J Biol Chem.* (2012) 287:36593–608. doi: 10.1074/jbc.M112.365999
113. Zhao L, Lim SY, Gordon-Weeks AN, Tapmeier TT, Im JH, Cao Y, et al. Recruitment of a myeloid cell subset (CD11b/Gr1 ^{mid}) via CCL2/CCR2 promotes the development of colorectal cancer liver metastasis*. *Hepatology.* (2013) 57:829–39. doi: 10.1002/hep.26094
114. Peña CG, Nakada Y, Saatcioglu HD, Aloisio GM, Cuevas I, Zhang S, et al. LKB1 loss promotes endometrial cancer progression via CCL2-dependent macrophage recruitment. *J Clin Invest.* (2015) 125:4063–76. doi: 10.1172/JCI82152
115. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget.* (2016) 7:28697–710. doi: 10.18632/oncotarget.7376
116. Majety M, Runza V, Lehmann C, Hoves S, Ries CH. A drug development perspective on targeting tumor-associated myeloid cells. *FEBS J.* (2018) 285:763–76. doi: 10.1111/febs.14277
117. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, et al. Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res.* (2013) 19:3404–15. doi: 10.1158/1078-0432.CCR-13-0525
118. Li M, Knight DA, Snyder AL, Smyth MJ, Stewart TJ. A role for CCL2 in both tumor progression and immunosurveillance. *Oncoimmunology.* (2013) 2:e25474. doi: 10.4161/onci.25474
119. Pienta KJ, Machiels J-P, Schrijvers D, Alekseev B, Shkolnik M, Crabb SJ, et al. Phase 2 study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 (CCL2), in metastatic castration-resistant prostate cancer. *Invest New Drugs.* (2013) 31:760–8. doi: 10.1007/s10637-012-9869-8
120. Fetterly GJ, Aras U, Meholick PD, Takimoto C, Seetharam S, McIntosh T, et al. Utilizing pharmacokinetics/pharmacodynamics modeling to simultaneously examine free CCL2, total CCL2 and carlumab (CNTO 888) concentration time data. *J Clin Pharmacol.* (2013) 53:1020–7. doi: 10.1002/jcph.140
121. Nywening Timothy M, Belt B, Cullinan D, Panni Roheena Z, Han BJ, Sanford D, et al. Targeting both tumour-associated CXCR2 + neutrophils and CCR2 + macrophages disrupts myeloid recruitment and improves chemotherapeutic responses in pancreatic ductal adenocarcinoma. *Gut.* (2018) 67:1112–23. doi: 10.1136/gutjnl-2017-313738
122. Bonapace L, Coissieux M-M, Wyckoff J, Mertz KD, Varga Z, Junt T, et al. Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. *Nature.* (2014) 515:130–3. doi: 10.1038/nature13862
123. Nywening TM, Wang-Gillam A, Sanford DE, Belt BA, Panni RZ, Cusworth BM, et al. Phase 1b study targeting tumour associated macrophages with CCR2 inhibition plus FOLFIRINOX in locally advanced and borderline resectable pancreatic cancer HHS Public Access. *Lancet Oncol.* (2016) 17:651–62. doi: 10.1016/S1470-2045(16)00078-4

124. Noel M, Lowery M, Ryan D, Wolpin B, Bullock A, Britten C, et al. 750PPhase Ib study of PF-04136309 (an oral CCR2 inhibitor) in combination with nab-paclitaxel/gemcitabine in first-line treatment of metastatic pancreatic adenocarcinoma. *Ann Oncol.* (2017) 28:132. doi: 10.1093/annonc/mdx369.132
125. Menne J, Eulberg D, Beyer D, Baumann M, Saudek F, Valkusz Z, et al. C-C motif-ligand 2 inhibition with emapticap pegol (NOX-E36) in type 2 diabetic patients with albuminuria. *Nephrol Dial Transpl.* (2017) 32:307–15. doi: 10.1093/ndt/gfv459
126. Bartneck M, Schrammen PL, Möckel D, Govaere O, Liepelt A, Krenkel O, et al. The CCR2+ macrophage subset promotes pathogenic angiogenesis for tumor vascularization in fibrotic livers. *CMGH.* (2019) 2019:7. doi: 10.1016/j.jcmgh.2018.10.007
127. Noel MS, Hezel AF, Linehan D, Wang-Gillam A, Eskens F, Sleijfer S, et al. Orally administered CCR2 selective inhibitor CCX872-b clinical trial in pancreatic cancer. *J Clin Oncol.* (2017) 35:276. doi: 10.1200/JCO.2017.35.4_suppl.276
128. Linehan D, Noel MS, Hezel AF, Wang-Gillam A, Eskens F, Sleijfer S, et al. Overall survival in a trial of orally administered CCR2 inhibitor CCX872 in locally advanced/metastatic pancreatic cancer: correlation with blood monocyte counts. *J Clin Oncol.* (2018) 36:92. doi: 10.1200/JCO.2018.36.5_suppl.92
129. Ban Y, Mai J, Li X, Mitchell-Flack M, Zhang T, Zhang L, et al. Targeting autocrine CCL5-CCR5 axis reprograms immunosuppressive myeloid cells and reinvigorates antitumor immunity. *Cancer Res.* (2017) 2017:2913. doi: 10.1158/0008-5472.CAN-16-2913
130. Cambien B, Richard-Fiardo P, Karimdjee BF, Martini V, Ferrua B. CCL5 neutralization restricts cancer growth and potentiates the targeting of PDGFRb in colorectal carcinoma. *PLoS ONE.* (2011) 6:28842. doi: 10.1371/journal.pone.0028842
131. Alfaro C, Teijeira A, Oñate C, Perez G, Sanmamed MF, Andueza MP, et al. Tumor-produced interleukin-8 attracts human myeloid-derived suppressor cells and elicits extrusion of Neutrophil Extracellular Traps (NETs). *Clin Cancer Res.* (2016) 2016:2463. doi: 10.1158/1078-0432.CCR-15-2463
132. Dominguez C, McCampbell KK, David JM, Palena C. Neutralization of IL-8 decreases tumor PMN-MDSCs and reduces mesenchymalization of claudin-low triple-negative breast cancer. *JCI Insight.* (2017) 2:94296. doi: 10.1172/jci.insight.94296
133. Dallos M, Aggen DH, Hawley J, Lim EA, Stein MN, Kelly WK, et al. A randomized phase Ib/II study of nivolumab with or without BMS-986253 in combination with a short course of ADT in men with castration-sensitive prostate cancer (MAGIC-8). *J Clin Oncol.* (2019) 37:TPS329. doi: 10.1200/JCO.2019.37.7_suppl.TPS329
134. Stanley ER, Chitu V. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb Perspect Biol.* (2014) 6:21857. doi: 10.1101/cshperspect.a021857
135. Ries CH, Hoves S, Cannarile MA, Rüttinger D. CSF-1/CSF-1R targeting agents in clinical development for cancer therapy. *Curr Opin Pharmacol.* (2015) 23:45–51. doi: 10.1016/j.coph.2015.05.008
136. Cannarile MA, Weisser M, Jacob W, Jegg A-M, Ries CH, Rüttinger D. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J Immunother Cancer.* (2017) 5:53. doi: 10.1186/s40425-017-0257-y
137. Brahmi M, Vinceneux A, Cassier PA. Current systemic treatment options for tenosynovial giant cell tumor/pigmented villonodular synovitis: targeting the CSF1/CSF1R axis. *Curr Treat Options Oncol.* (2016) 17:10. doi: 10.1007/s11864-015-0385-x
138. Giustini N, Bernthal NM, Bukata SV, Singh AS. Tenosynovial giant cell tumor: case report of a patient effectively treated with pexidartinib (PLX3397) and review of the literature. *Clin Sarcoma Res.* (2018) 8:14. doi: 10.1186/s13569-018-0101-2
139. Tap WD, Wainberg ZA, Anthony SP, Ibrahim PN, Zhang C, Healey JH, Chmielowski B, et al. Structure-guided blockade of CSF1R kinase in tenosynovial giant-cell tumor. *N Engl J Med.* (2015) 373:428–37. doi: 10.1056/NEJMoa1411366
140. West RB, Rubin BP, Miller MA, Subramanian S, Kaygusuz G, Montgomery K, et al. A landscape effect in tenosynovial giant-cell tumor from activation of CSF1 expression by a translocation in a minority of tumor cells. *Proc Natl Acad Sci USA.* (2006) 103:690–5. doi: 10.1073/pnas.0507321103
141. Shi G, Yang Q, Zhang Y, Jiang Q, Lin Y, Yang S, et al. Modulating the tumor microenvironment via oncolytic viruses and CSF-1R inhibition synergistically enhances anti-PD-1 immunotherapy. *Mol Ther.* (2019) 27:244–60. doi: 10.1016/j.ymthe.2018.11.010
142. Guan W, Hu J, Yang L, Tan P, Tang Z, West BL, et al. Inhibition of TAMs improves the response to docetaxel in castration-resistant prostate cancer. *Endocr Relat Cancer.* (2019) 26:131–40. doi: 10.1530/ERC-18-0284
143. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med.* (2013) 19:1264–72. doi: 10.1038/nm.3337
144. Mao Y, Eissler N, Le Blanc K, Johnsen JI, Kogner P, Kiessling R. Targeting suppressive myeloid cells potentiates checkpoint inhibitors to control spontaneous neuroblastoma. *Clin Cancer Res.* (2016) 22:3849–59. doi: 10.1158/1078-0432.CCR-15-1912
145. Harb WA, Johnson ML, Goldman JW, Weise AM, Call JA, Dudek AZ, et al. A phase 1b/2 study of ARRY-382, an oral inhibitor of colony stimulating factor 1 receptor (CSF1R), in combination with pembrolizumab (Pembro) for the treatment of patients (Pts) with advanced solid tumors. *J Clin Oncol.* (2018) 35:TPS3110. doi: 10.1200/jco.2017.35.15_suppl.tps3110
146. Bellovin D, Wondyfraw N, Levin A, Walker Q, Sennino B, Chao A, et al. *Antibody-Based Inhibtion of CSF1-R as a Component of Combination Immunotherapy in Preclinical Models*. AACR Poster (2017).
147. Wei S, Nandi S, Chitu V, Yeung Y-G, Yu W, Huang M, et al. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J Leukoc Biol.* (2010) 88:495–505. doi: 10.1189/jlb.1209822
148. Wainberg Z, Piha-Paul S, Luke J, Kim E, Thompson J, Pfanzelter N, et al. First-in-human phase 1 dose escalation and expansion of a novel combination, anti-CSF-1 receptor (cabiralizumab) plus anti-PD-1 (nivolumab), in patients with advanced solid tumors. *J Immunother Cancer.* (2017) 5:89. doi: 10.1186/s40425-017-0289-3
149. Wang-Gillam A, OReilly EM, Bendell J, Wainberg ZA, Borazanci E, Bahary N, et al. *A Randomized Phase 2 Study of Cabiralizumab + Nivolumab ± Chemotherapy in Advanced Pancreatic Ductal Adenocarcinoma*. ASCO Poster (2019).
150. Sankhala K, Sankhala KK, Blay J-Y, Ganjoo K, Italiano A, Hassan B, et al. *A Phase 1/2 Dose Escalation and Expansion Study of Cabiralizumab (FPA008), an anti-CSF1R antibody, in Tenosynovial Giant Cell Tumor (TGCT, Diffuse Pigmented Villonodular Synovitis D-PVNS).* Poster ASCO (2017).
151. Papadopoulos KP, Gluck L, Martin LP, Olszanski AJ, Tolcher AW, Ngarmchamnanrith G, et al. First-in-human study of AMG 820, a monoclonal anti-colony-stimulating factor 1 receptor antibody, in patients with advanced solid tumors. *Clin Cancer Res.* (2017) 23:5703–10. doi: 10.1158/1078-0432.CCR-16-3261
152. Cassier PA, Italiano A, Gomez-Roca CA, Le Tourneau C, Toulmonde M, Cannarile MA, et al. CSF1R inhibition with emactuzumab in locally advanced diffuse-type tenosynovial giant cell tumours of the soft tissue: a dose-escalation and dose-expansion phase 1 study. *Lancet Oncol.* (2015) 16:949–56. doi: 10.1016/S1470-2045(15)00132-1
153. Calvo A, Joensuu H, Sebastian M, Naing A, Bang Y-J, Martin M, et al. Phase Ib/II study of lacnotuzumab (MCS110) combined with spartalizumab (PDR001) in patients (pts) with advanced tumors. *J Clin Oncol.* (2018) 36:3014. doi: 10.1200/JCO.2018.36.15_suppl.3014
154. Gyori D, Lim EL, Grant FM, Spensberger D, Roychoudhuri R, Shuttleworth SJ, et al. Compensation between CSF1R+ macrophages and Foxp3+ Treg cells drives resistance to tumor immunotherapy. *JCI Insight.* (2018) 3:120631. doi: 10.1172/JCI.INSIGHT.120631
155. Weiskopf K. Cancer immunotherapy targeting the CD47/SIRPα axis. *Eur J Cancer.* (2017) 76:100–9. doi: 10.1016/j.ejca.2017.02.013
156. Majeti R, Alizadeh AA, van Rooijen N, Gibbs KD, Chao MP, Weissman IL, et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell.* (2009) 138:286–99. doi: 10.1016/j.cell.2009.05.045
157. Jaiswal S, Jamieson CHM, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 Is Upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell.* (2009) 138:271–85. doi: 10.1016/j.cell.2009.05.046

158. Mawby WJ, Holmes CH, Anstee DJ, Spring FA, Tanner MJ. Isolation and characterization of CD47 glycoprotein: a multispanning membrane protein which is the same as integrin-associated protein (IAP) and the ovarian tumour marker OA3. *Biochem J.* (1994) 304:525–30.
159. Kharitonenkov A, Chen Z, Sures I, Wang H, Schilling J, Ullrich A. A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature.* (1997) 386:181–6. doi: 10.1038/386181a0
160. Veillette A, Chen J. SIRPα-CD47 immune checkpoint blockade in anticancer therapy. *Trends Immunol.* (2018) 39:173–84. doi: 10.1016/j.it.2017.12.005
161. Russ A, Hua AB, Montfort WR, Rahman B, Riaz I, Khalid MU, et al. Blocking "don't eat me" signal of CD47-SIRPα in hematological malignancies, an in-depth review. *Blood Rev.* (2018) 32:480–9. doi: 10.1016/J.BLRE.2018.04.005
162. Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L, et al. Pre-clinical development of a humanized anti-CD47 antibody with anti-cancer therapeutic potential. *PLoS ONE.* (2015) 10:e0137345. doi: 10.1371/journal.pone.0137345
163. Advani R, Flinn I, Popplewell L, Forero A, Bartlett NL, Ghosh N, et al. CD47 Blockade by Hu5F9-G4 and rituximab in non-hodgkin's lymphoma. *N Engl J Med.* (2018) 379:1711–21. doi: 10.1056/NEJMoa1807315
164. Feng D, Gip P, Mckenna KM, Zhao F, Mata O, Mihardja S, et al. *Combination Treatment with 5F9 and Azacitidine Enhances Phagocytic Elimination of Acute Myeloid Leukemia Results*. Poster ASH (2018).
165. Sikic BI, Narayanan S, Colevas AD, Padda SK, Fisher GA, Supan D, et al. A first-in-human, first-in-class phase I trial of the anti-CD47 antibody Hu5F9-G4 in patients with advanced cancers. *J Clin Oncol.* (2016) 34:3019. doi: 10.1200/JCO.2016.34.15_suppl.3019
166. Chen JY, Mckenna KM, Choi TS, Duan J, Marjon K, Brown L, et al. *RBC-Specific CD47 Pruning Confers Protection and Underlies the Transient Anemia in 5F9 Anti-CD47 Treatment Results*. Poster ASH (2018).
167. Cekic C, Linden J. Purinergic regulation of the immune system. *Nat Rev Immunol.* (2016) 16:177–92. doi: 10.1038/nri.2016.4
168. Allard B, Longhi MS, Robson SC, Stagg J. The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. *Immunol Rev.* (2017) 276:121–44. doi: 10.1111/imr.12528
169. Leone RD, Emens LA. Targeting adenosine for cancer immunotherapy. *J Immunother Cancer.* (2018) 6:8. doi: 10.1186/s40425-018-0360-8
170. Fong L, Ii JP, Luke J, Hotson D, Sznol M, George S, et al. *Refractory Renal Cell Cancer (RCC) Exhibits High Adenosine A2A Receptor (A2AR) Expression and Prolonged Survival Following Treatment With the A2AR Antagonist CPI-444*. Poster SITC (2018).
171. Hoston A, Willingham S, Fong L, Powderly JI, Luke J, Sznol M, et al. *Adenosine Signature Genes Associate With Tumor Regression in Renal Cell Carcinoma (RCC) Patients Treated With the Adenosine A2A Receptor (A2AR) Antagonist, CPI-444*. Poster SITC (2018).
172. Laport G, Hotson AN, Sznol M, Miller R, Fong L, Kwei L, et al. Identification of adenosine pathway genes associated with response to therapy with the adenosine receptor antagonist CPI-444. *Ann Oncol.* (2018) 29:10. doi: 10.1093/annonc/mdy288.010
173. Willingham S, Hoston A, Laport G, Kwei L, Fong L, Sznol M, et al. *Identification of Adenosine Pathway Genes Associated With Response to Therapy With the Adenosine Receptor Antagonist CPI-444*. Poster ESMO (2018).
174. Antonioli L, Blandizzi C, Pacher P, Haskó G. Immunity, inflammation and cancer: a leading role for adenosine. *Nat Rev Cancer.* (2013) 13:842–57. doi: 10.1038/nrc3613
175. Sek K, Mølck C, Stewart GD, Kats L, Darcy PK, Beavis PA. Targeting adenosine receptor signaling in cancer immunotherapy. *Int J Mol Sci.* (2018) 19:19123837. doi: 10.3390/ijms19123837
176. Geoghegan JC, Diedrich G, Lu X, Rosenthal K, Sachsenmeier KF, Wu H, et al. Inhibition of CD73 AMP hydrolysis by a therapeutic antibody with a dual, non-competitive mechanism of action. *MAbs.* (2016) 8:3182. doi: 10.1080/19420862.2016.1143182
177. Hay CM, Sult E, Huang Q, Mulgrew K, Fuhrmann SR, Mcglinchey KA, et al. Targeting CD73 in the tumor microenvironment with MEDI9447. *Oncogene.* (2016) 5:12088751–10. doi: 10.1080/2162402X.2016.1208875
178. Overman MJ, LoRusso P, Strickler JH, Patel SP, Clarke SJ, Noonan AM, et al. Safety, efficacy and pharmacodynamics (PD) of MEDI9447 (oleclumab) alone or in combination with durvalumab in advanced colorectal cancer (CRC) or pancreatic cancer (panc). *J Clin Oncol.* (2018) 36:4123. doi: 10.1200/JCO.2018.36.15_suppl.4123
179. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol.* (2004) 5:987–95. doi: 10.1038/ni1112
180. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. *Nat Rev Cancer.* (2009) 9:57–63. doi: 10.1038/nrc2541
181. Kaczanowska S, Joseph AM, Davila E. TLR agonists: our best frenemy in cancer immunotherapy. *J Leukoc Biol.* (2013) 93:847–63. doi: 10.1189/jlb.1012501
182. Kobold S, Wiedemann G, Rothenfußer S, Endres S. Modes of action of TLR7 agonists in cancer therapy. *Immunotherapy.* (2014) 6:1085–95. doi: 10.2217/imt.14.75
183. Flowers C, Panizo C, Isufi I, Herrera AF, Okada C, Cull EH, et al. Intratumoral G100 induces systemic immunity and abscopal tumor regression in patients with follicular lymphoma: results of a phase 1/2 study examining G100 alone and in combination with pembrolizumab. *Blood.* (2017) 130:2771.
184. Flowers CR, Panizo C, Ai WZ, Isufi I, Herrera AF, Bartlett NL, et al. *Higher Dose Single-Agent Intratumoral G100 (A TLR4 Agonist) Results In Increased Biomarker Activity And Improved Clinical Outcomes In Patients With Follicular Lymphoma*. Poster SITC (2018).
185. Ter Meulen J, Lu H, Hsu FJ, Halwani A, Flowers C. *Toll-Like Receptor 4 Expression and Functional Signaling in Human and Murine B-Cell Lymphomas is Strongly Associated With Response to Treatment With the Synthetic TLR4 Agonist G100*. AACR Poster (2018).
186. Bhatia S, Miller NJ, Lu H, Longino NV, Ibrani D, Shinohara MM, et al. Intratumoral G100, a TLR4 agonist, induces antitumor immune responses and tumor regression in patients with merkel cell carcinoma. *Clin Cancer Res.* (2019) 25:1185–95. doi: 10.1158/1078-0432.CCR-18-0469
187. Ribas A, Medina T, Kummar S, Amin A, Kalbasi A, Drabick JJ, et al. SD-101 in combination with pembrolizumab in advanced melanoma: results of a phase Ib, multicenter study. *Cancer Discov.* (2018) 8:1250–7. doi: 10.1158/2159-8290.CD-18-0280
188. Wittig B, Schmidt M, Scheithauer W, Schmoll H-J. MGN1703, an immunomodulator and toll-like receptor 9 (TLR-9) agonist: from bench to bedside. *Crit Rev Oncol Hematol.* (2015) 94:31–44. doi: 10.1016/j.critrevonc.2014.12.002
189. Kapp K, Kleuss C, Schroff M, Wittig B. Genuine immunomodulation with dSLIM. *Mol Ther - Nucleic Acids.* (2014) 3:e170. doi: 10.1038/mtna.2014.28
190. Schmidt M, Hagner N, Marco A, König-Merediz SA, Schroff M, Wittig B. Design and structural requirements of the potent and safe TLR-9 agonistic immunomodulator MGN1703. *Nucleic Acid Ther.* (2015) 25:130–40. doi: 10.1089/nat.2015.0533
191. Weihrauch MR, Richly H, von Bergwelt-Baildon MS, Becker HJ, Schmidt M, Hacker UT, et al. Phase I clinical study of the toll-like receptor 9 agonist MGN1703 in patients with metastatic solid tumours. *Eur J Cancer.* (2015) 51:146–56. doi: 10.1016/j.ejca.2014.11.002
192. Schmoll H-J, Wittig B, Arnold D, Riera-Knorrenschild J, Nitsche D, Kroening H, et al. Maintenance treatment with the immunomodulator MGN1703, a Toll-like receptor 9 (TLR9) agonist, in patients with metastatic colorectal carcinoma and disease control after chemotherapy: a randomised, double-blind, placebo-controlled trial. *J Cancer Res Clin Oncol.* (2014) 140:1615–24. doi: 10.1007/s00432-014-1682-7
193. Thomas M, Ponce-Aix S, Navarro A, Riera-Knorrenschild J, Schmidt M, Wiegert E, et al. Immunotherapeutic maintenance treatment with toll-like receptor 9 agonist lefitolimod in patients with extensive-stage small-cell lung cancer: results from the exploratory, controlled, randomized, international phase II IMPULSE study. *Ann Oncol Off J Eur Soc Med Oncol.* (2018) 29:2076–84. doi: 10.1093/annonc/mdy326
194. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature.* (1998) 393:474–8. doi: 10.1038/30989
195. Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature.* (1998) 393:478–80. doi: 10.1038/30996
196. Schoenberger SP, Toes REM, van der Voort EIH, Offringa R, Melief CJM. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature.* (1998) 393:480–3. doi: 10.1038/31002

197. Beatty GL, Li Y, Long KB. Cancer immunotherapy: activating innate and adaptive immunity through CD40 agonists. *Expert Rev Anticancer Ther.* (2017) 2017:1270208. doi: 10.1080/14737140.2017.1270208
198. Johnson DH, Bentebibel SE, Lecagoonporn S, Bernatchez C, Haymaker CL, Murthy R, et al. Phase I/II dose escalation and expansion cohort safety and efficacy study of image guided intratumoral CD40 agonistic monoclonal antibody APX005M in combination with systemic pembrolizumab for treatment naive metastatic melanoma. *J Clin Oncol.* (2018) 36:TPS3133. doi: 10.1200/JCO.2018.36.15_suppl.TPS3133
199. O'Hara MH, O'Reilly EM, Rosemarie M, Varadhachary G, Wainberg ZA, Ko A, et al. *A Phase Ib Study of CD40 Agonistic Monoclonal Antibody APX005M Together With Gemcitabine (Gem) and Nab-Paclitaxel (NP) With or Without Nivolumab (Nivo) in Untreated Metastatic Ductal Pancreatic Adenocarcinoma (PDAC) Patients*. AACR Plenary Sess (2019).
200. Guerriero JL, Sotayo A, Ponichtera HE, Castrillon JA, Pourzia AL, Schad S, et al. Class IIa HDAC inhibition reduces breast tumours and metastases through anti-tumour macrophages. *Nature.* (2017) 543:428–32. doi: 10.1038/nature21409
201. Orillion A, Hashimoto A, Damayanti N, Shen L, Adelaiye-Ogala R, Arisa S, et al. Entinostat neutralizes myeloid-derived suppressor cells and enhances the antitumor effect of PD-1 inhibition in murine models of lung and renal cell carcinoma. *Clin Cancer Res.* (2017) 2017:741. doi: 10.1158/1078-0432.CCR-17-0741
202. Christmas BJ, Rafie CI, Hopkins AC, Scott BA, Ma HS, Cruz KA, et al. Entinostat converts immune-resistant breast and pancreatic cancers into checkpoint-responsive tumors by reprogramming tumor-infiltrating MDSCs. *Cancer Immunol Res.* (2018) 6:1561–77. doi: 10.1158/2326-6066.CIR-18-0070
203. Georgoudaki A-M, Prokopec KE, Boura VF, Hellqvist E, Sohn S, Östling J, et al. Reprogramming tumor-associated macrophages by antibody targeting inhibits cancer progression and metastasis. *Cell Rep.* (2016) 15:2000–11. doi: 10.1016/j.celrep.2016.04.084
204. Geraghty T, Shepard R, Rajagopalan A, Gupta S, Pathria P, Louis TL, et al. Integrin CD11b activation drives anti-tumor innate immunity. *Nat Commun.* (2018) 9:4. doi: 10.1038/s41467-018-07387-4
205. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem C, Gorjestani S, et al. PI3Kγ is a molecular switch that controls immune suppression HHS Public Access. *Nature.* (2016) 539:437–42. doi: 10.1038/nature19834
206. De Henau O, Rausch M, Winkler D, Campesato LF, Liu C, Cymerman DH, et al. Overcoming resistance to checkpoint blockade therapy by targeting PI3K-γ in myeloid cells. A developed the concepts, discussed experiments HHS Public Access. *Nature.* (2016) 539:443–7. doi: 10.1038/nature20554
207. Chmielowski B, Sullivan R, Postow M, Patnaik A, Shapiro G, Cohen EEW, et al. *The First Clinical/Translational Data from the Expansion Cohorts of Ph1/1b Study of IPI-549, a Tumor Macrophage-Reprogramming Small Molecule, in Combination with Nivolumab in Advanced Solid Tumors*. Poster SITC (2018).
208. Sullivan RJ, Hong DS, Tolcher AW, Patnaik A, Shapiro G, Chmielowski B, et al. Initial results from first-in-human study of IPI-549, a tumor macrophage-targeting agent, combined with nivolumab in advanced solid tumors. *J Clin Oncol.* (2018) 36:3013. doi: 10.1200/JCO.2018.36.15_suppl.3013
209. Spitzer MH, Nolan GP. Leading edge primer mass cytometry: single cells, many features. *Cell.* (2016) 2016:19. doi: 10.1016/j.cell.2016.04.019
210. Li B, Severson E, Pignon J-C, Zhao H, Li T, Novak J, et al. Comprehensive analyses of tumor immunity: implications for cancer immunotherapy. *Genome Biol.* (2016) 17:7. doi: 10.1186/s13059-016-1028-7
211. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles HHS Public Access. *Nat Methods.* (2015) 12:453–7. doi: 10.1038/nmeth.3337
212. Sautès-Fridman C, Lacroix L, Buttard B, Selves J, Fridman WH, Elarouci N, et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. *Genome Biol.* (2016) 17:5. doi: 10.1186/s13059-016-1070-5
213. Aran D, Hu Z, Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. *Genome Biol.* (2017) 18:1. doi: 10.1186/s13059-017-1349-1
214. Racle J, De Jonge K, Baumgaertner P, Speiser DE, Gfeller D. Simultaneous enumeration of cancer and immune cell types from bulk tumor gene expression data. *Elife.* (2017) 6:1. doi: 10.7554/eLife.26476.001
215. Ren X, Kang B, Zhang Z. Understanding tumor ecosystems by single-cell sequencing: promises and limitations. *Genome Biol.* (2018) 19:1593. doi: 10.1186/s13059-018-1593-z
216. Tirosh I, Izar B, Prakadan SM, Ii MHW, Treacy D, Trombetta JJ, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq HHS Public Access. *Science.* (2016) 352:189–96. doi: 10.1126/science.aad0501
217. Puram SV, Tirosh I, Parikh AS, Lin DT, Regev A, Bernstein BE. Single-cell transcriptomic analysis of primary and metastatic tumor ecosystems in head and neck cancer. *Cell.* (2017) 171:1611–24. doi: 10.1016/j.cell.2017.10.044
218. Li H, Courtois ET, Sengupta D, Tan Y, Chen KH, Goh JJL, et al. Reference component analysis of single-cell transcriptomes elucidates cellular heterogeneity in human colorectal tumors. *Nat Genet.* (2017) 49:708–18. doi: 10.1038/ng.3818
219. Venteicher AS, Tirosh I, Hebert C, Yizhak K, Neftel C, Filbin MG, et al. Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science.* (2017) 355:478. doi: 10.1126/science.aai8478
220. Sté phane Chevrier A, Harrison Levine J, Riccardo Tomaso Zanotelli V, Reis B, Pe D, Bodenmiller Correspondence B, et al. An immune atlas of clear cell renal cell carcinoma resource an immune atlas of clear cell renal cell carcinoma. *Cell.* (2017) 169:736–49. doi: 10.1016/j.cell.2017.04.016
221. Lambrechts D, Wauters E, Boeckx B, Aibar S, Nittner D, Burton O, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat Med.* (2018) 24:1277–89. doi: 10.1038/s41591-018-0096-5
222. Müller S, Kohanbash G, Liu SJ, Alvarado B, Carrera D, Bhaduri A, et al. Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biol.* (2017) 18:234. doi: 10.1186/s13059-017-1362-4
223. Chung W, Eum HH, Lee H-O, Lee K-M, Lee H-B, Kim K-T, et al. Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nat Commun.* (2017) 8:15081. doi: 10.1038/ncomms15081
224. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Resource transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity.* (2014) 40:274–88. doi: 10.1016/j.immuni.2014.01.006
225. Andrews TS, Hemberg M. Identifying cell populations with scRNASeq. *Mol Aspects Med.* (2018) 59:114–22. doi: 10.1016/j.mam.2017.07.002

Conflict of Interest Statement: MK is a founder, board member, and shareholder at Pionyr Immunotherapeutics. NJ, JP, MB, VS, and LR are shareholders at Pionyr Immunotherapeutics.

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

frontiers in Immunology

REVIEW
published: 06 August 2019
doi: 10.3389/fimmu.2019.01786

The Endless Saga of Monocyte Diversity

Stefania Canè[1], *Stefano Ugel*[1], *Rosalinda Trovato*[1], *Ilaria Marigo*[2], *Francesco De Sanctis*[1], *Silvia Sartoris*[1] *and Vincenzo Bronte*[1]*

[1] Section of Immunology, Department of Medicine, University of Verona, Verona, Italy, [2] Veneto Institute of Oncology IOV-IRCCS, Padua, Italy

Cancer immunotherapy relies on either restoring or activating the function of adaptive immune cells, mainly $CD8^+$ T lymphocytes. Despite impressive clinical success, cancer immunotherapy remains ineffective in many patients due to the establishment of tumor resistance, largely dependent on the nature of tumor microenvironment. There are several cellular and molecular mechanisms at play, and the goal is to identify those that are clinically significant. Among the hematopoietic-derived cells, monocytes are endowed with high plasticity, responsible for their pro- and anti-tumoral function. Indeed, monocytes are involved in several cancer-associated processes such as immune-tolerance, metastatic spread, neoangiogenesis, and chemotherapy resistance; on the other hand, by presenting cancer-associated antigens, they can also promote and sustain anti-tumoral T cell response. Recently, by high throughput technologies, new findings have revealed previously underappreciated, profound transcriptional, epigenetic, and metabolic differences among monocyte subsets, which complement and expand our knowledge on the monocyte ontogeny, recruitment during steady state, and emergency hematopoiesis, as seen in cancer. The subdivision into discrete monocytes subsets, both in mice and humans, appears an oversimplification, whereas continuum subsets development is best for depicting the real condition. In this review, we examine the evidences sustaining the existence of a monocyte heterogeneity along with functional activities, at the primary tumor and at the metastatic niche. In particular, we describe how tumor-derived soluble factors and cell-cell contact reprogram monocyte function. Finally, we point out the role of monocytes in preparing and shaping the metastatic niche and describe relevant targetable molecules altering monocyte activities. We think that exploiting monocyte complexity can help identifying key pathways important for the treatment of cancer and several conditions where these cells are involved.

Keywords: monocytes heterogeneity, monocyte continuum, primary tumor, metastatic niche, targeting of monocytes

OPEN ACCESS

Edited by:
Wei Zheng,
Albert Einstein College of Medicine, United States

Reviewed by:
Robin Parihar,
Baylor College of Medicine, United States
Shentong Fang,
Wihuri Research Institute, Finland

***Correspondence:**
Vincenzo Bronte
vincenzo.bronte@univr.it

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *17 May 2019*
Accepted: *16 July 2019*
Published: *06 August 2019*

Citation:
Canè S, Ugel S, Trovato R, Marigo I, De Sanctis F, Sartoris S and Bronte V (2019) The Endless Saga of Monocyte Diversity. Front. Immunol. 10:1786. doi: 10.3389/fimmu.2019.01786

INTRODUCTION

Monocyte diversity is well-recognized but the biologic and clinical significance of the different monocyte subtypes is far from being completely elucidated. The main hallmark of monocytes is their plastic nature, whereby they can exert multiple roles during the course of the immune response including cytokine production, pathogen clearance, antigen presentation, wound healing, and pro/anti-tumoral response (1–3). The original classification of monocytes into classical (in

humans: CD14^{high}, CD16^{-}; in mice: Ly6C^{high}), intermediate (in humans: CD14^{high}, CD16^{low}), and non-classical (in humans CD14^{low}, CD16^{high}; in mice: Ly6C^{low}) is currently being replaced by evidences supporting the existence of a "monocyte continuum" rather than stepwise differences between the different subtypes (4). Indeed, in mice under steady state, circulating classical monocyte subsets have been shown to switch into non-classical monocytes over time (5–7). However, it remains to be shown what relationship exists among the human monocytic subsets, and whether and how pathological conditions, like inflammation and cancer, impact this process.

Circulating monocytes have been viewed for many years, as precursor cells that provide tissue macrophages and dendritic cell (DCs) populations (8, 9); however, mounting evidence suggests that monocytes have their own effector functions in the blood and at peripheral sites throughout the body (10). The emerging data that distinct monocyte subsets, carrying different genetic, epigenetic, transcriptional, and metabolic arrangements, are committed to become macrophages and DCs seems to contradict the general accepted view of monocytes responding to a particular environmental stimuli and then differentiating into multifaceted macrophages and DCs. The intriguing evidence, both in mice and humans (11–13), of trained-monocytes, both present as mature and precursor cells, seems to strongly support the former hypothesis and reinvigorate the idea that monocytes have specific functions beyond being precursor cells. In this review, by combining the most recent advances in the field of monocytes' genetic, epigenetic, transcriptomic, and metabolomic, we outline and evaluate the changes occurring in monocyte subsets that underlie the aforementioned plasticity and heterogeneity. Secondly, we discuss new concepts in the monocyte field, like trained immunity and reprogramming and highlight the targetable pathways controlling monocyte fate and function.

We think that combining the information of single-cell transcriptome profiling, metabolomics array and epigenetic studies will elucidate complex relationships between cell types, thus solving limitations in the existing classification that relies on a relatively small number of markers.

MONOCYTE PHENOTYPICAL AND TRANSCRIPTIONAL PLASTICITY

Inflammatory and Patrolling Monocytes

Studies over the past two decades have delineated two major subsets of monocytes in mice and humans. Inflammatory monocytes (iMo), characterized by the high expression of the chemokine receptor CCR2, are repeatedly released from the bone marrow into the circulation. These cells, alternatively known as classical monocytes, are Ly6C^{hi} in mice and correspond to the CD14^{hi}CD16^{lo} monocyte subset in humans. The fate of these cells is strictly dependent on the state of the body. Under steady state conditions, extravasated iMo and their derived-cells enrich in nearly all tissues throughout the body, where they form a small yet significant group of the so called local tissue-resident macrophages (7). The gradual accumulation of monocyte-derived macrophages in tissues is generally associated with the slow but progressive replacement of embryonic macrophages, in both quantitative and qualitative fashion (14). Monocyte-derived macrophages present sustained gene modifications as compared to their circulating counterparts, as they shape to the tissue microenvironment. They acquire transcriptional signatures resembling resident macrophages of embryonic origin, even though a certain level of differences remains at the epigenetic, transcriptional and functional levels (15–17). Whether monocyte-derived macrophages that infiltrate tissues under steady-state condition gain a self-renewal ability, comparable to their embryonic counterparts, is still a matter of debate and seems to strictly depend on the type of infiltrated tissue. On the other hand, iMo can also conserve their monocyte-like state inside the tissues without differentiating into macrophages, thereby acting as a local monocyte reservoir (18) (**Table 1**).

Besides the aforementioned pathway of maturation, iMo can either remain in the blood, or transition into patrolling monocytes (pMo) by the setting up of *de novo* enhancers and activation of "frosted" enhancers (19, 20) (**Table 1**). The mechanisms driving the conversion of iMo into pMo are just beginning to be elucidated. It appears that Delta-like 1 (Dll1) signal from endothelial cells by interacting with NOTCH2 only iMo favors their switch into pMo cells (21). These data clearly indicate that iMo and pMo monocytes are biologically intertwined, corroborating observation obtained at the epigenetic level, which indicated that both monocyte subsets use the same promoter repertoire and minimally differ in their chromatin organization (19). Of course, this scenario raises several questions: are the iMo infiltrating the tissues able to reprogram into pMo? Is this switch tissue dependent? Can we interfere with this reprogramming to control the transition? Is there any factor maintaining iMo reservoir? Are pMo thus originated able to re-enter the blood stream? Are monocyte-derived macrophages transcriptionally similar to pMo? In mice, iMo can give rise to pMo, even though this does not rule out the presence of an alternative route for pMo development, independent from the iMo subset (8). Indeed, genetic evidence for this transition do exist. Two myeloid-determining transcription factors, like interferon regulatory factor 8 (IRF8), and the downstream Kruppel-like factor (KLF4), have been shown to regulate iMo generation without affecting the pMo numbers. Moreover, studies conducted on either global IRF8$^{-/-}$mice, or fetal liver transplant of KLF4$^{-/-}$cells into irradiated wild type mice, indicate a drastic reduced numbers of iMo in the bone marrow, while maintaining relatively normal pMo numbers (22). These findings suggest a pathway for pMo development untied from the iMo subset, probably originating directly from the common monocyte progenitor (cMoP). The identification of the transcription factors nerve growth factor IB (NR4A1) has helped to withstand the hypothesis that pMo can arise independently from iMo monocytes. On the other hand, recent single-cell RNA sequencing of murine and human monocytes indicate that circulating iMo and pMo represent, under physiological conditions, a nearly homogenous populations (19). Interestingly, data recently published (23) combining single

TABLE 1 | Summary of monocyte subsets presented in this review, highlighting their markers and function in both humans (top part of the table) and mice (bottom part of the table).

Monocyte subset	Markers of identification	Function
HUMAN		
Inflammatory monocytes (iMo)	$CCR2^{+}/CD14^{high}/CD16^{low/neg}$	Inflammatory response
Patrolling monocytes (pMo)	$CX3CR1^{+}/CD16^{high}/CD14^{low}$	Tissue repair
Immunosuppressive monocytes (M-MDSC)	$CD11b^{+}/CD14^{+}/CD124^{+}$/PD-$L1^{+}/CCR2^{+}$/HLA-$DR^{-}$/ARG1/IDO1/cFLIP/IL-6/IL-10/TGFβ/STAT3/cEPBβ/NF-κB	Immune dysfunction, tumor angiogenesis and vasculogenesis, promotion of metastasis, promotion of tumor cell stemness
Trained monocytes	$CD14^{+}$/Dectin$1^{+}/CD36^{+}/TLR4^{+}$/GM-$CSFR^{+}$/NOD/mTOR/ERK1/ERK2/NLPR3/HIF1α/aerobic glycolysis/TNFα/IL-6/IL-1β/H3K18Ac/H3K4me/H3K27ac	Innate immune memory that balance the equilibrium of balance of immune homeostasis, priming, training, and tolerance
SatM-expressing monocytes	Undefined	Not yet identified in humans
Neutrophil-like monocytes	Undefined	Not yet identified in humans
MOUSE		
Inflammatory monocytes (iMo)	$SSC^{int}/CD11b^{+}/F4/80^{+}/CD64^{+}/Ly6C^{high}/CD43^{low}/CD62L^{+}/CD115^{+}/CCR2^{+}/CX3CR1^{-}/MHCII^{low/-}$/IRF8/KLF4	Inflammatory response
Patrolling monocytes (pMo)	$SSC^{int}/CD11b^{+}/F4/80^{+}/CD64^{+}/Ly6C^{low}/CD43^{high}/CD62L^{-}/CD115^{+}/CCR2^{-}/CX3CR1^{+}/MHCII^{low}$/TREML4	Tissue repair
Immunosuppressive monocytes (M-MDSC)	$CD11b^{+}/Ly6C^{+}/Ly6G^{low/neg}/CD124^{+}$/PD-$L1^{+}/CCR2^{+}$/ARG1/NOS2/cFLIP/IL-6/IL-10/TGFβ/STAT3/STAT1/STAT6/cEPBβ/NF-κB/Chop/S100A8/S100A9	Immune dysfunction, tumor angiogenesis and vasculogenesis, promotion of metastasis, promotion of tumor cell stemness
Trained monocytes	$Ly6C^{low}$/Dectin$1^{+}/CD36^{+}/TLR4^{+}$/GM-$CSFR^{+}$/NOD/mTOR/ERK1/ERK2/NLPR3/HIF1α/aerobic glycolysis/lactate/mevalonate/TNFα/IL-6/IL-1β/H3K18Ac/H3K4me/H3K27ac	Innate immune memory that balance the equilibrium of balance of immune homeostasis, priming, training, and tolerance
SatM-expressing monocytes	$Ly6C^{low}/Flt3^{-}/Fc\varepsilon R1^{+}/CEACAM1^{+}/F4/80^{-}/Mac1^{+}/C5aR^{+}$/M-$CSFR^{+}/MSR1^{+}$/cEPBβ/MPO- and NE-containing granules	Fibrosis
Neutrophil-like monocytes	$Ly6C^{+}$/MPO- and NE-containing granules	Response to microbial components (i.e., LPS) and maintaining homeostasis at steady-state

profile and functional and phenotypic characterization, showed that monocytes subsets (defined as classical, intermediate, and non-classical) isolated from peripheral blood of both healthy mice and humans, can be further divided into two additional populations: one group expressing classical monocyte genes and also cytotoxic genes and the other one with undefined activity. Other studies conducted in human and mouse lung cancer samples (24) have showed that several tumor-infiltrating myeloid populations (TIM) and among those monocytes are uniquely associated with the disease and with clinical progress, highlighting the potential to use TIM as immunotherapeutic targets. We think that the multiple cell subsets identified in the aforementioned manuscripts, should be tested for their functional relevance in tumor progression, in disease progression and their abundance should be correlated with therapeutic response. Of course, the correlation between human and mouse TIM will help to achieve these goals with the ultimate purpose of gaining more insight into monocytes and monocyte-dependent therapies.

In contrast, patrolling monocytes (pMo) represent a more differentiated subset and are marked by the higher surface expression of CX3CR1. pMo express low levels of Ly6C in mice and are $CD14^{lo}CD16^{hi}$ in humans; they routinely check the vessels under physiological conditions through the engagement of an LFA/ICAM-dependent crawling mechanism with resting endothelial cells (25, 26). This patrolling behavior of pMo can be observed throughout the interdigitated system of capillaries, arterioles, and venules. Similarly, human $CD14^{lo}$ $CD16^{hi}$ pMo show patrolling behavior when adoptively transferred into immuno-compromised mice (27). The crawling features of pMo allows them to efficiently sense particles, on the one hand, and on the other hand to monitor of endothelial cell integrity. However, these cells are not restricted to the vessels as pMo also undergo diapedesis and can be identified within the parenchyma of multiple tissues (7). pMo display a longer lifespan at the steady state compared to iMo and they are, also for this reason, found in the blood, at any given time, more abundant than their counterpart (5, 28). Interestingly, pMo cells are strongly susceptible of the physiological status of the organism and therefore they might represent a potential diagnostic tool (29). Nevertheless, how the fine balance between iMo and pMo levels and differentiation capacity is maintained/regulated during severe inflammation, autoimmune diseases, and cancer is just began to be elucidated.

Under pathological conditions, such inflammation and cancer, the rapid recruitment of myeloid cells to sites of injury stimulates a constant development and mobilization of cells from the bone marrow, causing a state of "emergency" that

might generate monocytes from different ways or precursors. These include monocytes that have circumvented the canonical MDP-cMoP-monocyte developmental axes and resemble neutrophil-like iMo derived from GMP precursors (30). An additional example of a recently described monocyte subset that appears under inflammatory conditions is the segregated nucleus-containing atypical pMo (SatM) (31). SatM and neutrophil-like monocytes represent a minor pool of monocyte subsets under steady-state (19, 30, 31) (**Table 1**), yet become conspicuous during inflammation (30, 31). At present, the lack of reliable surface markers, associated with a deep epigenetic and transcriptional profile unable to make a clear distinguish between neutrophil-like iMo identified using GFI1/IRF8-reporter mice (30) from classical iMo and SatM (identified as $Ly6C^{low}Ceacam1^{hi}F4/80^{-}Mac1^{hi}$), from non-classical pMo (31). The limited whole-cell proteomic data available so far showed, for example, that SatM cells contain granules expressing granulocyte-related protein, like myeloperoxidase (MPO) and neutrophil elastase (NE) (31) (**Figure 1**). These cells are related to fibrosic responses; in fact, adoptive transfer of SatM monocytes into bleomycin-treated mice exacerbates fibrosis. Furthermore, it was shown that chimeric mice, lacking the CCAAT/enhancer-binding protein beta (Cebpb) in the hematopoietic progenitors were resistant to fibrosis when exposed to bleomycin but they had unaltered inflammatory response, further supporting the role of SatM in sustaining the mechanism of fibrosis (31). Nevertheless, more studies will be required to uncover the origin of these subsets and their involvement in different pathologies. To add more complexity, a recent work by Hanna et al. (32), showed that a subset of circulating pMo, but not iMo, accumulate at the site of tumor where they display an anti-tumoral role, by directly engulfing cancer cells and by releasing factors which in turn activate cytotoxic natural killer cells (NK). Are these extravasating pMo similar to their blood counterpart? How the findings from Hanna et al. (32) correlate with observations that extravasating pMo can differentiate into macrophages during cancer? Are pMo "corrupted" by tumor cells once they have extravasated within tissues and switched to pro-tumoral cells? Are iMo and pMo competing for the access to the tumor site? Are iMo suppressing the anti-tumor function of pMo? Indeed, this mechanism is also consistent with the data from the pMo adoptive transfer experiments since pMo appear to act early during seeding but not after establishment of metastatic foci despite their continued accumulation. Therefore, under pathological conditions it still remains possible that pMo derive from either blood iMo, via the formation of an intermediate $Ly6C^{int}$ monocyte, or from bone marrow $Ly6C^{+}$ monocytes, from an independent bone marrow monocyte progenitor, or from a combination of all these pathways (**Figure 1**). In order to solve all these issues a detailed understanding of the factors and pathways regulating the development and survival of both iMo and pMo populations in specific inflammatory settings is necessary.

As mentioned before, adoptive transfer and fate-mapping studies support the hypothesis that monocytes develop along a differentiation continuum in which inflammatory monocytes give rise to the patrolling subset in the circulation (3, 5). Development of monocytes from bone marrow progenitors combines the regulated expression of numerous transcription factors, with the contribution of growth factors and cytokines (33). Monocytes and neutrophils are both derived from hematopoietic stem cells (HSCs) via common myeloid progenitors (CMPs), which can originate from granulocyte-monocyte progenitor (GMP) and monocytes/macrophages, DC precursors (MDPs) (34, 35) (**Figure 1**). The commitment toward monocytes is characterized by three major lineage-determining factors (LDTF): PU.1, CCAAT/enhancer-binding protein beta (Cebpb) and Cebpa. PU.1 is a master regulator of myeloid and lymphoid cell development (36). The genetic ablation in mice of PU.1 promotes a lethal embryonic phenotype and the transfer of PU.1 mutated stem cells favors an altered myelopoiesis characterized by a robust contraction in both monocytes and DCs (37, 38). By binding closed chromatin through its C-terminal DNA-binding domain (39), PU.1 acts as a coordinator for the activation of selected genomic regions in collaboration with monocyte-associated transcriptional factors such as the IRF8 and KLF4 (40). Moreover, PU.1 synergistically cooperates with C/EBP-δ to activate the promoters of interleukin-6 (IL-6) and CCL5 (41) and transactivates the human macrophage colony-stimulating factor receptor (M-CSFR) promoter via the c-Jun pathway (42). Proteins of the C/EBP family radically impact the myeloid cells development. Since in *Cebpa*-deficient mice the transition from CMPs to GMPs is completely abrogated, these mice lack the granulocytic compartment (43, 44) indicating that C/EBPα is the master regulator of steady-state granulopoiesis.

Additionally, during myelopoiesis, C/EBPα controls and activates the myeloid-associated gene expression program by binding to either promoters or enhancers of myeloid-related genes, such as colony stimulating factor 3 receptor (CSF3R), growth factor independent-1 (GFI-1), interleukin 6 receptor (IL-6R), or C/EBPε, both in mice and in human stem cells (45). C/EBPα inhibits specific transcriptional factors attenuating the expression of non-myeloid lineage genes (46, 47). Moreover, genetically enforced inducible C/EBPα expression in GMPs by tamoxifen administration favors monocyte development demonstrating the critical role of this transcriptional factor during monopoiesis (48). By contrast, C/EBPβ is not necessary for steady-state granulopoiesis. However, C/EBPβ was recently identified as the key factor during the epigenetic default differentiation of iMo monocytes into pMo cells under steady state condition, highlighting the multifaceted role of this transcriptional factor during monocyte development (19). In this regard, a key contribution of C/EBPβ to mylopoiesis is highlighted by data showing that mice knockout for *Cebpb* have a dramatic reduction in circulating monocytes (19). C/EBPβ is also the master regulator of emergency myelopoiesis; in fact, inflammatory signals (i.e., cytokine stimulation) strongly induce the downregulation of all members of C/EBP family except for C/EBPβ (43). Under pathological condition, like fibrosis, a C/EBPβ-associated gene program in $Fc\varepsilon R1^{+}$ GMPs progenitors promote the development of alternative monocytes, like SatM, previously described. As we will discuss below, C/EBPβ-driven programs are also activated in cancer-educated myeloid cells.

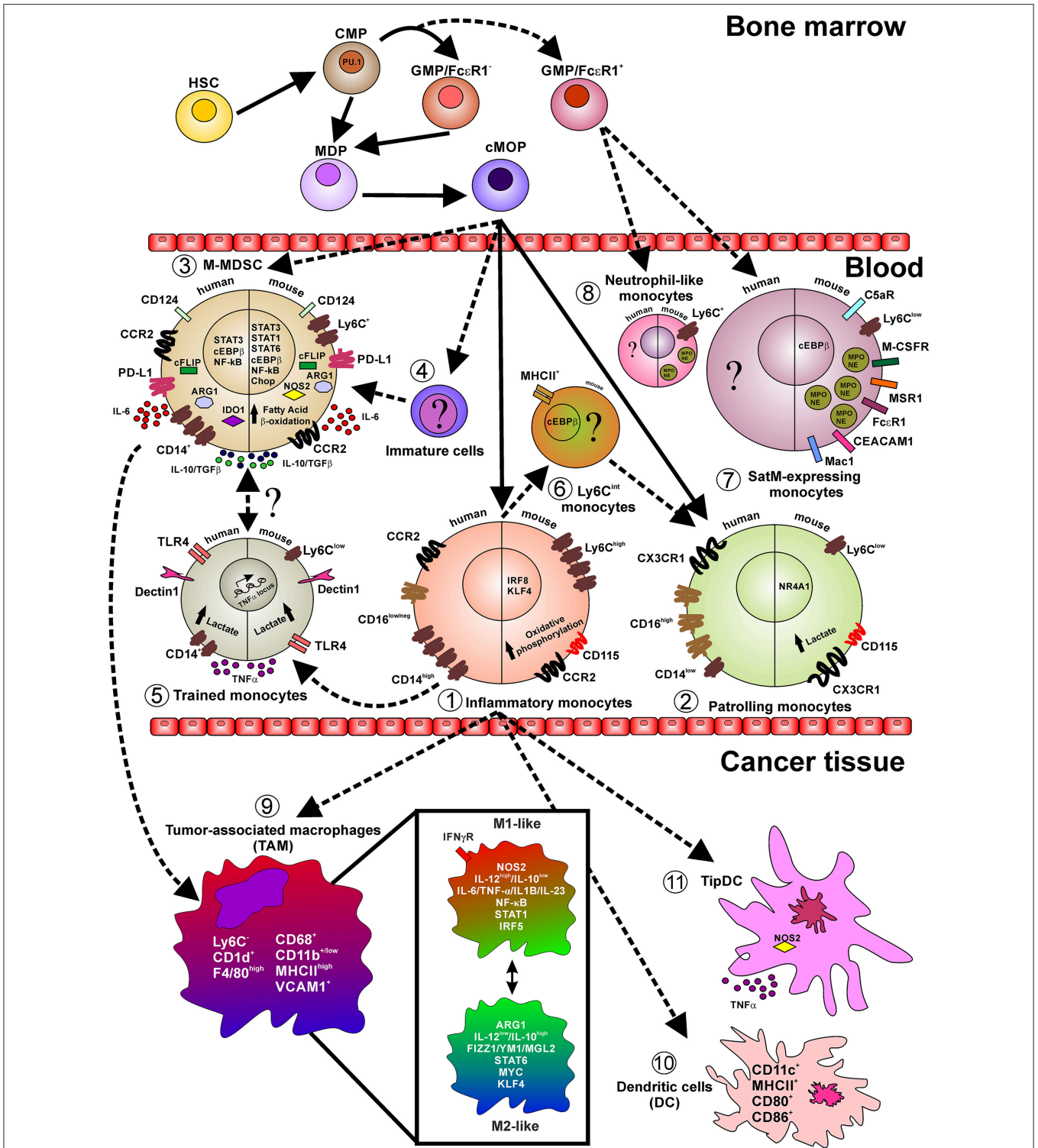


FIGURE 1 | Layout depicting the monocyte lineage precursors (on top), the monocyte subsets in the peripheral blood (center), and monocyte fate in cancer tissues. Indicated are relevant surface markers, transcription factors, secreted cytokines, intracellular mediators, and relevant metabolic pathways. Continuous lines indicate events occurring during normal myelopoiesis while shaded lines indicate events in emergency myelopiesis (e.g., cancer and inflammation). Briefly, under steady state cMop precursors originate both inflammatory (1) and patrolling (2) monocytes, both in humans and mice. However, it has been reported that during emergency myelopoiesis cMop precursors can also differentiate into M-MDSC (3) and into not yet defined immature cells (4). Particularly, during infection, inflammatory monocytes acquire a trained phenotype (5) and also switch into Ly6C^{int} cells (6) only identified in mice and with not fully defined function, transcriptional regulators,

(Continued)

FIGURE 1 | and markers. During fibrosis a novel subset of monocytes, so called SatM (8), have been characterized, in mice, defined as Ly6C$^+$ and expressing proteins typical of the neutrophil granules (MPO and NE). These cells, together with neutrophil-like monocytes (7), found in peripheral blood of mice during microbial infection and in the bone morrow in steady-state condition, originate from GMP/FcεRI$^+$ precursors cells in the bone marrow. In pathological conditions, like cancer, inflammatory monocytes infiltrating the tissue give rise to TAM (9) which in turn represent a fultifaced population of macrophages. Additionally, inflammatory monocytes can also differentiate into classical DC (10) expressing the costimulatory molecules CD80 and CD86 and TipDC (11) expressing high level of NOS2 and TNFα.

Myeloid-Derived Suppressor Cells

In cancer, tumor-derived soluble factors, such as growth factors, cytokines, chemokines, and tumor-derived exosomes, not only support an increased recruitment of monocytes from bone marrow to tumor-microenvironment bypassing the canonical monocyte development but, also, favor the acquisition of immunosuppressive features in myeloid cells. To highlight these acquired functional properties, myeloid cells comprising monocytes, neutrophils, and immature cells were named myeloid-derived suppressor cells (MDSCs) (49, 50). Although this terminology generated some controversies, it represents a useful ground for scientific researches on altered hematopoiesis. It relies on the concept that myelopoiesis in pathology might give rise to cellular subsets that can share some markers with the cells present under steady state but are functionally and molecularly distinct, sharing the property of negatively regulating effectors of adaptive and innate immunity. The monocytic-MDSC (M-MDSC) subset is broadly defined in mice as Ly6C$^+$CD11b$^+$ cells and in human as CD14$^+$HLA-DR$^-$ or CD14$^+$CD124$^+$ cells and is endowed with a stronger ability to arrest T cell response, when compared to the granulocytic-MDSC (G-MDSC) counterpart (51, 52), in part dependent on the activation of two enzymes, arginase 1 (ARG1) and inducible nitric oxide synthases (NOS2/iNOS), which are directly regulated by C/EBPβ expression (53) (**Figure 1**; **Table 1**). We demonstrated that, in tumor-bearing mice, both the expansion and the immunosuppressive function of MDSCs are abrogated in the absence of C/EBPβ, resulting in restricted tumor spread (53). These data confirm the central role of C/EBPβ in tumor-associated inflammation, underscoring it as promising therapeutic target to develop new approach to limit cancer progression. Both human and mouse M-MDSCs secrete immunoregulatory cytokines, like IL-10, TGFβ, and IL-6 and present and array of molecules, such as ARG1, FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP), indoleamine 2,3-dioxygenase 1 (IDO1), and nitric oxide synthase 2 (NOS2), which can contribute to the suppressive activity of these cells. Mechanistically, IL-6, for example, activates PI3Kγ, which stimulates mTOR, S6Kα, and C/EBPβ-mediated anti-inflammatory gene expression and inhibits NFkB-mediated pro-inflammatory gene expression, thereby promoting the immune suppressive function of these cells mediated by, but not limited to, IL-10, TGFβ, and ARG1 (54, 55). In particular, *Arg1* gene in MDSCs is strictly controlled by several inducible transcriptional factors able to recognize sequences characterized by high content in GC that impacts the nucleosomal stability (56), such as signal transducer and activator of transcription 3 (STAT3), IRF8, as well as CHOP, PU.1, KLF4, and activator-protein 1 (AP-1) (57). Moreover, STAT3 promotes both expansion and survival of M-MDSCs through Bcl-XL, c-Myc, and Cyclin D1 expression (58) as well as the induction of several immune regulatory mediators like bFGF, HGF, VEGF, IL-1β, MMP9, CCL2, and CXCL2 (50). Interestingly, phosphorylated STAT3 binds to multiple sites in the *Arg1* promoter, suggesting that STAT3 inhibitors, like Stattic, could reduce ARG1 dependent immunosuppression by dampening the expression of *Arg1* mRNA (59). Within the tumor environment, ARG1 can cooperate with NOS2 to produce high levels of superoxide anion (O_2^-) that can react with either nitric oxide (NO) or H_2O generating reactive–nitrogen species (RNS), such as peroxinitrites ($ONOO^-$), which damage both the function and migration of T cells to tumor site (60), and reactive-oxygen species (ROS), such as H_2O_2 which decreases T cellular CD3ζ expression limiting the activation of T cells, respectively (61). However, ARG1 has a hierarchically dominant negative role compared to NOS2 in developing an immunosuppressive tumor microenvironment by limiting the activity of monocyte-derived NOS2-expressing and TNF-producing dendritic cells (defined as Tip-DCs) that can sustain and favor the anti-tumor effect of transferred T lymphocytes (62). An alternative way to reprogram MDSC differentiation and function is through the expression of p53. It was recently demonstrated that M-MDSC can be driven to differentiate into potent antigen-presenting, defined as Ly6C$^+$CD103$^+$ DCs by inflammation-induced activation of p53. In fact, mice with a targeted deletion of p53 in myeloid cells specifically loose the Ly6C$^+$CD103$^+$ population and became unresponsive to different forms of immunotherapy and immunogenic chemotherapy (63).

Recently, we demonstrated the ability of c-FLIP, which controls the extrinsic apoptotic pathway and caspase 8 activation (64), to re-program monocytes into MDSC-like cells (65). In fact, FLIP-expressing monocytes displayed impressive regulatory features both *in vitro*, constraining the activated T cell proliferation, and *in vivo*, controlling the development of graft vs. host disease in a xenogeneic mouse model. Indeed, enforced expression of c-FLIP in monocytes up-regulates MDSC-associated immunosuppressive genes, such as CD273, CD124, IL-6, IL-10, CFS3, PTGS2, and IDO1, as a result of a "steered" NF-kB activation induced by the nuclear co-localization of c-FLIP with NF-kB p50 (65). During the course of a disease, like cancer, MDSCs infiltrate the tumor, differentiating into tumor-associated macrophages (TAMs) which can sustain primary tumor growth and contribute to the pre-metastatic niche formation (**Figure 1**).

Trained Immunity and Metabolic Landscape of Monocyte Subsets

Recent studies have shown that during infection with some pathogens iMo can undergo extensive epigenetic, transcriptional, and metabolic reprogramming, with the functional consequence

of an enhanced immune reactivity upon a second encounter, in other words they acquire an immunological memory. The existence of this innate immune memory was initially suggested by studies in mice deficient for functional T and B cells and exposed to mild *C. albicans* infection, which show protection against *C. albicans* reinfection by increased responsiveness of monocytes (66). Even though the requirements for monocyte training has been primarily investigated either *in vitro* or under *in vivo* steady state, trained monocytes seem to originate from iMo during emergency hematopoiesis by a profound epigenetic and metabolic rewiring (**Table 1**). Exposure of iMo to either *C. albicans* or β-glucan *in vitro*, induce profound genome-wide changes in epigenetic marks, including, but not restricted, histone H3 lysine 4 monomethylation (H3K4me1), trimethylation (H3K4me3), and H3 lysine 27 acetylation (H3K27ac) (67) as a consequence of Dectin-1/AKT/mTOR/HIF-1α signaling pathway activation and secretion of IL-6, IL-1β, and TNFα. Other studies instead, identified Bacille Calmette-Guérin (BCG) and peptidoglycan as potent inducers of the aforementioned trained-related epigenetic modifications, though a different mechanism dependent on nucleotide-binding oligomerization domain-containing protein 2 (NOD2) pathway and activation of NF-kB (68). Concomitantly to these epigenetic changes, a metabolic switch also occurs. Trained monocytes are mainly glycolytic (aerobic glycolysis) with impairment of the oxidative phosphorylation, production of lactate and disruption of the Krebs cycle at the level of both citrate, which is withdrawn for fatty acid biosynthesis, and succinate, which activates HIF-1α and consequently up regulates the expression of several pro-inflammatory cytokines, mainly IL-1β and TNF-α (69).

Beside microbial particles, products of the lipid metabolism were found to be activators of the trained immunity. Oxidized-low density lipoprotein (oxLDL), a damage-associated molecular pattern (DAMP), interacts with CD36 on myeloid cells leading to the activation of NLRP3 inflammasome and consequent production of IL-1β (70–72).

Although the role of epigenetic programing as a mechanism required to insure innate immune memory is becoming more clear, one crucial aspect still remains unanswered: what is the cellular process that induces and maintains such epigenetic changes? Initial evidences seem to suggest that metabolites might play a role since they can act as cofactors for the enzymes (mainly methylases, methyltransferases, histone deacetylases, and histone acetylases) involved in epigenetic modulation of gene transcription (67, 73, 74). Of course, more studies are required to fill the gap and also to deeply examine the role that different chromatin modifications have on the stability of the chromatin. It is expected that stable histone modifications (e.g., histone methylation) would be more suitable to maintain a functional modification than those with short half-life (e.g., histone acetylation). Thus, the long-lasting persistence of some histone modifications could reflect both the stability of such modifications or the persistent activation status of the signaling pathways and transcription factors upstream (75, 76). Understanding these regulations *is a sine qua non* for designing therapeutic intervention aimed at modulating trained immunity, to dampen it when in excess (e.g., organ rejection, autoimmunity, allergy, atherosclerosis) or enhance it when defective (e.g., cancer, infection).

A growing body of evidence suggests that the development of immune cells and their different effector functions are the results of a dynamic changes occurring at the metabolic level (77, 78). In mouse models of cancer, myeloid cells are metabolically influenced by tumor-derived factors to become MDSCs, helping to protect tumor from the effects of chemotherapy (79). Specifically, mouse MDSCs undergo a major metabolic reprogramming by switching off glycolysis and enhancing fatty acid β oxidation (FAO) pathway. This metabolic reprogramming is generally characterized by an up-regulation of lipid uptake receptors CD36 and Mrs1, an increase in FAO enzymes, mainly carnitine palmitoyltransferase 1 (CPT1) and 3-hydroxyacyl-Coa dehydrogenase (HADHA), and an increase in oxygen consumption. These events are associated with the activation of immunosuppressive pathways, namely upregulation of ARG1 and NOS2 synthesis and production of $ONOO^-$, contributing to dampen T cell proliferation and IFNγ secretion (80–82). Blockade of FAO, both *in vitro* and *in vivo* in different tumor models, decreased the incorporation of fatty acid and ATP production, holding up the development of suppressive MDSCs (82) and leading to increased efficacy of chemotherapy and adoptive T cell therapy. Interestingly, fatty acid oxidation also plays an important role in regulating the inflammatory properties of iMo. Increased intracellular level of unsaturated fatty acid (arachidonic acid) was shown to stimulate the secretion of pro-inflammatory IL-1α by uncoupling the mitochondrial respiration (83, 84) thus exacerbating the pathogenesis of atherosclerosis (85). The relevance of changes in the lipid metabolism occurring during myeloid cells differentiation, was also recently demonstrated by Mitroulis et al., in *an vivo* model of trained immunity (13). Treatment with β-glucan determines an increase in gene expression of several enzymes involved in cholesterol biosynthesis and decrease in expression of Abca1, a transporter regulating cholesterol efflux (86). Consistently, β-glucan administration in mice not only upregulates CD131, a subunit of the receptor for IL-3, IL-5, and GM-CSF, expression in myeloid precursors, but also activates downstream signaling, as demonstrated by STAT5 phosphorylation. The capacity of β-glucan to enhance the biosynthesis of cholesteryl esters and significantly decrease glycerophospholipid-containing arachidonic fatty acid chains highlight the capacity of cells to alter their lipidome and, thus, the physicochemical features of their membranes. This adaptive response has direct consequences in the composition of cellular membranes (87) and consequently in cell signaling (88). In this regard, alterations in the quantitative and qualitative cholesterol composition of the membrane can impact the localization of CD131, its signaling (86, 89) and consequently the differentiation of specific myeloid subsets.

Amino acids, besides being the building block of several molecules, serve as essential precursors of different metabolites. Different studies have shown that glutamine metabolism into glutamate, α-ketoglutarate and succinate semialdehyde can fuel the synthesis of fumarate and succinate for the tricarboxylic acid cycle (TCA) (90). Inhibition of glutaminolysis, in mice, down regulates the production of pro-inflammatory cytokines in

monocytes exposed to *C. albicans*, dampening the development of efficient trained monocytes triggered by β-glucan. In line with these observations, the biochemical catheterization of β-glucan trained monocytes has revealed that upon induction of the signaling cascade Dectin-1-Akt-mTOR-HIF-1α, a metabolic shift occurs leading to an increase aerobic glycolysis, glucose usage, lactate production, and TNF-α secretion (67).

Thus, it appears that modulation of metabolic landscape represents a fundamental step to unravel the functional consequences of different monocytes subsets helping to identify new strategies of intervention for the treatment of several pathophysiological conditions. It remains instead undefined whether cancer-derived factors can also generate trained monocytes and if these cells contribute to dampen the anti-tumor response or favor metastatic spread.

MONOCYTE FUNCTIONS

Monocytes at the Primary Tumor

Tumor derived factors (TDFs) are key mediators in the crosstalk between monocytes and tumor cells. They are involved in monocyte recruitment from the hematopoietic organs in adult life, i.e., bone marrow and in part the spleen, survival, and differentiation within the tumor site. Tumor-released monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) was identified as the major TDF involved in iMo recruitment, through the CCL2-CCR2 axis, into several mouse and human tumors (91). Indeed, inhibition of CCL2-CCR2 signaling in a mouse model of breast cancer significantly impair iMo infiltration and reduce tumor growth and metastases (92). Several studies have described the presence of other chemokines within the tumor microenvironment (TME), including CCL3, CCL4, CCL5, CXCL12, and growth factors such as colony stimulating factor-1 (CSF1), which may also contribute to monocyte recruitment to tumors (93). Indeed, in both mouse (94) and human (91) tumors, cells secrete high level of CSF1 that is involved in recruitment, survival, and differentiation of monocytes. The inhibition of CSF1 signaling in an experimental model of lung carcinoma significantly reduced the number of mature TAMs due to impaired recruitment, proliferation and maturation of iMo cells (95). Moreover, CSF1R signaling blockade can also reprogram immunosuppressive TAMs prompting the differentiation of iMo in anti-tumoral M1-like TAMs (96). However, abrogation of CSF1R signaling, by either small molecules (97) or monoclonal antibodies (98), even though appealing, have so far demonstrated a limited anti-tumor effects. Only a recent work done by Kumar et al. (99) highlighted that the CSF1-dependent cross-talk between tumor cells and cancer-associated fibroblasts (CAF) might explain the inefficacy of such a treatment. Thus, combinatorial therapy targeting both CSF1R and CXCR2 seems to have more chances to generate an effective anti-tumor T cell response. Thus, the therapeutic focus has shifted to combinations of CSF1R inhibitors with other agents. Indeed, treatment with CSF1 inhibitor in combination with either paclitaxel or radiotherapy, is showing to improve the survival of mouse model of breast or prostate cancer, respectively (79, 100) and improve the efficacy of ACT when combined with anti-PD-1 and anti-CTLA4 in a pancreatic mouse model (101). It is becoming clear that cytotoxic therapies, for example, induce mammary epithelial cells to produce monocyte/macrophage recruitment factors, including CSF1 and interleukin-34 (IL-34), which together enhance CSF1R-dependent monocytes/macrophage infiltration, making its inhibition more effective.

One of the strongest stimuli inducing the secretion of TDFs is hypoxia. Indeed, during tumor growth the level of available O_2 is significantly reduced, especially in the inner part of the neoplastic mass. This hypoxic microenvironment triggers HIF-1α stabilization in tumor cells and the consequent release of pro-angiogenic factors, such as growth factors (VEGF, PDGF, PIGF, ANG-2), chemokines (CXCL8, CXCL12), cytokines (TNFα, IL1β, TGFβ), and metalloproteases (MMPs), which results in the sprouting of new vessels supporting cancer cells growth (102, 103). In addition, hypoxia is a powerful monocyte and macrophage attractant. Through the release of VEGFB and PIGF, tumor cells can enhance haematopoiesis and monocyte recruitment (104). In addition, angiopoietin-2 (ANG-2) is able to recruit circulating Tie2-expressing monocytes (TEMs) that inhibit apoptosis in both tumor and endothelial cells, by mechanisms depending on TNFα release (105), and exhibits an essential pro-angiogenetic role with a not completely clarified mechanism. Interestingly, it was recently discovered that a small subgroup of recruited iMo can be educated by VEGF, and exert their proangiogenic function, supporting the formation of capillaries and larger vessels, as short-lived monocytes without becoming macrophages (106). Secreted VEGF promotes the acquisition of immunosuppressive features in monocytes generating M-MDSCs by upregulating both ARG1 and iNOS through hypoxia response elements and NF-kB (107). In addition, MDSCs can fuel this circuit by releasing MMP-9, which induces VEGF release from ECM (108). Therefore, targeting VEGF/VEGFR has received attention as a strategy to interfere with monocyte-driven angiogenesis. Moreover, blocking this axis will affect monocytes recruitment to the tumor site (109), and favor the conversion from predominant suppressive to anti-tumoral monocytes (110). An additional way to interfere with MDSC differentiation is by interfering with p53 expression, as mentioned before (63).

In the last few years, extracellular vesicles (EVs) emerged among the TDFs as additional determinants in the formation of TME, both at primary and metastatic sites (111). EVs are a heterogeneous group of membrane vesicles mainly composed of exosomes and microvesicles. Interestingly, EVs released by tumor cells (tEVs) and tumor-derived exosomes (TEX) interact with immune cells inducing their switch toward a pro-tumoral phenotype. Particularly, exosomes target monocytes altering their normal function by several mechanisms. In melanoma and colon cancer, TEX block peripheral iMo differentiation into DCs, and favor the acquisition of a peculiar phenotype reminiscent of M-MDSCs and characterized by decreased expression of HLA-DR and costimulatory molecules (112). A similar modulation of monocytes has been described in many other malignancies, including pancreatic cancer, bladder carcinoma, glioblastoma, and multiple myeloma (113–115) and it is often associated

with increased cytokine secretion, i.e., CCL2, CCL4, and IL-6, as well as programmed death-ligand 1 (PD-L1) expression (116). Moreover, glioblastoma-derived EVs may skew the differentiation of peripheral blood monocytes to alternatively activated M2 macrophages inducing the expression of elevated levels of VEGF, IL6, Cox2, ARG1, and PD-L1 through STAT3 activation (117). Interestingly, in gastric cancer, TEX effectively educated monocytes to differentiate into a peculiar type of M2 TAM expressing PD-1, which induce T cell dysfunction through IL-10 secretion by interacting directly with PD-L1$^+$ cells and thereby promote tumor progression (118). TEX have been described to contain miRNA which can be transferred to target cells and modulate cellular function (119). These data strongly highlight the role of TEX as additional mediators of monocyte dysfunction in TME. In the last few years, significant advances in understanding the mechanisms associated with exosome biogenesis/release have been obtained identifying some possible targets to interfere with this cell-cell communication. Recent studies in mouse models demonstrated that RAB27A or RAB35 inhibition significantly impair TEX secretion in HeLa cervical carcinoma and Oli-Neu oligodendroglial precursor cell lines (120), respectively. Moreover, RAB27A deficient tumor cell lines displayed reduced growth due to impaired recruitment of bone-marrow derived, pro-tumoral immune cells (121).

In addition to be modulated by soluble factors released by the tumor cells, monocytes can shape their effector function also in a contact-dependent manner. For instance, breast cancer stem cells express CD90 and Ephrin A4 receptor (EphA4R) that interact with CD11b and EphA4 present on tumor-associated monocytes and macrophages, respectively, leading to the secretion of inflammatory cytokine (IL-6, IL-8, GM-CSF), which in turn sustain tumor stem cell fate (122).

Among factors that could shape monocyte plasticity, TGFβ, a key multifunctional cytokine, involved in both cancer and inflammation, appears to play a key role. Besides being targeted in a number of human diseases (123), TGFβ has a very well-recognized ability to regulate T cell responses (124), supporting Th9 (125, 126) and Th17 (127) differentiation, and promoting regulatory T cell function (128–130). However, how TGFβ regulates innate immune responses just began to be appreciated. Many TME-associated cells, and among those monocytes and macrophages, express high amount of latent LTGFβ. The recent work by Kelly et al. (131), demonstrates that iMo, beside tumor cells, express high levels of αvβ8 integrin responsible for the activation TGFβ from LTGFβ form (131, 132). Additionally, monocyte-derived macrophages, integrin expression and TGFβ signaling are generally maintained in anti-inflammatory macrophages but down-modulated in pro-inflammatory macrophages. To sustain the immunosuppressive microenvironment, tumor cells exploit this regulatory mechanism upregulating the expression of integrin αvβ8 and activating TGFβ from LTGFβ-expressing monocytes and macrophages (133).

Monocytes at the Metastatic Niche

Cancer metastasis is a multi-step process of the neoplastic progression termed "invasion-metastasis cascade" (134, 135). Monocytes are, among other myeloid cells (e.g., neutrophils), corrupted to foster tumor progression and metastasis. Accumulating evidence indicates that monocytes (primarily iMo) are essential pre-metastatic promoters being rapidly recruited from the bone marrow to the pre-metastatic niche, mainly by CCL2/CCR2 axis (136, 137), where they promote tumor colonization by secreting angiogenic factors, like VEGFA (92, 138). Indeed, in the MMTV-PyMT mouse model of spontaneous breast cancer, CCL2, released by either tumor cells or stromal cells at the metastatic lung niche, induces the recruitment of CCR2-expressing iMo, which in turn favor the extravasation of tumor cells, through the release of VEGFA (92). Consequently, the inhibition of CCL2-CCR2 signaling axis abrogated the recruitment of monocytes thus reducing metastasis formation. Similarly, in a mouse model of metastatic melanoma (B16F10 model), accumulation of CXCR3$^+$monocytes/macrophages in the lung was a prerequisite to mediate melanoma engraftment and metastatic disease (139). However, how this process takes place remains undefined. In a different set of experiments employing B16F10 melanoma model, it was shown that M-MDSCs were recruited to the pre-metastatic niche, mainly by CCL12 expression. By releasing IL-1β, these cells promoted the expression of E-selectin on endothelial cells thus promoting the adhesion of tumor cells to the vascular endothelium (140).

At the metastatic targeted-organs, monocytes can offer survival stimuli for cancer cells. Metastatic cells in the lung, from either mouse or human breast cancer, overexpress vascular cell adhesion molecule-1 (VCAM-1) and shRNA-mediated depletion of VCAM-1 inhibited metastasis formation. Moreover, monocytic cells expressing α4-integrin can bind VCAM-1 present on the surface of tumor cells. Thus, upon α4-integrin engagement on monocytes, VCAM-1 delivers anti-apoptotic signals into breast cancer cells through the PI3K/Akt pathway favoring tumor cell survival (141).

EVs can cross the basal lamina of alveolar capillaries in the lung. In the lungs, alveolar and interstitial macrophages upon taking up these EVs start to secret CCL2 favoring the recruitment of iMo, which in turn differentiate into macrophages, mostly M2-like cells, promoting tumor growth by the secretion of IL-6 and deposition of fibrin (142). Similarly, in colorectal cancer (CRC) patients the high expression of serum exosomal-derived miR-203 was associated with increased probability to develop distant metastases. It was shown that TEX-derived miR-203 uptaken by monocytes promoted their differentiation into M2-like macrophages, *in vitro*. Furthermore, mice injected with CRC cells transfected with miR-203 developed significantly more liver metastases than the control group (143).

We have previously underlined the plasticity of monocytes and how iMo and pMo play different roles in cancer progression and surveillance. In line with these observations, it has been demonstrated that TEX from poorly-metastatic melanoma cells are taken up by bone-marrow monocytes, promoting their differentiation into pMo, which in turn migrate at the metastatic niche clearing tumor cells by direct engulfment or by activating cytotoxic NK cells (144). Interestingly, TEX from poorly-metastatic tumors caused macrophage alteration toward

M1-like cells expressing TRAIL, which competed with NK cells for tumor killing. These findings suggest that prior to the acquisition of the metastatic capacity, tumors continuously alert host immune system by producing vesicles that affect innate immune responses and support the concept of developing new cancer immunotherapeutic approach based on TEX to deliver specifically immune triggers.

FUTURE PROSPECTIVE: TARGETING OF MONOCYTES AS THE NEW FRONTIER FOR CANCER IMMUNOTHERAPY

Myeloid cells are extremely plastic and can develop specialized functions in response to micro-environmental pathologic conditions such as infections, autoimmunity or cancer (145). Myeloid cell polarization into either tumor-suppressive or tumor-promoting phenotypes is fundamental for shaping TME. Once at the tumor site, these myeloid cells generally acquire a pro-tumor phenotype (146). Thus, one of the major goals of contemporary tumor immunotherapy is targeting tumor-associated myeloid cells by depletion, recruitment inhibition or reprogramming their polarization/activation status.

As mentioned above, inhibition of the CCL2-CCR2 axis, used to prevent the egression of monocytes from bone marrow, improved the efficacy of chemo-, radio- and immune-therapy in several preclinical models. Nevertheless, the use of either CCL2 or CCR2 inhibitors, in clinical trials, gave disappointing results, indicating the need of supplementary studies considering the presence of potential TME-dependent compensatory mechanisms acting on tumor-resident myeloid cells (146, 147). Moreover, although the continuous blockade of macrophages constrains tumor progression, cessation of the CCL2 blocking therapy stimulates them to a rapid rebound, leading to accelerated metastatic disease via a mechanism dependent on VEGF-A and IL-6 production monocyte-derived by macrophages (102).

Targeting Trained Immunity

Trained immunity inducing factors were tested for their anti-tumor activity, both *in vitro* and *in vivo*, and some of them reached the clinical application. A β-glucan PAMP, Imprime PGG (Imprime), is currently in clinical development in combination with immune checkpoint inhibitors, tumor-targeting antibodies, and anti-angiogenic antibodies. The results from a randomized phase 2 clinical trial of Imprime in combination with bevacizumab and carboplatin/paclitaxel vs. bevacizumab and chemotherapy alone in the 1st-line treatment of stage IV non-small cell lung cancer showed promising efficacy in terms of both objective tumor response and survival (ClinicalTrials.gov NCT 00874107, EudraCT 2008-006780-37). Earlier results have shown that both the M2 macrophages and DCs derived from Imprime-trained monocytes have higher expression of PD-L1 and CD86, rendering these cells suitable for treatment with anti-PD-1 antibody. *Ex vivo* treatment of T cells with Nivolumab, an anti-PD-1 antibody, enhanced proliferation in response to αCD3/αCD28 stimulation and co-culture with Imprime-trained monocytes-derived M2 macrophages or DCs further improved T cell expansion and increased production of several cytokines, including IFNγ, IL-2, TNF-α, and GM-CSF. Results were further validated in syngeneic mouse model, like the CT26 colon carcinoma. Bacillus Calmette-Guerin (BCG), another trained immunity inducer, is currently the only agent approved by the US Food and Drug Administration for first line treatment of carcinoma *in situ* of the bladder. BCG therapy reduces the risk of recurrence and maintenance therapy with BCG decreases the risk of progression in patients with high-grade, non–muscle invasive bladder cancer (148, 149). It has been speculated that the mechanism of action involves the autophagy. In fact, pharmacologic or genetic inhibition of autophagy blocks the epigenetic reprogramming of monocytes at the level of H3K4 trimethylation, arresting the mechanism of trained immunity induced *in vitro* by BCG. Single nucleotide polymorphisms associated with bladder cancer progression and recurrence, in the autophagy genes ATG2B (rs3759601) and ATG5 (rs2245214), affected both the *in vitro* and *in vivo* training effect of BCG (150).

Muramyl dipeptide (MDP), a synthetic peptide of N-acetyl muramic acid attached to a short amino acid chain of L-Ala-D-isoGln, is a bacterial cell wall peptidoglycan active as NOD2 agonist and contributing to the generation of trained monocytes (151). Interestingly, Paclitaxel conjugated to MDP showed not only antitumor activity, but also immune enhancement capacity. In fact, compared with either paclitaxel or MDP alone, the combination significantly increased the expression and secretion of TNFα and IL-12 from mouse peritoneal monocytes (152). Moreover, it was shown that MDP can upregulate PD-L1 in healthy monocytes, but in patients with Crohn's disease, carrying the Leu1007 frameshift mutation of the NOD2 gene, such effect was completely lost (153) (**Table 2**).

Targeting Signaling Pathways

An alternative approach to target trained immunity is to inhibit the pathway Dectin-1-Akt-mTOR-HIF-1α. To this end, the beneficial effects of Metformin and Everolimus, an mTOR activator rapamycin analog, administration to patients with type 2 diabetes and cancer, respectively, were linked with the modulation of trained monocytes (67, 90, 154). Interestingly, inhibitors of other kinases, such as Raf-1, PI3K, and ERK are of particular interest in modulating trained monocytes because they represent downstream effectors of Dectin-1 and NOD2 activation (**Table 2**). In particular the knockout of PI3Kγ was reported to break tumor tolerance by MDSC reduction (54) as well as, the combination targeting of PI3Kδ in association with PD-L1- based immunotherapy better limited tumor progression (155, 156). Moreover, the pharmacological treatment using multi-kinase inhibitors carbozantinib and BEZ235, which limit MDSC accumulation, in combination with immune checkpoint therapy controlled more efficiently tumor growth in a castration-resistant prostate tumor model than the single agents (157) underlying the possibility to overcame *de novo* resistance to antibody blockade based therapy by limiting MDSCs.

The development of epigenetic modulators is acquiring increased interest due to the relevance of epigenetic changed in several diseases. The broad jumonji histone demethylase

TABLE 2 | Inhibitors and their corresponding targets found to impact pathways regulating different aspects of monocyte biology.

Effect	Target	Drug name	Targeted monocyte subset	References (PMID)
Recruitment abrogation	CCR2	PF04136309 Carlumab	Inflammatory monocytes M-MDSCs	27055731 22907596
	CFS1R	ARRY-382 FPA008 GW2580	Patrolling monocytes M-MDSCs	29872489 20008303 16249345
	IL-6R	mAb 15A7	Patrolling monocytes M-MDSCs	28235765 22653638
	Attenuating RNS generation	AT38	M-MDSCs	21930770
	Multi-kinase	carbozantinib BEZ235	M-MDSCs	28321130
	amino-biphosphonates	matrix metalloproteases	M-MDSCs	12912933
Apoptosis induction	FLIP	5-fluorouracil Gemcitabine Docetaxel Paclitaxel Oxaliplatin Cisplatin Irinotecan Etoposide	M-MDSCs	30518925
	Fas	IL-2 with anti-CD40 antibody (clone FGK115B3)	M-MDSCs	24808361
	IL1R	Anakinra	Monocytes	29808007
Inhibition of proliferation	GM-CSF	mAb clone MP1-22E9	M-MDSCs	22698406
	G-CSF	mAb clone MAB414	M-MDSCs	19346489
	VEGF	mAb clone G6.23	M-MDSCs	17664940
Metabolic alteration	Mevalonate-cholesterol pathway	Statins	Trained monocytes	29328908
	NOD2	Muramyl dipeptide	Trained monocytes	
	mTOR	Everolimus Metformin	Trained monocytes	25258083 27926861 23415113
	Bromodomains	I-BET151	Trained monocytes	27863248
	Histone deacetylase	JIB-04	Trained monocytes	23792809 29702467
	Glutamine-pathway	DON	Trained monocytes	30541099 297024
Immunosuppressive function	pSTAT3	Stattic	M-MDSCs	23454751
	COX2	Celecoxib	M-MDSCs	21324923
	IDO1	1-methyl-L-tryptophan Epacadostat	M-MDSCs	23440412
	ARG1	CB-1158 NCX 4016 NG-hydroxy-L-arginine [NOHA] Nω-hydroxy-nor-Arginine [Nor-NOHA]	M-MDSCs	29254508 29133913
	Phosphodiesterase (PDE5)	Sildenafil, tadalafil	M-MDSCs	27495172 25564570
	PD-L1/CTLA-4	Atezolizumab ipilimumab	M-MDSCs	28364000 30267200
Cell differentiation	Retinoic acid receptor	ATRA	M-MDSCs	18006848
	ENTPD2	POM-1	M-MDSCs	28894087

inhibitor JIB-04 decreased trained immunity response by modulating of the methylation status of H3K9 (158). Interestingly, a clinically relevant small molecule of the BET family of bromodomains, I-BET151, was shown to prevent monocyte tolerance when administered concomitantly with LPS, but it was ineffective when administered after LPS stimulation.

These results suggest that I-BET151 is not an effective treatment in monocytes that have already experienced an inflammatory response (159) (**Table 2**).

Targeting Metabolic Pathways

Metabolic modulation also represents another interesting approach to target both trained monocytes and M-MDSCs. Some metabolites and metabolic enzymes function as either substrates or cofactors for chromatin modifying enzymes, thereby influencing the epigenetic landscape of target cells. Accordingly, fumarate can increase trained immunity by increasing H3K4me3 and H3K27ac and inhibiting the degradation of HIF-1α (90). Moreover, the decreased expression of lysine demethylase 5 family of HDAC (KDM5), responsible for H3K4 demethylation, is also inhibited by fumarate, maintaining then the accessibility of the chromatin. On the other hand, it was shown that tolerant monocytes lack the activity of KDM5, whose function can be restored by its cofactor, α-ketoglutarate (90). Mevalonate, intermediate of the cholesterol pathway has been shown to induce trained immunity (74), consequently, statins can be used to prevent this process under conditions in which accumulation of trained monocytes is detrimental, like patient with hyper-IgD syndrome or inflammatory conditions. Additionally, given the enhanced glutamynolysis associated to trained immunity (90), administration of 6-diazo-5-oxo-L-norleucine (DON), which inhibits glutamine uptake and metabolism, was shown to have favorable effects after organ transplant preventing rejection (160) (**Table 2**). Even though targeting metabolic pathways, to modulate trained monocyte function, is feasible, toxicity, and side effects represent the main drawbacks of metabolic drugs. Overcoming this limitation, for example, by delivering drugs trough nanoparticles/nanocarriers, could open the access to a variety of molecules that have already demonstrated their efficacy *in vitro*.

Targeting Immunosuppressive Monocytes (M-MDSC)

Together with trained monocytes, MDSCs represent the other group of targetable cells. Proliferating T cells need a large supply of amino acid like L-arginine and L-tryptophan. MDSCs have developed a strategy to modulate local concentrations of these amino acids via the up regulation of enzymes involved in their degradation like ARG1, NOS2, and IDO. Developing inhibitors of these enzymes represent a field of intense research. To this end, nitroaspirin, consisting of a nitric oxide group covalently linked to aspirin, was shown to restore L-arginine levels in T cells, by suppressing the production of ROS and iNOS (161). Moreover, our laboratory showed that treatment with AT38 [3-(aminocarbonyl) furoxan-4-yl]methyl salicylate], decreased MDSC-induced nitration within the tumor environment, increasing CCL2 binding and T cell tumor infiltration in mice (60). N-hydroxy-L-arginine (NOHA) is an intermediate in the conversion of arginine to citrulline and NO by iNOS (162) (**Table 2**). It is a potent physiologic inhibitor of ARG1. Mice exposed to NOHA demonstrated inhibition of MDSC function, and mice with B cell lymphoma treated with NOHA had decreased numbers of circulating Treg cells and improved immune responses to the cancer (163). We also demonstrated that the production of polyamine by ARG1 activity promotes IDO1 activation through Src kinase signaling (164). Therefore, a combined targeting of ARG1 and IDO1 using pharmacological compounds (165) could be an effective treatment to constrain tumor-associated immunosuppression improving cancer immunotherapy. MDSCs generate an immunosuppressive environment also by producing prostaglandin E2 (PGE2), which levels are regulated through the enzyme Cox2 (166). PGE2 activates PGE2-R on MDSCs altering the differentiation of MDSCs. In the bone marrow, activation of PGE2-R hampers the differentiation of monocytes into antigen presenting cells, while increased PGE2 switch monocytes into MDSCs via increased expression of IDO, IL-4Rα, iNOS, and IL-10 (167, 168). In line with these findings, blocking the production of PGE2 in mice bearing lung carcinoma, with Cox2 inhibitors, decreased the expression of ARG1 in MDSC and tumor growth (169). Cox2 inhibitors may also provide other antitumor effects (**Table 2**). Celecoxib, a Cox2 inhibitors, was shown to decrease MDSC recruitment and increased $CD8^+$ T cell tumor infiltration in gliomas and colon carcinoma by decreasing CCL2 production (170).

Methionine, an essential amino acid for normal T cells function, is generally supplied by antigen presenting cells. DCs and macrophages import cysteine to create methionine, which is then secreted; they additionally release thioredoxin converting cysteine to methionine. However, in the TME, MDSCs transport cysteine intracellularly thus depleting T cells of methionine (171, 172). Consequently, blocking thioredoxin could prevent T cells proliferation arrest. In this situation small molecules or neutralizing antibodies targeting extracellularly released enzymes could be beneficial to restore T cells function and used in combination with ACT of tumor specific T cells or with check point inhibitors.

Constitutive activation of the JAK-STAT pathway has been implicated in the proliferation of MDSCs via anti-apoptotic and pro-proliferative genes (173). Moreover, ARG1 and iNOS, immunosuppressive enzymes in MDSCs, are controlled via STAT1 and STAT3 (174). Consequently, inhibition of the JAK-STAT pathway has been of great interest. Many inhibitors of STAT1/STAT3 have been discovered and several of them already enter clinical trials. Among those one, Stattic, an inhibitor of pSTAT3, reduces the suppressive activity of MDSC *in vitro* both in mice and in humans (175, 176) (**Table 2**).

In the last 10 years, several approaches to target immunosuppressive monocyte referred as M-MDSC were developed using a large spectrum of pharmacological compounds and immunotherapeutic approaches with the aim to limit MDSC proliferation, function, and recruitment to tumor site. For instance, non-therapeutic low doses of conventional chemotherapeutic drugs such as gemcitabine and 5-fluorouracil [which does not induce immunogenic cancer-cell death of tumor cells (177)] were able to limit $Ly6C^+$MDSC number and activity by inducing c-FLIP down-regulation (65). The M-MDSC elimination is essential to restore the immune response in tumor-bearing mice by rescuing the frequency of circulating anti-tumor T cells (51). These data obtained in preclinical cancer

models were also confirmed in tumor patients. In fact, renal cell cancer (RCC) patients with low frequency of M-MDSCs showed a better disease control after anti-tumor peptide-based vaccination in combination with chemotherapy (178); moreover, the HPV16 long-peptide-based vaccination during CarboTaxol treatment was able to reduce circulating MDSCs and generated a strong immune response, confirming the relevance of M-MDSC elimination as a valid approach to enforce anti-tumor immune response (179). Based on these data, several chemotherapeutic compounds with different mechanisms of action were listed as M-MDSC-targeting drugs (173). M-MDSC elimination can be also achieved using immune compounds such cytokines and antibodies. In this line, the combination of IL-2 with anti-CD40 antibody was effective on cancer growth control in two different mouse tumor models by inducing M-MDSC elimination trough Fas-mediated apoptosis (180). Similarly, to improve the efficacy of CAR T cells immunotherapy in leukemia setting, by limiting transferred T cells-associated life-threatening cytokine-release syndrome (CRS) and neurotoxicity, either monocyte-depletion or infusion of IL-1 receptor antagonist anakinra were reported as effective strategies (181). Other therapeutic approaches aim at favoring MDSC differentiation into anti-tumoral cell subsets such as macrophages and DCs. For example, all-trans-retinoic acid (ATRA) treatment was reported to promote the differentiation of MDSC into mature anti-tumoral myeloid cells via the activation of the ERK1/2 signaling pathway (182) as well as the treatment with 25-hydroxyvitamin D3 was reported to reduce the frequency of immature immune suppressive cells in peripheral blood of HNSCC patients (183). Moreover, M-MDSC differentiation toward mature anti-tumoral monocytes-derived DCs can be achieved using pharmacological inhibitor of ATP-converting ectoenzyme ENTPD2, thus mitigating cancer growth and enhancing the efficiency of immune checkpoint inhibitors (184). Finally, some targeting approaches aim at blocking M-MDSC migration from bone marrow to tumor site. For example, amino-biphosphonates were able to prevent the activation of matrix metalloproteases (MMPs) limiting cancer aggressiveness and distal spread (185). Along the same line, pharmacological antagonist of chemokine receptors (i.e., S-265610, CXCR2-specific antagonist) were able to drastically reduce M-MDSC in tumor-bearing mice (186).

CONCLUDING REMARKS

Monocytes are relevant immunological cells connecting the innate and adaptive immune compartments. Here, we have attempted to integrate recent advances in the molecular, metabolic, and functional aspects of monocyte biology with the current state of understanding about the role of these cells in cancer growth and metastatic spread. Although a large body of evidence supports the notion that circulating monocytes serve only as precursor cells that replenish tissue macrophages and DC populations, the overwhelming complexity underlined by high throughput technologies, is supporting a direct contribution of monocyte to cancer development. Targeting monocytes at the immunological, metabolic, epigenetic, and transcriptional level is a promising strategy to treat both disease with impaired immune function, like cancer, or with over-reactive immune response, like autoimmune diseases. The advances in our knowledge on monocyte development, response, and reprogramming, particularly during cancer evolution and metastatic spread, will pave the way for the development of new therapeutic strategy with specificity and limited toxicity.

AUTHOR CONTRIBUTIONS

SC, SU, RT, IM, FD, and SS wrote parts of the manuscript. SC and VB edited and finalized the manuscript.

FUNDING

This work was supported by the Italian Association for Cancer Research (18603 and 12182 that refers to Special Program Molecular Clinical Oncology 5 per mille), by the Cariverona Fondation (Project call, 2017), by the Cancer Research Institute (Clinic and Laboratory Integration Program, CLIP-2017), and by Euronanomed III (Joint Translational Call 2017, Project resolve) to VB.

REFERENCES

1. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol*. (2009) 27:669–92. doi: 10.1146/annurev.immunol.021908.132557
2. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. (2005) 5:953–64. doi: 10.1038/nri1733
3. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*. (2014) 14:392–404. doi: 10.1038/nri3671
4. Cignarella A, Tedesco S, Cappellari R, Fadini GP. The continuum of monocyte phenotypes: experimental evidence and prognostic utility in assessing cardiovascular risk. *J Leukoc Biol*. (2018) 103:1021–8. doi: 10.1002/JLB.5RU1217-477RR
5. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
6. Thomas GD, Hanna RN, Vasudevan NT, Hamers AA, Romanoski CE, McArdle S, et al. Deleting an Nr4a1 super-enhancer subdomain ablates Ly6C(low) monocytes while preserving macrophage gene function. *Immunity*. (2016) 45:975–87. doi: 10.1016/j.immuni.2016.10.011
7. Guilliams M, Mildner A, Yona S. Developmental and functional heterogeneity of monocytes. *Immunity*. (2018) 49:595–613. doi: 10.1016/j.immuni.2018.10.005
8. Segura E, Amigorena S. Inflammatory dendritic cells in mice and humans. *Trends Immunol*. (2013) 34:440–5. doi: 10.1016/j.it.2013.06.001
9. Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. *Annu Rev Immunol*. (2015) 33:643–75. doi: 10.1146/annurev-immunol-032414-112220
10. Mildner A, Yona S, Jung S. A close encounter of the third kind: monocyte-derived cells. *Adv Immunol*. (2013) 120:69–103. doi: 10.1016/B978-0-12-417028-5.00003-X
11. de Bree C, Marijnissen RJ, Kel JM, Rosendahl Huber SK, Aaby P, Benn CS, et al. Bacillus calmette-guerin-induced trained immunity is not protective

for experimental influenza A/Anhui/1/2013 (H7N9) infection in mice. *Front Immunol*. (2018) 9:869. doi: 10.3389/fimmu.2018.02471
12. Saz-Leal P, Del Fresno C, Brandi P, Martinez-Cano S, Dungan OM, Chisholm JD, et al. Targeting SHIP-1 in myeloid cells enhances trained immunity and boosts response to infection. *Cell Rep*. (2018) 25:1118–26. doi: 10.1016/j.celrep.2018.09.092
13. Mitroulis I, Ruppova K, Wang B, Chen LS, Grzybek M, Grinenko T, et al. Modulation of myelopoiesis progenitors is an integral component of trained immunity. *Cell*. (2018) 172:147–61 e12. doi: 10.1016/j.cell.2017.11.034
14. Bain CC, Bravo-Blas A, Scott CL, Perdiguero EG, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol*. (2014) 15:929–37. doi: 10.1038/ni.2967
15. Cronk JC, Filiano AJ, Louveau A, Marin I, Marsh R, Ji E, et al. Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia. *J Exp Med*. (2018) 215:1627–47. doi: 10.1084/jem.20180247
16. T'Jonck W, Guilliams M, Bonnardel J. Niche signals and transcription factors involved in tissue-resident macrophage development. *Cell Immunol*. (2018) 330:43–53. doi: 10.1016/j.cellimm.2018.02.005
17. van de Laar L, Saelens W, De Prijck S, Martens L, Scott CL, Van Isterdael G, et al. Yolk sac macrophages, fetal liver, and adult monocytes can colonize an empty niche and develop into functional tissue-resident macrophages. *Immunity*. (2016) 44:755–68. doi: 10.1016/j.immuni.2016.02.017
18. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. (2009) 325:612–6. doi: 10.1126/science.1175202
19. Mildner A, Schonheit J, Giladi A, David E, Lara-Astiaso D, Lorenzo-Vivas E, et al. Genomic characterization of murine monocytes reveals C/EBPbeta transcription factor dependence of Ly6C(-) cells. *Immunity*. (2017) 46:849–62 e7. doi: 10.1016/j.immuni.2017.04.018
20. Polletti S, Natoli G. Understanding spontaneous conversion: the case of the Ly6C(-) monocyte. *Immunity*. (2017) 46:764–6. doi: 10.1016/j.immuni.2017.04.010
21. Gamrekelashvili J, Giagnorio R, Jussofie J, Soehnlein O, Duchene J, Briseno CG, et al. Regulation of monocyte cell fate by blood vessels mediated by Notch signalling. *Nat Commun*. (2016) 7:12597. doi: 10.1038/ncomms12597
22. Kurotaki D, Osato N, Nishiyama A, Yamamoto M, Ban T, Sato H, et al. Essential role of the IRF8-KLF4 transcription factor cascade in murine monocyte differentiation. *Blood*. (2013) 121:1839–49. doi: 10.1182/blood-2012-06-437863
23. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. (2017) 356:eaah4573. doi: 10.1126/science.aah4573
24. Zilionis R, Engblom C, Pfirschke C, Savova V, Zemmour D, Saatcioglu HD, et al. Single-cell transcriptomics of human and mouse lung cancers reveals conserved myeloid populations across individuals and species. *Immunity*. (2019) 50:1317–34 e10. doi: 10.1016/j.immuni.2019.03.009
25. Shen JJ, Lee PH, Holden JJ, Shatkay H. Using cluster ensemble and validation to identify subtypes of pervasive developmental disorders. *AMIA Annu Symp Proc*. (2007) 666–70.
26. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell*. (2013) 153:362–75. doi: 10.1016/j.cell.2013.03.010
27. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*. (2010) 33:375–86. doi: 10.1016/j.immuni.2010.08.012
28. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med*. (2017) 214:1913–23. doi: 10.1084/jem.20170355
29. Selimoglu-Buet D, Wagner-Ballon O, Saada V, Bardet V, Itzykson R, Bencheikh L, et al. Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia. *Blood*. (2015) 125:3618–26. doi: 10.1182/blood-2015-01-620781
30. Yanez A, Coetzee SG, Olsson A, Muench DE, Berman BP, Hazelett DJ, et al. Granulocyte-monocyte progenitors and monocyte-dendritic cell progenitors independently produce functionally distinct monocytes. *Immunity*. (2017) 47:890–902 e4. doi: 10.1016/j.immuni.2017.10.021
31. Satoh T, Nakagawa K, Sugihara F, Kuwahara R, Ashihara M, Yamane F, et al. Identification of an atypical monocyte and committed progenitor involved in fibrosis. *Nature*. (2017) 541:96–101. doi: 10.1038/nature20611
32. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA, et al. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat Immunol*. (2011) 12:778–85. doi: 10.1038/ni.2063
33. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. (2003) 19:71–82. doi: 10.1016/S1074-7613(03)00174-2
34. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology*. (2006) 211:609–18. doi: 10.1016/j.imbio.2006.05.025
35. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. (2007) 317:666–70. doi: 10.1126/science.1142883
36. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*. (1994) 265:1573–7. doi: 10.1126/science.8079170
37. Nutt SL, Metcalf D, D'Amico A, Polli M, Wu L. Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *J Exp Med*. (2005) 201:221–31. doi: 10.1084/jem.20041535
38. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J*. (1996) 15:5647–58. doi: 10.1002/j.1460-2075.1996.tb00949.x
39. Ghisletti S, Barozzi I, Mietton F, Polletti S, De Santa F, Venturini E, et al. Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity*. (2010) 32:317–28. doi: 10.1016/j.immuni.2010.02.008
40. Glass CK, Natoli G. Molecular control of activation and priming in macrophages. *Nat Immunol*. (2016) 17:26–33. doi: 10.1038/ni.3306
41. Nagulapalli S, Pongubala JM, Atchison ML. Multiple proteins physically interact with PU.1. Transcriptional synergy with NF-IL6 beta (C/EBP delta, CRP3). *J Immunol*. (1995) 155:4330–8.
42. Zhang DE, Hetherington CJ, Chen HM, Tenen DG. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. *Mol Cell Biol*. (1994) 14:373–81. doi: 10.1128/MCB.14.1.373
43. Hirai H, Zhang P, Dayaram T, Hetherington CJ, Mizuno S, Imanishi J, et al. C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol*. (2006) 7:732–9. doi: 10.1038/ni1354
44. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol*. (1998) 18:4301–14. doi: 10.1128/MCB.18.7.4301
45. Avellino R, Havermans M, Erpelinck C, Sanders MA, Hoogenboezem R, van de Werken HJ, et al. An autonomous CEBPA enhancer specific for myeloid-lineage priming and neutrophilic differentiation. *Blood*. (2016) 127:2991–3003. doi: 10.1182/blood-2016-01-695759
46. Ma O, Hong S, Guo H, Ghiaur G, Friedman AD. Granulopoiesis requires increased C/EBPalpha compared to monopoiesis, correlated with elevated Cebpa in immature G-CSF receptor versus M-CSF receptor expressing cells. *PLoS ONE*. (2014) 9:e95784. doi: 10.1371/journal.pone.0095784
47. Rossi A, Sebastiani A. Photocoagulation treatment of diabetic retinopathy. *Minerva Endocrinol*. (1988) 13:63–6.
48. Wang D, D'Costa J, Civin CI, Friedman AD. C/EBPalpha directs monocytic commitment of primary myeloid progenitors. *Blood*. (2006) 108:1223–9. doi: 10.1182/blood-2005-12-008763
49. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nat Rev Cancer*. (2013) 13:739–52. doi: 10.1038/nrc3581

50. De Sanctis F, Bronte V, Ugel S. Tumor-induced myeloid-derived suppressor cells. *Microbiol Spectr*. (2016) 4:1–22. doi: 10.1128/microbiolspec.MCHD-0016-2015
51. Ugel S, Peranzoni E, Desantis G, Chioda M, Walter S, Weinschenk T, et al. Immune tolerance to tumor antigens occurs in a specialized environment of the spleen. *Cell Rep*. (2012) 2:628–39. doi: 10.1016/j.celrep.2012.08.006
52. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni V, Mocellin S, et al. IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. *J Immunol*. (2009) 182:6562–8. doi: 10.4049/jimmunol.0803831
53. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity*. (2010) 32:790–802. doi: 10.1016/j.immuni.2010.05.010
54. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kgamma is a molecular switch that controls immune suppression. *Nature*. (2016) 539:437–42. doi: 10.1038/nature19834
55. Kaneda MM, Cappello P, Nguyen AV, Ralainirina N, Hardamon CR, Foubert P, et al. Macrophage PI3Kgamma drives pancreatic ductal adenocarcinoma progression. *Cancer Discov*. (2016) 6:870–85. doi: 10.1158/2159-8290.CD-15-1346
56. Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, et al. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell*. (2009) 138:114–28. doi: 10.1016/j.cell.2009.04.020
57. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun*. (2016) 7:12150. doi: 10.1038/ncomms12150
58. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. (2012) 12:253–68. doi: 10.1038/nri3175
59. Vasquez-Dunddel D, Pan F, Zeng Q, Gorbounov M, Albesiano E, Fu J, et al. STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients. *J Clin Invest*. (2013) 123:1580–9. doi: 10.1172/JCI60083
60. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, et al. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med*. (2011) 208:1949–62. doi: 10.1084/jem.20101956
61. De Sanctis F, Sandri S, Ferrarini G, Pagliarello I, Sartoris S, Ugel S, et al. The emerging immunological role of post-translational modifications by reactive nitrogen species in cancer microenvironment. *Front Immunol*. (2014) 5:69. doi: 10.3389/fimmu.2014.00069
62. Marigo I, Zilio S, Desantis G, Mlecnik B, Agnellini AHR, Ugel S, et al. T cell cancer therapy requires CD40-CD40L activation of tumor necrosis factor and inducible nitric-oxide-synthase-producing dendritic cells. *Cancer Cell*. (2016) 30:377–90. doi: 10.1016/j.ccell.2016.08.004
63. Sharma MD, Rodriguez PC, Koehn BH, Baban B, Cui Y, Guo G, et al. Activation of p53 in immature myeloid precursor cells controls differentiation into Ly6c(+)CD103(+) monocytic antigen-presenting cells in tumors. *Immunity*. (2018) 48:91–106 e6. doi: 10.1016/j.immuni.2017.12.014
64. Safa AR. c-FLIP, a master anti-apoptotic regulator. *Exp Oncol*. (2012) 34:176–84.
65. Fiore A, Ugel S, De Sanctis F, Sandri S, Fracasso G, Trovato R, et al. Induction of immunosuppressive functions and NF-kappaB by FLIP in monocytes. *Nat Commun*. (2018) 9:5193. doi: 10.1038/s41467-018-07654-4
66. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe*. (2012) 12:223–32. doi: 10.1016/j.chom.2012.06.006
67. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science*. (2014) 345:1250684. doi: 10.1126/science.1250684
68. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille calmette-guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA*. (2012) 109:17537–42. doi: 10.1073/pnas.1202870109
69. Mourits VP, Wijkmans JC, Joosten LA, Netea MG. Trained immunity as a novel therapeutic strategy. *Curr Opin Pharmacol*. (2018) 41:52–58. doi: 10.1016/j.coph.2018.04.007
70. Christ A, Gunther P, Lauterbach MAR, Duewell P, Biswas D, Pelka K, et al. Western diet triggers NLRP3-dependent innate immune reprogramming. *Cell*. (2018) 172:162–75 e14. doi: 10.1016/j.cell.2017.12.013
71. Bekkering S, Quintin J, Joosten LA, van der Meer JW, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. *Arterioscler Thromb Vasc Biol*. (2014) 34:1731–8. doi: 10.1161/ATVBAHA.114.303887
72. Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat Immunol*. (2013) 14:812–20. doi: 10.1038/ni.2639
73. Dominguez-Andres J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, et al. The itaconate pathway is a central regulatory node linking innate immune tolerance and trained immunity. *Cell Metab*. (2019) 29:211–20 e5. doi: 10.1016/j.cmet.2018.09.003
74. Bekkering S, Arts RJW, Novakovic B, Kourtzelis I, van der Heijden C, Li Y, et al. Metabolic induction of trained immunity through the mevalonate pathway. *Cell*. (2018) 172:135–46 e9. doi: 10.1016/j.cell.2017.11.025
75. Ostuni R, Piccolo V, Barozzi I, Polletti S, Termanini A, Bonifacio S, et al. Latent enhancers activated by stimulation in differentiated cells. *Cell*. (2013) 152:157–71. doi: 10.1016/j.cell.2012.12.018
76. Kaikkonen MU, Spann NJ, Heinz S, Romanoski CE, Allison KA, Stender JD, et al. Remodeling of the enhancer landscape during macrophage activation is coupled to enhancer transcription. *Mol Cell*. (2013) 51:310–25. doi: 10.1016/j.molcel.2013.07.010
77. Groh L, Keating ST, Joosten LAB, Netea MG, Riksen NP. Monocyte and macrophage immunometabolism in atherosclerosis. *Semin Immunopathol*. (2018) 40:203–14. doi: 10.1007/s00281-017-0656-7
78. Stienstra R, Netea-Maier RT, Riksen NP, Joosten LAB, Netea MG. Specific and complex reprogramming of cellular metabolism in myeloid cells during innate immune responses. *Cell Metab*. (2017) 26:142–56. doi: 10.1016/j.cmet.2017.06.001
79. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. (2011) 1:54–67. doi: 10.1158/2159-8274.CD-10-0028
80. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity*. (2013) 38:633–43. doi: 10.1016/j.immuni.2013.04.005
81. Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, et al. Lipid accumulation and dendritic cell dysfunction in cancer. *Nat Med*. (2010) 16:880–6. doi: 10.1038/nm.2172
82. Hossain F, Al-Khami AA, Wyczechowska D, Hernandez C, Zheng L, Reiss K, et al. Inhibition of fatty acid oxidation modulates immunosuppressive functions of myeloid-derived suppressor cells and enhances cancer therapies. *Cancer Immunol Res*. (2015) 3:1236–47. doi: 10.1158/2326-6066.CIR-15-0036
83. Freigang S, Ampenberger F, Weiss A, Kanneganti TD, Iwakura Y, Hersberger M, et al. Fatty acid-induced mitochondrial uncoupling elicits inflammasome-independent IL-1alpha and sterile vascular inflammation in atherosclerosis. *Nat Immunol*. (2013) 14:1045–53. doi: 10.1038/ni.2704
84. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: a program of innate immune memory in health and disease. *Science*. (2016) 352:aaf1098. doi: 10.1126/science.aaf1098
85. Christ A, Bekkering S, Latz E, Riksen NP. Long-term activation of the innate immune system in atherosclerosis. *Semin Immunol*. (2016) 28:384–93. doi: 10.1016/j.smim.2016.04.004
86. Yvan-Charvet L, Pagler T, Gautier EL, Avagyan S, Siry RL, Han S, et al. ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. *Science*. (2010) 328:1689–93. doi: 10.1126/science.1189731
87. Sezgin E, Levental I, Mayor S, Eggeling C. The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat Rev Mol Cell Biol*. (2017) 18:361–74. doi: 10.1038/nrm.2017.16

88. Coskun U, Simons K. Cell membranes: the lipid perspective. *Structure*. (2011) 19:1543–8. doi: 10.1016/j.str.2011.10.010
89. Kaul S, Xu H, Zabalawi M, Maruko E, Fulp BE, Bluemn T, et al. Lipid-free apolipoprotein A-I reduces progression of atherosclerosis by mobilizing microdomain cholesterol and attenuating the number of CD131 expressing cells: monitoring cholesterol homeostasis using the cellular ester to total cholesterol ratio. *J Am Heart Assoc*. (2016) 5:e004401. doi: 10.1161/JAHA.116.004401
90. Arts RJ, Novakovic B, Ter Horst R, Carvalho A, Bekkering S, Lachmandas E, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. *Cell Metab*. (2016) 24:807–19. doi: 10.1016/j.cmet.2016.10.008
91. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget*. (2016) 7:28697–710. doi: 10.18632/oncotarget.7376
92. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*. (2011) 475:222–5. doi: 10.1038/nature10138
93. Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. *Nat Rev Immunol*. (2017) 17:559–72. doi: 10.1038/nri.2017.49
94. Scholl SM, Lidereau R, de la Rochefordiere A, Le-Nir CC, Mosseri V, Nogues C, et al. Circulating levels of the macrophage colony stimulating factor CSF-1 in primary and metastatic breast cancer patients. A pilot study. *Breast Cancer Res Treat*. (1996) 39:275–83. doi: 10.1007/BF01806155
95. Beck AH, Espinosa I, Edris B, Li R, Montgomery K, Zhu S, et al. The macrophage colony-stimulating factor 1 response signature in breast carcinoma. *Clin Cancer Res*. (2009) 15:778–87. doi: 10.1158/1078-0432.CCR-08-1283
96. Van Overmeire E, Stijlemans B, Heymann F, Keirsse J, Morias Y, Elkrim Y, et al. M-CSF and GM-CSF receptor signaling differentially regulate monocyte maturation and macrophage polarization in the tumor microenvironment. *Cancer Res*. (2016) 76:35–42. doi: 10.1158/0008-5472.CAN-15-0869
97. Ries CH, Cannarile MA, Hoves S, Benz J, Wartha K, Runza V, et al. Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. *Cancer Cell*. (2014) 25:846–59. doi: 10.1016/j.ccr.2014.05.016
98. Cannarile MA, Weisser M, Jacob W, Jegg AM, Ries CH, Ruttinger D. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J Immunother Cancer*. (2017) 5:53. doi: 10.1186/s40425-017-0257-y
99. Kumar V, Donthireddy L, Marvel D, Condamine T, Wang F, Lavilla-Alonso S, et al. Cancer-associated fibroblasts neutralize the anti-tumor effect of CSF1 receptor blockade by inducing PMN-MDSC infiltration of tumors. *Cancer Cell*. (2017) 32:654–68 e5. doi: 10.1016/j.ccell.2017.10.005
100. Xu J, Escamilla J, Mok S, David J, Priceman S, West B, et al. CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer Res*. (2013) 73:2782–94. doi: 10.1158/0008-5472.CAN-12-3981
101. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res*. (2014) 74:5057–69. doi: 10.1158/0008-5472.CAN-13-3723
102. Bonapace L, Coissieux MM, Wyckoff J, Mertz KD, Varga Z, Junt T, et al. Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. *Nature*. (2014) 515:130–3. doi: 10.1038/nature13862
103. Bernardini G, Ribatti D, Spinetti G, Morbidelli L, Ziche M, Santoni A, et al. Analysis of the role of chemokines in angiogenesis. *J Immunol Methods*. (2003) 273:83–101. doi: 10.1016/S0022-1759(02)00420-9
104. LaGory EL, Giaccia AJ. The ever-expanding role of HIF in tumour and stromal biology. *Nat Cell Biol*. (2016) 18:356–65. doi: 10.1038/ncb3330
105. Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat Rev Mol Cell Biol*. (2016) 17:611–25. doi: 10.1038/nrm.2016.87
106. Scavelli C, Nico B, Cirulli T, Ria R, Di Pietro G, Mangieri D, et al. Vasculogenic mimicry by bone marrow macrophages in patients with multiple myeloma. *Oncogene*. (2008) 27:663–74. doi: 10.1038/sj.onc.1210691
107. Avraham-Davidi I, Yona S, Grunewald M, Landsman L, Cochain C, Silvestre JS, et al. On-site education of VEGF-recruited monocytes improves their performance as angiogenic and arteriogenic accessory cells. *J Exp Med*. (2013) 210:2611–25. doi: 10.1084/jem.20120690
108. Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, DeNardo DG, et al. Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res*. (2010) 70:7465–75. doi: 10.1158/0008-5472.CAN-10-1439
109. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*. (2004) 6:409–21. doi: 10.1016/j.ccr.2004.08.031
110. Gabrilovich DI, Ishida T, Nadaf S, Ohm JE, Carbone DP. Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. *Clin Cancer Res*. (1999) 5:2963–70.
111. Du Four S, Maenhout SK, De Pierre K, Renmans D, Niclou SP, Thielemans K, et al. Axitinib increases the infiltration of immune cells and reduces the suppressive capacity of monocytic MDSCs in an intracranial mouse melanoma model. *Oncoimmunology*. (2015) 4:e998107. doi: 10.1080/2162402X.2014.998107
112. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell*. (2016) 30:836–48. doi: 10.1016/j.ccell.2016.10.009
113. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res*. (2006) 66:9290–8. doi: 10.1158/0008-5472.CAN-06-1819
114. Javeed N, Gustafson MP, Dutta SK, Lin Y, Bamlet WR, Oberg AL, et al. Immunosuppressive CD14(+)HLA-DR(lo/neg) monocytes are elevated in pancreatic cancer and "primed" by tumor-derived exosomes. *Oncoimmunology*. (2017) 6:e1252013. doi: 10.1080/2162402X.2016.1252013
115. Yuan XK, Zhao XK, Xia YC, Zhu X, Xiao P. Increased circulating immunosuppressive CD14(+)HLA-DR(-/low) cells correlate with clinical cancer stage and pathological grade in patients with bladder carcinoma. *J Int Med Res*. (2011) 39:1381–91. doi: 10.1177/147323001103900424
116. Brimnes MK, Vangsted AJ, Knudsen LM, Gimsing P, Gang AO, Johnsen HE, et al. Increased level of both CD4+FOXP3+ regulatory T cells and CD14+HLA-DR(-)/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand J Immunol*. (2010) 72:540–7. doi: 10.1111/j.1365-3083.2010.02463.x
117. de Vrij J, Maas SL, Kwappenberg KM, Schnoor R, Kleijn A, Dekker L, et al. Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells. *Int J Cancer*. (2015) 137:1630–42. doi: 10.1002/ijc.29521
118. Gabrusiewicz K, Li X, Wei J, Hashimoto Y, Marisetty AL, Ott M, et al. Glioblastoma stem cell-derived exosomes induce M2 macrophages and PD-L1 expression on human monocytes. *Oncoimmunology*. (2018) 7:e1412909. doi: 10.1080/2162402X.2017.1412909
119. Gershbein LL, Lieberman HL. Lactate dehydrogenase of concentric zones of human senile cataracts and calf lenses produced by ultrasound. *Biotechnol Appl Biochem*. (1989) 11:602–9.
120. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol*. (2010) 12:19–30. doi: 10.1038/ncb2000
121. Hsu C, Morohashi Y, Yoshimura S, Manrique-Hoyos N, Jung S, Lauterbach MA, et al. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. *J Cell Biol*. (2010) 189:223–32. doi: 10.1083/jcb.200911018
122. Lu H, Clauser KR, Tam WL, Frose J, Ye X, Eaton EN, et al. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. *Nat Cell Biol*. (2014) 16:1105–17. doi: 10.1038/ncb3041
123. Akhurst RJ, Hata A. Targeting the TGFbeta signalling pathway in disease. *Nat Rev Drug Discov*. (2012) 11:790–811. doi: 10.1038/nrd3810

124. Travis MA, Sheppard D. TGF-beta activation and function in immunity. *Annu Rev Immunol*. (2014) 32:51–82. doi: 10.1146/annurev-immunol-032713-120257
125. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol*. (2008) 9:1347–55. doi: 10.1038/ni.1677
126. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. (2008) 9:1341–6. doi: 10.1038/ni.1659
127. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol*. (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710
128. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. (2003) 198:1875–86. doi: 10.1084/jem.20030152
129. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol*. (2007) 178:4022–6. doi: 10.4049/jimmunol.178.7.4022
130. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol*. (2008) 9:632–40. doi: 10.1038/ni.1607
131. Kelly A, Gunaltay S, McEntee CP, Shuttleworth EE, Smedley C, Houston SA, et al. Human monocytes and macrophages regulate immune tolerance via integrin alphavbeta8-mediated TGFbeta activation. *J Exp Med*. (2018) 215:2725–36. doi: 10.1084/jem.20171491
132. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *J Cell Biol*. (2002) 157:493–507. doi: 10.1083/jcb.200109100
133. Takasaka N, Seed RI, Cormier A, Bondesson AJ, Lou J, Elattma A, et al. Integrin alphavbeta8-expressing tumor cells evade host immunity by regulating TGF-beta activation in immune cells. *JCI Insight*. (2018) 3:122591. doi: 10.1172/jci.insight.122591
134. Chiang AC, Massague J. Molecular basis of metastasis. *N Engl J Med*. (2008) 359:2814–23. doi: 10.1056/NEJMra0805239
135. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell*. (2011) 147:275–92. doi: 10.1016/j.cell.2011.09.024
136. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med*. (2006) 354:610–21. doi: 10.1056/NEJMra052723
137. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol*. (2006) 7:311–7. doi: 10.1038/ni1309
138. Zhang Y, Yang P, Sun T, Li D, Xu X, Rui Y, et al. miR-126 and miR-126* repress recruitment of mesenchymal stem cells and inflammatory monocytes to inhibit breast cancer metastasis. *Nat Cell Biol*. (2013) 15:284–94. doi: 10.1038/ncb2690
139. Butler KL, Clancy-Thompson E, Mullins DW. CXCR3(+) monocytes/macrophages are required for establishment of pulmonary metastases. *Sci Rep*. (2017) 7:45593. doi: 10.1038/srep45593
140. Shi H, Zhang J, Han X, Li H, Xie M, Sun Y, et al. Recruited monocytic myeloid-derived suppressor cells promote the arrest of tumor cells in the premetastatic niche through an IL-1beta-mediated increase in E-selectin expression. *Int J Cancer*. (2017) 140:1370–83. doi: 10.1002/ijc.30538
141. Chen Q, Zhang XH, Massague J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. *Cancer Cell*. (2011) 20:538–49. doi: 10.1016/j.ccr.2011.08.025
142. Zhang H, Yu Y, Zhou L, Ma J, Tang K, Xu P, et al. Circulating tumor microparticles promote lung metastasis by reprogramming inflammatory and mechanical niches via a macrophage-dependent pathway. *Cancer Immunol Res*. (2018) 6:1046–56. doi: 10.1158/2326-6066.CIR-17-0574
143. Takano Y, Masuda T, Iinuma H, Yamaguchi R, Sato K, Tobo T, et al. Circulating exosomal microRNA-203 is associated with metastasis possibly via inducing tumor-associated macrophages in colorectal cancer. *Oncotarget*. (2017) 8:78598–613. doi: 10.18632/oncotarget.20009
144. Plebanek MP, Angeloni NL, Vinokour E, Li J, Henkin A, Martinez-Marin D, et al. Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche. *Nat Commun*. (2017) 8:1319. doi: 10.1038/s41467-017-01433-3
145. Murray PJ. Immune regulation by monocytes. *Semin Immunol*. (2018) 35:12–18. doi: 10.1016/j.smim.2017.12.005
146. Bednar M, Kraemer R, Abraham NG, Mullane KM. Arachidonic acid monooxygenase and lipoxygenase activities in polymorphonuclear leukocytes. *Biochem Pharmacol*. (1987) 36:1741–7. doi: 10.1016/0006-2952(87)90063-3
147. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol*. (2019) 19:369–82. doi: 10.1038/s41577-019-0127-6
148. Kawai K, Miyazaki J, Joraku A, Nishiyama H, Akaza H. Bacillus Calmette-Guerin (BCG) immunotherapy for bladder cancer: current understanding and perspectives on engineered BCG vaccine. *Cancer Sci*. (2013) 104:22–7. doi: 10.1111/cas.12075
149. Redelman-Sidi G, Glickman MS, Bochner BH. The mechanism of action of BCG therapy for bladder cancer–a current perspective. *Nat Rev Urol*. (2014) 11:153–62. doi: 10.1038/nrurol.2014.15
150. Buffen K, Oosting M, Quintin J, Ng A, Kleinnijenhuis J, Kumar V, et al. Autophagy controls BCG-induced trained immunity and the response to intravesical BCG therapy for bladder cancer. *PLoS Pathog*. (2014) 10:e1004485. doi: 10.1371/journal.ppat.1004485
151. Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, et al. Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. *Clin Vaccine Immunol*. (2014) 21:534–45. doi: 10.1128/CVI.00688-13
152. Li X, Yu J, Xu S, Wang N, Yang H, Yan Z, et al. Chemical conjugation of muramyl dipeptide and paclitaxel to explore the combination of immunotherapy and chemotherapy for cancer. *Glycoconj J*. (2008) 25:415–25. doi: 10.1007/s10719-007-9095-3
153. Hewitt RE, Pele LC, Tremelling M, Metz A, Parkes M, Powell JJ. Immuno-inhibitory PD-L1 can be induced by a peptidoglycan/NOD2 mediated pathway in primary monocytic cells and is deficient in Crohn's patients with homozygous NOD2 mutations. *Clin Immunol*. (2012) 143:162–9. doi: 10.1016/j.clim.2012.01.016
154. Rojas LB, Gomes MB. Metformin: an old but still the best treatment for type 2 diabetes. *Diabetol Metab Syndr*. (2013) 5:6. doi: 10.1186/1758-5996-5-6
155. Davis RJ, Moore EC, Clavijo PE, Friedman J, Cash H, Chen Z, et al. Anti-PD-L1 efficacy can be enhanced by inhibition of myeloid-derived suppressor cells with a selective inhibitor of PI3Kdelta/gamma. *Cancer Res*. (2017) 77:2607–19. doi: 10.1158/0008-5472.CAN-16-2534
156. Ali K, Soond DR, Pineiro R, Hagemann T, Pearce W, Lim EL, et al. Inactivation of PI(3)K p110delta breaks regulatory T-cell-mediated immune tolerance to cancer. *Nature*. (2014) 510:407–11. doi: 10.1038/nature13444
157. Lu X, Horner JW, Paul E, Shang X, Troncoso P, Deng P, et al. Effective combinatorial immunotherapy for castration-resistant prostate cancer. *Nature*. (2017) 543:728–32. doi: 10.1038/nature21676
158. Wang L, Chang J, Varghese D, Dellinger M, Kumar S, Best AM, et al. A small molecule modulates Jumonji histone demethylase activity and selectively inhibits cancer growth. *Nat Commun*. (2013) 4:2035. doi: 10.1038/ncomms3035
159. Novakovic B, Habibi E, Wang SY, Arts RJW, Davar R, Megchelenbrink W, et al. β-glucan reverses the epigenetic state of LPS-induced immunological tolerance. *Cell*. (2016) 167:1354–68 e14. doi: 10.1016/j.cell.2016.09.034
160. Bettencourt IA, Powell JD. Targeting metabolism as a novel therapeutic approach to autoimmunity, inflammation, and transplantation. *J Immunol*. (2017) 198:999–1005. doi: 10.4049/jimmunol.1601318
161. Fiorucci S, Santucci L, Cirino G, Mencarelli A, Familiari L, Soldato PD, et al. IL-1 beta converting enzyme is a target for nitric oxide-releasing aspirin: new insights in the antiinflammatory mechanism of nitric oxide-releasing nonsteroidal antiinflammatory drugs. *J Immunol*. (2000) 165:5245–54. doi: 10.4049/jimmunol.165.9.5245

162. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. (2009) 9:162–74. doi: 10.1038/nri2506
163. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res*. (2004) 64:6337–43. doi: 10.1158/0008-5472.CAN-04-0757
164. Mondanelli G, Bianchi R, Pallotta MT, Orabona C, Albini E, Iacono A, et al. A Relay pathway between arginine and tryptophan metabolism confers immunosuppressive properties on dendritic cells. *Immunity*. (2017) 46:233–44. doi: 10.1016/j.immuni.2017.01.005
165. Mondanelli G, Ugel S, Grohmann U, Bronte V. The immune regulation in cancer by the amino acid metabolizing enzymes ARG and IDO. *Curr Opin Pharmacol*. (2017) 35:30–9. doi: 10.1016/j.coph.2017.05.002
166. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med*. (2005) 202:931–9. doi: 10.1084/jem.20050715
167. Eruslanov E, Daurkin I, Ortiz J, Vieweg J, Kusmartsev S. Pivotal advance: tumor-mediated induction of myeloid-derived suppressor cells and M2-polarized macrophages by altering intracellular PGE(2) catabolism in myeloid cells. *J Leukoc Biol*. (2010) 88:839–48. doi: 10.1189/jlb.1209821
168. Obermajer N, Kalinski P. Generation of myeloid-derived suppressor cells using prostaglandin E2. *Transplant Res*. (2012) 1:15. doi: 10.1186/2047-1440-1-15
169. Ye J, Ma C, Hsueh EC, Eickhoff CS, Zhang Y, Varvares MA, et al. Tumor-derived gammadelta regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence. *J Immunol*. (2013) 190:2403–14. doi: 10.4049/jimmunol.1202369
170. Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, et al. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res*. (2011) 71:2664–74. doi: 10.1158/0008-5472.CAN-10-3055
171. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res*. (2010) 70:68–77. doi: 10.1158/0008-5472.CAN-09-2587
172. Gmunder H, Eck HP, Droge W. Low membrane transport activity for cystine in resting and mitogenically stimulated human lymphocyte preparations and human T cell clones. *Eur J Biochem*. (1991) 201:113–7. doi: 10.1111/j.1432-1033.1991.tb16263.x
173. Ugel S, Delpozzo F, Desantis G, Papalini F, Simonato F, Sonda N, et al. Therapeutic targeting of myeloid-derived suppressor cells. *Curr Opin Pharmacol*. (2009) 9:470–81. doi: 10.1016/j.coph.2009.06.014
174. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends Immunol*. (2011) 32:19–25. doi: 10.1016/j.it.2010.10.002
175. Panni RZ, Sanford DE, Belt BA, Mitchem JB, Worley LA, Goetz BD, et al. Tumor-induced STAT3 activation in monocytic myeloid-derived suppressor cells enhances stemness and mesenchymal properties in human pancreatic cancer. *Cancer Immunol Immunother*. (2014) 63:513–28. doi: 10.1007/s00262-014-1527-x
176. Pinton L, Solito S, Damuzzo V, Francescato S, Pozzuoli A, Berizzi A, et al. Activated T cells sustain myeloid-derived suppressor cell-mediated immune suppression. *Oncotarget*. (2016) 7:1168–84. doi: 10.18632/oncotarget.6662
177. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res*. (2010) 70:3052–61. doi: 10.1158/0008-5472.CAN-09-3690
178. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med*. (2012) 18:1254–61. doi: 10.1038/nm.2883
179. Welters MJ, van der Sluis TC, van Meir H, Loof NM, van Ham VJ, van Duikeren S, et al. Vaccination during myeloid cell depletion by cancer chemotherapy fosters robust T cell responses. *Sci Transl Med*. (2016) 8:334ra52. doi: 10.1126/scitranslmed.aad8307
180. Weiss JM, Subleski JJ, Back T, Chen X, Watkins SK, Yagita H, et al. Regulatory T cells and myeloid-derived suppressor cells in the tumor microenvironment undergo Fas-dependent cell death during IL-2/alphaCD40 therapy. *J Immunol*. (2014) 192:5821–9. doi: 10.4049/jimmunol.1400404
181. Norelli M, Camisa B, Barbiera G, Falcone L, Purevdorj A, Genua M, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med*. (2018) 24:739–48. doi: 10.1038/s41591-018-0036-4
182. Nefedova Y, Fishman M, Sherman S, Wang X, Beg AA, Gabrilovich DI. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res*. (2007) 67:11021–8. doi: 10.1158/0008-5472.CAN-07-2593
183. Lathers DM, Clark JI, Achille NJ, Young MR. Phase IB study of 25-hydroxyvitamin D(3) treatment to diminish suppressor cells in head and neck cancer patients. *Hum Immunol*. (2001) 62:1282–93. doi: 10.1016/S0198-8859(01)00317-2
184. Chiu DK, Tse AP, Xu IM, Di Cui J, Lai RK, Li LL, et al. Hypoxia inducible factor HIF-1 promotes myeloid-derived suppressor cells accumulation through ENTPD2/CD39L1 in hepatocellular carcinoma. *Nat Commun*. (2017) 8:517. doi: 10.1038/s41467-017-00530-7
185. Santini D, Vincenzi B, Dicuonzo G, Avvisati G, Massacesi C, Battistoni F, et al. Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients. *Clin Cancer Res*. (2003) 9:2893–7.
186. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. (2008) 13:23–35. doi: 10.1016/j.ccr.2007.12.004

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

frontiers in Immunology

MINI REVIEW
published: 14 August 2019
doi: 10.3389/fimmu.2019.01912

Neutrophil Maturity in Cancer

John B. G. Mackey[1,2], *Seth B. Coffelt*[1,2]* *and Leo M. Carlin*[1,2]*

[1] *Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom,* [2] *Cancer Research UK Beatson Institute, Glasgow, United Kingdom*

Neutrophils are implicated in almost every stage of oncogenesis and paradoxically display anti- and pro-tumor properties. Accumulating evidence indicates that neutrophils display diversity in their phenotype resulting from functional plasticity and/or changes to granulopoiesis. In cancer, neutrophils at a range of maturation stages can be identified in the blood and tissues (i.e., outside of their developmental niche). The functional capacity of neutrophils at different states of maturation is poorly understood resulting from challenges in their isolation, identification, and investigation. Thus, the impact of neutrophil maturity on cancer progression and therapy remains enigmatic. In this review, we discuss the identification, prevalence, and function of immature and mature neutrophils in cancer and the potential impact of this on tumor progression and cancer therapy.

Keywords: neutrophil, cancer, myeloid, cancer inflammation, granulopoiesis

OPEN ACCESS

Edited by:
Wei Zheng,
Albert Einstein College of Medicine, United States

Reviewed by:
Siegfried Weiss,
Helmholtz Center for Infection Research, Germany
Viktor Umansky,
German Cancer Research Center (DKFZ), Germany

***Correspondence:**
Seth B. Coffelt
seth.coffelt@glasgow.ac.uk
Leo M. Carlin
leo.carlin@glasgow.ac.uk

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *09 May 2019*
Accepted: *29 July 2019*
Published: *14 August 2019*

Citation:
Mackey JBG, Coffelt SB and Carlin LM (2019) Neutrophil Maturity in Cancer. Front. Immunol. 10:1912. doi: 10.3389/fimmu.2019.01912

INTRODUCTION

Neutrophils in cancer have received very little attention until recently, despite contributing 50–70% and 10–25% of circulating leukocytes in humans and laboratory mice, respectively (1). However, recent progress has renewed interest in these cells. In experimental cancer models, neutrophils have been implicated in nearly every stage of the oncogenic process and their role has been reviewed in detail (2–4). Neutrophils are able to mediate a broad range of anti- and pro-tumor activities from direct cancer cell killing to tumor cell proliferation, angiogenesis, metastasis, and orchestrating other immune responses. These recent studies have highlighted the complexity of neutrophils in cancer progression, with novel information on their previously unappreciated plasticity and heterogeneity. While neutrophil plasticity can be directly affected by the local microenvironment, neutrophil heterogeneity is also influenced by their maturation (5), age (6), suppressive properties (7), function [e.g., phagocytosis (8)], and reverse transendothelial migration (rTEM) (9). An underexplored aspect of this is the appearance of immature neutrophils in cancer. Differences in the phenotype and functional capacities of immature and mature neutrophil populations are being identified, and their impact on cancer progression is emerging (10). However, the influence of neutrophil maturity on their anti- or pro-tumor properties remains understudied. In this review, we focus on the functional properties and relevance of immature neutrophils in cancer. We discuss methods used to identify neutrophils of different maturation states and explore their limitations. Finally, we postulate the impact that neutrophil maturity may have on the efficacy of cancer therapies.

GRANULOPOIESIS

After birth, neutrophil production occurs primarily in the bone marrow (BM) where they are derived from hematopoietic stem cells (HSCs). During neutrophil differentiation in mice and humans the nucleus progresses from a banded to segmented morphology, allowing the

identification of neutrophils at distinct stages of maturity (11). Stages of neutrophil differentiation are also characterized by their unique expression of the transcription factors PU.1 and CCAAT enhancer binding protein (C/EBP)-α (12), C/EBPβ (13), and C/EBPε (14). Mature neutrophils are mitotically inactive with cell cycle arrest occurring during the myelocyte to metamyelocyte transition (15). The post-mitotic BM transit of neutrophils and release into the circulation takes between 5 and 8 days in humans (16) and 1–2 days in mice during homeostasis (17). Neutrophil granules, termed azurophillic (primary), specific (secondary), and gelatinase (tertiary), in addition to secretory vesicles, are formed at specific stages of neutrophil differentiation. Each granule type is composed of distinct proteins synthesized at the time of formation (18) and granules are released in reverse sequential order following neutrophil activation (19). As such, the proteome composition of immature and mature neutrophils is greatly different. It is important to also acknowledge that in disease, including cancer, granulopoiesis can occur outside of the medullary spaces of the BM, termed extramedullary hematopoiesis (EMH); however, little is known about the mechanisms regulating EMH and its influence on neutrophil development (20).

ISOLATION AND IDENTIFICATION OF IMMATURE NEUTROPHILS

Despite the extensive data on neutrophils and their functions in homeostasis and disease, they remain a challenging cell population to study largely due to their short half-life [∼18.5 h in the circulation of humans during homeostasis (16)] and propensity for priming and activation. While neutrophil life span can be increased following their activation and extravasation, a small window of opportunity for *in vitro* experimentation remains in comparison to other cell types. Neutrophil properties derived from *ex vivo* experimentation can be difficult to accurately interpret and apply to their behavior *in vivo*. Developments of *in vivo* imaging techniques and identification of neutrophils (e.g., via *in vivo* injection of fluorescently conjugated anti-Ly6G antibody, clone 1A8 (9, 21, 22) and fluorescent reporter mice (23) have allowed their investigation without possible *ex vivo* manipulation-induced artifacts; however, these approaches still have their own caveats for example the undetermined function for Ly6G (23–25). Importantly, experimental analysis of immature neutrophil populations is an even greater challenge.

TABLE 1 | Methods for the identification of immature neutrophils in humans.

Immature population	Feature/Cell surface markers	References
Metamyelocyte Myelocyte	Sysmex IG	(27)
Myeloblast to mature	Low density	(28)
Immature	$CD10^{Low}CD15^{High}$	(29)
Myelocyte to band	Low density $SSC^{High}CD66b^{Pos}CD125^{Neg}$ Pappenheim staining	(30)
Myeloblast Promyelocyte	Blood smears Celltac ES hematology analyser	(31)
Band	$CD10^{Dim}CD16^{Dim}$	(32)
Band	$CD10^{Dim}CD16^{Dim}$	(33)
Metamyelocyte Myelocyte Promyelocyte	$CD11b^{Low}CD16^{Pos}$	(34)
Immature	BM resident Nuclear Morphology	(35)
Metamyelocyte Myelocyte Promyelocyte	XE 2100, Sysmex hematology analyser	(36)
Band	$CD16^{Dim}$	(37)
Metamyelocyte	$CD35^{Neg}CD49d^{Pos}$	(38)
Metamyelocyte Myelocyte Promyelocyte	Coulter Actdiff 5 automated hematology analyser	(39)
Immature	Nuclear morphology Number of nucleoli Cytoplasmic granularity	(40)

Density Properties

Neutrophil density changes during development as a result of their increased granularity and changes in cell size (26). Therefore, density gradient purification is useful for enriching neutrophil populations at certain stages of maturation and allows for down-stream analysis. Immature neutrophils are typically found in low density (LD) fractions, whereas mature neutrophils are found in the normal/high density (N/HD) fractions (5) (**Tables 1**, **2**). Nevertheless, the neutrophil populations obtained by density gradient purification are not pure as N/HDNs can become LDNs following activation (55), making interpretation of the functional properties of neutrophil maturity challenging by this technique. For instance, LDNs isolated from the peripheral blood of 4T1 tumor-bearing mice make up ∼40% of morphologically mature neutrophils (5), LDNs obtained from the peripheral blood of mice bearing breast cancer liver metastasis were composed of 80% neutrophils with an immature nuclear morphology (56), and the nuclear morphology of LDNs from lung cancer patients represent both mature and immature neutrophils (5). Overall, this technique can be useful for enriching neutrophil populations; although, more specific methods of identification of neutrophil maturity are required for accurate interpretation of downstream functional analysis.

Morphology and Cell Surface Markers

Nuclear segmentation is considered accurate for immature neutrophil identification in the peripheral blood of cancer patients (57) and mouse models of cancer (58) (**Tables 1**, **2**). However, cells cannot be isolated by this method for downstream experimentation. A major hindrance in neutrophil biology is the lack of a specific and robust marker of neutrophil maturity. Changes in cell surface receptor expression during maturation, such as the CXCR4:CXCR2 axis (59, 60), can be used to separate

TABLE 2 | Methods for the identification of immature neutrophils in mice.

Immature population	Feature/Cell surface markers	References
Myelocyte Meta-myelocyte	Nuclear morphology	(41)
Myeloblast	Nuclear morphology	(42)
Pro-myelocyte to band	Nuclear morphology	(43)
Band Meta-myelocyte	Nuclear Morphology Gr-1^{Low}BrdU ^{Dim}	(17)
Mature Myelocyte Promyelocyte	Gr-1^{Hi}CD11b^{Pos} Gr-1^{Low}CD11b ^{Pos}	(44)
Band/mature	Nuclear morphology	(45)
Immature	Reduced MPO Reduced oxidative burst	(46)
Band	CD11b^{Pos}Gr-1^{Pos}Ly6G^{Pos}Ly6C^{Pos}MDL-1^{Pos}	(47)
Band	Ly6G^{Int}	(21)
Immature	Ly6G$^{Low/Neg}$CD101^{Neg}	(48)
Immature Myelocyte Pro-myelocyte	Gr-1^{High}CD11b^{Low} Gr-1^{Int}CD11b ^{Int}	(49)
Mature Band Myelocyte	Gr-1^{Hi} Gr-1 ^{Low}	(50)
Neutrophil Precursors	Ly6G^{Low}Ly6B^{Int}CD115^{Neg} CD11b^{Pos}CD133^{Pos}	(51)
Mature Band	Gr-1^{Hi}CD11b^{Low-Hi} Gr-1^{Low-Hi}CD11b $^{Low-Hi}$	(52)
Mature Band Metamyelocyte Myelocyte	Ly6G^{Hi}CD11b^{Pos} Ly6G^{Low}CD11b ^{Pos}	(53)
Mature Band Myeloblast Pro-myelocyte Myelocytes Meta-myelocyte	Lin^{Neg}CD34$^{Low/Int}$c-KIT/CD117^{Neg}Ly6G^{High} Lin^{Neg}CD34$^{Low/Int}$c-KIT/CD117^{High}Ly6G^{Neg} Lin^{Neg}CD34$^{Low/Int}$c-KIT/CD117^{Int}Ly6G^{Neg} Lin^{Neg}CD34$^{Low/Int}$c-KIT/CD117^{Int}Ly6G^{Low} Lin^{Neg}CD34$^{Low/Int}$c-KIT/CD117^{Low}Ly6G ^{Int}	(13)
Metamyelocyte Myelocyte Promyelocyte Band Metamyelocyte Mature	Gr-1^{Int}CD11b^{Int} Gr-1^{Hi}CD11b^{Low} Gr-1^{Hi}CD11b ^{Hi}	(54)

immature and mature neutrophils (48) (**Table 2**). However, these surface receptors are prone to alteration following neutrophil activation [e.g., CD11b:CD18 (61)], tissue migration [e.g., CD62L (6, 62, 63)], and aging [e.g., CXCR4 (6)], resulting in a major challenge in the identification of efficient markers of maturity. In mice, immature and mature neutrophils can accurately be identified as Ly6G$^{Int/Low}$CD11b^{Pos} and Ly6G^{High}CD11b^{Pos} respectively (13, 21, 51) (**Table 2**). However, the limitations of using Ly6G as a maturity marker include relatively small differences in expression of this molecule between immature and mature neutrophils, compounding the technical issues associated with fluorescence intensity comparisons in some readouts. Despite this, recently identified markers of neutrophil maturity with larger differences in expression, for example CD101 (48), could be useful candidates for development of fluorescent reporter models and *in vivo* identification. Here, CD101 expression can be used to identify CD101^{Neg} (immature) and CD101^{Pos} (mature) neutrophils (48); however, this marker requires further validation to ensure its accuracy in a wide range of pathologies. Another example is c-KIT/CD117, the expression of which has been shown to associate with neutrophil maturity in naïve mice, mice undergoing candida-induced emergency granulopoiesis (13), and a mouse model of breast cancer (64) (**Table 2**). However, although in the *K14-Cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$, and 4T1 mouse mammary tumor models, neutrophil c-KIT expression is enriched on immature neutrophils, it fails to completely correlate with maturation status (58, 65). In humans, immature and mature neutrophils are commonly identified as CD16^{Low}CD10^{Neg} and CD16^{High}CD10^{Pos}, respectively (66) (**Table 1**). Expression of CD16 (FcγRIII) is initiated between the metamyelocyte and band stages of neutrophil maturation (67, 68). However, its expression can be reduced during apoptosis (69) and can be up-regulated on the cell surface following secretory granule cell membrane fusion (67). Distinct differences in the hematopoietic environment, local and systemic cytokine levels and the functional requirements for neutrophils will exist between naïve, emergency granulopoiesis and the more chronic "inflammation" present in cancer. Therefore, as neutrophils can exhibit plasticity in response to their environment, certain markers are likely to only be suitable in particular models and require efficient validation in each. Overall, the challenges associated with identifying and isolating populations of neutrophil maturity have hindered their study and our current understanding of their functional properties.

FUNCTIONAL PROPERTIES OF MATURE AND IMMATURE NEUTROPHILS

Immature Neutrophils in Cancer

The existence of immature neutrophils in the circulation and tissues is a consequence of cancer development in human patients and mouse models. For example, immature neutrophils are detectable in the circulation (and in some cases the primary tumors) of both injectable and transgenic mouse models of colon (70), skin (70), mammary (5, 58, 71, 72), lung cancer (5, 73), and mesothelioma (AB12) (5, 73). In humans, immature neutrophils have been described in patients with lung cancer (5, 74), breast cancer (5), and ovarian cancer (65).

Drivers of Immature Neutrophil Appearance Outside of the Hematopoietic Niche

Premature release from the BM as observed in states of emergency granulopoiesis, as reviewed by others (75), is

considered the main reason for the presence of immature neutrophils in the circulation. Emergency granulopoiesis commonly results from increased levels of granulocyte colony-stimulating factor (G-CSF; also known as CSF-3) (76) that promotes the differentiation of hematopoietic precursors down the neutrophil lineage and release of neutrophils into the circulation (60, 71, 72, 77) (**Figure 1A**). Production of G-CSF is controlled by interleukin (IL)-23 and IL-17 (58, 78, 79) and can be increased in many cancer models and patients (56, 58, 71, 72, 80). Enhanced levels of G-CSF drive excessive production and release of neutrophils and their precursors into the circulation, leading to neutrophilia (58, 71, 72, 81). G-CSF is dispensable for emergency granulopoiesis and other cytokines, including granulocyte/macrophage (GM)-CSF (also known as CSF-2) (43, 75), can drive neutrophil production and release. Furthermore, neutrophil precursors can seed distant tissues and produce neutrophils *in situ*, as has been observed in cancer patients (82) (**Figure 1B**). TGFβ is another cytokine that favors the presence of immature neutrophils, since its inhibition converts neutrophils to a mature phenotype in transplantable models of mesothelioma (73). The N1/N2 nomenclature—which mirrors the Th1/Th2 nomenclature of T helper cells—was coined in this study based on the influence of TGFβ to modulate neutrophil phenotype and function. Neutrophils were named pro-tumor N2 cells or anti-tumor N1 cells after Th1/Th2 CD4 T cells and M1/M2 macrophages. However, evidence that neutrophils mediate type 1 or type 2 immunity is lacking, and additionally, how these phenotypes relate to the neutrophils found in patients is still under investigation [recently reviewed in Shaul and Fridlender (83)]. Therefore, this nomenclature may be confusing in the context of cancer at this time and future work will determine its appropriateness. In contrast to TGFβ, expression of Type 1 interferons (IFNα and IFNβ) in tumors favors mature neutrophils over immature neutrophils (57). Most likely, there are many other tumor-derived factors that influence neutrophil maturity and their discovery could lead to opportunities for therapeutic intervention.

Functional Properties of Immature Neutrophils

The degree of functional difference between immature and mature neutrophils remains an open question in the field. Due to the importance of neutrophil differentiation in their effector mechanisms, (e.g., production of granule proteins), there is a strong argument for functional differences. Immature neutrophils may in many cases fall under the myeloid-derived suppressor cell (MDSC) umbrella, as these cells have been reported to inhibit T cells. MDSCs encompass a wide range of granulocytic and monocytic cell types at different stages of differentiation. Polymorphonuclear (PMN)-MDSCs are widely considered to be an immature neutrophil population, but methods for their identification, including with anti-Gr-1 (clone RB6-8C5) antibody—which recognizes both Ly6C and Ly6G epitopes—fail to accurately discriminate between mature and immature cells (84, 85). Recently, the classification and identification of MDSC subsets based on their phenotype and morphology has been improved, but these are still identified as $CD11b^{Pos}Ly6C^{Low}Ly6G^{Pos}$ (84). Nevertheless, we believe that the suppressive functions of immature and mature neutrophils is a pathological response to tumorigenesis rather than a completely separate granulocytic population, as discussed by others (86–88). Therefore, we refer to PMN-MDSCs as neutrophils in this article. *Ex vivo* suppression assays are the most common technique for identifying and analyzing suppressive neutrophils. Findings that have used this technique are challenging to interpret as they can be influenced by neutrophil survival, cytotoxicity, neutrophil:T cell ratio, and protocols used [e.g., CD3/CD28 microbeads or antibodies (89)]. Immature (90) and mature (91) neutrophils can be suppressive; however, differences in the suppressive capacities of these populations (66, 92) are likely influenced by disease, model, and neutrophil isolation and identification protocols used (**Figure 1D**). It should also be noted that not all tumor-infiltrating immature neutrophils possess T cell-suppressive abilities (93, 94).

Immunosuppression by neutrophils is not only important for primary tumor progression, but this mechanism can also promote metastasis formation. Neutrophils can be recruited by CXCR2 ligands to dampen anti-tumor immunity in pre-metastatic organs so that disseminated cancer cells can evade immune destruction (58, 95, 96). In these cases, it is the immature neutrophils that are thought to mediate immunosuppression and subsequent metastasis; although, this has not been formally shown. In addition, immature neutrophils and other myeloid progenitors can aid in the formation of the pre-metastatic niche via mechanisms other than T cell suppression (97–99). Interestingly, in models where immature neutrophils are absent, such as the *MMTV-PyMT* model of breast cancer, it is the mature neutrophils that drive metastasis (100). Together, these data indicate that neutrophil maturity may be irrelevant to their pro-metastatic functions.

ROS production is important in several neutrophil effector mechanisms including their microbicidal (101), phagocytic (102) and suppressive capacity and contributes to neutrophil anti- and pro-tumor functions [reviewed in Ohl and Tenbrock (103)]. One such pro-tumor function of neutrophil ROS in cancer is their promotion of tumor initiation at states of inflammation by damaging proliferating epithelial cells (104). In relation to neutrophil maturity the production of ROS can be variable between immature and mature cells. For example, immature neutrophils ($Ly6G^{Pos}CD101^{Neg}$) display reduced ROS production compared to mature ($Ly6G^{Pos}CD101^{Pos}$) in a mouse orthotopic pancreatic cancer model (48). Similarly, in a range of other transplantable mouse cancer models, LDNs—which are enriched in morphologically immature neutrophils—have reduced ROS production (5). However, the amount of ROS production may be context dependent and reliant on metabolism. In tumor-free mice ($Ly6G^{Int}c\text{-}Kit^{Pos}$) and ovarian cancer patients ($CD10^{Int}$), immature neutrophils are dependent on oxidative mitochondrial metabolism rather than glycolysis, for ROS production (65). Recently, LDNs from mice bearing 4T1 mammary tumor liver metastasis have also been shown to have an increased oxidative metabolism (56). This dependency may have implications in the glucose-limited tumor microenvironment

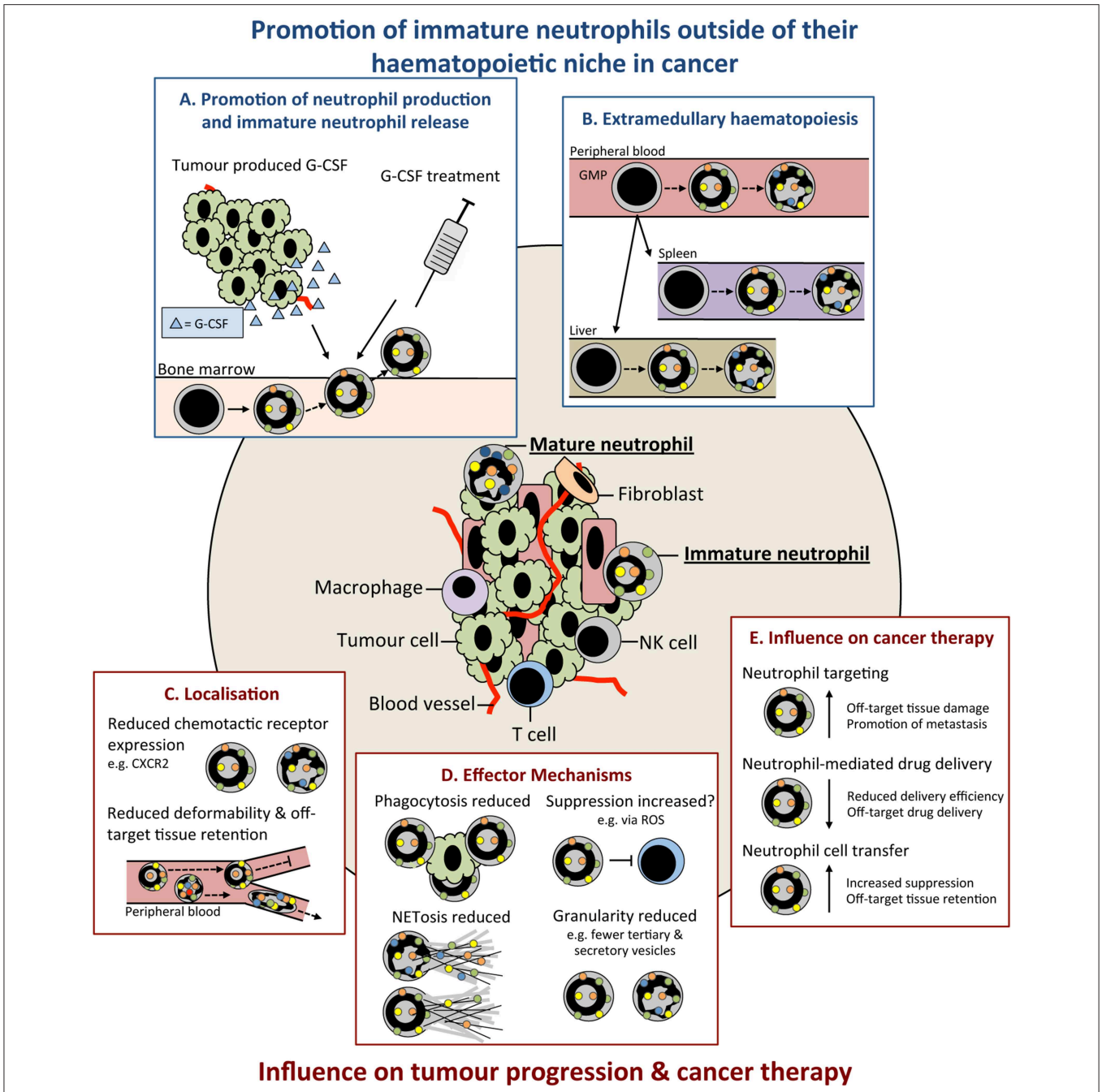


FIGURE 1 | Immature neutrophils are present in cancer and have an altered functional capacity compared to mature that may influence tumor progression. Immature neutrophils can be present and significantly increased in the peripheral blood and tissues of cancer patients. This increase may result from: **(A)** promotion of their early release from their bone marrow (BM) haematopoietic niche by increased systemic chemokines, such as granulocyte colony-stimulating (G-CSF), e.g., tumor produced or as therapy. **(B)** release of neutrophil precursors from the BM and their extramedullary proliferation in the circulation or tissues. Immature neutrophils may have both anti- and pro-tumor properties. These include **(C)** altered localization resulting from their differential cell surface marker expression influencing their chemotactic capacity and/or less segmented nuclear morphology compared to mature neutrophils reducing their deformability and **(D)** different functional capacity compared to mature neutrophils including their reduced phagocytic capacity, altered suppressive properties, reduced NETosis, and reduced granularity. **(E)** Together, these differences in the properties and functions of immature neutrophils could lead to their negative influence when targeting neutrophils in cancer therapy. G-CSF, colony stimulating factor-3; GMP, granulocyte monocyte progenitor; ROS, reactive oxygen species; NET, neutrophil extracellular trap; NK, natural killer cell.

and affect neutrophil function (65). Therefore, while immature neutrophils can have reduced ROS production compared to mature neutrophils, this appears to be dependent on stimulus, their localization and the tissue microenvironment.

Neutrophil extracellular traps (NETs) are extracellular fibers composed of nuclear, mitochondrial, cytoplasmic and granule contents that can be released by neutrophils following their activation (105). NETs can capture circulating cancer cells in the mouse lung promoting their extravasation and metastasis formation (106, 107). Neutrophils can also aid in formation of the omental pre-metastatic niche and capture of circulating ovarian cancer cells, promoting their metastasis at this site (108). The ability of immature human neutrophil populations to release NETs is reduced following interferon priming (35) (**Figure 1D**). In addition, when isolated from the peripheral blood of acute myeloid leukemia (AML) patients, morphologically immature neutrophils show decrease capacity for NET formation following phorbol 12-myristate 13-acetate (PMA) stimulation (109). As NETs have been proposed to arise from the inability of terminally differentiated neutrophils to re-enter mitosis (110), it could be inferred that the increased mitotic capacity of immature populations contributes to these differences. ROS contribute to NETosis by promoting granule release and rupture of the nuclear envelope, as highlighted by the inability of neutrophils from chronic granulomatous disease patients to undergo NETosis (111, 112). Differences in ROS production with neutrophil maturity may also influence NETosis (65). Differences in granule composition of neutrophils at different maturity may also influence the functional capacity of their NETs. The tertiary granule component MMP-9 (113) has been implicated in NET-induced dormant cancer cell reactivation (114) and its possible reduced abundance in banded neutrophils and earlier neutrophil precursors present in cancer could reduce their ability to promote this reactivation.

Multiple studies have indicated a reduced migratory capacity of immature compared to mature neutrophils (5, 48) (**Figure 1C**). This may result from lower expression of chemokine receptors, such as CXCR1 and CXCR2 (30), and other genes involved in chemotaxis (48). In mice, proliferating neutrophil precursors, identified as $Ly6G^{Low}CXCR2^{Neg}c\text{-}Kit^{Pos}CXCR4^{Pos}$, have reduced migration to laser-induced damage (48). High CXCR2 expression by neutrophils has been associated with poor outcome in human pancreatic ductal adenocarcinoma (PDAC) patients (95). Inhibition of CXCR2 in a mouse model of PDAC reduces neutrophil migration and delays tumor progression (95). Banded nuclear morphology, and thus reduced deformability, may promote immature neutrophil sequestration in capillaries and reduce their migratory capacity (115); although, banded nuclear morphology in immature human neutrophils does not affect transendothelial migration (TEM) when compared to segmented neutrophils *ex vivo* (62) (**Figure 1C**). It is therefore conceivable that their increased sequestration in off target tissues and ability to undergo TEM may result in unwanted immature neutrophil accumulation and the promotion of inflammation and/or metastasis. Additionally, neutrophil spontaneous migration is increased in the early compared to late stages of cancer in a mouse orthotopic lung cancer model (116). These changes in neutrophil function with tumor progression are present in BM cells, suggesting altered granulopoiesis over time (116). Therefore, while further investigation is required, differential trafficking of immature neutrophils could have the capacity to both antagonize and promote tumor development dependent on their localization.

The phagocytic capacity of immature, compared to mature, neutrophils is also reduced (5, 48) and could result from their altered cell surface receptor expression and decreased ROS production (**Figure 1D**). Fc receptors (FcRs) are important in mediating phagocytosis (117) with decreased expression of CD16 likely influencing their phagocytic capacity. Furthermore, immature neutrophils ($CD16^{Int}$) are unable to kill tumor cells via FcγRI, but exhibit cytotoxicity via FcαRI (118). Activation of FcRs, integrins and G-protein coupled receptors (GPCRs) can trigger neutrophil ROS production and its extracellular or intracellular release into the phagolysosome, as reviewed in more detail by others (102, 119). Unsurprisingly, immature neutrophils have also been shown to have an increased life span and can mature *ex vivo* (120). It will be interesting to determine if neutrophil maturation after their release from the BM contributes to heterogeneity within the mature neutrophil population. However, despite differences in the functional capacity of immature and mature neutrophils, they are still capable of mediating innate immune functions (120). Overall, the effect of neutrophil maturity in cancer remains enigmatic and further investigation, coupled with accurate identification, is required.

NEUTROPHIL MATURITY IN ANTI-CANCER THERAPY

Immunotherapy has shown great promise in cancer; however, only a minority of patients respond to certain therapies (121) and combinatorial therapies targeting a broad range of immune populations may be more beneficial. Therapies targeting neutrophils have received relatively little attention (**Figure 1E**). While the direct effect of therapies on neutrophils at different stages of maturation has not been investigated, we can consider ways in which the properties of immature neutrophils are relevant.

Neutrophils recruited to the tumor via CXCR2 can aid tumor progression (122) and inhibition of CXCR1 and CXCR2 has shown promise in mouse models (95) and human cancers (123). As CXCR2 expression increases with neutrophil maturation (48) inhibitors of CXCR2 may differentially influence immature and mature neutrophils affecting their efficacy. Therapies targeting immunosuppressive neutrophils enhance responses to checkpoint blockade by promoting tumor infiltration by T cells in mouse models (124–127). A greater understanding of the maturity composition of these cells could better aid targeting of this population. Furthermore, tyrosine kinase inhibitors that target the hepatocyte growth factor (HGF) receptor, cMET (e.g., Cabozantinib and Capmatinib) can extend survival by influencing neutrophil behavior in mouse melanoma and PTEN/p53-deficient prostate cancer models (128, 129). As tyrosine kinases (e.g., Bruton's tyrosine kinase; BTK) are important in regulating neutrophil development (49) and in neutrophil integrin signaling (130) it is possible that their inhibitors have altered effects on neutrophils of

different maturity. Similarly, monoclonal antibody (mAb) based therapies, for example anti-gp75 (TA99) (131), anti-HER2 (Trastuzumab) (131), and anti-SIRPα (KWAR23) (132, 133), promote neutrophil-mediated destruction of cancer cells. The reduced phagocytic capacity (48) of immature neutrophils and differences in their FcR expression (118) may reduce the efficacy of these therapies. Furthermore, these properties may hamper their ability to deliver therapeutics, such as in nanoparticles (134), to the tumor (**Figure 1E**). Finally, adoptively transferred neutrophils can aid in the killing of cancer cells (135) and can be isolated from G-CSF-treated donors (136) (**Figure 1E**). Here, the activation (137) and potential retention of transferred immature neutrophils in off-target organs [e.g., the lung (138)] needs to be considered. In addition, G-CSF-driven immature neutrophil release, neutrophil accumulation, and alterations to neutrophil function in cancer (66, 100, 139) need to be further deliberated when treating neutropenic cancer patients with G-CSF (140, 141).

CONCLUSIONS

To gain an accurate understanding of maturity on neutrophil functional capacity, consensus protocols for identification of neutrophil maturity are urgently required. However, as protocols and markers may not be transferable between models, detailed confirmation of maturity in each is essential (e.g., associated nuclear morphology, transcriptomics, proteomics, and surface protein expression data) allowing for proper comparison. Functional investigation needs to be further driven by *in vivo* investigation to remove concerns associated with *ex vivo* manipulation. Of particular importance, investigating the localization and suppressive capacity of immature neutrophils *in situ* will aid in determining their influence on immunotherapy. Furthermore, more research on immature neutrophils in cancer patients should be carried out to determine where these cells appear. Correlations between immature neutrophils and mutational drivers need investigation to understand how these cells occur outside the bone marrow and to identify additional biomarkers of disease. Changes in neutrophil maturation status before, during and after anti-cancer therapy may provide insight into how these cells are regulated. Taken together, the available evidence suggests immature neutrophils in cancer inevitably influence tumor development and we emphasize the importance of improving methodologies for their study.

AUTHOR CONTRIBUTIONS

JM, SC, and LC wrote and edited the manuscript.

FUNDING

This work was supported by Core funding from Cancer Research UK (A23983 and A17196 to LC), Cancer Research UK Glasgow Center (A25142 to SC), Breast Cancer Now (2018JulPR1101 to SC), Wellcome Trust (208990/Z/17/Z to SC), Tenovus Scotland (Project S17-17 to SC).

REFERENCES

1. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol*. (2004) 172:2731–8. doi: 10.4049/jimmunol.172.5.2731
2. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer*. (2016) 16:431–46. doi: 10.1038/nrc.2016.52
3. Grecian R, Whyte MKB, Walmsley SR. The role of neutrophils in cancer. *Br Med Bull*. (2018) 128:5–14. doi: 10.1093/bmb/ldy029
4. Galdiero MR, Varricchi G, Loffredo S, Mantovani A, Marone G. Roles of neutrophils in cancer growth and progression. *J Leukoc Biol*. (2018) 103:457–64. doi: 10.1002/JLB.3MR0717-292R
5. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep*. (2015) 10:562–73. doi: 10.1016/j.celrep.2014.12.039
6. Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chevre R, A-González N, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell*. (2013) 153:1025–35. doi: 10.1016/j.cell.2013.04.040
7. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*. (2008) 181:5791–802. doi: 10.4049/jimmunol.181.8.5791
8. Hellebrekers P, Hietbrink F, Vrisekoop N, Leenen LPH, Koenderman L. Neutrophil functional heterogeneity: identification of competitive phagocytosis. *Front Immunol*. (2017) 8:1498. doi: 10.3389/fimmu.2017.01498
9. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils *in vivo*. *Nat Immunol*. (2011) 12:761–9. doi: 10.1038/ni.2062
10. Liu Y, Wei G, Cheng WA, Dong Z, Sun H, Lee VY, et al. Targeting myeloid-derived suppressor cells for cancer immunotherapy. *Cancer Immunol Immunother*. (2018) 67:1181–95. doi: 10.1007/s00262-018-2175-3
11. Pillay J, Tak T, Kamp VM, Koenderman L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. *Cell Mol Life Sci*. (2013) 70:3813–27. doi: 10.1007/s00018-013-1286-4
12. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci USA*. (1997) 94:569–74. doi: 10.1073/pnas.94.2.569
13. Satake S, Hirai H, Hayashi Y, Shime N, Tamura A, Yao H, et al. C/EBPβ is involved in the amplification of early granulocyte precursors during candidemia-induced "emergency" granulopoiesis. *J Immunol*. (2012) 189:4546–55. doi: 10.4049/jimmunol.1103007
14. Yamanaka R, Barlow C, Lekstrom-Himes J, Castilla LH, Liu PP, Eckhaus M, et al. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci USA*. (1997) 94:13187–92. doi: 10.1073/pnas.94.24.13187
15. Theilgaard-Monch K, Jacobsen LC, Borup R, Rasmussen T, Bjerregaard MD, Nielsen FC., et al. The transcriptional program of terminal granulocytic differentiation. *Blood*. (2005) 105:1785–96. doi: 10.1182/blood-2004-08-3346
16. Lahoz-Beneytez J, Elemans M, Zhang Y, Ahmed R, Salam A, Block M, et al. Human neutrophil kinetics: modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood*. (2016) 127:3431–8. doi: 10.1182/blood-2016-03-700336
17. Basu S, Hodgson G, Katz M, Dunn AR. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood*. (2002) 100:854–61. doi: 10.1182/blood.V100.3.854

18. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunol Rev*. (2016) 273:11–28. doi: 10.1111/imr.12440
19. Hager M, Cowland JB, Borregaard N. Neutrophil granules in health and disease. *J Intern Med*. (2010) 268:25–34. doi: 10.1111/j.1365-2796.2010.02237.x
20. Johns JL, Christopher MM. Extramedullary hematopoiesis: a new look at the underlying stem cell niche, theories of development, and occurrence in animals. *Vet Pathol*. (2012) 49:508–23. doi: 10.1177/0300985811432344
21. Deniset JF, Surewaard BG, Lee WY, Kubes P. Splenic Ly6G(high) mature Ly6G(int) immature neutrophils contribute to eradication of S. pneumoniae. *J Exp Med*. (2017) 214:1333–50. doi: 10.1084/jem.20161621
22. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. (2008) 83:64–70. doi: 10.1189/jlb.0407247
23. Hasenberg A, Hasenberg M, Mann L, Neumann F, Borkenstein L, Stecher M, et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat Methods*. (2015) 12:445–52. doi: 10.1038/nmeth.3322
24. Wang JX, Bair AM, King SL, Shnayder R, Huang YF, Shieh CC, et al. Ly6G ligation blocks recruitment of neutrophils via a β2-integrin-dependent mechanism. *Blood*. (2012) 120:1489–98. doi: 10.1182/blood-2012-01-404046
25. Yipp BG, Kubes P. Antibodies against neutrophil LY6G do not inhibit leukocyte recruitment in mice *in vivo*. *Blood*. (2013) 121:241–2. doi: 10.1182/blood-2012-09-454348
26. Cowland JB, Borregaard N. Isolation of neutrophil precursors from bone marrow for biochemical and transcriptional analysis. *J Immunol Methods*. (1999) 232:191–200. doi: 10.1016/S0022-1759(99)00176-3
27. Bernstein LH, Rucinski J. Measurement of granulocyte maturation may improve the early diagnosis of the septic state. *Clin Chem Lab Med*. (2011) 49:2089–95. doi: 10.1515/CCLM.2011.688
28. Bjerregaard MD, Jurlander J, Klausen P, Borregaard N, Cowland JB. The *in vivo* profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood*. (2003) 101:4322–32. doi: 10.1182/blood-2002-03-0835
29. Blazkova J, Gupta S, Liu Y, Gaudilliere B, Ganio EA, Bolen CR, et al. Multicenter systems analysis of human blood reveals immature neutrophils in males and during pregnancy. *J Immunol*. (2017) 198:2479–88. doi: 10.4049/jimmunol.1601855
30. Brandau S, Trellakis S, Bruderek K, Schmaltz D, Steller G, Elian M, et al. Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. *J Leukoc Biol*. (2011) 89:311–7. doi: 10.1189/jlb.0310162
31. Che YQ, Shen D, Zhang SM, Qi J. Identification of immature granulocytes in cancer chemotherapy patients by cell counting vs. microscopic examination of blood smears. *Mol Clin Oncol*. (2014) 2:207–11. doi: 10.3892/mco.2014.243
32. Demaret J, Venet F, Friggeri A, Cazalis MA, Plassais J, Jallades L, et al. Marked alterations of neutrophil functions during sepsis-induced immunosuppression. *J Leukoc Biol*. (2015) 98:1081–90. doi: 10.1189/jlb.4A0415-168RR
33. Guerin E, Orabona M, Raquil MA, Giraudeau B, Bellier R, Gibot S, et al. Circulating immature granulocytes with T-cell killing functions predict sepsis deterioration*. *Crit Care Med*. (2014) 42:2007–18. doi: 10.1097/CCM.0000000000000344
34. Makoni M, Eckert J, Anne Pereira H, Nizet V, Lawrence SM. Alterations in neonatal neutrophil function attributable to increased immature forms. *Early Hum Dev*. (2016) 103:1–7. doi: 10.1016/j.earlhumdev.2016.05.016
35. Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al. Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. *J Biol Chem*. (2004) 279:44123–32. doi: 10.1074/jbc.M405883200
36. Nierhaus A, Klatte S, Linssen J, Eismann NM, Wichmann D, Hedke J, ET AL. Revisiting the white blood cell count: immature granulocytes count as a diagnostic marker to discriminate between SIRS and sepsis–a prospective, observational study. *BMC Immunol*. (2013) 14:8. doi: 10.1186/1471-2172-14-8
37. Pillay J, Ramakers BP, Kamp VM, Loi AL, Lam SW, Hietbrink F, et al. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *J Leukoc Biol*. (2010) 88:211–20. doi: 10.1189/jlb.1209793
38. Pliyev BK, Shmidt EI, Ivanova AV, Menshikov M. Circulating CD35(-)/CD49d(+) neutrophils in influenza virus infection patients. *Hum Immunol*. (2012) 73:1087–90. doi: 10.1016/j.humimm.2012.07.327
39. Senthilnayagam B, Kumar T, Sukumaran J, J M, Rao KR. Automated measurement of immature granulocytes: performance characteristics and utility in routine clinical practice. *Patholog Res Int*. (2012) 2012:483670. doi: 10.1155/2012/483670
40. Taneja R, Sharma AP, Hallett MB, Findlay GP, Morris MR. Immature circulating neutrophils in sepsis have impaired phagocytosis and calcium signaling. *Shock*. (2008) 30:618–22. doi: 10.1097/SHK.0b013e318173ef9c
41. Akagi T, Thoennissen NH, George A, Crooks G, Song JH, Okamoto R, et al. *In vivo* deficiency of both C/EBPβ and C/EBPε results in highly defective myeloid differentiation and lack of Cytokine response. *PLoS ONE*. (2010) 5:e15419. doi: 10.1371/journal.pone.0015419
42. Alberich-Jorda M, Wouters B, Balastik M, Shapiro-Koss C, Zhang H, Di Ruscio A, et al. C/EBPγ deregulation results in differentiation arrest in acute myeloid leukemia. *J Clin Invest*. (2012) 122:4490–504. doi: 10.1172/JCI65102
43. Basu S, Hodgson G, Zhang HH, Katz M, Quilici C, Dunn AR. "Emergency" granulopoiesis in G-CSF-deficient mice in response to Candida albicans infection. *Blood*. (2000) 95:3725–33. Available online at: http://www.bloodjournal.org/content/95/12/3725.long?
44. Boettcher S, Ziegler P, Schmid MA, Takizawa H, van Rooijen N, Kopf M, et al. Cutting edge: LPS-induced emergency myelopoiesis depends on TLR4-expressing nonhematopoietic cells. *J Immunol*. (2012) 188:5824–8. doi: 10.4049/jimmunol.1103253
45. Boxio R, Bossenmeyer-Pourie C, Steinckwich N, Dournon C, Nusse O. Mouse bone marrow contains large numbers of functionally competent neutrophils. *J Leukoc Biol*. (2004) 75:604–11. doi: 10.1189/jlb.0703340
46. Break TJ, Witter AR, Indramohan M, Mummert ME, Dory L, Berg RE. Extracellular superoxide dismutase enhances recruitment of immature neutrophils to the liver. *Infect Immun*. (2016) 84:3302–12. doi: 10.1128/IAI.00603-16
47. Cheung R, Shen F, Phillips JH, McGeachy MJ, Cua DJ, Heyworth PG, et al. Activation of MDL-1 (CLEC5A) on immature myeloid cells triggers lethal shock in mice. *J Clin Invest*. (2011) 121:4446–61. doi: 10.1172/JCI57682
48. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J, et al. Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking, and effector functions. *Immunity*. (2018) 48:364–79.e8. doi: 10.1016/j.immuni.2018.02.002
49. Fiedler K, Sindrilaru A, Terszowski G, Kokai E, Feyerabend TB, Bullinger L, et al. Neutrophil development and function critically depend on Bruton tyrosine kinase in a mouse model of X-linked agammaglobulinemia. *Blood*. (2011) 117:1329–39. doi: 10.1182/blood-2010-04-281170
50. Hestdal K, Ruscetti FW, Ihle JN, Jacobsen SE, Dubois CM, Kopp WC, et al. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J Immunol*. (1991) 147:22–8.
51. Kim MH, Yang D, Kim M, Kim SY, Kim D, Kang SJ. A late-lineage murine neutrophil precursor population exhibits dynamic changes during demand-adapted granulopoiesis. *Sci Rep*. (2017) 7:39804. doi: 10.1038/srep39804
52. Lagasse E, Weissman IL. Flow cytometric identification of murine neutrophils and monocytes. *J Immunol Methods*. (1996) 197:139–50. doi: 10.1016/0022-1759(96)00138-X
53. Sanchez A, Relano C, Carrasco A, Contreras-Jurado C, Martin-Duce A, Aranda A, et al. Map3k8 controls granulocyte colony-stimulating factor production and neutrophil precursor proliferation in lipopolysaccharide-induced emergency granulopoiesis. *Sci Rep*. (2017) 7:5010. doi: 10.1038/s41598-017-04538-3
54. Ueda Y, Kondo M, Kelsoe G. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. *J Exp Med*. (2005) 201:1771–80. doi: 10.1084/jem.20041419

55. Pember SO, Barnes KC, Brandt SJ, Kinkade JM Jr. Density heterogeneity of neutrophilic polymorphonuclear leukocytes: gradient fractionation and relationship to chemotactic stimulation. *Blood*. (1983) 61:1105–15.
56. Hsu BE, Tabaries S, Johnson RM, Andrzejewski S, Senecal J, Lehuede C, et al. Immature low-density neutrophils exhibit metabolic flexibility that facilitates breast cancer liver metastasis. *Cell Rep*. (2019) 27:3902–15.e6. doi: 10.1016/j.celrep.2019.05.091
57. Andzinski L, Kasnitz N, Stahnke S, Wu CF, Gereke M, von Kockritz-Blickwede M. et al. Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. *Int J Cancer*. (2016) 138:1982–93. doi: 10.1002/ijc.29945
58. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. (2015) 522:345–8. doi: 10.1038/nature14282
59. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity*. (2003) 19:583–93. doi: 10.1016/S1074-7613(03)00263-2
60. Strydom N, Rankin SM. Regulation of circulating neutrophil numbers under homeostasis and in disease. *J Innate Immun*. (2013) 5:304–14. doi: 10.1159/000350282
61. Hoglund M, Hakansson L, Venge P. Effects of *in vivo* administration of G-CSF on neutrophil functions in healthy volunteers. *Eur J Haematol*. (1997) 58:195–202. doi: 10.1111/j.1600-0609.1997.tb00947.x
62. van Grinsven E, Textor J, Hustin LSP, Wolf K, Koenderman L, Vrisekoop N. Immature neutrophils released in acute inflammation exhibit efficient migration despite incomplete segmentation of the nucleus. *J Immunol*. (2019) 202:207–17. doi: 10.4049/jimmunol.1801255
63. Rzeniewicz K, Newe A, Rey Gallardo A, Davies J, Holt MR, Patel A, et al. L-selectin shedding is activated specifically within transmigrating pseudopods of monocytes to regulate cell polarity *in vitro*. *Proc Natl Acad Sci USA*. (2015) 112:E1461–70. doi: 10.1073/pnas.1417100112
64. Kuonen F, Laurent J, Secondini C, Lorusso G, Stehle JC, Rausch T, et al. Inhibition of the Kit ligand/c-Kit axis attenuates metastasis in a mouse model mimicking local breast cancer relapse after radiotherapy. *Clin Cancer Res*. (2012) 18:4365–74. doi: 10.1158/1078-0432.CCR-11-3028
65. Rice CM, Davies LC, Subleski JJ, Maio N, Gonzalez-Cotto M, Andrews C, et al. Tumour-elicited neutrophils engage mitochondrial metabolism to circumvent nutrient limitations and maintain immune suppression. *Nat Commun*. (2018) 9:5099. doi: 10.1038/s41467-018-07505-2
66. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature $CD10^{(+)}$ and immature $CD10^{(-)}$ neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood*. (2017) 129:1343–56. doi: 10.1182/blood-2016-04-713206
67. Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells Mol Dis*. (2002) 28:260–74. doi: 10.1006/bcmd.2002.0513
68. Terstappen LW, Safford M, Loken MR. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. *Leukemia*. (1990) 4:657–63.
69. Dransfield I, Buckle AM, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J Immunol*. (1994) 153:1254–63.
70. Yang XD, Ai W, Asfaha S, Bhagat G, Friedman RA, Jin G, et al. Histamine deficiency promotes inflammation-associated carcinogenesis through reduced myeloid maturation and accumulation of CD11b+Ly6G+ immature myeloid cells. *Nat Med*. (2011) 17:87–95. doi: 10.1038/nm.2278
71. Casbon J, Reynaud D, Park C, Khuc E, Gan DD, Schepers K, et al. Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. *Proc Natl Acad Sci USA*. (2015) 112:E566–75. doi: 10.1073/pnas.1424927112
72. Waight D, Hu Q, Miller A, Liu S, Abrams SI. Tumor-derived G-CSF facilitates neoplastic growth through a granulocytic myeloid-derived suppressor cell-dependent mechanism. *PLoS ONE*. (2011) 6:e27690. doi: 10.1371/journal.pone.0027690
73. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
74. McCoach CE, Rogers JG, Dwyre DM, Jonas BA. Paraneoplastic leukemoid reaction as a marker of tumor progression in non-small cell lung cancer. *Cancer Treat Commun*. (2015) 4:15–8. doi: 10.1016/j.ctrc.2015.03.003
75. Manz MG, Boettcher S. Emergency granulopoiesis. *Nat Rev Immunol*. (2014) 14:302–14. doi: 10.1038/nri3660
76. Boettcher S, Gerosa RC, Radpour R, Bauer J, Ampenberger F, Heikenwalder M, et al. Endothelial cells translate pathogen signals into G-CSF-driven emergency granulopoiesis. *Blood*. (2014) 124:1393–403. doi: 10.1182/blood-2014-04-570762
77. Semerad CL, Liu F, Gregory AD, Stumpf K, Link DC. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity*. (2002) 17:413–23. doi: 10.1016/S1074-7613(02)00424-7
78. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 IL-17. *Immunity*. (2005) 22:285–94. doi: 10.1016/j.immuni.2005.01.011
79. Smith E, Zarbock A, Stark MA, Burcin TL, Bruce AC, Foley P, et al. IL-23 is required for neutrophil homeostasis in normal and neutrophilic mice. *J Immunol*. (2007) 179:8274–9. doi: 10.4049/jimmunol.179.12.8274
80. Morris T, Khan H, Ahmad A, Weston LL, Nofchissey RA, Pinchuk IV, et al. G-CSF and G-CSFR are highly expressed in human gastric and colon cancers and promote carcinoma cell proliferation and migration. *Br J Cancer*. (2014) 110:1211–20. doi: 10.1038/bjc.2013.822
81. Price TH, Chatta GS, Dale DC. Effect of recombinant granulocyte colony-stimulating factor on neutrophil kinetics in normal young and elderly humans. *Blood*. (1996) 88:335–40.
82. Wu C, Ning H, Liu M, Lin J, Luo S, Zhu W, et al. Spleen mediates a distinct hematopoietic progenitor response supporting tumor-promoting myelopoiesis. *J Clin Invest*. (2018) 128:3425–38. doi: 10.1172/JCI97973
83. Shaul E, Fridlender ZG. Tumour-associated neutrophils in patients with cancer. *Nat Rev Clin Oncol*. (2019). doi: 10.1038/s41571-019-0222-4. [Epub ahead of print].
84. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun*. (2016) 7:12150. doi: 10.1038/ncomms12150
85. Damuzzo V, Pinton L, Desantis G, Solito S, Marigo I, Bronte V, et al. Complexity and challenges in defining myeloid-derived suppressor cells. *Cytometry B Clin Cytom*. (2015) 88:77–91. doi: 10.1002/cyto.b.21206
86. Budhwar S, Verma P, Verma R, Rai S, Singh K. The Yin and Yang of Myeloid Derived Suppressor Cells. *Front Immunol*. (2018) 9:2776. doi: 10.3389/fimmu.2018.02776
87. Minns D, Smith KJ, Findlay EG. Orchestration of adaptive T cell responses by neutrophil granule contents. *Mediators Inflamm*. (2019) 2019:8968943. doi: 10.1155/2019/8968943
88. Negorev D, Beier UH, Zhang T, Quatromoni JG, Bhojnagarwala P, Albelda SM, et al. Human neutrophils can mimic myeloid-derived suppressor cells (PMN-MDSC) and suppress microbead or lectin-induced T cell proliferation through artefactual mechanisms. *Sci Rep*. (2018) 8:3135. doi: 10.1038/s41598-018-21450-6
89. Davis RJ, Silvin C, Allen CT. Avoiding phagocytosis-related artifact in myeloid derived suppressor cell T-lymphocyte suppression assays. *J Immunol Methods*. (2017) 440:12–8. doi: 10.1016/j.jim.2016.11.006
90. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J Immunol*. (2005) 175:4583–92. doi: 10.4049/jimmunol.175.7.4583
91. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A Subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. (2012) 122:327–36. doi: 10.1172/JCI57990
92. Rahman S, Sagar D, Hanna RN, Lightfoot YL, Mistry P, Smith CK, et al. Low-density granulocytes activate T cells and demonstrate a non-suppressive role in systemic lupus erythematosus. *Ann Rheum Dis*. (2019) 78:957–66. doi: 10.1136/annrheumdis-2018-214620
93. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell*. (2010) 17:121–34. doi: 10.1016/j.ccr.2009.12.019

94. Tomihara K, Guo M, Shin T, Sun X, Ludwig SM, Brumlik MJ, et al. Antigen-specific immunity and cross-priming by epithelial ovarian carcinoma-induced CD11b(+)Gr-1(+) cells. *J Immunol*. (2010) 184:6151–60. doi: 10.4049/jimmunol.0903519
95. Steele CW, Karim SA, Leach JDG, Bailey P, Upstill-Goddard R, Rishi L, et al. CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma. *Cancer Cell*. (2016) 29:832–45. doi: 10.1016/j.ccell.2016.04.014
96. Wang D, Sun H, Wei J, Cen B, DuBois RN. CXCL1 Is Critical for premetastatic niche formation and metastasis in colorectal cancer. *Cancer Res*. (2017) 77:3655–65. doi: 10.1158/0008-5472.CAN-16-3199
97. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. (2005) 438:820–7. doi: 10.1038/nature04186
98. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1^{+}CD11b^{+} myeloid cells that promote metastasis. *Cancer Cell*. (2008) 13:23–35. doi: 10.1016/j.ccr.2007.12.004
99. Yan HH, Pickup M, Pang Y, Gorska AE, Li Z, Chytil A, et al. Gr-1+CD11b+ myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. *Cancer Res*. (2010) 70:6139–49. doi: 10.1158/0008-5472.CAN-10-0706
100. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature*. (2015) 528:413–7. doi: 10.1038/nature16140
101. Warnatsch A, Tsourouktsoglou TD, Branzk N, Wang Q, Reincke S, Herbst S, et al. Reactive oxygen species localization programs inflammation to clear microbes of different size. *Immunity*. (2017) 46:421–432. doi: 10.1016/j.immuni.2017.02.013
102. Dupre-Crochet S, Erard M, Nubetae O. ROS production in phagocytes: why, when, and where? *J Leukoc Biol*. (2013) 94:657–70. doi: 10.1189/jlb.1012544
103. Ohl K, Tenbrock K. Reactive oxygen species as regulators of MDSC-mediated immune suppression. *Front Immunol*. (2018) 9:2499. doi: 10.3389/fimmu.2018.02499
104. Antonio N, Bonnelykke-Behrndtz ML, Ward LC, Collin J, Christensen IJ, Steiniche T, et al. The wound inflammatory response exacerbates growth of pre-neoplastic cells and progression to cancer. *EMBO J*. (2015) 34:2219–36. doi: 10.15252/embj.201490147
105. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. (2004) 303:1532–5. doi: 10.1126/science.1092385
106. Park J, Wysocki RW, Amoozgar Z, Maiorino L, Fein MR, Jorns J, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci Transl Med*. (2016) 8:361ra138. doi: 10.1126/scitranslmed.aag1711
107. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J Clin Invest*. (2013) 123:67484. doi: 10.1158/1538-7445.AM2012-2972
108. Lee W, Ko SY, Mohamed MS, Kenny HA, Lengyel E, Naora H. Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. *J Exp Med*. (2019) 216:176–94. doi: 10.1084/jem.20181170
109. Lukasova E, Koristek Z, Klabusay M, Ondrej V, Grigoryev S, Bacikova A, et al. Granulocyte maturation determines ability to release chromatin NETs and loss of DNA damage response; these properties are absent in immature AML granulocytes. *Biochim Biophys Acta*. (2013) 1833:767–79. doi: 10.1016/j.bbamcr.2012.12.012
110. Amulic B, Knackstedt SL, Abu Abed U, Deigendesch N, Harbort CJ, Caffrey BE, et al. Cell-cycle proteins control production of neutrophil extracellular traps. *Dev Cell*. (2017) 43:449–62.e5 doi: 10.1016/j.devcel.2017.10.013
111. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. (2007) 176:231–41. doi: 10.1083/jcb.200606027
112. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med*. (2016) 22:146–53. doi: 10.1038/nm.4027
113. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*. (2003) 5:1317–27. doi: 10.1016/j.micinf.2003.09.008
114. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:aao4227. doi: 10.1126/science.aao4227
115. van Eeden SF, Kitagawa Y, Klut ME, Lawrence E, Hogg JC. Polymorphonuclear leukocytes released from the bone marrow preferentially sequester in lung microvessels. *Microcirculation*. (1997) 4:369–80. doi: 10.3109/10739689709146801
116. Patel S, Fu S, Mastio J, Dominguez GA, Purohit A, Kossenkov A, et al. Unique pattern of neutrophil migration and function during tumor progression. *Nat Immunol*. (2018) 19:1236–47. doi: 10.1038/s41590-018-0229-5
117. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. *Microbes Infect*. (2003) 5:1299–306. doi: 10.1016/j.micinf.2003.09.014
118. Otten MA, Rudolph E, Dechant M, Tuk CW, Reijmers RM, Beelen RH. et al. Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors. *J Immunol*. (2005) 174:5472–80. doi: 10.4049/jimmunol.174.9.5472
119. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: mechanisms of NADPH oxidase activation and bacterial resistance. *Front Cell Infect Microbiol*. (2017) 7:373. doi: 10.3389/fcimb.2017.00373
120. Drifte G, Dunn-Siegrist I, Tissieres P, Pugin J. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. *Crit Care Med*. (2013) 41:820–32. doi: 10.1097/CCM.0b013e318274647d
121. Ventola CL. Cancer immunotherapy, Part 2: Efficacy, Safety, Other Clinical Considerations. *P T*. (2017) 42:452–63. Available online at: http://www.ptcommunity.com/journal/article/full/2017/7/452/cancer-immunotherapy-part-2-efficacy-safety-and-other-clinical
122. Shang K, Bai YP, Wang C, Wang Z, Gu HY, Du X, et al. Crucial involvement of tumor-associated neutrophils in the regulation of chronic colitis-associated carcinogenesis in mice. *PLoS ONE*. (2012) 7:e51848. doi: 10.1371/journal.pone.0051848
123. Varney ML, Singh S, Li A, Mayer-Ezell R, Bond R, Singh RK. Small molecule antagonists for CXCR2 and CXCR1 inhibit human colon cancer liver metastases. *Cancer Lett*. (2011) 300:180–8. doi: 10.1016/j.canlet.2010.10.004
124. Yang L, Wang B, Qin J, Zhou H, Majumdar APN, Peng F, Blockade of CCR5-mediated myeloid derived suppressor cell accumulation enhances anti-PD1 efficacy in gastric cancer. *Immunopharmacol Immunotoxicol*. (2018) 40:91–97. doi: 10.1080/08923973.2017.1417997
125. Guan Y, Zhang R, Peng Z, Dong D, Wei G, Wang Y. Inhibition of IL-18-mediated myeloid derived suppressor cell accumulation enhances anti-PD1 efficacy against osteosarcoma cancer. *J Bone Oncol*. (2017) 9:59–64. doi: 10.1016/j.jbo.2017.10.002
126. Davis RJ, Moore EC, Clavijo PE, Friedman J, Cash H, Chen Z, et al. Anti-PD-L1 efficacy can be enhanced by inhibition of myeloid-derived suppressor cells with a selective inhibitor of PI3Kδ/γ. *Cancer Res*. (2017) 77:2607–19. doi: 10.1158/0008-5472.CAN-16-2534
127. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci USA*. (2014) 111:11774–9. doi: 10.1073/pnas.1410626111
128. Patnaik A, Swanson KD, Csizmadia E, Solanki A, Landon-Brace N, Gehring MP, et al. Cabozantinib eradicates advanced murine prostate cancer by activating antitumor innate immunity. *Cancer Discov*. (2017) 7:750–65. doi: 10.1158/2159-8290.CD-16-0778
129. Glodde N, Bald T, van den Boorn-Konijnenberg D, Nakamura K, O'Donnell JS, Szczepanski S, et al. Reactive neutrophil responses dependent on the receptor tyrosine kinase c-met limit cancer immunotherapy. *Immunity*. (2017) 47:789–802 e9. doi: 10.1016/j.immuni.2017.09.012
130. Futosi K, Mocsai A. Tyrosine kinase signaling pathways in neutrophils. *Immunol Rev*. (2016) 273:121–39. doi: 10.1111/imr.12455

131. Albanesi M, Mancardi DA, Jonsson F, Iannascoli B, Fiette L, Di Santo JP, et al. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood*. (2013) 122:3160–4. doi: 10.1182/blood-2013-04-497446
132. Ring NG, Herndler-Brandstetter D, Weiskopf K, Shan L, Volkmer JP, George B, et al. Anti-SIRPα antibody immunotherapy enhances neutrophil and macrophage antitumor activity. *Proc Natl Acad Sci USA*. (2017) 114:E10578–85. doi: 10.1073/pnas.1710877114
133. Matlung HL, Babes L, Zhao XW, van Houdt M, Treffers LW, van Rees DJ, et al. Neutrophils kill antibody-opsonized cancer cells by trogoptosis. *Cell Rep*. (2018) 23:3946–59 e6. doi: 10.1016/j.celrep.2018.05.082
134. Chu D, Zhao Q, Yu J, Zhang F, Zhang H, Wang Z. Nanoparticle targeting of neutrophils for improved cancer immunotherapy. *Adv Healthc Mater*. (2016) 5:1088–93. doi: 10.1002/adhm.201500998
135. Hicks M, Riedlinger G, Willingham MC, Alexander-Miller MA, Von Kap-Herr C, Pettenati MJ, et al. Transferable anticancer innate immunity in spontaneous regression/complete resistance mice. *Proc Natl Acad Sci USA*. (2006) 103:7753–8. doi: 10.1073/pnas.0602382103
136. Maharaj D, Vianna PG, Ward W, Messina AJ, Rayborn T, Gouvea JV,et al. Young donor white blood cell immunotherapy induces extensive tumor necrosis in advanced-stage solid tumors. *Heliyon*. (2017) 3:e00438. doi: 10.1016/j.heliyon.2017.e00438
137. Peters M. Just how big is the pulmonary granulocyte pool? *Clin Sci*. (1998) 94:7–19. doi: 10.1042/cs0940007
138. Worthen GS, Schwab B III, Elson EL, Downey GP. Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. *Science*. (1989) 245:183–6. doi: 10.1126/science.2749255
139. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci USA*. (2010) 107:21248–55. doi: 10.1073/pnas.1015855107
140. Voloshin T, Gingis-Velitski S, Bril R, Benayoun L, Munster M, Milsom C, et al. G-CSF supplementation with chemotherapy can promote revascularization and subsequent tumor regrowth: prevention by a CXCR4 antagonist. *Blood*. (2011) 118:3426–35. doi: 10.1182/blood-2010-11-320812
141. Pickup MW, Owens P, Gorska AE, Chytil A, Ye F, Shi C, et al. Development of aggressive pancreatic ductal adenocarcinomas depends on granulocyte colony stimulating factor secretion in carcinoma cells. *Cancer Immunol Res*. (2017) 5:718–729. doi: 10.1158/2326-6066.CIR-16-0311

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

frontiers in Immunology

REVIEW
published: 16 August 2019
doi: 10.3389/fimmu.2019.01817

Context Drives Diversification of Monocytes and Neutrophils in Orchestrating the Tumor Microenvironment

Juhee Jeong[1], *Yoorock Suh*[1] *and Keehoon Jung*[1,2,3]*

[1] *Lab of Cancer Immunology and In Vivo Imaging, Department of Biomedical Sciences, BK21 Plus Biomedical Science Project, Seoul National University College of Medicine, Seoul, South Korea,* [2] *Department of Anatomy and Cell Biology, Seoul National University College of Medicine, Seoul, South Korea,* [3] *Institute of Allergy and Clinical Immunology, Seoul National University Medical Research Center, Seoul, South Korea*

Recent preclinical/clinical studies have underscored the significant impact of tumor microenvironment (TME) on tumor progression in diverse scenarios. Highly heterogeneous and complex, the tumor microenvironment is composed of malignant cancer cells and non-malignant cells including endothelial cells, fibroblasts, and diverse immune cells. Since immune compartments play pivotal roles in regulating tumor progression via various mechanisms, understanding of their multifaceted functions is crucial to developing effective cancer therapies. While roles of lymphoid cells in tumors have been systematically studied for a long time, the complex functions of myeloid cells have been relatively underexplored. However, constant findings on tumor-associated myeloid cells are drawing attention, highlighting the primary effects of innate immune cells such as monocytes and neutrophils in disease progression. This review focuses on hitherto identified contextual developments and functions of monocytes and neutrophils with a special interest in solid tumors. Moreover, ongoing clinical applications are discussed at the end of the review.

Keywords: monocytes, neutrophils, tumor microenvironment, myeloid cell heterogeneity, innate immunity, cancer immunology

OPEN ACCESS

Edited by:
Wei Zheng,
Albert Einstein College of Medicine, United States

Reviewed by:
Markus Munder, Johannes Gutenberg University Mainz, Germany
Raymond B. Birge, Rutgers University, The State University of New Jersey, United States

***Correspondence:**
Keehoon Jung
keehoon.jung@snu.ac.kr

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *03 May 2019*
Accepted: *18 July 2019*
Published: *16 August 2019*

Citation:
Jeong J, Suh Y and Jung K (2019) Context Drives Diversification of Monocytes and Neutrophils in Orchestrating the Tumor Microenvironment. Front. Immunol. 10:1817. doi: 10.3389/fimmu.2019.01817

MONOCYTES: FROM DEVELOPMENT TO DEPLOYMENT

Monocyte Development

Monocytes originally stem from the bone marrow and constitute 10% of leukocytes in human blood and 4% of leukocytes in mouse blood, respectively (1). The development of blood monocytes is dependent on colony-stimulating factor 1 receptor, CSF-1R (also known as M-CSFR; macrophage colony-stimulating factor receptor) (1, 2). CSF-1R is a hematopoietic growth factor receptor expressed on monocytes, macrophages, dendritic cells and their progenitors (1, 2). CSF-1R interacts with its ligands CSF-1 (M-CSF) and IL-34 to regulate the development of monocytes in the bone marrow (1, 2). In mice deficient in CSF-1R and CSF-1, monocyte development is inhibited, and therefore the number of monocytes in blood is remarkably reduced (1, 2).

With knowledge of CSF-1R, it is possible to navigate the development process of monocytes. From the bone marrow, hematopoietic stem cells (HSCs) give rise to heterogeneous multipotent progenitors (MPPs) generating common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs) in a CSF-1 dependent manner (3). While lymphoid cells such as T

lymphocytes, B lymphocytes, and natural killer cells are derived from CLPs, CMPs generate megakaryocyte and erythrocyte progenitors (MEPs) or granulocyte and macrophage progenitors (GMPs). Generated GMPs further go through a series of differentiation, firstly into macrophage, and DC progenitors (MDPs), then into common monocyte progenitors (cMoPs), and finally into monocytes (3). Differentiated monocytes can be divided into two main subpopulations defined as $Ly6C^{hi}CX_3CR1^{low}$ and $Ly6C^{low}CX_3CR1^{hi}$ cells in mice and as $CD14^{hi}CD16^{+/-}$ and $CD14^{low}CD16^{hi}$ cells in humans (4–8).

$Ly6C^{hi}CX_3CR1^{low}$ populations (hereinafter referred to as $Ly6C^{hi}$ monocytes) are named "classical" or "inflammatory" monocytes, whereas $Ly6C^{low}CX_3CR1^{hi}$ populations (hereinafter referred to as $Ly6C^{lo}$ monocytes) are named "non-classical" or "patrolling" monocytes for their preferential patrolling behavior while circulating the blood stream (9, 10). Development of $Ly6C^{hi}$ monocytes occurs during the cMoP stage, dependent on GM-CSF, c-FLIP, IRF8, and KLF4 (10). The widely accepted hypothesis on $Ly6C^{lo}$ monocyte differentiation is that after generation of $Ly6C^{hi}$ monocytes from the bone marrow, a proportion of them differentiate into $Ly6C^{lo}$ monocytes as downregulation of Ly6C and upregulation of Nr4a1, C/EBPβ, CSF-1R, and CX_3CR1 (4, 10, 11). However, this was questioned for some time in that deletion of transcription factors KLF4 and IRF8 hinders the development of $Ly6C^{hi}$ monocytes but not $Ly6C^{lo}$ monocytes (12–14). This finding led to controversy on whether $Ly6C^{lo}$ monocytes originate from $Ly6C^{hi}$ monocytes or not. The latter argues that $Ly6C^{lo}$ monocytes might have a distinct differentiation lineage in a $Ly6C^{hi}$ monocyte-independent way, namely direct differentiation from cMoPs.

Single-cell RNA sequencing provided an additional clue, reasserting that $Ly6C^{hi}$ monocyte population is the source of $Ly6C^{lo}$ monocytes (15). Application of such advanced technology revealed that steady-state $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes are homogenous populations, and C/EBPβ regulates the differentiation of $Ly6C^{hi}$ monocytes into $Ly6C^{lo}$ monocytes (15). This is also in line with remarkable expression/function of Nr4a1 on $Ly6C^{lo}$ monocyte development (16), as it was found that regulation of Nr4a1 is mediated by the expression of C/EBPβ and also KLF2 assisting conversion of $Ly6C^{hi}$ monocytes to $Ly6C^{lo}$ monocytes (12, 15–17). Besides Nr4a1, Ten-Eleven-Translation-3 (TET3), a target of hsa-miR-150, regulates differentiation of classical monocytes into non-classical monocytes in K562 human chronic myeloid leukemia and U937 human lymphoma (18). Upregulation of TET3 expression in classical monocytes following downregulation of hsa-miR150 rarely generates non-classical monocytes, but does not affect the survival of non-classical monocytes (18). Recently, single-cell RNA-seq has also led to the identification of two additional monocyte populations and their distinct relationships with other immune cells in human blood, highlighting the heterogeneity of myeloid cells (19). High-dimensional mass cytometry has further revealed heterogeneity within human non-classical monocytes, and has allowed distinguishing between two different non-classical monocyte subsets, $Slan^{+}$ and $Slan^{-}$, with functional differences based on Slan expression (20).

Some developed monocytes can enter non-lymphoid organs such as skin and lung without differentiation and orchestrate the physiological condition, while some portion of developed monocytes undergoes differentiation into macrophages or dendritic cells (21–24). Of note, differentiated macrophages are conventionally classified into pro-inflammatory M1 type and anti-inflammatory (pro-tumoral) M2 type, and these macrophages differentially regulate tumor progressions and metastases (25). However, this binary classification of macrophages is insufficient to represent their multifaceted and plastic functions (25). On the other hand, monocyte-derived dendritic cells have been mainly regarded as immune activators in the tumor microenvironment, recruiting and stimulating immune effector cells (26). Nevertheless, dendritic cells are also highly heterogeneous, and cancer cells can recruit the immunosuppressive subset of dendritic cells and/or suppress their anti-tumoral functions (26). All this flexibility appears in a context-dependent manner. Likewise, differing individual functions of monocytes might result from different contexts of development. While it is well-accepted that the bone marrow is the primary source of production and supply of monocytes in physiological condition (1), there is substantial controversy whether the bone marrow serves the same role in cancer-derived pathological conditions. Splenic progenitor cells are reinforced to generate monocytes during KP lung carcinoma progression, which suggests that the spleen could be a critical organ to produce and amplify monocytes (27). The pivotal role of spleen as a source of monocytes has also been highlighted in a different inflammatory condition (28). Angiotensin II plays a central role in amplifying $Ly6C^{hi}$ monocytes and their precursors in the spleen red pulp of KP lung carcinoma-bearing mice as well as releasing monocytes from their splenic reservoir (28, 29). However, a conflicting view has been suggested in a different lung tumor model. During the development of Lewis lung carcinoma (LLC), the bone marrow primarily promotes monocyte production while the spleen plays a minor role in monocyte production (30). Monocytes produced from the bone marrow are more favored to migrate into and to be accumulated in the tumor region than those from the spleen (30). Although an increased accumulation of monocytes in the spleen is also detected in the LLC model, it is because the bone marrow primarily accelerates monocyte production and transfers the newly formed monocytes to the spleen; the spleen is not the primary source (30). As such, different context might have yielded the controversy on tumor monocyte development. Therefore, further studies need to be conducted in as many types of tumors as possible (31).

Monocytes: Pro-tumoral vs. Anti-tumoral Functions in Solid Tumors

Other than the well-known feature as precursors of macrophage and dendritic cell populations, monocytes play a significant role *per se* in orchestrating the immune system not only in homeostatic condition (21), but also in tumor progression (7, 8, 32–35). Generally, high rate of monocyte infiltration into the tumor milieu indicates poor clinical prognosis of cancers (36, 37).

Since each subset of monocytes has different functions in tumor progression depending on the context, it is momentous to decide which subset of monocytes should be targeted in each tumor. Distinct functions of Ly6C^{hi} monocytes and Ly6C^{lo} monocytes in solid tumors have been explored (**Table 1**; **Figure 1**). These monocytes play pro-tumoral or anti-tumoral roles, regulating diverse mechanisms ranging from angiogenesis to immune modulation in a context-dependent manner (**Table 1**; **Figure 1**).

Recruitment of Classical Monocytes and Their Functions in Solid Tumors

Ly6C^{hi} classical monocytes have been mostly reported to play pro-tumoral functions once recruited to the tumor microenvironment (**Table 1**; **Figure 1**). Ly6C^{hi} monocytes express high levels of CCR2 on their surface (32). CCR2 mediates the migration of Ly6C^{hi} monocytes from the bone marrow to CCL2-secreting tumor milieu in PyMT spontaneous breast carcinoma, KCKO pancreatic carcinoma, and MC38 colorectal carcinoma (29, 45, 46). These recruited classical monocytes release VEGFA (a major stimulator of angiogenesis) to facilitate tumor cell extravasation and lung metastasis (32, 47). In human pancreatic tumor as well as murine pancreatic lesion model, the tumor microenvironment releases CCL2 and thereby actively recruits CCR2-expressing CD14^{+}CD16^{-} classical monocytes from bone marrow to blood stream, which is a prognostic factor of worse outcome (45). In contrast, CCR2 inhibition attenuates the mobilization and thus leads to forming an anti-tumoral immune environment in KCKO pancreatic carcinoma and MC38 colorectal carcinoma (45, 46). In human RCC patients and xenograft models, the IL-1β/IL-1R interaction activates the MyD88-NF-kB signaling pathway, and thereby enables classical monocytes with pro-tumoral phenotypes to upregulate pro-tumoral genes such as VEGF, MMP-10, IL-8, TNF-α, and PTGS2 (38). Ly6C^{hi} monocytes/CD14^{+}CD16^{-} monocytes also facilitate cancer cell invasion and metastases via expressing F13a1 to promote fibrin cross-linking not only in murine KLN205 lung squamous cell carcinoma but also in human lung cancer, implicating poor survivals (39). As such, in hepatocellular carcinoma (HCC), Gr-1+ myeloid cells which contain Ly6C^{hi} monocyte population play pro-tumoral function supporting tumor fibrosis by secreting platelet-derived growth factor-beta (PDGF-β), a pro-fibrotic growth factor (40).

Moreover, classical monocytes play a major role in establishing a cancer therapy-resistant microenvironment (34, 48, 49). Doxorubicin treatment on MMTV-PyMT breast carcinoma, for induction of necrotic cell death, triggers the enhanced infiltration of CCR2-expressing monocytes. At later stages of cancer, this backfires; these monocytes have been revealed responsible for resistance against doxorubicin, promoting tumor relapse after treatment (48). In 4T1 and MMTV-PyMT breast carcinoma, paclitaxel treatment induces the secretion of tumor-derived extracellular vesicles (EVs), and these EVs upregulate pulmonary CCL2 expression to elicit classical monocyte expansion establishing a lung pre-metastatic niche (34). Applying radiotherapy on KPC pancreatic carcinoma also leads to a significant increase in CCL2 production by tumor cells. Subsequent recruitment of classical monocytes thereby endows the tumor with resistance against the cancer treatment (49). Use of anti-CCL2 antibodies selectively restrains radiotherapy-dependent recruitment of classical monocytes, impeding tumor progression when combined with radiotherapy (49).

Based on these findings, treatment with anti-CCL2 antibody might sound attractive for tumor regression. However, the following study has proposed a caution for anti-CCL2 mono-treatment. During anti-CCL2 treatment in 4T1, J110, and Met-1 mammary carcinoma, a large population of the classical monocytes is retained within the bloodstream, and their homing to the primary tumor or to the metastatic site is attenuated (50). However, after anti-CCL2 treatment cessation, monocytes initiate their migration to the lungs, and the level of IL-6 rises within the lungs. The increased level of IL-6 augments pro-angiogenic VEGF-A expression in classical monocytes, and thereby accelerates tumor metastasis (50). IL-6RA is largely expressed in Ly6C^{hi} monocytes, and anti-IL-6R antibodies effectively target Ly6C^{hi} monocytes (51, 52). Notably, IL-6-IL-6R interaction not only promotes VEGF-A secretion from classical monocytes but also activates the STAT3 signaling pathway in cancer cells, which enhances tumor cell proliferation in pancreatic ductal adenocarcinoma (PDAC) (50–53). IL-6 is also strongly induced in adipocytes and tumor-infiltrated myeloid cells after anti-VEGF treatment on overweight breast cancer patients. The upregulated IL-6 mediates resistance to anti-VEGF therapy, leading to the proliferation of cancer cells and dysfunctional angiogenesis (54). IL-6 inhibition increases the tumor microenvironment's sensitivity to chemotherapy and anti-angiogenic therapy and promotes tumor cell death (52, 54).

Contrary to the pro-tumoral properties of Ly6C^{hi}/CD14^{+}CD16^{-} monocytes explicated above, it has also been reported that these classical monocytes play anti-tumoral functions in certain treatments (**Table 1**; **Figure 1**). Tumor fibrosis promotes tumor progression by increasing collagen deposition, reducing T cell infiltration, and inducing pro-tumoral macrophage polarization (33, 55–58). A distinct class of Mac1^{+}F4/80^{-}Msr1^{+}ceacam1^{+}Ly6C^{lo} monocytes has been recently discovered to promote fibrosis in C/EBPβ dependent manner (59). Meanwhile, Ly6C^{hi} monocyte infiltration into KPC pancreatic adenocarcinoma via IFN-γ and CCL2 following anti-CD40 treatment has been reported to facilitate degradation of tumor fibrosis, increasing the efficacy of the chemotherapy on PDAC while Ly6C^{hi} monocyte-containing Gr-1^{+} myeloid cells in HCC play pro-fibrotic roles (33, 40, 55).

Recruitment of Non-classical Monocytes and Their Functions in Solid Tumors

On the other hand, Ly6C^{lo}/CD14^{-}CD16^{+} non-classical monocytes have independent mechanisms for infiltration to tumors, and their functions are context-dependent. In models of colorectal cancer, Jung et al. have firstly revealed immunosuppressive functions of non-classical monocytes in any context, including cancers (7, 8). Anti-angiogenic therapy leads to non-classical monocyte influx to CX_3CL1-secreting tumor milieu. Then these recruited non-classical monocytes secrete CXCL5, and mediate a massive infiltration of CXCR2-expressing neutrophils through the highly specific chemokine axis (7). This

TABLE 1 | Context-derived heterogeneous functions of monocyte subsets.

Type of monocytes	Function		Factor	Model	Cancer type/treatment	References
Classical monocytes	Protumoral	Metastasis; Tumor cell extravasation	VEGFA	Mouse	MMTV-PyMT breast cancer	(32)
			VEGF, MMP-10, IL-8, TNF-α, PTGS2	Human	Renal cell carcinoma	(38)
		Metastasis; Cancer cell invasion	F13a1	Mouse	KLN205 lung squamous cell carcinoma	(39)
				Human	Lung cancer	
		Tumor fibrosis	PDGF-β	Mouse	Hepatocellular carcinoma	(40)
	Antitumoral	Degradation of tumor fibrosis	MMPs	Mouse	KPC pancreatic adenocarcinoma w/anti-CD40 treatment	(33)
Non-classical monocytes	Protumoral	Immunosuppression	CXCL5, IL-10	Mouse	CT26, SL4 colorectal cancer w/anti-VEGFR2 therapy	(7, 8)
		Angiogenesis	MMP-9	Human cancer cell xenograft	DLD1, HCT116 human colorectal carcinoma	(41)
	Antitumoral	NK cell recruitment	CCL3, CCL4, CCL5	Mouse, Human cancer cell xenograft, Human	B16F10 melanoma, A375 human melanoma, MMTV-PyMT breast cancer, Human lung cancer specimen (early stage)	(35, 42, 43)
		NK cell activation	IL-15	Mouse	B16F10, B16F0 melanoma	(44)

Summarizes diverse protumoral and antitumoral functions of monocyte subsets (classical and non-classical monocytes) and their related factors in each model.

finding echoes a previous finding also showing that non-classical monocytes recruit neutrophils, albeit mediated by CXCL1—not CXCL5—in a different disease condition outside oncology (60). These tumor-infiltrating non-classical monocytes and neutrophils release immunosuppressive cytokines including IL-10 which inhibits infiltration and activity of cytotoxic T lymphocytes in tumors (7, 8) (**Table 1**; **Figure 1**). Jung et al. also successfully developed several therapeutic strategies targeting these non-classical monocyte-mediated cascades by blocking their infiltration and activity (7, 8). Through a series of *in silico* and *in vitro* screening, novel siRNA sequences against CX_3CL1 with potent knock-down efficacy were identified. The siRNA was formulated with nanoparticles particularly designed for endothelial cell-specific delivery, which resulted in inhibiting $Ly6C^{lo}$ monocyte infiltration and subsequently reduced tumor growth (7). Notably, CXCR4 was discovered to be a critical chemokine receptor expressed on non-classical monocytes and neutrophils (8). CXCL12/CXCR4 axis in these cells mediates restrained cytotoxic T cell infiltration and builds up immunosuppressive tumor microenvironment in CT26, SL4 colorectal carcinoma, and E0771, MCa-M3C mammary carcinoma (8, 61). Supporting this finding, AMD3100 which is a potent CXCR4 inhibitor, also known as plerixafor, efficiently hinders the recruitment of non-classical monocytes, improving the treatment efficacy of anti-VEGFR2 therapy. This suggests the potential of rapid clinical translation, since AMD3100 is already an FDA-approved CXCR4 blocker being used in the clinic for other uses (8, 61).

Despite the several pro-tumoral features of $Ly6C^{lo}/CD14^{-}CD16^{+}$ non-classical monocytes, these monocytes also display anti-tumoral properties in different tumor/treatment conditions (**Table 1**; **Figure 1**). In B16F10 melanoma and MMTV-PyMT spontaneous mammary carcinoma, non-classical monocytes play a pivotal role in engulfing tumor material in the lung and attenuating tumor metastasis and activating NK cells (17, 35). In B16F10 and B16F0 melanoma, non-classical monocytes also activate NK cells by releasing IL-15, which is a determinant cytokine for NK cells' homeostasis, activation and effector function, preventing lung metastases in primary tumor-bearing mice (44). In B16F10 melanoma and A375 human melanoma xenograft models, exosomes secreted from non-metastatic cancer cells promoted the expansion of non-classical monocytes in the bone marrow (42). The expanded population of the non-classical monocytes leads to recruiting NK cells which function in cancer cell clearance at the pre-metastatic niche (42). This NK cell-recruiting function of non-classical monocytes have been reconfirmed in early stage lung cancer patients (43). Based on these findings, reduced $CD16^{+}$ non-classical monocytes might be correlated with NK cell paucity in this lung tumor lesions (43). According to *ex vivo* study of patients with stage IV cutaneous melanoma, $CD14^{-}CD16^{+}$ non-classical monocytes kill regulatory T lymphocytes (Tregs) by assisting ipilimumab, anti-cytotoxic T lymphocyte associated antigen 4 (CTLA4) monoclonal antibody, -mediated ADCC (antibody-dependent cell-mediated cytotoxicity) (62).

Importantly, it had been widely believed that non-classical monocytes are not able to extravasate out of blood vessels. Instead, they were known to stay inside vasculature and patrol the endothelium, which gave these monocytes the nickname "patrolling monocytes" (9). However, recent studies strongly suggest that they do have the capability of transmigration and actively infiltrate into tissues, proven by state-of-the-art *in vivo* imaging techniques (7, 8). Supporting this, in DLD1

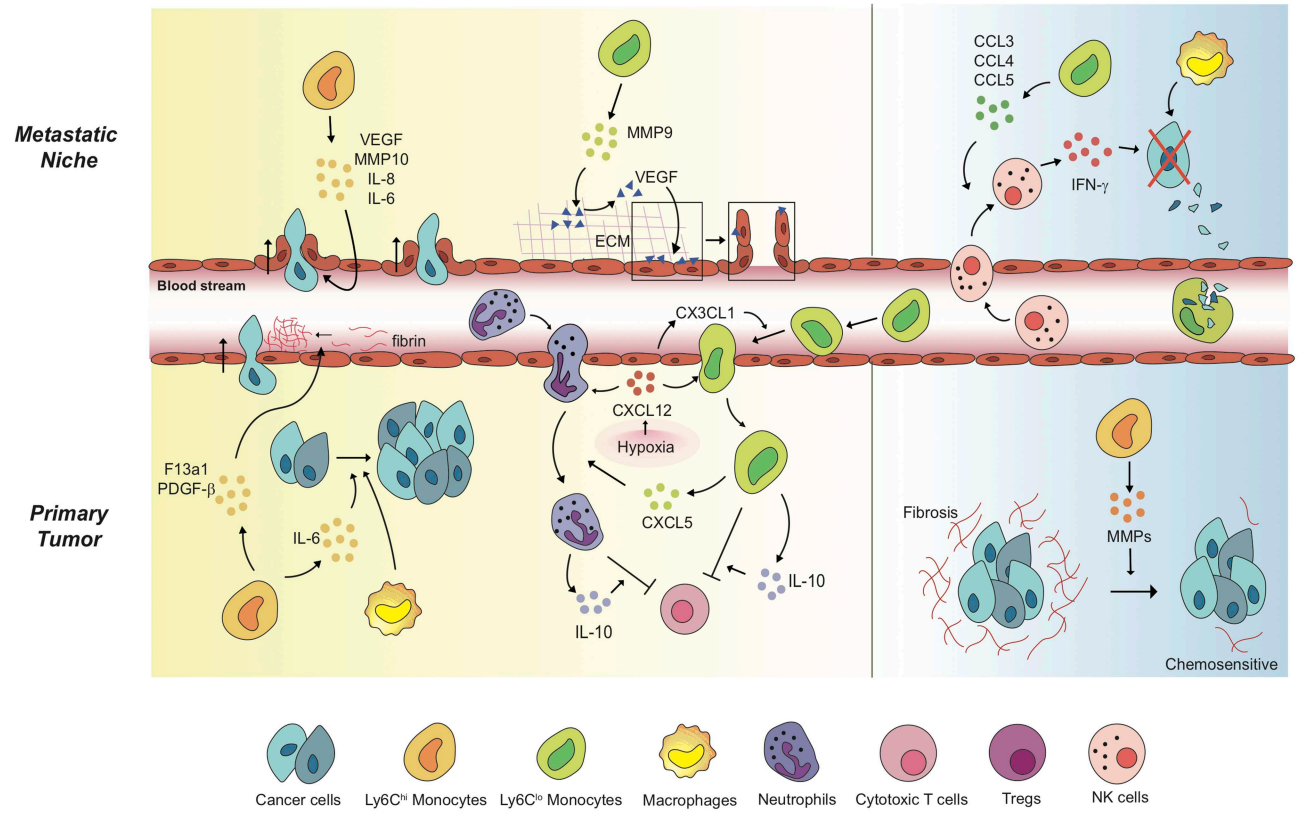


FIGURE 1 | Monocytes mediate a variety of pro-tumoral and anti-tumoral mechanisms in a context-dependent way. In primary tumors, Ly6C^{hi} monocytes exert pro-tumoral effects to promote cancer cell proliferation and cancer cell intravasation. Of note, anti-angiogenic therapy induces Ly6C^{lo} monocyte-mediated immunosuppressive tumor microenvironment and triggers resistance against the therapy. Under the treatment, non-classical Ly6C^{lo} monocytes have been revealed to extravasate to primary tumor regions. The tumor-infiltrated Ly6C^{lo} monocytes significantly contribute to inhibition of cytotoxic T cell function. In metastatic niches, Ly6C^{hi} monocytes and Ly6C^{lo} monocytes facilitate cancer cell extravasation by secreting pro-angiogenic molecules and by mediating the release of ECM-bounded VEGF molecules. In contrast, these monocytes display anti-tumoral functions in different settings. In the lung metastatic sites, Ly6C^{lo} monocytes recruit tumor-killing NK cells, and scavenge tumor materials in the lung vasculature. Meanwhile, Ly6C^{hi} monocytes degrade fibrosis around cancer cells, which have the cancer cells acquire chemosensitivity upon treatments.

and HCT116 human colorectal carcinoma, recruited human patrolling monocytes in tumors secrete matrix metalloproteinase 9 (MMP9), a proteolytic enzyme fostering angiogenesis, triggering a release of matrix-bound VEGFA. This accelerates the extravasation and accumulation of these pro-angiogenic patrolling monocytes, promoting tumor progression (41). This also validates the first finding of non-classical monocyte extravasation directly visualized by intravital microscopic imaging (7, 8).

Tie2-Expressing Monocytes

Other than the traditional classification of monocytes by Ly6C expression level, another classification method by Tie2 (angiopoietin receptor) expression exists. Tie2-expressing monocytes (TEMs) are a monocyte population present in both human and mouse peripheral blood and tumor, and are localized in perivascular spaces but not incorporated with vascular endothelial cells (63, 64). Angiopoietin-1 (Ang-1), a Tie2 ligand, is likely to promote the recruitment of TEMs to tumor vasculature before the turn-on of the angiogenic switch in early stages of N202 breast carcinoma, Rip1-Tag2 pancreatic insulinoma and U87 human glioma (63, 65, 66). In a following study, it was also elucidated that Angiopoietin-2 (Ang-2), another Tie2 ligand upregulated in tumor hypoxia, can also recruit TEMs. The TEMs are then reprogrammed to show proangiogenic phenotypes (67, 68). Meanwhile, Collagen triple-helix repeat-containing 1 (CTHRC1) secreted by several malignant tumors has been reported to recruit TEMs to the tumor microenvironment through upregulation of Ang-2 in endothelial cells and promote metastasis in human MiaPaCa-2, CFPAC-1, and Panc-1 pancreatic cancers (69). Recruited TEMs promote angiogenesis via secretion of a proangiogenic molecule, basic fibroblast growth factor (bFGF) (63–66). Also, Ang-2 and hypoxia cause TEM influx into the tumor microenvironment, and the TEMs mediate downregulation of TNF-α supporting cancer cell survival and causing metastasis of the primary tumor

(63, 67, 70). Blockade of Ang-2 impedes tumor angiogenesis in MMTV-PyMT breast carcinoma and Rip1-Tag2 pancreatic insulinoma through downregulation of Tie2 in TEMs (71).

NEUTROPHILS: FROM DEVELOPMENT TO DEPLOYMENT

Neutrophil Development

Neutrophils are another myeloid compartment which plays critical roles both in homeostatic condition and tumor context. There is a train of precursors to be passed through to generate mature neutrophils in the bone marrow (72). Hematopoietic stem cells (HSCs) give rise to multipotent progenitors (MPPs), lymphoid primed multipotent progenitors (LMPPs), and granulocyte/macrophage progenitors (GMPs) in this very order (72). There are several more stages to go to be differentiated to neutrophils, namely a series of myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, and finally neutrophils (72). These steps for neutrophil generation occur under major regulation by the granulocyte-colony stimulating factor (G-CSF), granulocyte–macrophage-colony stimulating factor (GM-CSF) and also minor regulation by other molecules such as IL-6 and KIT ligand (KITL) (73). Differentiating neutrophils express the G-CSF receptor (G-CSFR) throughout the myeloid lineage (73). During development in the bone marrow, neutrophils acquire three types of granules sequentially; azurophil (primary) granules which retain myeloperoxidase regulated by transcription factors C/EBPα and Gfi-1, specific (secondary) granules which contain lactoferrin mostly regulated by C/EBPε, and gelatinase (tertiary) granules which contain MMP9 regulated by C/EBPβ, C/EBPδ, C/EBPγ, and PU.1 (74, 75). Of note, mass cytometry has recently found new proliferative precursors of neutrophils after GMP stage which further differentiate to immature neutrophils and mature neutrophils with regulation of C/EBPε (76). Although the bone marrow is primarily responsible for the neutrophil formation, the spleen can be an alternative source of neutrophils during emergency granulopoiesis derived from cancer progression (73). In KP lung adenocarcinoma, splenic hematopoietic stem cells, and progenitor cells produce neutrophils during tumor progression (27). Presence of cancer cells upregulates the expression of several factors accelerating neutrophil development. The expression of CXCL1, CXCL2, CXCL5, and CXCL8, which are CXCR2 ligands, and the expression of KITL and GM-CSF are strongly enhanced by KRAS signaling in cancer cells and tumor-derived hypoxia (73). Moreover, IL-1β-producing macrophages and IL-17-producing γδ T cells secrete G-CSF to promote neutrophil development in the tumor (73). Cancer cells accelerate secretion of these cytokines and chemokines to instigate overactive granulopoiesis and neutrophilia (73). The accelerated secretion of these factors promotes the release of immature neutrophils to the blood stream, resulting in an increased number of circulating neutrophils (73). In 4T07, 4T1 mammary carcinoma, LLC, and Kras-driven pancreatic carcinoma, G-CSF production is also facilitated via RAS/MEK/ERK pathway in cancer cells, promoting recruitment of neutrophils (77). Meanwhile, type I IFNs from tumor trigger differentiation of neutrophils to achieve an anti-tumoral phenotype, reducing not only CXCR4 expression in neutrophils which mediates tumor-homing, but also VEGF and MMP9 expression (78, 79). Moreover, type I IFNs suppress G-CSF signaling pathways in neutrophils, thereby reducing expression of Bv8, S100A8, S100A9, and MMP9 so that they can attenuate the formation of the pre-metastatic niche (78, 79). Inhibition of type I IFNs impairs cytotoxicity of neutrophils and promotes metastasis of B16F10 melanoma, MCA205 fibrosarcoma, 4T1 mammary carcinoma and LLC mediated by neutrophils (78, 79).

Neutrophils: Pro-tumoral vs. Anti-tumoral Functions in Solid Tumors

Functions of neutrophils in the tumor microenvironment vary by context including types of tumor, stages of tumor progression, and different therapies (**Table 2**; **Figure 2**).

Recruitment of Neutrophils to Tumor Milieu

As mentioned above, tumors promote the early release of neutrophils yet with immature phenotypes from the bone marrow. There are several ligand-receptor axes studied for neutrophil recruitment into the tumor. Upon research on diverse tumor models, it has been revealed that CXCR2 is a pressing chemokine receptor which recruits neutrophils to the tumor (7, 8, 119, 120). In KPC pancreatic carcinoma and inflammation-driven and spontaneous intestinal adenocarcinoma, the migration of myeloid cells, especially neutrophils, to the tumor microenvironment is impaired when CXCR2 signaling is suppressed (119, 120). This enhances tumor cell apoptosis and restrains tumorigenesis, resulting in a failure to set up a metastatic niche (119, 120). Moreover, activated neutrophils also express CCR7 on their membrane, which pushes those cells to tumor sites in response to CCL19, CCL21, and GM-CSF secretion (121). IL-17 also triggers neutrophil recruitment to tumor sites in 4T1 breast carcinoma (122), KRAS mutated lung carcinoma (123), and ovarian carcinoma (124). The recruited neutrophils present high expression of tumor-promoting genes such as TNF-α, CXCL1, MMP9, and VEGF (122). In zebrafish larvae model of glioblastoma initiation, neutrophils are actively recruited to KRAS-transformed cells very early in oncogenesis via the CXCL8-CXCR1 signaling axis, and this recruitment contributes to the proliferation of tumor-initiating cells (125). Gastrin-releasing peptide (GRP)-GRP receptor (GRPR) axis can also induce neutrophil migration in the tumor (126, 127). In A375 and M24met human melanoma, CXCL5 overexpression by tumor cells enhances neutrophil recruitment and infiltration into primary tumors and tumor lymphatic vessels (128). It triggers the proximal interaction between neutrophils and cancer cells near the lymphatic endothelial cells in order to help trans-endothelial migration of the cancer cells (128). In SL4 and CT26 colorectal cancer, tumor-infiltrated Ly6C^{lo} monocytes induced by anti-VEGF therapy can also recruit CXCR2-expressing neutrophils to the tumor site via the CXCL5-CXCR2 and CXCL12-CXCR4 axes (7, 8). Albeit in a different disease setting, it has been

TABLE 2 | Context-dependent multifaceted functions of neutrophils.

	Function		Factor	Model	Cancer type/treatment	References
Neutrophils	Protumoral	Tumor initiation	Neutrophil elastase	Mouse	Kras mutant	(80)
			ROS, RNS	Mouse	Colon cancer	(81)
		Cancer cell proliferation	NETs (Neutrophil elastase traps), HMGB-1	Mouse	MC38 colorectal cancer w/ischemia and reperfusion injury	(82)
			Neutrophil elastase	Mouse	A549 lung adenocarcinoma	(83)
			IL-6, IL-1β	Mouse	4T1 breast cancer	(84)
			Transferrin	Mouse	4T1 breast cancer	(85)
		Cancer cell colonization; Differentiation from monocytes to fibrocytes	MMP-9	Mouse	CMT93 colon carcinoma	(86, 87)
		Fibrosis	MAP kinase pathway	Mouse	HCA-1 hepatocellular carcinoma w/Sorafenib treatment	(88)
			IL-1β	Mouse	AK4.4, Pan02, KPC, iKRAS pancreatic adenocarcinoma	(58)
		Macrophage recruitment	MAP kinase pathway	Mouse	TRAMP-C1 prostate cancer, E0771 breast cancer w/VEGF blockade	(89)
		T cell suppression	IL-10	Mouse, Human cancer cell xenograft	CT26, SL4 colorectal cancer w/anti-VEGFR2 therapy/LS174T human colorectal cancer	(7, 8, 90)
			PD-L1	Mouse	H22-generated hepatoma	(91)
			IL-10, LGALS9, ARG1, MFGE8	Mouse	KP lung carcinoma	(92)
			Nos2	Mouse	KEP breast carcinoma, AB12 mesothelioma, LKRM lung carcinoma, LLC	(93, 94)
			ARG1	Human	Non-small cell lung cancer	(95)
		Regulatory T cell attraction	CCL17	Mouse	LLC, AB12 mesothelioma	(96)
		Angiogenesis	Bv8	Mouse	Rip-Tag pancreatic insulinoma	(97, 98)
			MMP-9	Mouse, Human cancer cell xenograft	Rip1-Tag2 pancreatic insulinoma, L929 fibrosarcoma, B16-F10 melanoma, LLC, HPV-15-induced squamouse carcinoma, HT-1080 fibrosarcoma/PC-3 human prostate carcinoma	(99–102)
			VEGF	Human	Oral cavity cancer	(103)
			FGF2	Mouse, Human cancer cell xenograft	Pan02, KPC pancreatic carcinoma/HT29, HCT-116, Lovo human colon cancer	(104)
		Metastasis; Tumor cell extravasation	IL-1β, Leukotriene, IL-8	Mouse, Human cancer cell xenograft	4T1, D2A1 breast cancer/Human MDA-MB-231 breast cancer, human A375-MA2, WM35, C8161.C19, UACC903 melanoma	(105–107)
		Metastasis; Epithelial-mesenchymal transition (EMT)	IL-17α	*In vitro* human cancer cell	(*In vitro*) Human MKN45, MKN74 gastric cancer	(108)
		Metastasis; Bridge between ICAM-1-expressing cancer cells and endothelial cells	MAC-1	Mouse, Human cancer cell xenograft	H50 Lewis Lung carcinoma/Human A549 lung carcinoma	(109)
		Cancer cell retention	NETs (Neutrophil elastase traps)	Mouse	H59 Lewis lung carcinoma w/cecal ligation and puncture/MC38 colorectal cancer w/ischemia and reperfusion injury	(82, 110)
		Activation of dormant cancer cell		Mouse	D2.0R breast cancer	(111)

(Continued)

TABLE 2 | Continued

	Function		Factor	Model	Cancer type/treatment	References
	Antitumoral	Tumor cell death	TNF-α, NO, H_2O_2	Mouse	LLC, AB12 mesothelioma	(112)
			Granzyme B		CT26 colon cancer	(113)
			H_2O_2		AT3, 4T1, MMTV-PyMT breast cancer	(114, 115)
		IL-17+ $\gamma\delta$ T cell suppression	ROS	Mouse	B16F10 melanoma, Hepa1-6 hepatoma	(116)
		Impairment of tumor cell proliferation	Tsp-1	Human cancer cell xenograft	Human PC3 prostate cancer, human MDA- MB-231 breast cancer	(117)
		Stimulation of T cell response	CD54, CD86, OX40L, and 4-1BBL	Human	Lung cancer (early stage)	(118)

Summarizes multiple protumoral and antitumoral functions of neutrophils and their responsible factors in each different context.

also reported that monocytes recruit neutrophils in a TLR7-dependent manner through CXCL1 (60), different from the case of cancer context where non-classical monocytes-derived CXCL5 was newly discovered as the key chemokine attracting neutrophils (7, 8).

Pro-tumoral Functions of Neutrophils in Solid Tumors

Recruited neutrophils from the blood stream have potent influences on various components of tumor progression and metastasis, including tumor initiation, cancer cell survival/proliferation, immune modulation, angiogenesis, and intra/extravasation of cancer cells (**Table 2**; **Figure 2**).

Neutrophils and tumor initiation

In Kras mutant mice, airway inflammation induces secretion of IL-8 by lung keratinocytes, recruiting CXCR2-expressing neutrophils to the inflammation site (80). Neutrophil elastase (NE), a powerful serine protease exclusively found in primary granules of neutrophils, facilitates inflammation-mediated tumor initiation in the lung (80). H. hepaicus-induced colitis triggers tissue infiltration of MPO^+ neutrophils and macrophages into the infected sites (81). These neutrophils and macrophages generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) which subsequently cause molecular damage, promoting tumorigenesis (81). Transcriptional analysis reveals that genes involved in repairing DNA damage were downregulated, but genes associated with reactive chemical species generation were upregulated in infected colons (with no impact on cell proliferation) (81).

Neutrophils and cancer cell survival/proliferation

Beyond tumorigenic functions of neutrophils, their roles in cancer cell survival and proliferation have been also elucidated well. Overexpression of insulin receptor substrate 1 (IRS-1) is known to reduce tumor growth (83). In A549 lung adenocarcinoma, neutrophil elastase (NE) degrades IRS-1 in cancer cells, thereby causing tumor cell proliferation. PI3K signaling pathway alteration mediates this process by fostering the interaction with PDGF-receptor (83). In 4T1 breast carcinoma, transferrin, an iron-transporting protein secreted by neutrophils, binds to its receptor expressed on cancer cells (85). Then the transferrin supplies iron to the cancer cells for their proliferation (85). As tumor growth and metastasis are promoted, transferrin secretion by neutrophils increases (85). Sorafenib, a rapidly accelerated fibrosarcoma (RAF) inhibitor, is commonly used as treatment for HCC. However, the use of sorafenib causes side effects and resistance such as tumor desmoplasia. $Gr\text{-}1^+$ myeloid cells including neutrophils have been revealed to be responsible for the resistance (40, 88, 129, 130). Sorafenib treatment induces tumor hypoxia, which upregulates CXCL12 expression in HCA-1 cancer cells and stromal cells. Then, CXCR4-expressing $Gr\text{-}1^+$ myeloid cells are promoted to infiltrate to CXCL12-secreting tumor sites, and the infiltrated cells support differentiation and activation of hepatic stellate cells via the MAP kinase pathway and fibrosis in HCC (40). Of note, CXCL12-CXCR4 axis triggers increased infiltration of Tregs and M2 type macrophages and upregulation of intratumoral PD-L1 in HCA-1 HCC (88). Moreover, in Ak4.4, Pan02, KPC, and iKRAS pancreatic adenocarcinoma, adipocytes of obese population secrete increased levels of IL-1β to recruit neutrophils to the tumor along with enhancing Treg infiltration, and hindrance of $CD8^+$ T cell infiltration (58). The recruited neutrophils then activate pancreatic stellate cells via IL-1β secretion to accelerate fibrosis, which promotes tumor growth and reduces sensitivity to chemotherapy (58).

Neutrophils and immune modulation

Neutrophils play essential roles in tumor growth and metastasis not only to regulate cancer cell proliferation and survival, but also to modulate innate and adaptive immunity. The recruited neutrophils via CXCL12-CXCR4 axis secrete IL-10 that suppresses cytotoxic T cell function on tumor cells, which then causes anti-VEGF therapy resistance in SL4 and CT26 colorectal carcinoma (7, 8). Similar findings have been recently reported in LS174T human colorectal carcinoma (90), which confirms the previous observations in preclinical murine models (7, 8). The CXCL12-CXCR4 axis in myeloid cells including neutrophils is also responsible for NK cell apoptosis and inactivation by enhancing the Fas signaling pathway and restraining IL-18 production in neutrophils, respectively, in metastatic B16F0 melanoma, PyMT breast carcinoma, and YAC-1 lymphoma

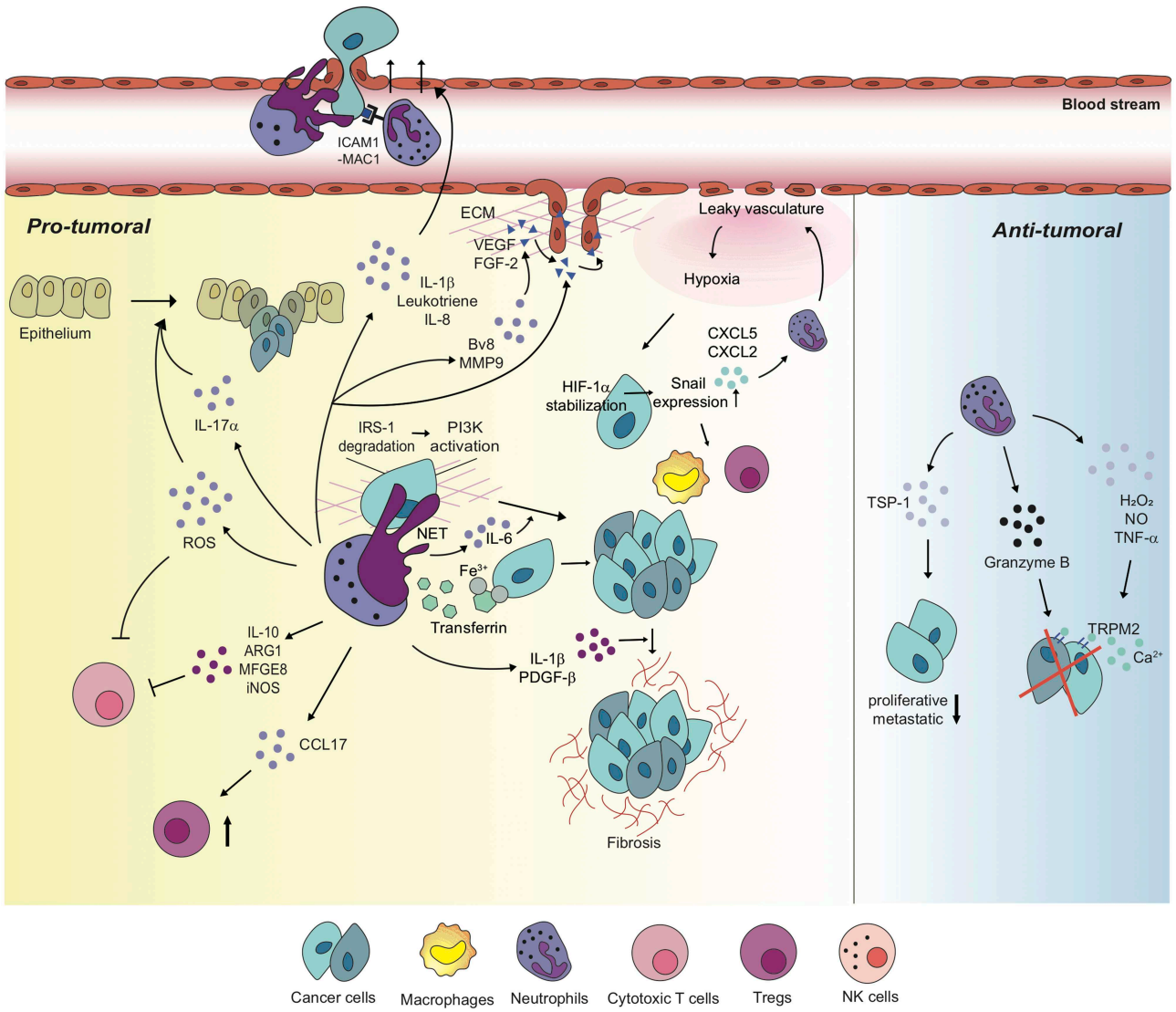


FIGURE 2 | Neutrophils differentially regulate tumor microenvironment with diverse mechanisms. Neutrophils perform pro-tumoral roles in most tumor settings, promoting tumorigenesis, and cancer cell proliferation via diverse mechanisms. Moreover, neutrophils regulate the functions of other immune cells including cytotoxic T cells and Tregs in order to build up tumor-favorable tumor microenvironment. On the one hand, neutrophils stimulate tumor angiogenesis via inducing the release of VEGF and FGF-2 from ECM or secreting pro-angiogenic molecules, themselves. Furthermore, metastatic competence of cancer cells can be achieved by physical interaction with neutrophils and neutrophil-derived secretory molecules, facilitated to extravasate to secondary tumor sites. Neutrophils also create a positive feedback loop with cancer cells toward the formation of the tumor-supportive microenvironment, developing dysfunctional vasculature around the tumor, leading to hypoxia which recruits more neutrophils and pro-tumoral immune cells into tumor milieu. On the other hand, a couple of studies indicate anti-tumoral functions of neutrophils in different contexts. In these contexts, neutrophils perform a cytotoxic function on cancer cells, and have cancer cells lose proliferative and metastatic properties.

(131). Neutrophil-mediated modulation of NK cells has been confirmed in 4T1 and D2A1 metastatic mammary carcinoma context (D2A1 inoculation after 4T1 injection) as well (105). The expanded population of neutrophils near metastatic sites inhibits functional activation of NK cells, and thus the NK cells lose their ability to clear intraluminal tumor cells (105). This consequently comes across with a favorable environment for cancer cell survival and metastasis (105). CXCR4 depletion in those myeloid cells recovers the tumor-killing capacity of NK cells (131). In H22-generated hepatoma-bearing mice, PD-L1 is upregulated in tumor-infiltrating neutrophils (TINs), induced by GM-CSF and TNF-α secretion from the tumor microenvironment (91). The overexpressed PD-L1 of neutrophils suppresses proliferation and activation of PD-1$^+$ T cells, dampening anti-tumor immunity (91). In STK11/LKB1-deficient KP lung carcinoma, recruited neutrophils produce suppressive factors such as IL-10, LGALS9, Arginase1 (ARG1), and Milk fat globulin EGF factor (MFGE8) which are also involved in cytotoxic T cell suppression as well as the tumor-promoting cytokine IL-6 (92). In KEP breast carcinoma, Nos2, the gene encoding iNOS, is largely upregulated

in neutrophils (93). Then the neutrophils suppress $CD8^+$ T cell activity via NO from iNOS, promoting lung metastasis (93). Effect of neutrophil NO production on $CD8^+$ T cell apoptosis has been also confirmed in AB12 mesothelioma, LKRM lung carcinoma, and LLC (94). TNFα-mediated iNOS upregulation and NO secretion in neutrophils induce the apoptosis of non-activated $CD8^+$ T cells via direct contact between cells in these tumor contexts (94). ARG I secretion by neutrophils has been also uncovered to affect T cell suppression by degrading extracellular arginine in non-small cell lung carcinoma patients (95). IL-8 and TNF-α secretions are enhanced in non-small cell lung carcinoma patients, and these cytokines induce ARG I release from exocytosis of granules in neutrophils (95). In 4T1 mammary carcinoma, SCF-c-kit signaling increases c-kit+ neutrophil frequency in the circulatory system. Even in nutrient-limited tumor microenvironments, these neutrophils exploit fatty acid metabolism to maintain mitochondrial function and support ROS production, resulting in T cell suppression (132). This also echoes the formal observations of immunosuppressive neutrophils in colon cancers (7, 8). Meanwhile, a research adopting mass cytometry and single-cell RNA sequencing has recently revealed that a unipotent precursor of neutrophils promotes B16F10 melanoma progression via inhibition of pro-inflammatory T cell activation, eliciting an immunosuppressive microenvironment around the tumor (133).

Neutrophils affect tumor progression by regulating other immune cells beyond NK cells and cytotoxic T cells. In LLC and AB12 mesothelioma, neutrophils attract Tregs via CCL17 secretion and thus scupper the formation of an anti-tumor immune microenvironment (96). Furthermore, blockade of VEGF in TRAMP-C1 prostate carcinoma and E0771 breast carcinoma triggers $Gr\text{-}1^+$ myeloid cell recruitment which mediates macrophage recruitment to the tumor microenvironment via activation of p38 mitogen-activated protein kinase (MAPK) to promote lung metastasis (89).

Myeloid-derived suppressor cells (MDSCs) are immunophenotypically defined as $CD11b^+$ $Gr\text{-}1^+$ cells (in mice) and possess pro-tumorigenic functions including immune suppression (134). These MDSCs can be further classified into granulocytic (or polymorphonuclear) MDSCs [gMDSCs or PMN-MDSCs] ($CD11b^+$ $Ly6C^-$ $Ly6G^+$ in mouse, $CD11b^+$ $CD14^-$ $CD15^+$ in human) and monocytic MDSCs [mMDSCs] ($CD11b^+$ $Ly6C^+$ $Ly6G^-$ in mouse, $CD11b^+$ $CD14^+$ $HLA\text{-}DR^{low}$ $CD15^-$ in human) (134). Although we do not doubt that MDSCs play important roles in regulating tumor progression, the definition of MDSCs is still under debate since it is difficult to clearly discriminate the heterogeneous myeloid cell mixtures only with markers being currently used (7, 8). Indeed, phenotypical and functional features of MDSCs are considerably overlapped with those of monocytes and neutrophils, which we discuss in depth throughout this review. Therefore, we would rather not go into details of MDSCs here.

Neutrophils and angiogenesis

Even though cancer cells and cancer-associated fibroblasts are considerably responsible for the source of angiogenic factors, tumor-infiltrated myeloid cells including neutrophils also exert potent properties in tumor angiogenesis over diverse tumor settings (135). Upregulation of Bv8 following STAT3 activation is responsible for neutrophil-mediated tumor angiogenesis in the early stages of Rip-Tag pancreatic insulinoma (97, 98). In Rip1-Tag2 pancreatic insulinoma, L929 fibrosarcoma, B16-F10 melanoma, LLC, HPV-15-induced squamous carcinoma, HT-1080 fibrosarcoma, and PC-3 human prostate carcinoma, neutrophils infiltrated to the tumors majorly secrete MMP9 remodeling the ECM to release VEGF and FGF-2, and activating them to trigger chronic angiogenesis and thereby promotes tumor progression (99–102, 135). Meanwhile, in oral cavity cancer patients, neutrophils actively secrete VEGF, and promote tumor angiogenesis and metastasis (103). By studying HT29, HCT-116, LoVo human colon carcinoma and Pan02, KPC murine pancreatic carcinoma, it has also been studied that neutrophils are the main source of FGF2 (104). Here, these neutrophils play a proangiogenic role to develop unsystematic tumor vasculature and prompt liver metastasis, facilitating endothelial cell proliferation and migration (104). Hence, inhibition of FGF2 delays tumor growth via normalizing the vasculature (104). In KP lung carcinoma, neutrophils alter angiogenesis around tumor tissue, causing a hypoxic environment (136). HIF1α stabilization induced by hypoxia increases expression of the Snail gene in cancer cells (136). The Snail-expressing cancer cells secrete increased levels of CXCL5 and CXCL2 to recruit more pro-tumoral neutrophils to the tumor, creating a positive amplifying loop to facilitate tumor growth (136, 137). Notably, it has been revealed that Snail has a pro-tumorigenic influence via recruiting pro-tumoral M2 macrophages as well in 4T1 breast cancer and LLC1 lung cancer (137). Snail also induces Treg differentiation and impairs the activity of dendritic cells in B16F10 melanoma (138).

Neutrophils and metastasis

While affecting primary tumor cell proliferation, modulation of the immune microenvironment, and angiogenesis, neutrophils also play significant roles in supervising tumor metastasis. It has been reported that neutrophil infiltration is essential to endow non-malignant BMT-11 fibrosarcoma cancer cells with malignant and metastatic phenotypes (139). The metastatic incidence is significantly reduced with the anti-Gr1 antibody-mediated neutrophil depletion in blood circulation or integrin β2 knockout mice lacking in neutrophil extravasation (139). Of note, integrin β2 mediates neutrophil adhesion on activated endothelium with high affinity, which leads to transmigration of neutrophils across the endothelium (140, 141). A number of subsequent researches have endeavored to illuminate diverse factors and mechanisms which can explain functions of neutrophils on tumor metastasis. In 4T1 and D2A1 metastatic mammary carcinoma (D2A1 injection after 4T1 injection), recruited neutrophils activate endothelial cells via secretion of IL-1β. This, in turn, facilitates trans-endothelial migration of intraluminal tumor cells, forming small protrusions of the cell bodies across the endothelial layer (105, 106). In 4T1 breast carcinoma and human MDA-MB-231 breast carcinoma, leukotrienes derived from neutrophils transform cancer

cell populations to acquire highly metastatic competence in lung pre-metastatic sites (106). The metastatic competence of cancer cells can be also acquired by IL-17α secretion of neutrophils in gastric cancer (108). Of note, epithelial-mesenchymal transition (EMT) endows cancer cells with invasive properties and high-grade malignancy (108, 142). Coculture of gastric cancer patient-derived neutrophils and human MKN45, MKN74 gastric cancer cells has proven that IL-17α activates JAK2/STAT3 axis in the cancer cells following by their acquisition of mesenchymal characteristics, and the IL-17α is mostly derived from tumor-associated neutrophils (TANs) (108).

Employment of a multiplexed microfluidic model of the human microvasculature has revealed that neutrophils also secrete IL-8 by themselves (143). The self-secreted IL-8 induces not only neutrophil sequestration in A375-MA2 human melanoma cells but the interference of endothelial barrier function, supporting cancer cell extravasation (143). In human WM35, A375, C8161.C19, and UACC903 melanoma, IL-8 secreted from entrapped melanoma cells attracts neutrophils and increases integrin β2, specifically MAC-1, on the neutrophils. This leads to the enhancement of neutrophil-melanoma cell interaction, facilitating lung metastasis (107). There is another research subsequently conducted which confirms the interaction between MAC-1 and ICAM1 (neutrophils and cancer cells, respectively) (109). In H59 Lewis lung carcinoma and A549 human lung carcinoma, MAC-1 on neutrophils acts as a bridge between ICAM-1-expressing cancer cells and endothelial cells in favor of liver metastasis (109). Even though both pieces of research by Huh et al. (107) and Spicer et al. (109) have elucidated the MAC-1-ICAM-1 interaction and highlighted the significant function of neutrophils on tumor metastasis, the finding of Spicer et al. (109), is incompatible with Huh et al. (107), in that neutrophils come first to metastatic sites, and then circulating tumor cells directly adhere to the arrested neutrophils in the early step of metastasis. Interaction between neutrophils and circulating tumor cells in the bloodstream has also been elucidated to be mediated via VCAM-1 in 4T1 breast carcinoma (84). Also, the neutrophils physically clustered with circulating 4T1 breast cancer cells support the cancer cell cycle progression, secreting IL-6 and IL-1β, and promote metastasis of cancer cells (84). In MCF-7 human ER+ breast cancer, estradiol alters the neutrophil phenotype to overexpress integrin LFA-1, promoting ER^+ cancer cell dissemination by activating cell-cell interaction (144). Meanwhile, it has been recently studied that neutrophils regulate diurnal transcription profiles in the lung, and promote the migration of B16F1 melanoma cells to the lungs (145). In CCL9-expressing CMT93 colon carcinoma, $CCR1^+$ neutrophils secrete MMP9 to foster cancer foci, and in late phases of tumor the neutrophils recruit fibrocytes or induce differentiation from monocytes to fibrocytes which secrete MMP2, accommodating tumor cell colonization (86, 87). In short, the collaborative work of CCR1, MMP9, and MMP2 at metastatic sites promotes cancer metastasis (86, 87).

Neutrophil elastase traps (NETs) consist of extracellular decondensed DNA with granules and histones derived from neutrophils (146). Through a myriad of studies, it was explored that upon activation of neutrophils, neutrophil-derived NETs degrade virulence factors and trap bacteria within the vasculature, eventually killing them. Thus, NETs work as antimicrobial substances (146–148). It has also been elucidated that NETs play potent roles in tumor cell migration by trapping circulating cancer cells in vasculature and releasing secretory molecules by themselves (110). A study on the progression of H59 Lewis lung carcinoma after cecal ligation and puncture (CLP), represented as an alternative model of postsurgical infection, has proven that systemic sepsis induces neutrophil-derived NET formation in the hepatic sinusoid (110). Then the NETs enable stable retention of tumor cells and accelerate tumor growth within the liver (110). The link between trapped cancer cells by NETs and their proliferation in metastatic sites has been explicated in a metastatic MC38 tumor model followed by ischemia and reperfusion (I/R) injury, which is in an inevitable state after liver resection (82). Tumor hypoxia promotes NET formation within the metastatic site, and the NETs release the High mobility group box 1 (HMGB-1) protein (82). Secreted HMGB-1 activates TLR9, which encourages tumor progression via activation of related intracellular growth signaling pathways, involving phosphorylation of p38, Stat3, JNK and p65 of NF-kB (82). Moreover, it has been recently elucidated that NETs are involved in activation of dormant cancer cells in D2.0R mammary carcinoma (111). NET formation driven by LPS inflammation mediates laminin cleavage and thrombospondin-1 (Tsp-1) modulation by neutrophil elastase and NET-associated proteases (111, 149). This stimulates integrin α3β1 on dormant cancer cells and activates the FAK/ERK/MLCK/YAP signaling pathway to awaken cancer cells (111). Even in the absence of infection, 4T1 mammary cancer cells induce neutrophils to form NETs once they arrive at lung metastatic sites, promoting the expansion of disseminated cells (150). In ID8 ovarian cancer, the cancer cell-derived factors such as IL-8, GRO-a, GRO-b, and G-CSF enhance neutrophil influx to premetastatic omental niche and promote NET formation. In sequence, the NETs support tumor metastasis throughout trapping circulating ovarian cancer cells (151).

Meanwhile, the importance of considering cancer as a systemic disease has been highlighted again through its interaction with bones (152). In KP lung adenocarcinoma, the lung tumor activates Ocn+ osteoblasts via secretion of the soluble receptor for advanced glycation end products (sRAGE), which induces tumor infiltration of $siglecF^{high}$ neutrophils and promotes tumor growth (152). These neutrophils represent a tumor-promoting transcriptional profile with upregulated expression of genes associated with angiogenesis (VEGFA, HIF1α, and SEMA4d), myeloid cell differentiation and recruitment (CSF1, CCL3, and MIF), extracellular matrix remodeling (ADAMDEC1, ADAM17, and many cathepsins), T cell suppression (PD-L1, FCGR2b, and HAVCR2), and tumor cell proliferation (TNF, TGFβ1, and IL-1α) (152). In contrast, the $siglecF^{high}$ neutrophils downregulate genes involved in cytotoxicity (CD244, ITGAL, and Fas) (152). Furthermore, these neutrophils increase ROS production and foster monocyte differentiation into macrophages (152).

Anti-tumoral Functions of Neutrophils in Solid Tumors

The majority of the hitherto conducted researches indicate that neutrophils can only serve to promote tumor progression (**Table 2**; **Figure 2**). However, depending on the context, neutrophils suppress tumor metastasis by inhibiting malignant progression. In CT26 colon carcinoma, neutrophils inhibit the growth of G-CSF-producing cancer cells via contact-mediated cytostatic activity, but not G-CSF-nonproducing cancer cells (153). It has been recently revealed that the H_2O_2 secreted by neutrophils leads to tumor cell death, and TRPM2-mediated calcium influx acts as a go-between for this tumor killing process by neutrophils in AT3 and 4T1 breast cancer (114). In 4T1 mammary carcinoma and MMTV-PyMT spontaneous mammary carcinoma, entrained in the pre-metastatic lung prior to the arrival of metastatic cancer cells from primary sites, neutrophils play a cytotoxic function via physical contacts with cancer cells, secreting H_2O_2 and inhibiting the seeding of the cancer cells (115). Neutrophil-derived ROS secretion in B16F10 melanoma and Hepa1-6 hepatoma suppress IL-17$^+$ γδ T cells which have pro-tumoral features, but not CD8$^+$ T cells, in tumor niches (116). Neutrophils also have cytotoxic activity against CT26 colon cancer cells via production of granzyme B (113). Meanwhile, in B16F10 melanoma, T241 fibrosarcoma, LLC, and MMTV-PyMT-derived lung adenocarcinoma, tumor-induced TNF-α stimulates the NF-kB signaling pathway to express proto-oncogene MET in neutrophils (154). This enables the hepatocyte growth factor (HGF), also driven by the tumor, to bind to MET (154). HGF/MET signaling promotes neutrophil extravasation, induces iNOS and NO production, and thereby supports tumoricidal neutrophil function (154). In human PC3 prostate cancer and MDA-MB-231 breast cancer, bone marrow-derived CD11b$^+$ Gr1$^+$ cells which contain neutrophil populations mainly induce thrombospondin-1 (Tsp-1) in lung premetastatic sites, impairing tumor cell proliferation at the sites (117). It has been also reported that tumor-infiltrated neutrophils undergo functional changes and acquire an anti-tumoral phenotype, supporting T cell responses against tumor in early stages of human lung cancer (118). Photodynamic therapy (PDT) augments anti-tumor immunity and tumor regression by regulating the anti-tumoral functions of neutrophils (155).

Furthermore, neutrophils regulate pro-tumoral or anti-tumoral mechanisms depending on tumor stage. In LLC and AB12 mesothelioma, TANs from the early tumors are more cytotoxic toward tumor cells and produce higher levels of TNF-α, NO, and H_2O_2, while these expressions are downregulated in late stages of tumors in which TANs acquire an enhanced pro-tumoral phenotype (112). Although depletion of neutrophils in the early stages of tumor has no effect on tumor growth, depletion of neutrophils in late stages of tumor dramatically decreases tumor growth (112).

Polarization of Tumor-Associated Neutrophils

According to a myriad of aforementioned studies on functions of neutrophils in diverse tumor circumstances, it has been well-established that TANs acquire pro-tumoral phenotype or anti-tumoral phenotype depending on related factors (147, 156). In AB12 mesothelioma and LKR lung carcinoma, TGF-β secreted by the tumor induces neutrophil polarization toward a pro-tumorigenic phenotype (156). Blockade of TGF-β attracts anti-tumorigenic neutrophils which release a large number of proinflammatory cytokines to infiltrate into the tumor microenvironment (156). Moreover, as the tumor develops, neutrophils display different functions regarding tumor growth through pro-tumoral or anti-tumoral mechanisms. IFN-β (type I IFN) differentiates neutrophils to achieve an anti-tumoral phenotype, reducing VEGF, and MMP9 expression (78, 79, 157). Inhibition of IFN-β endows TANs with pro-tumoral properties, and promotes growth and metastasis of B16F10 melanoma, MCA205 fibrosarcoma, 4T1 mammary carcinoma, CT26 colon carcinoma and Lewis lung carcinoma (78, 79, 157). *In vitro* study of BGC-823, MGC80-3, SGC-7901, and HGC-27 human gastric cancer cells has elucidated that interaction between HMGB1 secreted by the cancer cell-derived exosomes and toll-like receptor 4 (TLR4) on neutrophils fosters the formation of the autophagosome, inhibition of ROS production, and upregulation of MMP9 and VEGF in neutrophils, inducing polarization of neutrophils, promoting cancer cell migration (158).

CLINICAL ASPECTS

Prognostic Biomarkers

There have been a number of trials to predict cancer prognosis, including the TNM staging system established by The American Joint Committee on Cancer/Union Internationale Contre Ie Cancer (AJCC/UICC) (159). Through the TNM staging system, tumor prognostic information can be provided depending on tumor burden, the presence of cancer cells in lymph nodes (N) and event of distant metastases (M). Nonetheless, TNM provides limited capacity for accurate prediction (159). Cancer is a multidimensional disease, beyond difficulties in cure and prediction, which incurs many systemic alternations to be considered for effective treatment (159). One of the alternations emanates from the immune microenvironment. Reflecting the considerable impact of the immune system on tumor progression, the application of the immune parameter (Immunescore) has been introduced in disease classification to overcome the limitations of the traditional TNM staging system (159). As described above, presence of monocytes and neutrophils can be a double-edged sword, pro-tumoral or anti-tumoral, depending on the characteristics of tumors and applied therapies. In lung cancer, increased amount of monocytes within the tumor is associated with a poor survival rate, represented by progression-free survival (PFS) and overall survival (OS) of patients (36). In patients with colorectal cancer, profound influx of CCR2$^+$ classical monocytes from the bone marrow to the circulatory system is correlated with worse clinical outcomes, showing accelerated liver metastasis (46). Reversely, in patients with melanoma, high frequency of classical monocytes allows us to predict favorable treatment response to anti-PD1 therapy and increased survival rates (160). Presence of TEMs and M2-polarized macrophages infiltrated in PDAC is associated with a high possibility of tumor recurrence and poor survival rates

(161). In hepatitis B virus related hepatocellular carcinoma, high percentage of TEMs in peripheral blood monocytes represent poor overall survival and a shorter time to disease recurrence after resection (162). Changes in abundance between TEMs before and at 1 month after initial therapy also could serve as a biomarker in order to predict overall survival of hepatocellular carcinoma patients treated with sorafenib, a multi-kinase inhibitor of tumor angiogenesis (163). In breast cancer, endometrial cancer, prostate cancer, bladder cancer, ovary cancer, and urothelial cancer patients, high density of tumor-associated monocytes/macrophages (TAMs) has been reported to correlate with poor overall survival rates, while high density of TAMs in colorectal cancer patients shows longer overall survival (164). High density of TAMs is also associated with advanced tumor stages (III+IV) rather than with early stages (I+II) in breast cancer, oral cancer, and bladder cancer patients (164). However, there was no observed relation between TAMs and disease free survival rate in this clinical study (164).

Despite the controversial functions of neutrophils, neutrophil lymphocyte ratio (NLR) could be a potential biomarker for clinical use in some cases. After surgical removal of colorectal cancer (CRC), esophageal squamous cell carcinoma (ESCC), and PDAC, patients with lower values of NLR have a greater survival rate and reduced disease progression compared to patients with high NLR (165–167). When using everolimus for treatment of metastatic renal cell carcinoma (RCC), patients with low NLR also represent increased levels of both overall survival and PFS (168). Meanwhile, NLR inversely correlates with prostate-specific antigen (PSA) responsiveness to abiraterone acetate (abiraterone), a medication for metastatic castration-resistant prostate cancer patients (169, 170). In hepatocellular patients, tumor-infiltrated neutrophils represent upregulated PD-L1 expression (91). The ratio of PD-L1$^+$ neutrophils to PD-1$^+$ T cells helps better predict the disease-free survival of HCC patients (91). The NLR system is still under investigation across various cancer types, and it would be safe to be cautious to make an interpretation of disease prognosis with this system.

On one hand, counting TINs indicates controversial clinical outcomes. In RCC, presence of TINs has a negative impact on survival rates (171) and in melanoma patients, high amount of TINs mediated by activated pSTAT3 is linked to poor disease prognosis (172). Robust tumor infiltration of neutrophils also presents a negative disease progression of head and neck squamous cell carcinoma (HNSCC) (173). In the same manner, colorectal cancer patients with increased level of TINs are more likely to acquire a malignant phenotype of cancer and show adverse prognosis (174). Moreover, upon bevacizumab treatment (anti-VEGF therapy) for metastatic colorectal cancer patients, neutrophil infiltration engenders drastically low survival rates and represents a hostile clinical response against bevacizumab treatment (90). However, according to a couple of other clinical researches regarding influence of TINs on colorectal cancer prognosis, neutrophil infiltration to tumor tissue positively associates with favorable disease prognosis (175, 176) and with better responses to 5-FU-based chemotherapy (177). Interestingly, level of TINs may affect tumor prognosis differently depending on the sex of gastric cancer patients. Extensive amount of TINs reduces mortality risk of female patients while it does not affect male patients (178). Meanwhile, in non-small cell lung cancer (NSCLC) TINs do not represent any immediate impact on recurrence-free survival and overall survival (179).

Tests of functional single-nucleotide polymorphisms in genes regulating TAMs also enable us to predict clinical treatment outcomes (180). Through related trials, TBK1 rs7486100, CCL2 rs4586, CCL18 rs14304, and IRF3 rs2304205 have also been revealed to correlate with overall survival and progression free survival of metastatic colorectal cancer patients treated with bevacizumab (180).

Therapeutic Applications

The CCL2-CCR2 chemokine axis plays a major role in recruitment of TAMs, which renders the immunosuppressive tumor microenvironment immunosuppressive and thereby promotes tumor progression (46). Conversely, inhibition of this axis restores anti-tumor immunity (46). Combination therapy of CCR2 inhibitor PF-04136309 with FOLFIRINOX chemotherapy for PDAC restores the anti-tumor immune microenvironment, preventing CCR2+ monocytes from emerging from the bone marrow (181). Carlumab is a human immunoglobulin $G_{1\kappa}$ monoclonal antibody which specifically binds to human CCL2 with high affinity, leading to CCL2-CCR2 axis disruption (182–184). Clinical trials conducted for Carlumab in ovarian cancer, prostate cancer and other solid tumors with and without other chemotherapies such as docetaxel, gemcitabine, paclitaxel+carboplatin, or PLD has proven that Carlumab is well-tolerated but unfortunately fails to trigger significant tumor responses, since it could not sustain the long-term blockade of CCL2 (182–184). Since the CSF-1/CSF-1R axis is responsible for differentiation and survival of pro-tumoral TAMs, incessant efforts have been made to target CSF-1R to eliminate or repolarize TAMs (185). There are several CSF-1R inhibitors currently in clinical trials in many tumor types (186). Emactuzumab (RG7155) is a recombinant, humanized monoclonal antibody of IgG1 subclass, targeting CSF-1R expressed on macrophages (186). Clinical treatment of emactuzumab to patients with tenosynovial giant cell tumor shows durable tumor responses and functional improvement of patients with significant reduction of infiltrated macrophages in the tumor (186).

Another CSF-1R inhibitor is pexidartinib (PLX3397), a small-molecule inhibitor (187). As delineated above, preclinical studies in diverse solid tumors including mammary carcinoma, melanoma, lung carcinoma, pancreatic carcinoma, and glioma have proven that this molecule effectively blocks CSF-1R signaling, suppresses infiltration of macrophages into tumors, and accordingly restrains tumor progression (187–191). The dramatic tumor response to PLX3397 has provided a rationale to begin work on its clinical applications, currently ongoing in many solid tumors with and without combination with pembrolizumab, a monoclonal antibody targeting PD-1. According to a clinical case report, the progression of tenosynovial giant cell tumor was inhibited during non-surgical management with pexidartinib treatment (192).

In the context of tumor where neutrophils exert detrimental influence, the activation and homing of neutrophils need to be interrupted for better prognosis. Repertaxin is a small molecule inhibitor of CXCR1 and CXCR2 for blocking neutrophil trafficking (193). In patients with HER-2 negative metastatic breast cancer, treatment of repertaxin in combination with paclitaxel shows a durable tumor response with fine safety and tolerance. In this setting, an increased rate of neutropenia has not been observed, which needs to be evaluated further (194). Meanwhile, myeloid cell-derived IDO could be another attractive target for tumor regression since it shows suppressive activity on T cells (195). Preclinical research using MMTV-Neu breast tumor model has revealed that indoximod, a small molecule inhibitor of IDO, in combined use of paclitaxel, successfully induces tumor regression (195). As a clinical trial, targeting IDO with a peptide vaccine elicits long-lasting disease stabilization in lung cancer patients along with reduction of Treg frequency and increased cytotoxicity of $CD8^+$ T cells to kill cancer cells (196). Clinical application of indoximod is also ongoing in metastatic solid tumor patients (197). However, it may be asked whether IDO is an effective target, since phase III ECHO301 trial of epacadostat, another inhibitor of IDO, with pembrolizumab for melanoma as a combination therapy failed, missing the first primary endpoint of improving PFS vs. pembrolizumab alone (198).

CONCLUDING REMARKS

As thoroughly discussed in this review, tumor-associated monocytes and neutrophils are highly heterogeneous in a context dependent manner. Setting aside the need for the fine-tuning, we still have limited knowledge of their versatile functions in diverse tumor scenarios: cancer types, stages of disease, and applied therapies. In order to decipher these multifaceted roles of monocytes and neutrophils, there are several demands to be considered. First, we strongly suggest establishing orthotopic tumor models for preclinical studies. Ectopic tumor implantation has been conducted in many pieces of researches without consideration of organ settings. However, since the organ specific microenvironment, including different immune landscape, differently regulate tumor growth and progression, neglecting it undermines the validation of ectopic tumor models. Second, we also urge that researches be further progressed with development of applicable technologies such as single-cell RNA sequencing, intravital imaging, and mass cytometry. Application of advanced technological methods not only help in systemically understanding the heterogeneous and dynamic tumor microenvironment, but will also let us forecast disease prognosis and make therapeutic decisions with minimal side effects. Lastly, more clinical studies are required to validate prognostic markers and therapeutic agents.

With the fulfillment of these methodological and practical suggestions, we will be able to heighten our understanding of heterogeneous functions of monocytes and neutrophils in various tumor contexts, and further establish effective tumor therapies based on the comprehensive understanding.

AUTHOR CONTRIBUTIONS

KJ conceived the concept. JJ, YS, and KJ wrote the manuscript.

FUNDING

This work was supported by Research Resettlement Fund for the new faculty of Seoul National University (KJ) and by Creative-Pioneering Researchers Program through Seoul National University (SNU) (KJ) and also by SNUH Research Fund 03-2018-0290 (KJ).

ACKNOWLEDGMENTS

We thank all members of the Jung Lab for helpful comments on the manuscript.

REFERENCES

1. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol.* (2009) 27:669–92. doi: 10.1146/annurev.immunol.021908.132557
2. Dai MX, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood.* (2002) 99:111–20. doi: 10.1182/blood.V99.1.111
3. Guilliams M, Mildner A, Yona S. Developmental and functional heterogeneity of monocytes. *Immunity.* (2018) 49:595–613. doi: 10.1016/j.immuni.2018.10.005
4. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
5. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity.* (2010) 33:375–86. doi: 10.1016/j.immuni.2010.08.012
6. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity.* (2003) 19:71–82. doi: 10.1016/S1074-7613(03)00174-2
7. Jung K, Heishi T, Khan OF, Kowalski PS, Incio J, Rahbari NN, et al. Ly6Clo monocytes drive immunosuppression and confer resistance to anti-VEGFR2 cancer therapy. *J Clin Invest.* (2017) 127:3039–51. doi: 10.1172/JCI93182
8. Jung K, Heishi T, Incio J, Huang Y, Beech EY, Pinter M, et al. Targeting CXCR4-dependent immunosuppressive Ly6C(low) monocytes improves antiangiogenic therapy in colorectal cancer. *Proc Natl Acad Sci USA.* (2017) 114:10455–60. doi: 10.1073/pnas.1710754114
9. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science.* (2007) 317:666–70. doi: 10.1126/science.1142883
10. Murray PJ. Immune regulation by monocytes. *Semin Immunol.* (2018) 35:12–8. doi: 10.1016/j.smim.2017.12.005
11. Tamura A, Hirai H, Yokota A, Kamio N, Sato A, Shoji T, et al. C/EBPβ is required for survival of Ly6C(-) monocytes. *Blood.* (2017) 130:1809–18. doi: 10.1182/blood-2017-03-772962

12. Thomas G, Tacke R, Hedrick CC, Hanna RN. Nonclassical patrolling monocyte function in the vasculature. *Arterioscler Thromb Vasc Biol.* (2015) 35:1306–16. doi: 10.1161/ATVBAHA.114.304650
13. Alder JK, Georgantas RW, Hildreth RL, Kaplan IM, Morisot S, Yu X, et al. Kruppel-like factor 4 is essential for inflammatory monocyte differentiation *in vivo*. *J Immunol.* (2008) 180:5645–52. doi: 10.4049/jimmunol.180.8.5645
14. Kurotaki D, Osato N, Nishiyama A, Yamamoto M, Ban T, Sato H, et al. Essential role of the IRF8-KLF4 transcription factor cascade in murine monocyte differentiation. *Blood.* (2013) 121:1839–49. doi: 10.1182/blood-2012-06-437863
15. Mildner A, Schonheit J, Giladi A, David E, Lara-Astiaso D, Lorenzo-Vivas E, et al. Genomic characterization of murine monocytes reveals C/EBPβ transcription factor dependence of Ly6C(-) cells. *Immunity.* (2017) 46:849–62.e7. doi: 10.1016/j.immuni.2017.04.018
16. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA, et al. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat Immunol.* (2011) 12:778–85. doi: 10.1038/ni.2063
17. Olingy CE, Dinh HQ, Hedrick CC. Monocyte heterogeneity and functions in cancer. *J Leukoc Biol.* (2019) 1–14. doi: 10.1002/JLB.4RI0818-311R
18. Selimoglu-Buet D, Riviere J, Ghamlouch H, Bencheikh L, Lacout C, Morabito M, et al. A miR-150/TET3 pathway regulates the generation of mouse and human non-classical monocyte subset. *Nat Commun.* (2018) 9:5455. doi: 10.1038/s41467-018-07801-x
19. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science.* (2017) 356:eaah4573. doi: 10.1126/science.aah4573
20. Hamers AJ, Dinh HQ, Thomas GD, Marcovecchio P, Blatchley A, Nakao CS, et al. Human monocyte heterogeneity as revealed by high-dimensional mass cytometry. *Arterioscler Thromb Vasc Biol.* (2019) 39:25–36. doi: 10.1161/ATVBAHA.118.311022
21. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity.* (2013) 39:599–610. doi: 10.1016/j.immuni.2013.08.007
22. Kim KW, Williams JW, Wang YT, Ivanov S, Gilfillan S, Colonna M, et al. MHC II+ resident peritoneal and pleural macrophages rely on IRF4 for development from circulating monocytes. *J Exp Med.* (2016) 213:1951–9. doi: 10.1084/jem.20160486
23. Lavin Y, Mortha A, Rahman A, Merad M. Regulation of macrophage development and function in peripheral tissues. *Nat Rev Immunol.* (2015) 15:731. doi: 10.1038/nri3920
24. Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. *Immunity.* (2016) 44:439–49. doi: 10.1016/j.immuni.2016.02.024
25. Aras S, Zaidi MR. TAMeless traitors: macrophages in cancer progression and metastasis. *Br J Cancer.* (2017) 117:1583–91. doi: 10.1038/bjc.2017.356
26. Tran Janco JM, Lamichhane P, Karyampudi L, Knutson KL. Tumor-infiltrating dendritic cells in cancer pathogenesis. *J Immunol.* (2015) 194:2985–91. doi: 10.4049/jimmunol.1403134
27. Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, et al. Origins of tumor-associated macrophages and neutrophils. *Proc Natl Acad Sci USA.* (2012) 109:2491–6. doi: 10.1073/pnas.1113744109
28. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science.* (2009) 325:612–6. doi: 10.1126/science.1175202
29. Cortez-Retamozo V, Etzrodt M, Newton A, Ryan R, Pucci F, Sio SW, et al. Angiotensin II drives the production of tumor-promoting macrophages. *Immunity.* (2013) 38:296–308. doi: 10.1016/j.immuni.2012.10.015
30. Shand FH, Ueha S, Otsuji M, Koid SS, Shichino S, Tsukui T, et al. Tracking of intertissue migration reveals the origins of tumor-infiltrating monocytes. *Proc Natl Acad Sci USA.* (2014) 111:7771–6. doi: 10.1073/pnas.1402914111
31. Salmon H, Remark R, Gnjatic S, Merad M. Host tissue determinants of tumour immunity. *Nat Rev Cancer.* (2019) 19:215–27. doi: 10.1038/s41568-019-0125-9
32. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature.* (2011) 475:222–5. doi: 10.1038/nature10138
33. Long KB, Gladney WL, Tooker GM, Graham K, Fraietta JA, Beatty GL. IFNγ and CCL2 cooperate to redirect tumor-infiltrating monocytes to degrade fibrosis and enhance chemotherapy efficacy in pancreatic carcinoma. *Cancer Discov.* (2016) 6:400–13. doi: 10.1158/2159-8290.CD-15-1032
34. Keklikoglou I, Cianciaruso C, Guc E, Squadrito ML, Spring LM, Tazzyman S, et al. Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models. *Nat Cell Biol.* (2019) 21:190–202. doi: 10.1038/s41556-018-0256-3
35. Hanna RN, Cekic C, Sag D, Tacke R, Thomas GD, Nowyhed H, et al. Patrolling monocytes control tumor metastasis to the lung. *Science.* (2015) 350:985–90. doi: 10.1126/science.aac9407
36. Lin GN, Peng JW, Xiao JJ, Liu DY, Xia ZJ. Prognostic impact of circulating monocytes and lymphocyte-to-monocyte ratio on previously untreated metastatic non-small cell lung cancer patients receiving platinum-based doublet. *Med Oncol.* (2014) 31:70. doi: 10.1007/s12032-014-0070-0
37. Feng F, Zheng G, Wang Q, Liu S, Liu Z, Xu G, et al. Low lymphocyte count and high monocyte count predicts poor prognosis of gastric cancer. *BMC Gastroenterol.* (2018) 18:148. doi: 10.1186/s12876-018-0877-9
38. Chittezhath M, Dhillon MK, Lim JY, Laoui D, Shalova IN, Teo YL, et al. Molecular profiling reveals a tumor-promoting phenotype of monocytes and macrophages in human cancer progression. *Immunity.* (2014) 41:815–29. doi: 10.1016/j.immuni.2014.09.014
39. Porrello A, Leslie PL, Harrison EB, Gorentla BK, Kattula S, Ghosh SK, et al. Factor XIIIA-expressing inflammatory monocytes promote lung squamous cancer through fibrin cross-linking. *Nat Commun.* (2018) 9:1988. doi: 10.1038/s41467-018-04355-w
40. Chen Y, Huang Y, Reiberger T, Duyverman AM, Huang P, Samuel R, et al. Differential effects of sorafenib on liver versus tumor fibrosis mediated by stromal-derived factor 1 alpha/C-X-C receptor type 4 axis and myeloid differentiation antigen-positive myeloid cell infiltration in mice. *Hepatology.* (2014) 59:1435–47. doi: 10.1002/hep.26790
41. Sidibe A, Ropraz P, Jemelin S, Emre Y, Poittevin M, Pocard M, et al. Angiogenic factor-driven inflammation promotes extravasation of human proangiogenic monocytes to tumours. *Nat Commun.* (2018) 9:355. doi: 10.1038/s41467-017-02610-0
42. Plebanek MP, Angeloni NL, Vinokour E, Li J, Henkin A, Martinez-Marin D, et al. Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche. *Nat Commun.* (2017) 8:1319. doi: 10.1038/s41467-017-01433-3
43. Lavin Y, Kobayashi S, Leader A, Amir ED, Elefant N, Bigenwald C, et al. Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses. *Cell.* (2017) 169:750–65.e17. doi: 10.1016/j.cell.2017.04.014
44. Kubo H, Mensurado S, Gonçalves-Sousa N, Serre K, Silva-Santos B. Primary tumors limit metastasis formation through induction of IL15-mediated cross-talk between patrolling monocytes and NK cells. *Cancer Immunol Res.* (2017) 5:812–20. doi: 10.1158/2326-6066.CIR-17-0082
45. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, et al. Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res.* (2013) 19:3404–15. doi: 10.1158/1078-0432.CCR-13-0525
46. Grossman JG, Nywening TM, Belt BA, Panni RZ, Krasnick BA, DeNardo DG, et al. Recruitment of CCR2(+) tumor associated macrophage to sites of liver metastasis confers a poor prognosis in human colorectal cancer. *Oncoimmunology.* (2018) 7:e1470729. doi: 10.1080/2162402X.2018.1470729
47. Harney AS, Arwert EN, Entenberg D, Wang Y, Guo P, Qian BZ, et al. Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage-derived VEGFA. *Cancer Discov.* (2015) 5:932–43. doi: 10.1158/2159-8290.CD-15-0012
48. Nakasone ES, Askautrud HA, Kees T, Park JH, Plaks V, Ewald AJ, et al. Imaging tumor-stroma interactions during chemotherapy reveals contributions of the microenvironment to resistance. *Cancer Cell.* (2012) 21:488–503. doi: 10.1016/j.ccr.2012.02.017
49. Kalbasi A, Komar C, Tooker GM, Liu M, Lee JW, Gladney WL, et al. Tumor-derived CCL2 mediates resistance to radiotherapy in

pancreatic ductal adenocarcinoma. *Clin Cancer Res.* (2017) 23:137–48. doi: 10.1158/1078-0432.CCR-16-0870
50. Bonapace L, Coissieux MM, Wyckoff J, Mertz KD, Varga Z, Junt T, et al. Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. *Nature.* (2014) 515:130–3. doi: 10.1038/nature13862
51. Nagathihalli NS, Castellanos JA, Shi C, Beesetty Y, Reyzer ML, Caprioli R, et al. Signal transducer and activator of transcription 3, mediated remodeling of the tumor microenvironment results in enhanced tumor drug delivery in a mouse model of pancreatic cancer. *Gastroenterology.* (2015) 149:1932–43.e9. doi: 10.1053/j.gastro.2015.07.058
52. Long KB, Tooker G, Tooker E, Luque SL, Lee JW, Pan X, et al. IL6 receptor blockade enhances chemotherapy efficacy in pancreatic ductal adenocarcinoma. *Mol Cancer Ther.* (2017) 16:1898–908. doi: 10.1158/1535-7163.MCT-16-0899
53. Corcoran RB, Contino G, Deshpande V, Tzatsos A, Conrad C, Benes CH, et al. Plays a critical role in -induced pancreatic tumorigenesis. *Cancer Res.* (2011) 71:5020–9. doi: 10.1158/0008-5472.CAN-11-0908
54. Incio J, Ligibel JA, McManus DT, Suboj P, Jung K, Kawaguchi K, et al. Obesity promotes resistance to anti-VEGF therapy in breast cancer by up-regulating IL-6 and potentially FGF-2. *Sci Transl Med.* (2018) 10:eaag0945. doi: 10.1126/scitranslmed.aag0945
55. Jiang H, Hegde S, DeNardo DG. Tumor-associated fibrosis as a regulator of tumor immunity and response to immunotherapy. *Cancer Immunol Immunother.* (2017) 66:1037–48. doi: 10.1007/s00262-017-2003-1
56. Cox TR, Erler JT. Molecular pathways: connecting fibrosis and solid tumor metastasis. *Clin Cancer Res.* (2014) 20:3637–43. doi: 10.1158/1078-0432.CCR-13-1059
57. Rahbari NN, Kedrin D, Incio J, Liu H, Ho WW, Nia HT, et al. Anti-VEGF therapy induces ECM remodeling and mechanical barriers to therapy in colorectal cancer liver metastases. *Sci Transl Med.* (2016) 8:360ra135. doi: 10.1126/scitranslmed.aaf5219
58. Incio J, Liu H, Suboj P, Chin SM, Chen IX, Pinter M, et al. Obesity-induced inflammation and desmoplasia promote pancreatic cancer progression and resistance to chemotherapy. *Cancer Discov.* (2016) 6:852–69. doi: 10.1158/2159-8290.CD-15-1177
59. Satoh T, Nakagawa K, Sugihara F, Kuwahara R, Ashihara M, Yamane F, et al. Identification of an atypical monocyte and committed progenitor involved in fibrosis. *Nature.* (2017) 541:96–101. doi: 10.1038/nature20611
60. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell.* (2013) 153:362–75. doi: 10.1016/j.cell.2013.03.010
61. Chen IX, Chauhan VP, Posada J, Ng MR, Wu MW, Adstamongkonkul P, et al. Blocking CXCR4 alleviates desmoplasia, increases T-lymphocyte infiltration, and improves immunotherapy in metastatic breast cancer. *Proc Natl Acad Sci USA.* (2019) 116:4558–4566. doi: 10.1158/1538-7445.SABCS18-2744
62. Romano E, Kusio-Kobialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells *ex vivo* by nonclassical monocytes in melanoma patients. *Proc Natl Acad Sci USA.* (2015) 112:6140–5. doi: 10.1073/pnas.1417320112
63. De Palma M, Murdoch C, Venneri MA, Naldini L, Lewis CE. Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends Immunol.* (2007) 28:519–24. doi: 10.1016/j.it.2007.09.004
64. De Palma M, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med.* (2003) 9:789. doi: 10.1038/nm871
65. De Palma M, Venneri MA, Galli R, Sergi LS, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell.* (2005) 8:211–26. doi: 10.1016/j.ccr.2005.08.002
66. Venneri MA, Palma MD, Ponzoni M, Pucci F, Scielzo C, Zonari E, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood.* (2007) 109:5276–85. doi: 10.1182/blood-2006-10-053504
67. Murdoch C, Tazzyman S, Webster S, Lewis CE. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J Immunol.* (2007) 178:7405–11. doi: 10.4049/jimmunol.178.11.7405
68. Coffelt SB, Tal AO, Scholz A, De Palma M, Patel S, Urbich C, et al. Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. *Cancer Res.* (2010) 70:5270–80. doi: 10.1158/0008-5472.CAN-10-0012
69. Lee J, Song J, Kwon ES, Jo S, Kang MK, Kim YJ, et al. CTHRC1 promotes angiogenesis by recruiting Tie2-expressing monocytes to pancreatic tumors. *Exp Molecul Med.* (2016) 48:e261. doi: 10.1038/emm.2016.87
70. Lewis CE, De Palma M, Naldini L. Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin-2. *Cancer Res.* (2007) 67:8429–32. doi: 10.1158/0008-5472.CAN-07-1684
71. Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell.* (2011) 19:512–26. doi: 10.1016/j.ccr.2011.02.005
72. Nicolas-Avila JA, Adrover JM, Hidalgo A. Neutrophils in homeostasis, immunity, and cancer. *Immunity.* (2017) 46:15–28. doi: 10.1016/j.immuni.2016.12.012
73. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer.* (2016) 16:431–46. doi: 10.1038/nrc.2016.52
74. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol.* (2013) 13:159–75. doi: 10.1038/nri3399
75. Fiedler K, Brunner C. Mechanisms controlling hematopoiesis. In: Lawrie CH, editor. *Hematology-Science and Practice*. London: IntechOpen (2012). doi: 10.5772/33749
76. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J, et al. Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking, and effector functions. *Immunity.* (2018) 48:364–379.e8. doi: 10.1016/j.immuni.2018.02.002
77. Phan VT, Wu X, Cheng JH, Sheng RX, Chung AS, Zhuang G, et al. Oncogenic RAS pathway activation promotes resistance to anti-VEGF therapy through G-CSF-induced neutrophil recruitment. *Proc Natl Acad Sci USA.* (2013) 110:6079–84. doi: 10.1073/pnas.1303302110
78. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest.* (2010) 120:1151–64. doi: 10.1172/JCI37223
79. Wu CF, Andzinski L, Kasnitz N, Kroger A, Klawonn F, Lienenklaus S, et al. The lack of type I interferon induces neutrophil-mediated pre-metastatic niche formation in the mouse lung. *Int J Cancer.* (2015) 137:837–47. doi: 10.1002/ijc.29444
80. Gong F, Peng X, Luo C, Shen G, Zhao C, Zou L, et al. Cathepsin B as a potential prognostic and therapeutic marker for human lung squamous cell carcinoma. *Molecul Cancer.* (2013) 12:125. doi: 10.1186/1476-4598-12-125
81. Mangerich A, Knutson CG, Parry NM, Muthupalani S, Ye W, Prestwich E, et al. Infection-induced colitis in mice causes dynamic and tissue-specific changes in stress response and DNA damage leading to colon cancer. *Proc Natl Acad Sci USA.* (2012) 109:E1820–9. doi: 10.1073/pnas.1207829109
82. Tohme S, Yazdani HO, Al-Khafaji AB, Chidi AP, Loughran P, Mowen K, et al. Neutrophil extracellular traps promote the development and progression of liver metastases after surgical stress. *Cancer Res.* (2016) 76:1367–80. doi: 10.1158/0008-5472.CAN-15-1591
83. Houghton AM, Rzymkiewicz DM, Ji H, Gregory AD, Egea EE, Metz HE, et al. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nat Med.* (2010) 16:219–23. doi: 10.1038/nm.2084
84. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature.* (2019) 566:553–57. doi: 10.1038/s41586-019-0915-y
85. Liang W, Li Q, Ferrara N. Metastatic growth instructed by neutrophil-derived transferrin. *Proc Natl Acad Sci USA.* (2018) 115:11060–5. doi: 10.1073/pnas.1811717115
86. Hirai H, Fujishita T, Kurimoto K, Miyachi H, Kitano S, Inamoto S, et al. CCR1-mediated accumulation of myeloid cells in the liver microenvironment promoting mouse colon cancer metastasis. *Clin Exp Metastasis.* (2014) 31:977–89. doi: 10.1007/s10585-014-9684-z
87. Kitamura T, Fujishita T, Loetscher P, Revesz L, Hashida H, Kizaka-Kondoh S, et al. Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model. *Proc Natl Acad Sci USA.* (2010) 107:13063–8. doi: 10.1073/pnas.1002372107

88. Chen Y, Ramjiawan RR, Reiberger T, Ng MR, Hato T, Huang Y, et al. CXCR4 inhibition in tumor microenvironment facilitates anti-programmed death receptor-1 immunotherapy in sorafenib-treated hepatocellular carcinoma in mice. *Hepatology.* (2015) 61:1591–602. doi: 10.1002/hep.27665
89. Hiratsuka S, Duda DG, Huang Y, Goel S, Sugiyama T, Nagasawa T, et al. C-X-C receptor type 4 promotes metastasis by activating p38 mitogen-activated protein kinase in myeloid differentiation antigen (Gr-1)-positive cells. *Proc Natl Acad Sci USA.* (2011) 108:302–7. doi: 10.1073/pnas.1016917108
90. Schiffmann LM, Fritsch M, Gebauer F, Gunther SD, Stair NR, Seeger JM, et al. Tumour-infiltrating neutrophils counteract anti-VEGF therapy in metastatic colorectal cancer. *Br J Cancer.* (2019) 120:69–78. doi: 10.1038/s41416-018-0198-3
91. He G, Zhang H, Zhou J, Wang B, Chen Y, Kong Y, et al. Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res.* (2015) 34:141. doi: 10.1186/s13046-015-0256-0
92. Koyama S, Akbay EA, Li YY, Aref AR, Skoulidis F, Herter-Sprie GS, et al. STK11/LKB1 deficiency promotes neutrophil recruitment and proinflammatory cytokine production to suppress T-cell activity in the lung tumor microenvironment. *Cancer Res.* (2016) 76:999–1008. doi: 10.1158/0008-5472.CAN-15-1439
93. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature.* (2015) 522:345–8. doi: 10.1038/nature14282
94. Michaeli J, Shaul ME, Mishalian I, Hovav AH, Levy L, Zolotriov L, et al. Tumor-associated neutrophils induce apoptosis of non-activated CD8 T-cells in a TNFα and NO-dependent mechanism, promoting a tumor-supportive environment. *Oncoimmunology.* (2017) 6:e1356965. doi: 10.1080/2162402X.2017.1356965
95. Rotondo R, Barisione G, Mastracci L, Grossi F, Orengo AM, Costa R, et al. IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. *Int J Cancer.* (2009) 125:887–93. doi: 10.1002/ijc.24448
96. Mishalian I, Bayuh R, Eruslanov E, Michaeli J, Levy L, Zolotarov L, et al. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17–a new mechanism of impaired antitumor immunity. *Int J Cancer.* (2014) 135:1178–86. doi: 10.1002/ijc.28770
97. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc Natl Acad Sci USA.* (2008) 105:2640–5. doi: 10.1073/pnas.0712185105
98. Qu X, Zhuang G, Yu L, Meng G, Ferrara N. Induction of Bv8 expression by granulocyte colony-stimulating factor in CD11b+Gr1+ cells: key role of Stat3 signaling. *J Biol Chem.* (2012) 287:19574–84. doi: 10.1074/jbc.M111.326801
99. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci USA.* (2006) 103:12493–8. doi: 10.1073/pnas.0601807103
100. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol.* (2000) 2:737–44. doi: 10.1038/35036374
101. Deryugina EI, Zajac E, Juncker-Jensen A, Kupriyanova TA, Welter L, Quigley JP. Tissue-infiltrating neutrophils constitute the major in vivo source of angiogenesis-inducing MMP-9 in the tumor microenvironment. *Neoplasia.* (2014) 16:771–88. doi: 10.1016/j.neo.2014.08.013
102. Bekes EM, Schweighofer B, Kupriyanova TA, Zajac E, Ardi VC, Quigley JP, et al. Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. *Am J Pathol.* (2011) 179:1455–70. doi: 10.1016/j.ajpath.2011.05.031
103. Jablonska E, Piotrowski L, Jablonski J, Grabowska Z. VEGF in the culture of PMN and the serum in oral cavity cancer patients. *Oral Oncology.* (2002) 38:605–9. doi: 10.1016/S1368-8375(01)00110-5
104. Gordon-Weeks AN, Lim SY, Yuzhalin AE, Jones K, Markelc B, Kim KJ, et al. Neutrophils promote hepatic metastasis growth through fibroblast growth factor 2-dependent angiogenesis in mice. *Hepatology.* (2017) 65:1920–35. doi: 10.1002/hep.29088
105. Spiegel A, Brooks MW, Houshyar S, Reinhardt F, Ardolino M, Fessler E, et al. Neutrophils suppress intraluminal NK cell-mediated tumor cell clearance and enhance extravasation of disseminated carcinoma cells. *Cancer Discov.* (2016) 6:630–49. doi: 10.1158/2159-8290.CD-15-1157
106. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature.* (2015) 528:413–7. doi: 10.1038/nature16140
107. Huh SJ, Liang S, Sharma A, Dong C, Robertson GP. Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. *Cancer Res.* (2010) 70:6071–82. doi: 10.1158/0008-5472.CAN-09-4442
108. Li S, Cong X, Gao H, Lan X, Li Z, Wang W, et al. Tumor-associated neutrophils induce EMT by IL-17a to promote migration and invasion in gastric cancer cells. *J Exp Clin Cancer Res.* (2019) 38:177. doi: 10.1186/s13046-019-1168-1
109. Spicer JD, McDonald B, Cools-Lartigue JJ, Chow SC, Giannias B, Kubes P, et al. Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells. *Cancer Res.* (2012) 72:3919–27. doi: 10.1158/0008-5472.CAN-11-2393
110. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J Clin Invest.* (2013) 123:3446–3458. doi: 10.1158/1538-7445.AM2012-2972
111. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science.* (2018) 361:eaao4227. doi: 10.1126/science.aao4227
112. Mishalian I, Bayuh R, Levy L, Zolotarov L, Michaeli J, Fridlender ZG. Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression. *Cancer Immunol Immunother.* (2013) 62:1745–56. doi: 10.1007/s00262-013-1476-9
113. Martin A, Seignez C, Racoeur C, Isambert N, Mabrouk N, Scagliarini A, et al. Tumor-derived granzyme B-expressing neutrophils acquire antitumor potential after lipid A treatment. *Oncotarget.* (2018) 9:28364. doi: 10.18632/oncotarget.25342
114. Gershkovitz M, Caspi Y, Fainsod-Levi T, Katz B, Michaeli J, Khawaled S, et al. TRPM2 mediates neutrophil killing of disseminated tumor cells. *Cancer Res.* (2018) 78:2680–90. doi: 10.1158/0008-5472.CAN-17-3614
115. Granot Z, Henke E, Comen EA, King TA, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell.* (2011) 20:300–14. doi: 10.1016/j.ccr.2011.08.012
116. Mensurado S, Rei M, Lanca T, Ioannou M, Goncalves-Sousa N, Kubo H, et al. Tumor-associated neutrophils suppress pro-tumoral IL-17+ γδ T cells through induction of oxidative stress. *PLoS Biol.* (2018) 16:e2004990. doi: 10.1371/journal.pbio.2004990
117. Catena R, Bhattacharya N, El Rayes T, Wang S, Choi H, Gao D, et al. Bone marrow-derived Gr1+ cells can generate a metastasis-resistant microenvironment via induced secretion of thrombospondin-1. *Cancer Discov.* (2013) 3:578–89. doi: 10.1158/2159-8290.CD-12-0476
118. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *J Clin Invest.* (2014) 124:5466–80. doi: 10.1172/JCI77053
119. Steele CW, Karim SA, Leach JDG, Bailey P, Upstill-Goddard R, Rishi L, et al. CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma. *Cancer Cell.* (2016) 29:832–45. doi: 10.1016/j.ccell.2016.04.014
120. Jamieson T, Clarke M, Steele CW, Samuel MS, Neumann J, Jung A, et al. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. *J Clin Invest.* (2012) 122:3127–44. doi: 10.1172/JCI61067
121. Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry ML, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood.* (2011) 117:1196–204. doi: 10.1182/blood-2009-11-254490
122. Benevides L, da Fonseca DM, Donate PB, Tiezzi DG, De Carvalho DD, de Andrade JM, et al. IL17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment. *Cancer Res.* (2015) 75:3788–99. doi: 10.1158/0008-5472.CAN-15-0054

123. Akbay EA, Koyama S, Liu Y, Dries R, Bufe LE, Silkes M, et al. Interleukin-17A promotes lung tumor progression through neutrophil attraction to tumor sites and mediating resistance to PD-1 blockade. *J Thorac Oncol.* (2017) 12:1268–79. doi: 10.1016/j.jtho.2017.04.017
124. Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, Schultheis A, et al. The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J Clin Invest.* (2009) 119:3011–23. doi: 10.1172/JCI39065
125. Powell D, Lou M, Barros Becker F, Huttenlocher A. Cxcr1 mediates recruitment of neutrophils and supports proliferation of tumor-initiating astrocytes *in vivo*. *Sci Rep.* (2018) 8:13285. doi: 10.1038/s41598-018-31675-0
126. Czepielewski RS, Porto BN, Rizzo LB, Roesler R, Abujamra AL, Pinto LG, et al. Gastrin-releasing peptide receptor (GRPR) mediates chemotaxis in neutrophils. *Proc Natl Acad Sci USA*. (2012) 109:547–52. doi: 10.1073/pnas.1110996109
127. Cornelio D, Roesler R, Schwartsmann G. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. *Ann Oncol.* (2007) 18:1457–66. doi: 10.1093/annonc/mdm058
128. Soler-Cardona A, Forsthuber A, Lipp K, Ebersberger S, Heinz M, Schossleitner K, et al. CXCL5 facilitates melanoma cell-neutrophil interaction and lymph node metastasis. *J Invest Dermatol.* (2018) 138:1627–35. doi: 10.1016/j.jid.2018.01.035
129. Chen Y, Liu YC, Sung YC, Ramjiawan RR, Lin TT, Chang CC, et al. Overcoming sorafenib evasion in hepatocellular carcinoma using CXCR4-targeted nanoparticles to co-deliver MEK-inhibitors. *Sci Rep.* (2017) 7:44123. doi: 10.1038/srep44123
130. Sung YC, Liu YC, Chao PH, Chang CC, Jin PR, Lin TT, et al. Combined delivery of sorafenib and a MEK inhibitor using CXCR4-targeted nanoparticles reduces hepatic fibrosis and prevents tumor development. *Theranostics.* (2018) 8:894–905. doi: 10.7150/thno.21168
131. Yang J, Kumar A, Vilgelm AE, Chen SC, Ayers GD, Novitskiy SV, et al. Loss of CXCR4 in Myeloid cells enhances antitumor immunity and reduces melanoma growth through NK cell and FASL mechanisms. *Cancer Immunol Res.* (2018) 6:1186–98. doi: 10.1158/2326-6066.CIR-18-0045
132. Rice CM, Davies LC, Subleski JJ, Maio N, Gonzalez-Cotto M, Andrews C, et al. Tumour-elicited neutrophils engage mitochondrial metabolism to circumvent nutrient limitations and maintain immune suppression. *Nat Commun.* (2018) 9:5099. doi: 10.1038/s41467-018-07505-2
133. Zhu YP, Padgett L, Dinh HQ, Marcovecchio P, Blatchley A, Wu R, et al. Identification of an early unipotent neutrophil progenitor with pro-tumoral activity in mouse and human bone marrow. *Cell Rep.* (2018) 24:2329–41.e8. doi: 10.1016/j.celrep.2018.07.097
134. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun.* (2016) 7:12150. doi: 10.1038/ncomms12150
135. Mazzone M, Bergers G. Regulation of blood and lymphatic vessels by immune cells in tumors and metastasis. *Annu Rev Physiol.* (2019) 81:535–60. doi: 10.1146/annurev-physiol-020518-114721
136. Faget J, Groeneveld S, Boivin G, Sankar M, Zangger N, Garcia M, et al. Neutrophils and snail orchestrate the establishment of a pro-tumor microenvironment in lung cancer. *Cell Rep.* (2017) 21:3190–204. doi: 10.1016/j.celrep.2017.11.052
137. Hsu DS, Wang HJ, Tai SK, Chou CH, Hsieh CH, Chiu PH, et al. Acetylation of snail modulates the cytokinome of cancer cells to enhance the recruitment of macrophages. *Cancer Cell.* (2014) 26:534–48. doi: 10.1016/j.ccell.2014.09.002
138. Kudo-Saito C, Shirako H, Takeuchi T, Kawakami Y. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell.* (2009) 15:195–206. doi: 10.1016/j.ccr.2009.01.023
139. Tazawa H, Okada F, Kobayashi T, Tada M, Mori Y, Une Y, et al. Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression. *Am J Pathol.* (2003) 163:2221–32. doi: 10.1016/S0002-9440(10)63580-8
140. Fan Z, McArdle S, Marki A, Mikulski Z, Gutierrez E, Engelhardt B, et al. Neutrophil recruitment limited by high-affinity bent β2 integrin binding ligand in cis. *Nat Commun.* (2016) 7:12658. doi: 10.1038/ncomms12658
141. Ley K, Hoffman HM, Kubes P, Cassatella MA, Zychlinsky A, Hedrick CC, et al. Neutrophils: new insights and open questions. *Sci Immunol.* (2018) 3:eaat4579. doi: 10.1126/sciimmunol.aat4579
142. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
143. Chen MB, Hajal C, Benjamin DC, Yu C, Azizgolshani H, Hynes RO, Kamm RD. Inflamed neutrophils sequestered at entrapped tumor cells via chemotactic confinement promote tumor cell extravasation. *Proc Natl Acad Sci USA.* (2018) 115:7022–7. doi: 10.1073/pnas.1715932115
144. Vazquez Rodriguez G, Abrahamsson A, Jensen LD, Dabrosin C. Estradiol promotes breast cancer cell migration via recruitment and activation of neutrophils. *Cancer Immunol Res.* (2017) 5:234–47. doi: 10.1158/2326-6066.CIR-16-0150
145. Casanova-Acebes M, Nicolas-Avila JA, Li JL, Garcia-Silva S, Balachander A, Rubio-Ponce A, et al. Neutrophils instruct homeostatic and pathological states in naive tissues. *J Exp Med.* (2018) 215:2778–95. doi: 10.1084/jem.20181468
146. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
147. Shaul ME, Fridlender ZG. Cancer-related circulating and tumor-associated neutrophils – subtypes, sources and function. *FEBS J.* (2018) 285:4316–42. doi: 10.1111/febs.14524
148. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med.* (2007) 13:463–9. doi: 10.1038/nm1565
149. El Rayes T, Catena R, Lee S, Stawowczyk M, Joshi N, Fischbach C, et al. Lung inflammation promotes metastasis through neutrophil protease-mediated degradation of Tsp-1. *Proc Natl Acad Sci USA.* (2015) 112:16000–5. doi: 10.1073/pnas.1507294112
150. Park J, Wysocki RW, Amoozgar Z, Maiorino L, Fein MR, Jorns J, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci Transl Med.* (2016) 8:361ra138. doi: 10.1126/scitranslmed.aag1711
151. Lee W, Ko SY, Mohamed MS, Kenny HA, Lengyel E, Naora H. Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. *J Exp Med.* (2019) 216:176–94. doi: 10.1084/jem.20181170
152. Engblom C, Pfirschke C, Zilionis R, Da Silva Martins J, Bos SA, Courties G, et al. Osteoblasts remotely supply lung tumors with cancer-promoting SiglecF(high) neutrophils. *Science.* (2017) 358:eaal5081. doi: 10.1126/science.aal5081
153. Colombo MP, Lombardi L, Stoppacciaro A, Melani C, Parenza M, Bottazzi B, et al. Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition *in vivo*. Neutrophils discriminate between G-CSF-producing and G-CSF-nonproducing tumor cells. *J Immunol.* (1992) 149:113–9.
154. Finisguerra V, Di Conza G, Di Matteo M, Serneels J, Costa S, Thompson AR, et al. MET is required for the recruitment of anti-tumoural neutrophils. *Nature.* (2015) 522:349–53. doi: 10.1038/nature14407
155. Kousis PC, Henderson BW, Maier PG, Gollnick SO. Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. *Cancer Res.* (2007) 67:10501–10. doi: 10.1158/0008-5472.CAN-07-1778
156. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell.* (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
157. Andzinski L, Kasnitz N, Stahnke S, Wu CF, Gereke M, von Kockritz-Blickwede M, et al. Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. *Int J Cancer.* (2016) 138:1982–93. doi: 10.1002/ijc.29945
158. Zhang X, Shi H, Yuan X, Jiang P, Qian H, Xu W. Tumor-derived exosomes induce N2 polarization of neutrophils to promote gastric cancer cell migration. *Mol Cancer.* (2018) 17:146. doi: 10.1186/s12943-018-0898-6
159. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol.* (2014) 232:199–209. doi: 10.1002/path.4287
160. Krieg C, Nowicka M, Guglietta S, Schindler S, Hartmann FJ, Weber LM, et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat Med.* (2018) 24:144–53. doi: 10.1038/nm.4466

161. Atanasov G, Pötner C, Aust G, Schierle K, Dietel C, Benzing C, et al. TIE2-expressing monocytes and M2-polarized macrophages impact survival and correlate with angiogenesis in adenocarcinoma of the pancreas. *Oncotarget.* (2018) 9:29715. doi: 10.18632/oncotarget.25690
162. He YF, Wang CQ, Yu Y, Qian J, Song K, Sun QM, et al. Tie2-expressing monocytes are associated with identification and prognoses of hepatitis B virus related hepatocellular carcinoma after resection. *PLoS ONE.* (2015) 10:e0143657. doi: 10.1371/journal.pone.0143657
163. Shoji H, Yoshio S, Mano Y, Doi H, Sugiyama M, Osawa Y, et al. Pro-angiogenic TIE-2-expressing monocytes/TEMs as a biomarker of the effect of sorafenib in patients with advanced hepatocellular carcinoma. *Int J Cancer.* (2017) 141:1011–7. doi: 10.1002/ijc.30804
164. Zhang QW, Liu L, Gong CY, Shi HS, Zeng YH, Wang XZ, et al. Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PLoS ONE.* (2012) 7:e50946. doi: 10.1371/journal.pone.0050946
165. Guthrie GJ, Roxburgh CS, Farhan-Alanie OM, Horgan PG, McMillan DC. Comparison of the prognostic value of longitudinal measurements of systemic inflammation in patients undergoing curative resection of colorectal cancer. *Br J Cancer.* (2013) 109:24–8. doi: 10.1038/bjc.2013.330
166. Wang J, Jia Y, Wang N, Zhang X, Tan B, Zhang G, et al. The clinical significance of tumor-infiltrating neutrophils and neutrophil-to-CD8+ lymphocyte ratio in patients with resectable esophageal squamous cell carcinoma. *J Transl Med.* (2014) 12:7. doi: 10.1186/1479-5876-12-7
167. Nywening TM, Belt BA, Cullinan DR, Panni RZ, Han BJ, Sanford DE, et al. Targeting both tumour-associated CXCR2(+) neutrophils and CCR2(+) macrophages disrupts myeloid recruitment and improves chemotherapeutic responses in pancreatic ductal adenocarcinoma. *Gut.* (2018) 67:1112–23. doi: 10.1136/gutjnl-2017-313738
168. Santoni M, De Giorgi U, Iacovelli R, Conti A, Burattini L, Rossi L, et al. Pre-treatment neutrophil-to-lymphocyte ratio may be associated with the outcome in patients treated with everolimus for metastatic renal cell carcinoma. *Br J Cancer.* (2013) 109:1755–9. doi: 10.1038/bjc.2013.522
169. Leibowitz-Amit R, Templeton A, Omlin A, Pezaro C, Atenafu E, Keizman D, et al. Clinical variables associated with PSA response to abiraterone acetate in patients with metastatic castration-resistant prostate cancer. *Ann Oncol.* (2014) 25:657–62. doi: 10.1093/annonc/mdt581
170. Lorente D, Mateo J, Templeton A, Zafeiriou Z, Bianchini D, Ferraldeschi R, et al. Baseline neutrophil–lymphocyte ratio (NLR) is associated with survival and response to treatment with second-line chemotherapy for advanced prostate cancer independent of baseline steroid use. *Ann Oncol.* (2014) 26:750–5. doi: 10.1093/annonc/mdu587
171. Jensen TO, Schmidt H, Moller HJ, Hoyer M, Maniecki MB, Sjoegren P, et al. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J Clin Oncol.* (2009) 27:3330–7. doi: 10.1200/JCO.2008.19.9919
172. Jensen TO, Schmidt H, Møller HJ, Donskov F, Høyer M, Sjoegren P, Christensen IJ, et al. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer.* (2012) 118:2476–85. doi: 10.1002/cncr.26511
173. Trellakis S, Farjah H, Bruderek K, Dumitru CA, Hoffmann TK, Lang S, et al. Peripheral blood neutrophil granulocytes from patients with head and neck squamous cell carcinoma functionally differ from their counterparts in healthy donors. *Int J Immunopathol Pharmacol.* (2011) 24:683–93. doi: 10.1177/039463201102400314
174. Rao SP, Sancho J, Campos-Rivera J, Boutin PM, Severy PB, Weeden T, et al. Human peripheral blood mononuclear cells exhibit heterogeneous CD52 expression levels and show differential sensitivity to alemtuzumab mediated cytolysis. *PLoS ONE.* (2012) 7:e39416. doi: 10.1371/journal.pone.0039416
175. Droeser RA, Hirt C, Eppenberger-Castori S, Zlobec I, Viehl CT, Frey DM, et al. High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. *PLoS ONE.* (2013) 8:e64814. doi: 10.1371/journal.pone.0064814
176. Governa V, Trella E, Mele V, Tornillo L, Amicarella F, Cremonesi E, et al. The interplay between neutrophils and CD8(+) T cells improves survival in human colorectal cancer. *Clin Cancer Res.* (2017) 23:3847–58. doi: 10.1158/1078-0432.CCR-16-2047
177. Galdiero MR, Bianchi P, Grizzi F, Di Caro G, Basso G, Ponzetta A, et al. Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer. *Int J Cancer.* (2016) 139:446–56. doi: 10.1002/ijc.30076
178. Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C. Prognostic Value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in Northern Italy. *Modern Pathol.* (2002) 15:831. doi: 10.1097/01.MP.0000020391.98998.6B
179. Carus A, Ladekarl M, Hager H, Pilegaard H, Nielsen PS, Donskov F. Tumor-associated neutrophils and macrophages in non-small cell lung cancer: no immediate impact on patient outcome. *Lung Cancer.* (2013) 81:130–7. doi: 10.1016/j.lungcan.2013.03.003
180. Sunakawa Y, Stintzing S, Cao S, Heinemann V, Cremolini C, Falcone A, et al. Variations in genes regulating tumor-associated macrophages (TAMs) to predict outcomes of bevacizumab-based treatment in patients with metastatic colorectal cancer: results from TRIBE and FIRE3 trials. *Ann Oncol.* (2015) 26:2450–6. doi: 10.1093/annonc/mdv474
181. Nywening TM, Wang-Gillam A, Sanford DE, Belt BA, Panni RZ, Cusworth BM, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. *Lancet Oncol.* (2016) 17:651–62. doi: 10.1016/S1470-2045(16)00078-4
182. Sandhu SK, Papadopoulos K, Fong PC, Patnaik A, Messiou C, Olmos D, et al. A first-in-human, first-in-class, phase I study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 in patients with solid tumors. *Cancer Chemother Pharmacol.* (2013) 71:1041–50. doi: 10.1007/s00280-013-2099-8
183. Pienta KJ, Machiels JP, Schrijvers D, Alekseev B, Shkolnik M, Crabb SJ, et al. Phase 2 study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 (CCL2), in metastatic castration-resistant prostate cancer. *Invest New Drugs.* (2013) 31:760–8. doi: 10.1007/s10637-012-9869-8
184. Brana I, Calles A, LoRusso PM, Yee LK, Puchalski TA, Seetharam S, et al. Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody, in combination with four chemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study. *Target Oncol.* (2015) 10:111–23. doi: 10.1007/s11523-014-0320-2
185. Cannarile MA, Weisser M, Jacob W, Jegg MA, Ries CH, Rüttinger D. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J Immuno Ther Cancer.* (2017) 5:53. doi: 10.1186/s40425-017-0257-y
186. Cassier PA, Italiano A, Gomez-Roca CA, Le Tourneau C, Toulmonde M, Cannarile MA, et al. CSF1R inhibition with emactuzumab in locally advanced diffuse-type tenosynovial giant cell tumours of the soft tissue: a dose-escalation and dose-expansion phase 1 study. *Lancet Oncol.* (2015) 16:949–56. doi: 10.1016/S1470-2045(15)00132-1
187. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* (2011) 1:54–67. doi: 10.1158/2159-8274.CD-10-0028
188. Mok S, Koya RC, Tsui C, Xu J, Robert L, Wu L, et al. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. *Cancer Res.* (2014) 74:153–61. doi: 10.1158/0008-5472.CAN-13-1816
189. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* (2014) 74:5057–69. doi: 10.1158/0008-5472.CAN-13-3723
190. Cuccarese MF, Dubach JM, Pfirschke C, Engblom C, Garris C, Miller MA, et al. Heterogeneity of macrophage infiltration and therapeutic response in lung carcinoma revealed by 3D organ imaging. *Nat Commun.* (2017) 8:14293. doi: 10.1038/ncomms14293
191. Yan D, Kowal J, Akkari L, Schuhmacher AJ, Huse JT, West BL, et al. Inhibition of colony stimulating factor-1 receptor abrogates microenvironment-mediated therapeutic resistance in gliomas. *Oncogene.* (2017) 36:6049–58. doi: 10.1038/onc.2017.261
192. Giustini N, Bernthal NM, Bukata SV, Singh AS. Tenosynovial giant cell tumor: case report of a patient effectively treated with pexidartinib

(PLX3397) and review of the literature. *Clin Sarcoma Res.* (2018) 8:14. doi: 10.1186/s13569-018-0101-2
193. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases. *Theranostics.* (2017) 7:1543–88. doi: 10.7150/thno.15625
194. Schott AF, Goldstein LJ, Cristofanilli M, Ruffini PA, McCanna S, Reuben JM, et al. Phase Ib pilot study to evaluate reparixin in combination with weekly paclitaxel in patients with HER-2-negative metastatic breast cancer. *Clin Cancer Res.* (2017) 23:5358–65. doi: 10.1158/1078-0432.CCR-16-2748
195. Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. *Nat Med.* (2005) 11:312. doi: 10.1038/nm1196
196. Iversen TZ, Engell-Noerregaard L, Ellebaek E, Andersen R, Larsen SK, Bjoern J, et al. Long-lasting disease stabilization in the absence of toxicity in metastatic lung cancer patients vaccinated with an epitope derived from indoleamine 2,3 dioxygenase. *Clin Cancer Res.* (2014) 20:221–32. doi: 10.1158/1078-0432.CCR-13-1560
197. Soliman HH, Jackson E, Neuger T, Dees EC, Harvey RD, Han H, et al. A first in man phase I trial of the oral immunomodulator, indoximod, combined with docetaxel in patients with metastatic solid tumors. *Oncotarget.* (2014) 5:8136–46. doi: 10.18632/oncotarget.2357
198. Komiya T, Huang CH. Updates in the clinical development of epacadostat and other indoleamine 2,3-dioxygenase 1 inhibitors (IDO1) for human cancers. *Front Oncol.* (2018) 8:423. doi: 10.3389/fonc.2018.00423

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

frontiers in Immunology

REVIEW
published: 12 September 2019
doi: 10.3389/fimmu.2019.02178

Tumors vs. Chronic Wounds: An Immune Cell's Perspective

Yichao Hua[1] *and Gabriele Bergers*[1,2]*

[1] *Laboratory of Tumor Microenvironment and Therapeutic Resistance, Department of Oncology, VIB-Center for Cancer Biology, KU Leuven, Leuven, Belgium,* [2] *Department of Neurological Surgery, UCSF Comprehensive Cancer Center, UCSF, San Francisco, CA, United States*

The wound repair program is tightly regulated and coordinated among different cell constituents including epithelial cells, fibroblasts, immune cells and endothelial cells following consecutive steps to ensure timely, and proper wound closure. Specifically, innate and adaptive immune cells are pivotal participants that also closely interact with the vasculature. Tumors are portrayed as wounds that do not heal because they undergo continuous stromal remodeling and vascular growth with immunosuppressive features to ensure tumor propagation; a stage that is reminiscent of the proliferative resolution phase in wound repair. There is increasing evidence from mouse model systems and clinical trials that targeting both the immune and vascular compartments is an attractive therapeutic approach to reawaken the inflammatory status in the "tumor wound" with the final goal to abrogate tumor cells and invigorate tissue homeostasis. In this review, we compare the implication of immune cells and the vasculature in chronic wounds and tumor wounds to underscore the conceptual idea of transitioning tumors into an inflammatory wound-like state with antiangiogenic immunotherapies to improve beneficial effects in cancer patients.

Keywords: myeloid cells, macrophages, neutrophils, endothelial cells, tumor vessels, wound repair, antiangiogenic immunotherapy

OPEN ACCESS

Edited by:
Wei Zheng,
Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Zoltan Vereb,
University of Szeged, Hungary
Shentong Fang,
Wihuri Research Institute, Finland

***Correspondence:**
Gabriele Bergers
gabriele.bergers@kuleuven.vib.be

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *20 May 2019*
Accepted: *29 August 2019*
Published: *12 September 2019*

Citation:
Hua Y and Bergers G (2019) Tumors vs. Chronic Wounds: An Immune Cell's Perspective. Front. Immunol. 10:2178. doi: 10.3389/fimmu.2019.02178

INTRODUCTION

Neoplastic conversion of cells into a malignant tumor with metastatic properties acquires not only multiple intrinsic traits but also necessitates the participation of the tumor microenvironment with its diverse cellular and matrix constituents (1). Notably, innate immune cells, and specifically macrophages, are functionally involved in nearly every stage of the multistep cascade of tumorigenesis (2). There is also increasing evidence that neutrophils functionally contribute to distinct stages, which includes angiogenesis, escape of tumor dormancy, and metastatic seeding (3, 4). Of the many cancer hallmarks, the onset of tumor neovascularization, and escape of immunosurveillance are two environmental traits that are codependent. They encompass endothelial and mural cells constituting the vasculature as well as innate and adaptive immune cells that partake in heterotypic interactions with one another (5). This crosstalk is not tumor-specific but attributed to their traditional roles in tissue repair where immune cells also affect vascular properties while endothelial cells direct immune cell trafficking and survival.

IMMUNE CELLS IN WOUND HEALING

Acute wound healing, being extensively studied in the skin and gut, follows a well-coordinated multistep process that constitutes inflammation, proliferation and remodeling phases to restore tissue homeostasis, regain function, and protect from infection (6–9) (**Figure 1**, upper panel). Following immediate hemostasis to impede bleeding, and as a first defense mechanism, neutrophils, and then $CCR2^+$ monocytes and macrophages are recruited to the wound and activated by proinflammatory cytokines (e.g., TNFα, IL1) and chemokines (e.g., CXCL-1,5,8; CCL-2) -secreting epithelial cells and fibroblasts and cellular contents (e.g., DNA, RNA, uric acid, metabolites, HMGB1) from dying cells that serve as danger signals (DAMPs) (10, 11). During this inflammation period, neutrophils secrete reactive oxygen species (ROS), nitric oxide (NO), and antimicrobial proteins (AMPs) and deploy web-like extracellular traps (NETs) in order to phagocytose and kill contaminating microorganisms (12, 13). Neutrophils also produce TNFα, IL1β, IL-6, CXCL2/8 as well as MCP-1 (monocyte attracting protein-1) that recruit macrophages, T cells as well as additional neutrophils to the wound thus amplifying a Th1 proinflammatory response. Inflammatory macrophages predominantly serve as scavengers removing dead cells and cellular debris. They also produce similar cytokines, including IL-12/23 as well as IFNγ that recruit T-cells and natural killer cells (NK), and stimulate their proinflammatory responses (14, 15). In addition, endothelial cells in dermal venules upregulate the lymphocyte adhesion molecules V-CAM-1, I-CAM-1, E- and P-selectins, which regulate lymphocyte rolling and tethering, and thus augment lymphocyte infiltration into the wound (7, 16). Consequently, T cells in the wound produce interleukin (IL)-17, IL-22, and tumor necrosis factor a (TNFα), which further intensifies the defense response of the immune system (**Figure 1**, upper panel). In addition, plasmacytoid dendritic cells (pDC) infiltrate the wound and recognize nucleic acids from injured cells leading to the production of type I interferons (17). Further, dermal conventional dendritic cell type 1 (cDC1s) can cross-present antigens (6, 18, 19) to facilitate T cell function, and control the generation of commensal-specific $CD8^+$ $IL\text{-}17^+$ T cells in the skin (20). As soon as neutrophils complete their mission, they undergo apoptosis and are removed by macrophages (21). This phagocytotic activity instigates the transition to an anti-inflammatory Th2-like phenotype in macrophages and ends the inflammatory period (21). The conversion from a "Th1" to "Th2" state is indeed an essential and critical step to impede inflammation and necessary to initiate the proliferative and resolution phase for efficient wound repair (**Figure 1**, upper panel) (22). If the wound repair cannot proceed beyond the inflammation phase, it will generate a chronic wound with barrier defects (8, 9, 23). During the proliferative resolution phase, granulation tissue fills the wound with connective tissue, and keratinocytes, fibroblasts, and endothelial cells expand to enable a proper wound closure. Therefore, anti-inflammatory Th2-like "repair" macrophages activate fibroblasts that in turn incite keratinocyte proliferation and migration and together promote neovascularization by directly secreting Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor β1 (TGF-β1), and IL8 as well as other factors including metalloproteinases (24). During wound healing, the generation of a new vascular network is predominantly caused by sprouting by which new vessel growth is initiated from activated preexisting capillary endothelial cells. In addition, but to a much lesser extent, bone marrow-derived hematopoietic precursors, and even dendritic cells and monocytes, can also be recruited to the growing vasculature where they differentiate into endothelial cells (25–29).

Expanding vascular sprouts exist of proliferating endothelial stalk cells and migrating tip cells at the leading edge which follow a gradient of proangiogenic factors produced by various cells including keratinocytes and stromal cells. Tip cells of different sprouts connect by anastomosis under the chaperon of macrophages, followed by maturation of the new vessel to enable blood flow (30). The entire process is tightly regulated by several proangiogenic factors (e.g., VEGF, PIGF, FGF, IL8) as well as antiangiogenic factors (e.g., Sprouty2, pigment epithelium-derived factor (PEDF), CXCL10) displaying a fine balance of both vascular growth and remodeling until vessels become covered with pericytes, form a basement membrane and mature (24, 31, 32). Although the implication of macrophages has been well-established in the distinct steps of wound healing, the role of neutrophils in the later stages, specifically in angiogenesis has not been appreciated until recently. Like macrophages, neutrophils can polarize from an immunostimulating N1 phenotype to an immunosuppressive N2 status in which they, like macrophages, produce VEGF and MMPs and other angiogenic factors (3, 33). For example, neutrophil-produced VEGF appears to be crucial in the healing process of an injured cornea in mice because antibody-mediated neutrophil depletion substantially impaired neovascularization (34). Also, dendritic cell expansion in the skin can enhance wound healing by DC-produced factors that promote re-epithelialization, angiogenesis, granulation tissue formation, growth factor production (35). Finally, during the last phase of wound repair, the immune cell composition reverses back to normal levels, and the extracellular matrix in the wound undergoes further remodeling to properly close the wound, a process that can persist for weeks to months (8, 9).

TUMORS ARE NON-HEALING WOUNDS BUT DIFFER FROM CHRONIC WOUNDS

While the acute wound healing cascade is tightly regulated and coordinated, chronic wounds (like in diabetes or ulcers) develop when the repair process is trapped, most commonly in the inflammatory response phase being unable to trigger the repair program in macrophages to move to the next phase. Consequently, an excessive immune response develops that leads to further tissue damage rather than tissue restoration (23). In the late 80s, Harold Dvorak compared tumors to wounds that never heal (36). The difference to chronic wounds, however, is that "tumor wounds" avoid the inflammatory phase

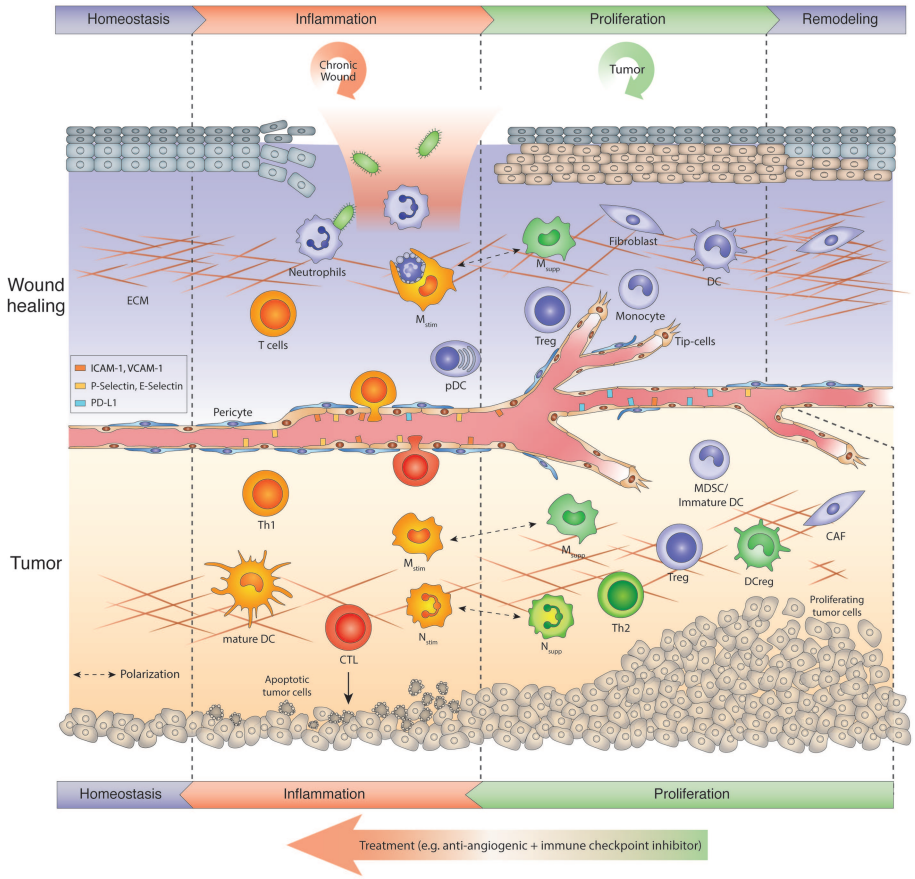


FIGURE 1 | Tumors hijack the wound repair program: Chronic wound vs. tumor wound. Usually wound healing is manifested in several sequential steps after injury referred to as inflammation, proliferation-resolution, and remodeling phase. Immune cells are key regulators in the wound repair program. In the inflammation phase, neutrophils kill microbes and macrophages phagocytose apoptosing neutrophils, while skin-resident or infiltrating T cells produce IL-17, IL-22, and TNF α to amplify the host defense response. During the proliferation phase, macrophages switch to an anti-inflammatory phenotype (M_{supp}). M_{supp} macrophages, N_{supp} neutrophils (or tumor-associated neutrophil, TANs), Tregs and other immunosuppressive cells may help to attenuate the inflammation response and facilitate resolution and tissue remodeling. Chronic wounds get trapped in the inflammation phase, exacerbate inflammation and thus, hinder tissue repair. Tumors, on the other hand, hijack the proliferative/remodeling program and provide signals that create a continuous angiogenic and immunosuppressive environment enabling tumors to grow and escape immune surveillance. Therefore, tumors remain in the proliferative phase upon the onset of angiogenesis. Antiangiogenic immunotherapies induce an inflammation program in tumors that reawakens and boosts an anti-tumor response. The ultimate goal is to create a homeostatic situation in which tumor cells are eliminated and a normal tissue architecture is achieved. CAF, Cancer-associated fibroblast; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DCreg, regulatory DC; ECM, extracellular matrix; MDSC, myeloid-derived suppressor cells; Mono, monocyte; M_{stim}, immunostimulatory macrophage (M1-like); M_{supp}, immunosuppressive macrophage (M2-like); N_{stim}, immunostimulatory neutrophil (N1-like); N_{supp}, immunosuppressive neutrophil (TAN); pDC, plasmacytoid DC; Th, T helper cell; Treg, regulatory T cell.

to escape immunosurveillance, and hijack the proliferative resolution program of the wound repair to induce a vascular-rich stroma with immunosuppressive and angiogenesis-promoting cell constituents conducive to tumor propagation (**Figure 1**) (36). Similar to the processes in the resolution phase of wounds, tumors instigate several remodeling processes that include increased vascular permeability, the onset of angiogenesis and deposition of an extravascular fibrin-enriched provisional stroma which is replaced by a vascular connective granulation tissue causing desmoplasia in certain tumor types (37). Concomitantly, tumors polarize innate immune cells from an immunostimulating to an immunosuppressive and angiogenic state and thus, not only escape immunosurveillance but also take advantage of myeloid-produced angiogenic factors that help to expand its tumor vasculature accommodating the needs of a growing tumor (**Figure 1**, lower panel) (38). Notably, the process of angiogenesis in wounds and tumors is regulated by similar factors, but in contrast to the tight regulation of angiogenesis in acute wounds, the production of angiogenesis-promoting and inhibiting molecules in tumors is imbalanced (39, 40). Tumors continue to stimulate neovascularization, which results in an expanding tumor vasculature with an abnormal phenotype displaying hyperdilated tumor vessels with poor pericyte coverage and leaky and sluggish blood flow (41). Subsequently, a hypoxic and acidic environment in tumors with increased interstitial pressure evolves that further elevates the production of proangiogenic factors and thus exacerbates a proangiogenic response (40, 42).

INNATE IMMUNE CELLS PROMOTE ANGIOGENESIS

Like in wounds, myeloid cells present a prominent population in tumors where they can make up to 30% of the entire population dependent on tumor type and stage (5, 43–45). As soon as myeloid cells reach the tumor, some of the immature innate immune cells will differentiate into tumor-associated macrophages (TAMs) and neutrophils while others remain in an immature stage resembling monocytic myeloid-derived suppressor cells (M-MDSCs) and immature DCs or granulocytic MDSCs (G-MDSCs) (46). In addition, the presence of regulatory (reg) DCs has also been described which suppress T cell activation and proliferation and enable Treg differentiation and expansion (47–49).

Importantly, however, the cytokine milieu to which myeloid cells are exposed, and the specific tumor microenvironment in which they reside will dictate which phenotype these plastic cells will display. IFNγ, TNFα, and IL-12 promote an immunostimulatory polarization in innate immune cells while TGF-β, IL-4, IL-10, and CSF-1 are prominent factors that skew macrophages and neutrophils toward an immunosuppressive and angiogenic phenotype, and promote Treg proliferation (3, 50, 51).

Thus, although macrophages and neutrophils have the ability to inhibit angiogenesis and attack whatever they consider foreign, in tumors, they commonly promote an escape of immunosurveillance, and new vessel formation. There is strong evidence of the functional significance of TAMs in tumor angiogenesis in multiple systems. One of the first seminal studies demonstrating the relevance of TAM-directed tumor angiogenesis was achieved in the mammary virus-polyoma middle T- antigen (PyMT) breast tumor model, and then confirmed in other tumor model systems (52–54). Thereby, macrophages were depleted in tumors by genetically or pharmacologically impairing CSF1-CSF1R signaling, which is essential for macrophage differentiation and survival, or by broad elimination of myeloid cells with clodronate liposomes. As a result, macrophage-deficiency in these various murine tumor models reduced angiogenic activity (52, 54–56). Again, however, the pro-angiogenic capacity of TAMs is dependent on the cytokine milieu, which in part is triggered by a hypoxic and acidic microenvironment (40, 57). These conditions induce the secretion of chemotactic factors such as VEGF, colony-stimulating factor 1-3 (CSF1-3), the CX chemokines CXCL12 (aka SDF1a) and CX3CL1, the CC-chemokines CCL2, CCL5, CCL22, interleukin IL6, semaphoring 3A and others that recruit immune cells to the tumor where they become programmed to facilitate angiogenesis by secreting proangiogenic factors (40, 43, 46, 54, 58–63) (**Figure 2**). In this pro-tumor state, myeloid cells represent a crucial source of angiogenic factors producing VEGF, fibroblast growth factor 2 (FGF2), CXCL8 (CXC-chemokine ligand 8), WNT7B, and BV8. In addition, they produce PDGF-B, PIGF, Neuropilin-1, IL-6, and several proteinases, including matrix metalloproteinases (MMPs) and cathepsins, which also have pro-angiogenic properties (64, 65) (**Figure 2**). Certainly, hypoxia via HIF-1α also augments the secretion of proangiogenic cytokines in tumor cells, specifically VEGF, which is the most prominent angiogenic factor being highly expressed in a variety of different tumor types (66). This makes tumor cells the major source of VEGF and raises the question as to why myeloid cells also induce VEGF in response to hypoxia (67–70). As it is well-established that VEGF contributes to tumorigenesis (71), Stockmann et al. made a surprising observation that myeloid cell-specific VEGF deletion in mice enhanced the development of spontaneous mammary PYMT tumors and tumors of several subcutaneous isograft models (53). Interestingly, VEGF depletion in macrophages promoted tumor vessel normalization and thus enhanced the exposure of tumors to chemotherapeutic cytotoxicity (53). This is an important study that supports the notion that not only total VEGF levels but also the location of VEGF within the tumor regulate vascular characteristics. It appears that likely perivascular macrophages secrete VEGF to fine-tune angiogenic properties of blood vessels by closely interacting with endothelial cells. Congruent with these observation of location-dependent effects of VEGF, myeloid cell-produced VEGF has also been shown to promote the intravasation of tumor cells into the blood stream by enhancing vascular permeability (72). Besides producing VEGF, myeloid cells regulate VEGF bioavailability by releasing matrix metalloproteinase MMP-9 to liberate sequestered VEGF from the extracellular matrix. This enables VEGF binding to and activation of VEGFR2 on endothelial cells at sites of neovascularization (59, 73) (**Figure 2**). Another example of location-dependent regulation and function of TAM activity has been described for semaphorin 3A (Sema 3A). Sema 3A is induced by hypoxia and was found to recruit macrophages by binding to neuropilin-1 (Nrp-1) and PlexinA1/A4 co-receptors and signaling through VEGFR1. As soon as macrophages localized in low-oxygen conditions, expression of Nrp-1, but not PlexinA1/A4, was repressed in macrophages, which trapped macrophages in these hypoxic areas where they facilitated angiogenic and immunosuppressive properties (63). Congruently, genetic deletion of Nrp-1 in macrophages was sufficient to impair TAM recruitment and accumulation in hypoxic regions, resulting in impaired neovascularization, improved antitumor immunity and consequently, delayed tumor growth (63). TIE2-expressing macrophages (TEMs) have highly angiogenic characteristics which, like TAMs, correlate with vascular density in various murine and human tumors (74, 75). TEMs are preferentially found in close association with blood vessels being recruited by angiopoietin 2 (ANGPT2)-secreting endothelial cells. ANGPT2 promotes angiogenesis in an autocrine manner by binding to the TIE2 receptor on endothelial cells and mediates interactions between endothelial cells and TEMs to support vessel sprouting and macrophage -directed anastomosis (30, 46, 76). Albeit TEMs compose a minor subset of TAMs, they have been found to be highly relevant in promoting tumor angiogenesis because TEM depletion experiments using antibody-mediated neutralization of the Tie2 ligand Ang2 or Tie2 promoter-driven thymidine kinase both reduced angiogenesis and tumor propagation in mammary, pancreatic neuroendocrine and brain tumor mouse models (76, 77). Besides macrophages, neutrophils have now also been recognized to be important

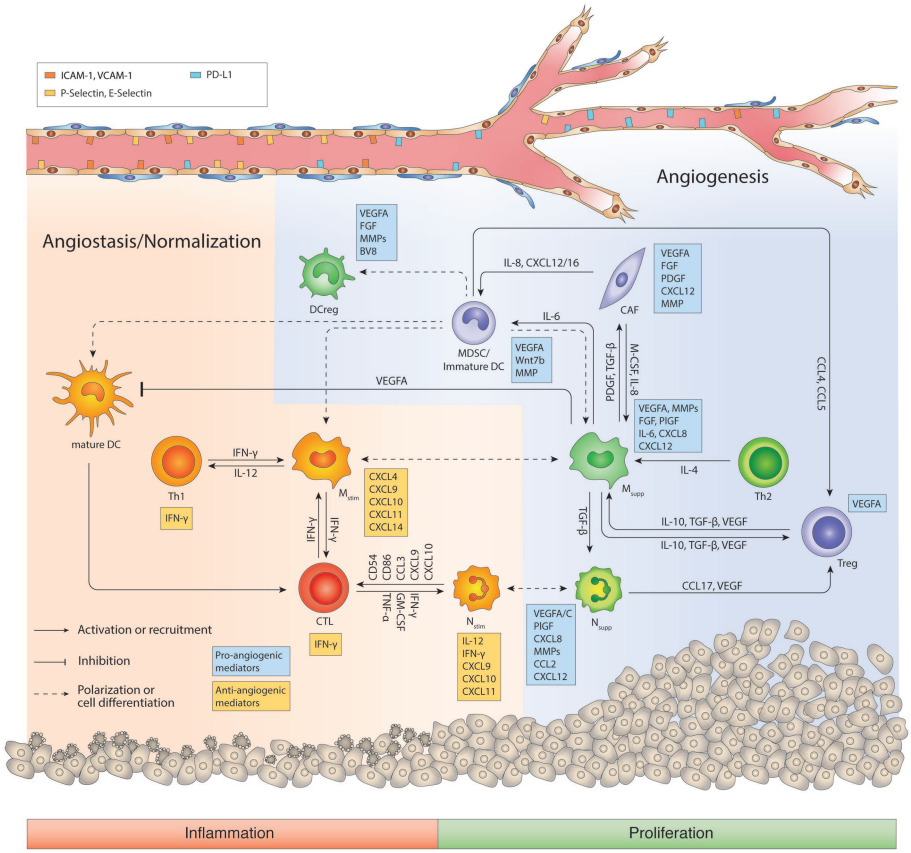


FIGURE 2 | Regulatory network of the tumor immune microenvironment. The tumor microenvironment facilitates cross-talks between immunosuppressive macrophages, MDSCs, N_{supp}, Tregs and $CD4^+$ Th2 cells that promotes angiogenesis, immunosuppression, and tumor progression. During the course of antiangiogenic and/or immunotherapy, myeloid cells including macrophages can switch to an inflammatory phenotype, and cooperate with $CD8^+$ CTLs and $CD4^+$ Th1 cells to generate an anti-tumor response and promote vessel pruning and normalization. Ang2, angiopoietin 2; CAF, Cancer-associated fibroblast; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DCreg, regulatory DC; FGF, Fibroblast growth factor; IFN, interferon; IL, interleukin; M-CSF, Macrophage colony-stimulating factor; MDSC, myeloid-derived suppressor cells; MMP, Matrix metalloproteinases; M_{stim}, immunostimulatory macrophage (M1-like); M_{supp}, immunosuppressive macrophage (M2-like); N_{stim}, immunostimulatory neutrophil (N1-like); N_{supp}, immunosuppressive neutrophil (TAN); PDGF, Platelet-derived growth factor; PlGF, Placental growth factor; TAN, Tumor-associated neutrophil; TGF-β, Transforming growth factor beta; Th, T helper cell; Treg, regulatory T cell; VEGFA, Vascular endothelial growth factor A. Cells in orange or red color represent immunostimulation/type 1 immunity/anti-angiogenic status, while cells in green/blue represent immunosuppression/type 2 immunity/pro-angiogenic status.

mediators of tumorigenesis but the TAN-dependent mechanisms of tumor progression are not fully understood (3, 4, 78). G-CSF mediates neutrophil proliferation and differentiation by binding to CSF3R and activating downstream Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) pathways. CXCL chemokines including CXCL8 as well as IL-1β, IL17, and IL-6 predominantly mediate the recruitment of neutrophils to tumors (3, 79–81) (**Figure 2**). In contrast to injuries, where neutrophils are the first cells to enter the wound to fight contaminants and then undergo efferocytosis, neutrophils in tumors (TAN) do not appear to apoptose but like macrophages become polarized to an immunosuppressive and angiogenic phenotype. These observations of phenotypic neutrophil modulation have led to the notion that the functional plasticity seen in other immune cells, such as TAMs, may also be reflected in TANs (3). In support, cytokine-driven polarization of neutrophils in murine models of cancer have provided evidence that the cytokine TGF-β and type I interferons are key effectors of neutrophil polarization. TGF-β skews neutrophils toward an N2 phenotype. It blocks neutrophil production of ROS, reactive nitrogen intermediates, and IL-1β and impedes neutrophil degranulation in response to LPS. Conversely, TGF-β inhibition or the presence of type I interferons polarize neutrophils to an N1 phenotype while inhibiting type I interferon signaling unleashes N2 properties in neutrophils (82). N2 conversion, similar to M2 macrophage polarization, may in part be caused by hypoxia, which has been shown to delay neutrophil apoptosis (83). Mechanistically, hypoxia induced neutrophil survival through HIF-1α-dependent NF-κB activity under low-oxygen tension in a PHD3-dependent manner (57,

84). Like TAMs, TANs produce similar proangiogenic factors and proteases like VEGF, FGF, BV8, and MMP9, which is in part regulated by STAT3 signaling (81, 85–87). The angiogenic expression profile appears to be very conserved because in zebrafish, transcriptomic profiling of liver tumor-associated neutrophils revealed up-regulation of similar gene transcripts promoting angiogenesis (88). VEGF is the prominent angiogenic factor that neutrophils, like TAMs, not only express and secrete but they also carry it in granules which are released upon TNF stimulation (89). TANs, like TAMs, provide another quick route of VEGF accessibility to activate endothelial cells by releasing MMP-9 to release sequestered VEGF from the extracellular matrix (ECM) (90, 91). Indeed, this neutrophil-dependent mechanism was critical to instigate the angiogenic switch in the dysplastic stage of pancreatic islets in the Rip1Tag2 endogenous pancreatic neuroendocrine tumor model because not only MMP-9 inhibition but also neutrophil depletion was sufficient to diminish the angiogenic switch (73, 90). Further, GM-CSF stimulated tumor-associated neutrophils to produce the angiogenic factor Bv8 in several murine tumor models, which in turn attracted more neutrophils, thus, providing a forward loop for neutrophil recruitment and activation (92). Consequently, pharmacological or genetic blockade of CSF3, CSF3R, or BV8 decreased the number of TANs and inhibited tumor angiogenesis and growth (81). It is notable that in addition to the identification of intratumoral neutrophils, three distinct neutrophil populations have recently been described in the blood circulation, both in mice and in patients with advanced cancer (93). High- density neutrophils are reminiscent of cancer-killing N1 neutrophils while mature LDNs are not cytotoxic and display impaired functionality and immunosuppressive properties. The third population consists of morphologically immature LDNs which show characteristics of granulocytic myeloid-derived immunosuppressive cells (MDSCs). They are also observed in tumors, and thus suggest the other circulating neutrophil populations may be present in tumors as well (93). MDSCs are immature myeloid cells of granulocytic (G-MDSC) or monocytic (M-MDSC) origin, first discovered in tumors, that not only strongly suppress CD4 and CD8 T cells but also convey angiogenic features (43, 94, 95). MDSCs, as well as reg-DCs, secrete proangiogenic factors similar to M2-like TAMs and N2-like TANs, such as VEGF, FGF2, BV8, and MMP9 (79). Tumor -produced CSF3, IL-1β, and IL-6 activate STAT3 in MDSCs which leads to their expansion but hinders MDSC maturation into macrophages or neutrophils. Notably, the proangiogenic expression profile of MDSCs conceivably overlaps with those of TAMs and TANs (85, 87, 94). Indeed, it has become apparent from several studies that the different innate immune cell populations produce several but similar angiogenic molecules to facilitate neovascularization. Given the functional redundancy in their angiogenic properties, it is conceivable that myeloid cells can compensate for the lack of other myeloid cell constituents to regulate tumor angiogenesis. Indeed, neutrophils can compensate for macrophages to support tumor angiogenesis in tumor-bearing CCR2-knockout mice (91). Further, neutrophils and macrophages are implicated in adaptive resistance to anti-angiogenic therapy in the Rip1Tag2 pancreatic neuroendocrine tumors. Therapeutic targeting of either population caused enhanced infiltration of the other myeloid cell population compensating for the loss of neutrophils and macrophages, respectively, which created an oscillating pattern of distinct immune-cell populations to facilitate adequate neovascularization (87).

Finally, innate lymphoid cells (ILCs) represent a recently identified heterogeneous family of mononuclear hematopoietic cells. Based on their lymphoid morphology, surface antigens, transcription factor expression, and cytokine productions (TH1, TH2, and TH17-like), ILCs have been classified into three major groups, termed ILC1, ILC2, and ILC3 (96). ILC3s elicit tumorigenic and angiogenic properties in part by secreting IL-17 (79, 97, 98). Notably, a subset of ILC1s share features with Natural killer (NK) cells, which are bone marrow-derived large granular effector lymphocytes. Cancer infiltrating NK cells have been shown to release angiogenic factors and immunosuppressive cytokines like VEGF, PlGF, and IL-8, similar to proangiogenic NK cells found in the developing endometrium (99). $CD56^{+}CD16^{-}$ NK cells from peripheral blood of patients with non-small cell lung cancer (NSCLC), especially squamous cell carcinoma (SCC) subtype, produce higher levels of VEGF, IL-8, and PlGF than those from healthy donors (100)

ADAPTIVE IMMUNE CELLS REGULATE ANGIOGENESIS

While adaptive immune cells are predominantly associated with immune surveillance, there is increasing evidence that they also regulate angiogenesis, although their exact functions in this process are just beginning to be revealed. In tumors, T-cells, due to their heterogeneous nature, appear to negatively or positively regulate tumor angiogenesis. Conditioned medium from Th2 and Th17 T-cells contained factors that enhanced angiogenesis *in vitro* in an endothelial sprouting assay and in a murine model of ischemia when released from an injectable alginate biomaterial. In contrast, Th1 conditioned medium induced regression of vascular tubes *in vitro* and was inefficient to instigate angiogenesis *in vivo* (101). In several mouse tumor model systems, $CD8^{+}$ T-cells and $CD4^{+}$ T-helper 1 cells have been shown to secrete IFNγ, which blocks vascular growth and triggers TAMs and TANs to produce the angiostatic chemokines CXCL 9,10, and 11 (3, 102, 103). In contrast, Treg cells suppress INFγ -expressing $CD4^{+}$ Th1 cells and secrete VEGF via hypoxia-induced CCL28, that both promote an angiogenic tumor environment (104). The importance of VEGF production by T-cells was recently underscored by the finding that genetic deletion of VEGF in $CD8^{+}$ T-cells enhanced tumorigenesis while it also exhibited hallmarks of tumor vessel normalization, with typical features of increased pericyte coverage of tumor blood vessels and decreased vessel tortuosity (105). Interestingly, the overall level of hypoxia was decreased consistently with better perfusion, a phenotype that was also observed when VEGF was deleted in TAMs (53). The lower numbers of infiltrating T-cells in tumors of VEGF mutant mice suggests that VEGF secreted by $CD8^{+}$ T cells

may affect T cell homing through the endothelial barrier and thus, its lack may be in part responsible for the augmented tumor growth (105). In support of these observations, human breast cancer tissues revealed an inverse correlation between VEGF levels and $CD8^+$ T cell infiltration, and congruently linked T cell infiltration with the stage of vascularization (105). In further support, depletion of intratumoral $CD4^+$ and $CD8^+$ T-cell in mouse tumor models generated a more dysfunctional tumor vasculature with an increase in hypoxic areas. These effects could be reverted by CD8 influx and activity through checkpoint immunotherapy (anti-PD1 and/or anti-CTLA4), or by adoptive TH1 transfer, both invigorating tumor vessel normalization and reducing hypoxia (106). While these data provide evidence of T-cells in regulating vascular properties, the implication of B-cells remains somewhat elusive. Analysis of the overall B-cell population in tumors revealed that B-cells can secrete proangiogenic factors such as VEGF, FGF2, and MMP-9 and that they are able to promote immunosuppressive and proangiogenic properties in macrophages in an IgG-dependent manner (107, 108).

HEALING TUMOR WOUNDS

The studies described above support the proposition that tumors generate a cytokine and chemokine milieu that stimulates an immunosuppressive and angiogenic environment displaying characteristics of the proliferative resolution phase in the wound repair process. Among the multifarious participants in this "wound scenario" are immune cells and blood vessels, which are functionally interconnected by mediators and molecules that commonly regulate both immunity and angiogenesis. Strategies to impede neovascularization were first developed with the intention to restrain tumor growth and "starve a tumor to death" (109). Antiangiogenic therapy targeting the VEGF-VEGFR and/or Ang-Tie2 pathway, however, has so far only provided beneficial effects in a subset of patients eliciting progression-free but not overall survival (77, 110, 111) because tumors find alternative strategies to adapt to the restrictions of vascular growth and reinstate growth (112). A major resistance mechanism is prompted by treatment-induced hypoxia and relies on recruiting distinct innate immune cells from the bone marrow to the tumor where they stimulate vascular growth in a VEGF-independent manner (5, 57, 59, 77). Importantly, the seminal observation of "vessel normalization" in responding tumors that pruned tumor vessels exhibited a more functional morphology with proper pericyte alignment improving blood flow and oxygenation also revealed a more immunostimulating environment with enhanced CD8 T cell influx (113, 114). Congruent with these studies, angiokinase inhibitors and anti-VEGFR antibodies facilitating vessel normalization in responding Rip1Tag2 PNET tumors converted intratumoral myeloid cells to an angiostatic and immunostimulating phenotype which was associated with an enhanced influx of cytotoxic CD8 cells (87). Due to continuous vessel pruning, however, hypoxic areas formed, leading to enhanced influx as well as proangiogenic and immunosuppressive polarization of innate immune cells concomitant with a drop of intratumoral CD8 cells. Mechanistically, CXCL12 and IL6 induction activated PI3Kγ signaling in intratumoral macrophages, neutrophils and MDSCs rendering them proangiogenic and immunosuppressive. PI3K-activated myeloid cells negated the antiangiogenic blockade and promoted tumor relapse (87). Further support stems from the observation that myeloid PI3Kγ signaling inhibits NFκB while it promotes C/EBPβ activation, thereby inducing a transcriptional program that favors immunosuppression (115). Importantly, therapeutic inhibition of myeloid PI3Kγ/δ was able to sustain the efficacy of antiangiogenic therapy. It polarized all myeloid cells to an angiostatic and immunostimulatory phenotype and enhanced CD8 T cell infiltration and activity in tumors (87). Tumors relapsing from antiangiogenic therapy did not only convert myeloid cells into a Th2 state, but they also enhanced the levels of the negative immune checkpoint regulator PD-L1 in tumor and stromal cells (116, 117). This displayed another mechanism of escaping immune surveillance because PD-L1 binds PD-1 on the surface of activated T-cells and thus blocks T-cell activity. Similarly to antiangiogenic therapy combined with a myeloid PI3K inhibitor, combined antiangiogenic (either anti-VEGF or anti-VEGF/Ang2 antibodies) and anti-PD-L1 immunotherapy had superior beneficial effects than respective monotherapies because the immunostimulating therapy blocked evasion from antiangiogenic therapy, while antiangiogenic-induced vascular normalization enhanced cytotoxic T cell infiltration and activation (116, 117). Notably, successful antiangiogenic immunotherapy could not only normalize tumor vessels but also generate high-endothelial venule (HEV)-like structures in some tumors that further enhanced lymphocyte infiltration to eradicate tumor cells (117). Another example demonstrating the benefits of antiangiogenic immunotherapy was demonstrated with the combination of the angiokinase inhibitor axitinib and anti-CTLA4 treatment. The drug combination provided extensive survival benefits in a mouse model of melanoma because it increased effector T-cell influx and dendritic cell maturation, and it reduced intratumoral MDSCs while the monotherapies failed (118). These observations resemble only a few examples for the support of targeting both the vascular and immune cell compartment to elicit enduring effects. Besides immune checkpoint inhibitors, there are certainly a variety of different drugs that have been developed for targeting signaling pathways in myeloid cells, including the inhibition of CSF1R, CXCR4, PI3Kγ/δ, CD47/SIRPα, and CCL2/CCR2 as well as the activation of CD40 and TLR7/9 (2, 119, 120) that could be combined with antiangiogenic therapies. From a mechanistic point of view, these results reveal a communality, i.e., the attempt to transit tumors from their proangiogenic and immunosuppressive phase into an immunostimulatory and angiostatic state similar to those phases observed during wound repair (**Figures 1**, **2**). However, while the wound repair program transitions from an inflammatory stage to a proliferative resolution phase in order to properly close the wound, antiangiogenic immunotherapy in tumors attempts to do the opposite by awakening an inflammatory status in the "tumor wound" to abrogate tumor cells and invigorate tissue homeostasis.

CONCLUSIONS

Ongoing clinical trials that combine antiangiogenic agents and immunotherapies like ICB or those targeting and modulating innate immune cells as well as strategies to directly enhance infiltration and activation of CD8 T-cells validate the concept of enhancing an immunostimulating environment in cancer. For example, several clinical trials are currently evaluating combined VEGF/VEGFR and PD-1/PD-L1 inhibitors for various cancer types including renal cell carcinoma, recurrent glioblastoma, ovarian cancer and colorectal cancer (NCT03024437, NCT02659384, NCT02873962, NCT02017717). The clinical trial IMmotion150 (NCT01984242) in patients with naïve renal cell cancer (mRCC) assessed the combination of anti-PD-L1 (atezolizumab) with or without bevacizumab, against the standard-of-care angiokinase inhibitor, sunitinib (121). Combining anti-PD-L1 with bevacizumab was more efficacious than sunitinib in patients with PDL1-positive tumors. Interestingly, the mutational rate and neoantigen burden of tumors did not correlate with progression-free survival (PFS), but angiogenesis and myeloid inflammatory gene expression signatures associated strongly with PFS within and across the treatments arguing that these signatures could be utilized as prospective biomarkers (121, 122). Similar to the results obtained in preclinical tumor models described above, myeloid-driven inflammation in tumors appeared to be a resistance mechanism to anti-PD-L1 monotherapy in mRCC which could be overturned by bevacizumab (87, 116, 117). These first clinical results are certainly promising and together with upcoming clinical trials, will be able to thoroughly assess the effectiveness of antiangiogenic immunotherapies in improving and enduring survival of cancer patients.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by grants from the Flemish cancer society Kom op tegen Kanker (2018/11321/2822 to YH), the Flemish government FWO (G0A0818N to GB), and the National Institute of Health NIH/NCI (R01CA201537 to GB).

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
2. Cassetta L, Pollard JW. Targeting macrophages: therapeutic approaches in cancer. *Nat Rev Drug Discov*. (2018) 17:887–904. doi: 10.1038/nrd.2018.169
3. Shaul ME, Fridlender ZG. Neutrophils as active regulators of the immune system in the tumor microenvironment. *J Leukoc Biol*. (2017) 102:343–9. doi: 10.1189/jlb.5MR1216-508R
4. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:eaao4227. doi: 10.1126/science.aao4227
5. Rivera LB, Bergers G. Intertwined regulation of angiogenesis and immunity by myeloid cells. *Trends Immunol*. (2015) 36:240–9. doi: 10.1016/j.it.2015.02.005
6. Nguyen AV, Soulika AM. The dynamics of the skin's immune system. *Int J Mol Sci*. (2019) 20:1811. doi: 10.3390/ijms20081811
7. MacLeod AS, Mansbridge JN. The innate immune system in acute and chronic wounds. *Adv Wound Care*. (2016) 5:65–78. doi: 10.1089/wound.2014.0608
8. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med*. (2014) 6:265sr6. doi: 10.1126/scitranslmed.3009337
9. Brazil JC, Quiros M, Nusrat A, Parkos CA. Innate immune cell–epithelial crosstalk during wound repair. *J Clin Invest*. (2019) 129:2983–93. doi: 10.1172/JCI124618
10. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol*. (2008) 8:279–89. doi: 10.1038/nri2215
11. Willenborg S, Lucas T, Van Loo G, Knipper JA, Krieg T, Haase I, et al. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. *Blood*. (2012) 120:613–25. doi: 10.1182/blood-2012-01-403386
12. Ellis S, Lin EJ, Tartar D. Immunology of wound healing. *Curr Dermatol Rep*. (2018) 7:350–8. doi: 10.1007/s13671-018-0234-9
13. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. (2013) 13:159–75. doi: 10.1038/nri3399
14. Brancato SK, Albina JE. Wound macrophages as key regulators of repair: origin, phenotype, and function. *Am J Pathol*. (2011) 178:19–25. doi: 10.1016/j.ajpath.2010.08.003
15. Trinchieri G, Lyakh L, Trinchieri G, Provezza L, Carra G, Gerosa F. Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. *Immunol Rev*. (2008) 226:112–31. doi: 10.1111/j.1600-065X.2008.00700.x
16. Issekutz AC, Issekutz TB. The role of E-selectin, P-selectin, and very late activation antigen-4 in T lymphocyte migration to dermal inflammation. *J Immunol*. (2002) 168:1934–9. doi: 10.4049/jimmunol.168.4.1934
17. Gregorio J, Meller S, Conrad C, Di Nardo A, Homey B, Lauerma A, et al. Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. *J Exp Med*. (2010) 207:2921–30. doi: 10.1084/jem.20101102
18. Granja AG, Leal E, Pignatelli J, Castro R, Abós B, Kato G, et al. Identification of teleost skin CD8α+ dendritic-like cells, representing a potential common ancestor for mammalian cross-presenting dendritic cells. *J Immunol*. (2015) 195:1825–37. doi: 10.4049/jimmunol.1500322
19. del Rio M-L, Bernhardt G, Rodriguez-Barbosa J-I, Förster R. Development and functional specialization of CD103+ dendritic cells. *Immunol Rev*. (2010) 234:268–81. doi: 10.1111/j.0105-2896.2009.00874.x
20. Naik S, Bouladoux N, Linehan JL, Han S-J, Harrison OJ, Wilhelm C, et al. Commensal-dendritic-cell interaction specifies a unique protective skin immune signature. *Nature*. (2015) 520:104–8. doi: 10.1038/nature14052
21. Elliott MR, Koster KM, Murphy PS. Efferocytosis signaling in the regulation of macrophage inflammatory responses. *J Immunol*. (2017) 198:1387–94. doi: 10.4049/jimmunol.1601520
22. Landén NX, Li D, Ståhle M. Transition from inflammation to proliferation: a critical step during wound healing. *Cell Mol Life Sci*. (2016) 73:3861–85. doi: 10.1007/s00018-016-2268-0
23. Larouche J, Sheoran S, Maruyama K, Martino MM. Immune regulation of skin wound healing: mechanisms and novel therapeutic targets. *Adv Wound Care*. (2018) 7:209–31. doi: 10.1089/wound.2017.0761
24. DiPietro LA. Angiogenesis and wound repair: when enough is enough. *J Leukoc Biol*. (2016) 100:979–84. doi: 10.1189/jlb.4MR0316-102R
25. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med*. (2003) 9:702–12. doi: 10.1038/nm0603-702

26. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*. (2001) 7:1194–201. doi: 10.1038/nm1101-1194
27. Li B, Pozzi A, Young PP. TNFalpha accelerates monocyte to endothelial transdifferentiation in tumors by the induction of integrin alpha5 expression and adhesion to fibronectin. *Mol Cancer Res*. (2011) 9:702–11. doi: 10.1158/1541-7786.MCR-10-0484
28. Kopp HG, Ramos CA, Rafii S. Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Curr Opin Hematol*. (2006) 13:175–81. doi: 10.1097/01.moh.0000219664.26528.da
29. Bosisio D, Ronca R, Salvi V, Presta M, Sozzani S. Dendritic cells in inflammatory angiogenesis and lymphangiogenesis. *Curr Opin Immunol*. (2018) 53:180–6. doi: 10.1016/j.coi.2018.05.011
30. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S, et al. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood*. (2010) 116:829–40. doi: 10.1182/blood-2009-12-257832
31. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology*. (2005) 69 (Suppl. 3):4–10. doi: 10.1159/000088478
32. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol*. (2007) 8:464–78. doi: 10.1038/nrm2183
33. Wang X, Qiu L, Li Z, Wang XY, Yi H. Understanding the multifaceted role of neutrophils in cancer and autoimmune diseases. *Front Immunol*. (2018) 9:1–10. doi: 10.3389/fimmu.2018.02456
34. Gong Y, Koh DR. Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an *in vivo* corneal model. *Cell Tissue Res*. (2010) 339:437–48. doi: 10.1007/s00441-009-0908-5
35. Vinish M, Cui W, Stafford E, Bae L, Hawkins H, Cox R, et al. Dendritic cells modulate burn wound healing by enhancing early proliferation. *Wound Repair Regen*. (2016) 24:6–13. doi: 10.1111/wrr.12388
36. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med*. (1986) 315:1650–9. doi: 10.1056/NEJM198612253152606
37. Dvorak HF. Tumors: wounds that do not heal-redux. *Cancer Immunol Res*. (2015) 3:1–11. doi: 10.1158/2326-6066.CIR-14-0209
38. Motz GT, Coukos G. The parallel lives of angiogenesis and immunosuppression: cancer and other tales. *Nat Rev Immunol*. (2011) 11:702–11. doi: 10.1038/nri3064
39. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer*. (2003) 3:401–10. doi: 10.1038/nrc1093
40. Mazzone M, Bergers G. Regulation of blood and lymphatic vessels by immune cells in tumors and metastasis. *Annu Rev Physiol*. (2019) 81:535–60. doi: 10.1146/annurev-physiol-020518-114721
41. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev*. (2005) 15:102–11. doi: 10.1016/j.gde.2004.12.005
42. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med*. (2018) 24:541–50. doi: 10.1038/s41591-018-0014-x
43. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer*. (2008) 8:618–31. doi: 10.1038/nrc2444
44. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res*. (1996) 56:4625–9.
45. Lewis CE, Harney AS, Pollard JW. The multifaceted role of perivascular macrophages in tumors. *Cancer Cell*. (2016) 30:18–25. doi: 10.1016/j.ccell.2016.05.017
46. De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer*. (2017) 17:457–74. doi: 10.1038/nrc.2017.51
47. Sato K, Uto T, Fukaya T, Takagi H. Regulatory dendritic cells. *Curr Top Microbiol Immunol*. (2017) 410:47–71. doi: 10.1007/82_2017_60
48. Ma Y, Shurin GV, Gutkin DW, Shurin MR. Tumor associated regulatory dendritic cells. *Semin Cancer Biol*. (2012) 22:298–306. doi: 10.1016/j.semcancer.2012.02.010
49. Shurin GV, Ma Y, Shurin MR. Immunosuppressive mechanisms of regulatory dendritic cells in cancer. *Cancer Microenviron*. (2013) 6:159–67. doi: 10.1007/s12307-013-0133-3
50. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. (2010) 22:231–7. doi: 10.1016/j.coi.2010.01.009
51. Laoui D, Van Overmeire E, De Baetselier P, Van Ginderachter JA, Raes G. Functional relationship between tumor-associated macrophages and macrophage colony-stimulating factor as contributors to cancer progression. *Front Immunol*. (2014) 5:489. doi: 10.3389/fimmu.2014.00489
52. Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, et al. Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. *Blood*. (2010) 115:1461–71. doi: 10.1182/blood-2009-08-237412
53. Stockmann C, Doedens A, Weidemann A, Zhang N, Takeda N, Greenberg JI, et al. Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature*. (2008) 456:814–8. doi: 10.1038/nature07445
54. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res*. (2006) 66:11238–46. doi: 10.1158/0008-5472.CAN-06-1278
55. Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K, Schwendener RA. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *Br J Cancer*. (2006) 95:272–81. doi: 10.1038/sj.bjc.6603240
56. Lohela M, Casbon A-J, Olow A, Bonham L, Branstetter D, Weng N, et al. Intravital imaging reveals distinct responses of depleting dynamic tumor-associated macrophage and dendritic cell subpopulations. *Proc Natl Acad Sci USA*. (2014) 111:E5086–95. doi: 10.1073/pnas.1419899111
57. Palazon A, Goldrath AW, Nizet V, Johnson RS. HIF transcription factors, inflammation, and immunity. *Immunity*. (2014) 41:518–28. doi: 10.1016/j.immuni.2014.09.008
58. Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL. Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J Pathol*. (2000) 190:430–6. doi: 10.1002/(SICI)1096-9896(200003)190:4<430::AID-PATH538>3.0.CO;2-6
59. Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, et al. HIF1 alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell*. (2008) 13:206–20. doi: 10.1016/j.ccr.2008.01.034
60. Dineen SP, Lynn KD, Holloway SE, Miller AF, Sullivan JP, Shames DS, et al. Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. *Cancer Res*. (2008) 68:4340–6. doi: 10.1158/0008-5472.CAN-07-6705
61. Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, et al. Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp Med*. (2003) 198:1391–402. doi: 10.1084/jem.20030267
62. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature*. (2003) 425:307–11. doi: 10.1038/nature01874
63. Casazza A, Laoui D, Wenes M, Rizzolio S, Bassani N, Mambretti M, et al. Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell*. (2013) 24:695–709. doi: 10.1016/j.ccr.2013.11.007
64. Squadrito ML, De Palma M. Macrophage regulation of tumor angiogenesis: implications for cancer therapy. *Mol Aspects Med*. (2011) 32:123–45. doi: 10.1016/j.mam.2011.04.005
65. Missiaen R, Mazzone M, Bergers G. The reciprocal function and regulation of tumor vessels and immune cells offers new therapeutic opportunities in cancer. *Semin Cancer Biol*. (2018) 52:107–16. doi: 10.1016/j.semcancer.2018.06.002
66. Apte RS, Chen DS, Ferrara N. VEGF in signaling and disease: beyond discovery and development. *Cell*. (2019) 176:1248–64. doi: 10.1016/j.cell.2019.01.021

67. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. (1992) 359:843–5.
68. Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, et al. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell*. (2003) 112:645–57. doi: 10.1016/s0092-8674(03)00154-5
69. Eubank TD, Roda JM, Liu H, O'Neil T, Marsh CB. Opposing roles for HIF-1α and HIF-2α in the regulation of angiogenesis by mononuclear phagocytes. *Blood*. (2011) 117:323–32. doi: 10.1182/blood-2010-01-261792
70. Roda JM, Wang Y, Sumner LA, Phillips GS, Marsh CB, Eubank TD. Stabilization of HIF-2 induces sVEGFR-1 production from tumor-associated macrophages and decreases tumor growth in a murine melanoma model. *J Immunol*. (2012) 189:3168–77. doi: 10.4049/jimmunol.1103817
71. Goel HL, Mercurio AM. VEGF targets the tumour cell. *Nat Rev Cancer*. (2013) 13:871–82. doi: 10.1038/nrc3627
72. Harney AS, Arwert EN, Entenberg D, Wang Y, Guo P, Qian BZ, et al. Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage-derived VEGFA. *Cancer Discov*. (2015) 5:932–43. doi: 10.1158/2159-8290.CD-15-0012
73. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol*. (2000) 2:737–44. doi: 10.1038/35036374
74. De Palma M, Murdoch C, Venneri MA, Naldini L, Lewis CE. Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends Immunol*. (2007) 28:519–524. doi: 10.1016/j.it.2007.09.004
75. Matsubara T, Kanto T, Kuroda S, Yoshio S, Higashitani K, Kakita N, et al. TIE2-expressing monocytes as a diagnostic marker for hepatocellular carcinoma correlates with angiogenesis. *Hepatology*. (2013) 57:1416–25. doi: 10.1002/hep.25965
76. De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*. (2005) 8:211–26. doi: 10.1016/j.ccr.2005.08.002
77. Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell*. (2011) 19:512–26. doi: 10.1016/j.ccr.2011.02.005
78. Coffelt SB, Wellenstein MD, De Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer*. (2016) 16:431–46. doi: 10.1038/nrc.2016.52
79. Albini A, Bruno A, Noonan DM, Mortara L. Contribution to tumor angiogenesis from innate immune cells within the tumor microenvironment: implications for immunotherapy. *Front Immunol*. (2018) 9:527. doi: 10.3389/fimmu.2018.00527
80. Benevides L, De Carvalho DD, Tiezzi DG, de Andrade JM, da Fonseca DM, Donate PB, et al. IL17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment. *Cancer Res*. (2015) 75:3788–99. doi: 10.1158/0008-5472.can-15-0054
81. Liang W, Ferrara N. The complex role of neutrophils in tumor angiogenesis and metastasis. *Cancer Immunol Res*. (2016) 4:83–91. doi: 10.1158/2326-6066.CIR-15-0313
82. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
83. Mecklenburgh KI, Walmsley SR, Cowburn AS, Wiesener M, Reed BJ, Upton PD, et al. Involvement of a ferroprotein sensor in hypoxia-mediated inhibition of neutrophil apoptosis. *Blood*. (2002) 100:3008–16. doi: 10.1182/blood-2002-02-0454
84. Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T, et al. Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. *J Exp Med*. (2005) 201:105–15. doi: 10.1084/jem.20040624
85. Coffelt SB, de Visser KE. Immune-mediated mechanisms influencing the efficacy of anticancer therapies. *Trends Immunol*. (2015) 36:198–216. doi: 10.1016/j.it.2015.02.006
86. Kujawski M, Kortylewski M, Lee H, Herrmann A, Kay H, Yu H. Stat3 mediates myeloid cell-dependent tumor angiogenesis in mice. *J Clin Invest*. (2008) 118:3367–77. doi: 10.1172/JCI35213
87. Rivera LB, Meyronet D, Hervieu V, Frederick MJ, Bergsland E, Bergers G. Intratumoral myeloid cells regulate responsiveness and resistance to antiangiogenic therapy. *Cell Rep*. (2015) 11:577–91. doi: 10.1016/j.celrep.2015.03.055
88. Huo X, Li H, Li Z, Yan C, Agrawal I, Mathavan S, et al. Transcriptomic profiles of tumor-associated neutrophils reveal prominent roles in enhancing angiogenesis in liver tumorigenesis in zebrafish. *Sci Rep*. (2019) 9:1–11. doi: 10.1038/s41598-018-36605-8
89. Gaudry M, Bregerie O, Andrieu V, El Benna J, Pocidalo MA, Hakim J, et al. Intracellular pool of vascular endothelial growth factor in human neutrophils. *Blood*. (1997) 90:4153–61. Available online at: http://www.bloodjournal.org/content/90/10/4153.long
90. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci USA*. (2006) 103:12493–8. doi: 10.1073/pnas.0601807103
91. Pahler JC, Tazzyman S, Erez N, Chen YY, Murdoch C, Nozawa H, et al. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. *Neoplasia*. (2008) 10:329–40. doi: 10.1593/neo.07871
92. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, et al. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature*. (2007) 450:825–31. doi: 10.1038/nature06348
93. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep*. (2015) 10:562–73. doi: 10.1016/j.celrep.2014.12.039
94. Kumar V, Patel S, Tcyganov E, Gabrilovich DI, Kumar V, Patel S, et al. The nature of myeloid-derived suppressor cells in the tumor microenvironment. *Trends Immunol*. (2016) 37:208–20. doi: 10.1016/j.it.2016.01.004
95. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*. (2008) 111:4233–44. doi: 10.1182/blood-2007-07-099226
96. Mjösberg J, Spits H. Human innate lymphoid cells. *J Allergy Clin Immunol*. (2016) 138:1265–76. doi: 10.1016/j.jaci.2016.09.009
97. Chen X, Xie Q, Cheng X, Diao X, Cheng Y, Liu J, et al. Role of interleukin-17 in lymphangiogenesis in non-small-cell lung cancer: enhanced production of vascular endothelial growth factor C in non-small-cell lung carcinoma cells. *Cancer Sci*. (2010) 101:2384–90. doi: 10.1111/j.1349-7006.2010.01684.x
98. Huang Q, Duan L, Qian X, Fan J, Lv Z, Zhang X, et al. IL-17 promotes angiogenic factors IL-6, IL-8, and vegf production via Stat1 in lung adenocarcinoma. *Sci Rep*. (2016) 6:36551. doi: 10.1038/srep36551
99. Bruno A, Ferlazzo G, Albini A, Noonan DM. A think tank of TINK/TANKs: tumor-infiltrating/tumor-associated natural killer cells in tumor progression and angiogenesis. *J Natl Cancer Inst*. (2014) 106:dju200. doi: 10.1093/jnci/dju200
100. Bruno A, Focaccetti C, Pagani A, Imperatori AS, Spagnoletti M, Rotolo N, et al. The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer. *Neoplasia*. (2013) 15:133–42. doi: 10.1593/neo.121758
101. Kwee BJ, Budina E, Najibi AJ, Mooney DJ. CD4 T-cells regulate angiogenesis and myogenesis. *Biomaterials*. (2018) 178:109–21. doi: 10.1016/j.biomaterials.2018.06.003
102. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity*. (2005) 23:344–6. doi: 10.1016/j.immuni.2005.10.001
103. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, et al. CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell*. (2009) 16:91–102. doi: 10.1016/j.ccr.2009.06.018
104. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature*. (2011) 475:226–30. doi: 10.1038/nature10169
105. Palazon A, Tyrakis PA, Macias D, Velica P, Rundqvist H, Fitzpatrick S, et al. An HIF-1alpha/VEGF-A axis in cytotoxic T cells regulates tumor progression. *Cancer Cell*. (2017) 32:669–83 e5. doi: 10.1016/j.ccell.2017.10.003
106. Tian L, Goldstein A, Wang H, Ching Lo H, Sun Kim I, Welte T, et al. Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming. *Nature*. (2017) 544:250–4. doi: 10.1038/nature21724

107. Yang C, Lee H, Pal S, Jove V, Deng J, Zhang W, et al. B cells promote tumor progression via STAT3 regulated-angiogenesis. *PLoS ONE*. (2013) 8:e64159. doi: 10.1371/journal.pone.0064159
108. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell*. (2010) 17:121–34. doi: 10.1016/j.ccr.2009.12.019
109. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med*. (1971) 133:275–88.
110. Jain RK, Duda DG, Willett CG, Sahani DV, Zhu AX, Loeffler JS, et al. Biomarkers of response and resistance to antiangiogenic therapy. *Nat Rev Clin Oncol*. (2009) 6:327–38. doi: 10.1038/nrclinonc.2009.63
111. Ebos JM, Kerbel RS. Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nat Rev Clin Oncol*. (2011) 8:210–21. doi: 10.1038/nrclinonc.2011.21
112. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer*. (2008) 8:592–603. doi: 10.1038/nrc2442
113. Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med*. (2001) 7:987–9. doi: 10.1038/nm0901-987
114. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer Cell*. (2014) 26:605–22. doi: 10.1016/j.ccell.2014.10.006
115. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kgamma is a molecular switch that controls immune suppression. *Nature*. (2016) 539:437–42. doi: 10.1038/nature19834
116. Schmittnaegel M, Rigamonti N, Kadioglu E, Cassara A, Wyser Rmili C, Kiialainen A, et al. Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci Transl Med*. (2017) 9:eaak9670. doi: 10.1126/scitranslmed.aak9670
117. Allen E, Jabouille A, Rivera LB, Lodewijckx I, Missiaen R, Steri V, et al. Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci Transl Med*. (2017) 9:eaak9679. doi: 10.1126/scitranslmed.aak9679
118. Du Four S, Maenhout SK, Niclou SP, Thielemans K, Neyns B, Aerts JL. Combined VEGFR and CTLA-4 blockade increases the antigen-presenting function of intratumoral DCs and reduces the suppressive capacity of intratumoral MDSCs. *Am J Cancer Res*. (2016) 6:2514–31.
119. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol*. (2019) 19:369–82. doi: 10.1038/s41577-019-0127-6
120. Toor SM, Elkord E. Therapeutic prospects of targeting myeloid-derived suppressor cells and immune checkpoints in cancer. *Immunol Cell Biol*. (2018) 96:888–97. doi: 10.1111/imcb.12054
121. Motzer RJ, Tannir NM, McDermott DF, Aren Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *N Engl J Med*. (2018) 378:1277–90. doi: 10.1056/NEJMoa1712126
122. McDermott DF, Drake CG, Sznol M, Choueiri TK, Powderly JD, Smith DC, et al. Survival, durable response, and long-term safety in patients with previously treated advanced renal cell carcinoma receiving nivolumab. *J Clin Oncol*. (2015) 33:2013–20. doi: 10.1200/jco.2014.58.1041

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

frontiers in Oncology

ORIGINAL RESEARCH
published: 20 September 2019
doi: 10.3389/fonc.2019.00907

Bexarotene Reduces Production of CCL22 From Tumor-Associated Macrophages in Cutaneous T-Cell Lymphoma

Kayo Tanita[1†], Taku Fujimura[1†], Yota Sato[1], Chunbing Lyu[1], Yumi Kambayashi[1], Dai Ogata[2], Satoshi Fukushima[3], Azusa Miyashita[3], Hideki Nakajima[4], Motoki Nakamura[5], Akimichi Morita[5] and Setsuya Aiba[1]*

[1] Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan, [2] Department of Dermatology, Saitama Medical University, Saitama, Japan, [3] Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan, [4] Department of Dermatology, Kochi Medical School, Kochi University, Kochi, Japan, [5] Department of Geriatric and Environmental Dermatology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

OPEN ACCESS

Edited by:
Maija Hollmén,
University of Turku, Finland

Reviewed by:
Graham Robert Leggatt,
University of Queensland, Australia
Nahum Puebla-Osorio,
University of Texas MD Anderson Cancer Center, United States

***Correspondence:**
Taku Fujimura
tfujimura1@mac.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: 30 April 2019
Accepted: 02 September 2019
Published: 20 September 2019

Citation:
Tanita K, Fujimura T, Sato Y, Lyu C, Kambayashi Y, Ogata D, Fukushima S, Miyashita A, Nakajima H, Nakamura M, Morita A and Aiba S (2019) Bexarotene Reduces Production of CCL22 From Tumor-Associated Macrophages in Cutaneous T-Cell Lymphoma. Front. Oncol. 9:907. doi: 10.3389/fonc.2019.00907

Bexarotene is a third-generation retinoid X receptor-selective retinoid that has been approved for use in the treatment of both early and advanced cutaneous T-cell lymphoma (CTCL). Although bexarotene has been used for decades in the treatment of CTCL, little is known about the mechanisms underlying its anti-tumor effects in CTCL patients. This study therefore focused on the immunomodulatory effects of bexarotene *in vivo* using an EL4 mouse T-cell lymphoma model, followed by investigation in CTCL patients treated with bexarotene. Intraperitoneal injection of bexarotene significantly decreased expressions of CCL22, CXCL5, CXCL10, and p19 in the tumor microenvironment. Based on those results, we then evaluated serum levels of CCL22, CXCL5, and CXCL10 in 25 patients with CTCL, revealing that CCL22 was significantly increased in advanced CTCL compared with early CTCL. Next, we evaluated serum levels of CCL22, CXCL5, and CXCL10 in CTCL patients treated with bexarotene. Serum levels of CCL22 were significantly decreased in 80% of CTCL patients who responded to bexarotene therapy. In addition, immunofluorescence staining revealed $CD163^{+}$ M2 macrophages as the main source of CCL22. Moreover, bexarotene decreased the production of CCL22 by M2 macrophages generated from monocytes *in vitro*. Our findings suggest that the clinical benefits of bexarotene are partially attributable to suppressive effects on the production of CCL22 by M2-polarized tumor-associated macrophages.

Keywords: advanced CTCL, bexarotene, tumor-associated macrophages, CCL22, immunomodulation

INTRODUCTION

Most cutaneous T-cell lymphomas (CTCLs) start as an indolent disease that progresses slowly, but finally advances to skin tumors followed by lymph node and visceral involvements (1). Since CTCL is a rare disease, and since established criteria for staging and response evaluation for CTCL are limited, few prospective clinical trials for advanced CTCL have been reported, and guidelines for the treatment of CTCL have yet to be established (2, 3). Instead, several preclinical studies have

been used to determine the optimal therapy for CTCL (4–6). Among them, Shono et al. reported that mycosis fungoides (MF), the most common subtype of CTCL, shows high expression of CCR4 on the cell surface, correlating with poor prognosis of MF (4).

Moreover, Kim et al. reported that mogamulizumab therapy significantly prolonged progression-free survival (PFS) compared with vorinostat therapy for recurrent, advanced CTCL patients [hazard ratio, 0.53; 95% confidence interval (CI), 0.41–0.69; $p < 0.0001$) (3), and suggested immunotherapy as a promising option for the treatment of advanced CTCL. However, the optimal first-line therapy for advanced CTCL has remained unclear. Such reports suggested the importance of evaluating the production of CCR4 ligands CCL17 and CCL22 in the tumor microenvironment of CTCL. In addition, according to these preclinical studies, malignant T cells in CTCL have been shown to exhibit features of the regulatory T-cell (Treg) phenotype, Th2 phenotype, and Th17 phenotype (5), suggesting that not only Tregs and Th2-related factors, but also Th17-related factors are important in understanding the immunological background of CTCL.

Bexarotene is a third-generation retinoid X receptor (RXR)-selective retinoid that has been approved for use in the treatment of both early and advanced CTCL (7–9). Although bexarotene has been used for decades in the treatment of CTCL, and several preclinical studies have suggested anti-CTCL mechanisms are involved in the efficacy of this drug (10–12), little is known about the exact mechanisms underlying its anti-tumor effects in CTCL patients *in vivo* (7–9, 11, 12). Since bexarotene is useful for both early and advanced CTCL, bexarotene is applied in the real world to ultraviolet-tolerant early CTCL patients as a first-line therapy. Most such patients will subsequently need another type of therapy (2). Evaluating the immunological background of CTCL is therefore important, and this study focused on the immunomodulatory effects of bexarotene *in vivo* using an EL4 mouse T-cell lymphoma model, followed by investigation in CTCL patients treated with bexarotene.

MATERIALS AND METHODS

Ethics Statement for Animal and Human Experiments

The protocol for the animal study was approved by the ethics committee at Tohoku University Graduate School of Medicine for Animal Experimentation, Sendai, Japan (permit number: 2017MdLMO-216). The research complied with the Tohoku University Graduate School of Medicine's Animal Experimentation Ethics guidelines and policies. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol for the human study was approved by the ethics committee at Tohoku University Graduate School of Medicine, Sendai, Japan (permit number: 2018-1-772). All patients provided written informed consent to participate.

Animals and T-Cell Lymphoma Cell Line

C57BL/6 mice (5–8 weeks old) were purchased from Japan Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in the animal facility at Tohoku University Graduate School of Medicine. The EL4 murine T-cell lymphoma cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum. All mice were bred under specific pathogen-free conditions at Tohoku University Graduate School of Medicine.

Tumor Inoculation and Treatment

EL4 T-cell lymphoma cells (100 μl of 2 $\times 10^6$ cells/ml) were subcutaneously injected into female C57BL/6 mice (13). For quantitative (q)RT-PCR and enzyme-linked immunosorbent assay (ELISA), 0.15 mg bexarotene was intraperitoneally injected on day 12, and the tumor was harvested on day 14. For qRT-PCR, the whole tumor was frozen with liquid nitrogen, then crushed with a Cryo-Press (MICROTEC, Chiba, Japan), as described previously (14). Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. For the therapeutic experiments, we measured the size of established tumors with calipers (Mitsutoyo, Utsunomiya, Japan) and estimated tumor volume using the following formula: $\pi/6 \times \text{length} \times \text{width}$ (14). Starting on day 6, we intraperitoneally injected 0.15 mg of bexarotene or 0.30 mg of anti-CCL22 antibodies (R&D Systems, Minneapolis, MN) on day 6 and day 12. Tumor-bearing animals were sacrificed when the tumor resulted in severe ulceration or reached a size of 1,000 mm^3.

RNA Extraction and Quantitative Real-Time PCR Experiments

Total RNA was extracted using an RNeasy Micro kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's instructions. RNA was eluted with 14 μl of RNase-free water. DNase I treatment (RNase-Free DNase Set; Qiagen) was performed to remove contaminating genomic DNA. Reverse transcription was performed with the SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Amplification reactions were performed using an Mx 3000P Real-Time Quantitative PCR System (Stratagene, San Diego, CA). Relative mRNA expression levels were calculated for each gene and each time point after normalization against GAPDH using the ΔCt method or ΔΔCt method.

Patients

Data from 25 CTCL patients were collected from five clinical sites in Japan. Pathologists and dermatologists in each institute had diagnosed these patients with CTCL both clinically and pathologically. No patients who were administered bexarotene had received any systemic therapies previously. We have summarized the clinical information in **Table 1**.

Reagents

The following antibodies were used for immunofluorescence (IF): mouse anti-human CD163 phycoerythrin-conjugated

TABLE 1 | Characteristics of patients with CTCL.

	Age	Sex	Subtype	Stage	Therapy	Response for bexarotene	CCR4
Case 1	46	F	MF	T3N0M0B0 stage IIB	Bexarotene	PR	+
Case 2	46	M	MF	T3N0M0B0 stage IIB	Bexarotene	PR	+
Case 3	37	M	MF	T1aN0M0B0 stage IA	NB-UVB	N.A.	N.A.
Case 4	45	M	PCPTCL	T3bN1M0 stage IIIA	Bexarotene	SD	–
Case 5	44	M	MF	T4N0M0B0 Stage IIIA	Bexarotene	PD	+
Case 6	60	M	MF	T2bN0M0B0 stage IB	Bexarotene	PD	+
Case 7	81	M	ALCL	T3bN0M0 ALCL stage IIIB	Bexarotene	PD	N.A.
Case 8	78	M	MF	T2bN0M0 stage IB	Bexarotene	CR	N.A.
Case 9	84	F	MF	T1bN0M0 stage IA	Bexarotene	PR	N.A.
Case 10	51	F	MF	T3N3M0B0 stage IVA2	Bexarotene	PD	+
Case 11	75	F	MF	T1aN0M0 stage IA	Topical steroid	N.A.	N.A.
Case 12	67	M	MF	T2bN0M0 stage IB	NB-UVB	N.A.	N.A.
Case 13	44	M	MF	T1bN3M0 Stage IVA2	Bexarotene	PR	+
Case 14	48	F	LyP	T2aN0M0 stage IIA	Bexarotene	CR	–
Case 15	63	F	MF	T2bNxM0B0 stage IIA	NB-UVB	N.A.	+
Case 16	26	F	PCALCL	T1bN0M0 stage IA	Topical steroid	N.A.	–
Case 17	86	F	ATLL	stage IV (Ann Arbor)	Bexarotene	PR	+
Case 18	69	M	MF	T3N0M0 stage IIB	Bexarotene	PR	+
Case 19	67	F	PCPTCL	T3aN3M0B0 stage IIIB	Bexarotene	CR	–
Case 20	76	M	NKTL	T3bNxM0B0 stage IIIA	Bexarotene	PD	–
Case 21	70	M	MF	T3N0M0B0 stage IIB	Bexarotene	PD	+
Case 22	42	F	MF	T4N0M0 Stage IIIA	Bexarotene	PD	+
Case 23	70	M	MF	T2bN0M0B0 Stage IB	Bexarotene	PR	+
Case 24	59	M	MF	T1aN0M0B0 stage IA	Topical steroid	N.A.	N.A.
Case 25	62	F	MF	T3N0M0 stage IIB	Bexarotene plus NB-UVB	PR	+

MF, mycosis fungoides; PCPTCL, primary cutaneous peripheral T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; PCALCL, primary cutaneous anaplastic large-cell lymphoma; ATLL, adult T-cell leukemia/lymphoma; NKTL, natural killer T-cell lymphoma; NB-UVB, narrowband ultraviolet B.

monoclonal antibody (R&D Systems), rabbit polyclonal anti-CCL22 antibody (Biorbyt, Cambridge, UK), rabbit polyclonal anti-CXCL5 antibody (Lifespan Bioscience, Seattle, WA), mouse anti-CXCL10 antibody (Lifespan Bioscience), Alexa Fluor 488-conjugated anti-mouse rat immunoglobulin (Ig)G (Abcam, Tokyo, Japan), and Alexa Fluor 488-conjugated anti-rabbit goat IgG (Abcam). We used the following antibodies for immunohistochemical staining: rabbit polyclonal antibodies for human CCL22 (R&D Systems) and human CXCL5 (LifeSpan Bioscience).

Tissue Samples and Immunohistochemical Staining

Each sample was processed for single staining of CCL22 and CXCL5, and developed with liquid permanent red (DAKO, Santa Clara, CA). Briefly, formalin-fixed, paraffin-embedded tissue samples were sectioned at 4 μm and deparaffinized. After protease treatment for antigen retrieval, sections were blocked with goat serum for 10 min, then exposed to primary antibodies at 4°C overnight. Sections were developed with 3-Amino-9-ethylcarbazole (AEC).

Tissue Samples and IF Staining

For cryosections, each sample was frozen in optimal cutting temperature embedding medium, and 6-μm sections were fixed with cold acetone for 10 min and blocked with IF buffer (PBS, 5% bovine serum albumin). Each section was therefore incubated with relevant antibodies. Slides were mounted in DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) and examined using an LSM 700 microscope equipped with a SPOT digital camera (Zeiss, Oberkochen, Germany).

Cytokine ELISA

Secretion of CCL22 in each tumor was determined using ELISA kits (R&D Systems), according to the manufacturer's instructions. Serum levels of CCL22, CXCL5, and CXCL10 in each CTCL patient were measured according to the manufacturer's instructions. For patients treated with bexarotene, serum was obtained at 0 days and/or 4 weeks after bexarotene administration (300 mg/m^2), then stored for ELISA analysis of serum levels of each chemokine. For peripheral blood monocytes (PBMo)-derived M2 macrophages, after the 7-day culture, supernatants were collected and secretion of CCL22, CXCL5, and sCSD163 was determined by ELISA kits (R&D Systems), according to the manufacturer's instructions.

Data from each donor were obtained as the mean of duplicate assays.

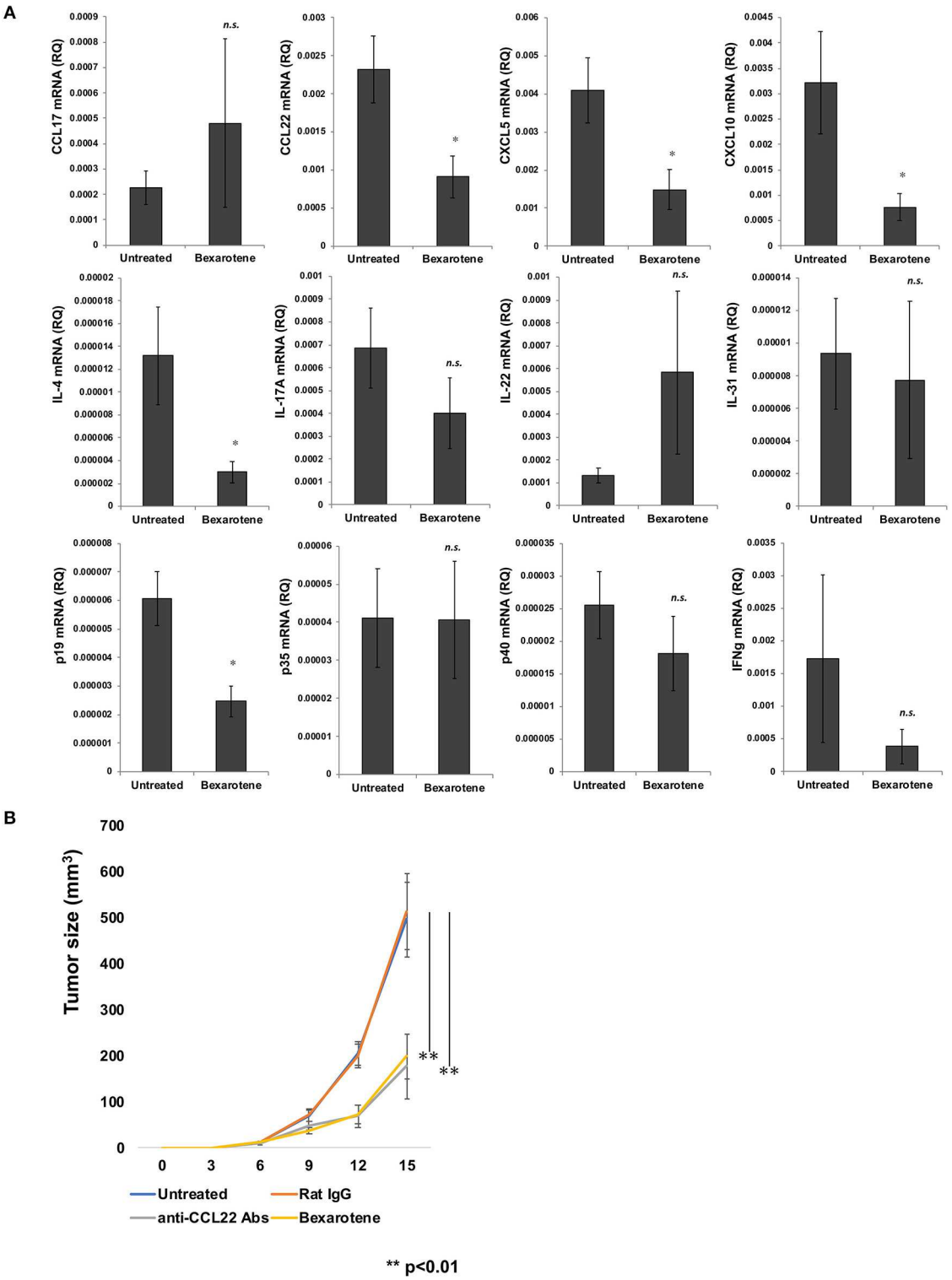


FIGURE 1 | Intraperitoneally administered bexarotene modifies chemokine and cytokine expression in mouse EL4 T-cell lymphoma. Expression of chemokines and cytokines in EL4 T-cell lymphoma was analyzed by quantitative RT-PCR using the ΔCt method ($n = 5$). Averages of five independent experiments are shown **(A)**. We subcutaneously injected 100 μl of 2×10^6 cells/ml of EL4 T-cell lymphoma cells, and intraperitoneally injected 0.15 mg bexarotene or 0.30 mg anti-CCL22 antibodies (R&D Systems, Minneapolis, MN) at day 6 and day 12 ($n = 5$ for each treated group) Error bar represents ± standard deviation. **(B)** One representative experiment of two is shown $^*p < 0.05$, $^{**}p < 0.01$. Error bar represents ± standard deviation.

Culture of M2 Macrophages From Human Peripheral Blood Monocytes

$CD14^+$ monocytes were isolated from peripheral blood mononuclear cells from healthy donors using MACS beads (CD14 microbeads; Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's protocol. $CD14^+$ monocytes (2×10^5/ml) were cultured in complete medium containing 100 ng/ml of recombinant human M-CSF for 5 days, as previously reported (15, 16). On day 5, monocyte-derived macrophages were treated with recombinant human IL-4 (20 ng/ml) with or without bexarotene (10 ng/ml−1 μg/ml) for 48 h, and culture supernatant was harvested.

Flow Cytometry

The surface expression of CD163 and arginase 1 on macrophages was analyzed by flow cytometry. Cell staining was conducted with PE-conjugated anti-CD163 (R & D system), FITC-conjugated anti-arginase 1 (R & D system), PE-conjugated isotype control Ab (BD Bioscience, Tokyo, Japan), or FITC-conjugated isotype control Ab (BD Bioscience). The cells were analyzed with a C6 flow cytometer (Acuri Cytometers Inc., Ann Arbor, MI).

Statistical Analysis

For a single comparison of two groups, the Mann–Whitney *U*-test was used. The level of significance was set at $p < 0.05$.

RESULTS

Immunomodulatory Effects of Intraperitoneal Injection of Bexarotene on the Tumor Microenvironment of EL4 T-Cell Lymphoma

Since the immunological microenvironment of CTCL resembles that of atopic dermatitis (16–18), and as tumor-associated macrophages (TAMs) have been reported to play a significant role in stimulating the developing tumor microenvironment by periostin and IL-4 in lesional skin of mycosis fungoides (MF) (16), we hypothesized that bexarotene might affect the immunological functions of TAMs in tumor sites of CTCL. To investigate the immunomodulatory effects of bexarotene on the tumor microenvironment *in vivo*, we used the mouse EL4 T-cell lymphoma model. First, we evaluated TAM-related chemokines, Th1/Th2-related cytokines and proinflammatory cytokines. Intraperitoneal administration of bexarotene significantly decreased expressions of CCL22, CXCL5, CXCL10, IL-4, and p19 mRNA in the tumor microenvironment (**Figure 1A**). No significant differences were seen in the expressions of CCL17, IL-17A, p35, or p40 mRNA.

Bexarotene Suppresses the Growth of EL4 T-Cell Lymphoma *in vivo*

Since bexarotene significantly decreased expression of CCL22 mRNA in the tumor microenvironment, we hypothesized that bexarotene, as well as anti-CCL22 antibody (Ab) could suppress the growth of EL-4 T cell lymphoma *in vivo*. We examined the therapeutic effects of bexarotene *in vivo* using the EL-4 murine T-cell lymphoma model. We treated EL-4 murine T-cell lymphoma (3–4 mm in diameter) on the backs of mice by intraperitoneal injection of bexarotene (0.15 mg/mouse) or anti-CCL22 Ab (0.30 mg/mouse) on days 6 and 12. For the control antibody, we used rat IgG (0.30 mg/mouse). Both bexarotene and anti-CCL22 Ab significantly suppressed the growth of EL-4 T-cell lymphoma (**Figure 1B**).

Serum Levels of CCL22, CXCL5, and CXCL10 in Patients With CTCL

Since intraperitoneal injection of bexarotene decreased expression of CCL22, CXCL5, and CXCL10 mRNA in EL4 mouse T-cell lymphoma, we hypothesized that serum levels of CCL22, CXCL5, or CXCL10 might be associated with response in CTCL patients treated with bexarotene. We therefore first evaluated serum levels of CCL22, CXCL5, and CXCL10 in 9 patients with early CTCL and 16 patients with advanced CTCL (**Table 1**). Serum levels of CCL22 were significantly increased in advanced CTCL compared with early CTCL (**Figure 2**). In contrast, no significant difference in serum levels of CXCL5 and CXCL10 were seen between early and advanced CTCL (**Figure 2**). Next, we evaluated serum levels of CCL22, CXCL5, and CXCL10 before and after administration of oral bexarotene. All patients were administered bexarotene at 300 mg/m^2/day. Serum levels of CCL22 were decreased in CTCL patients who

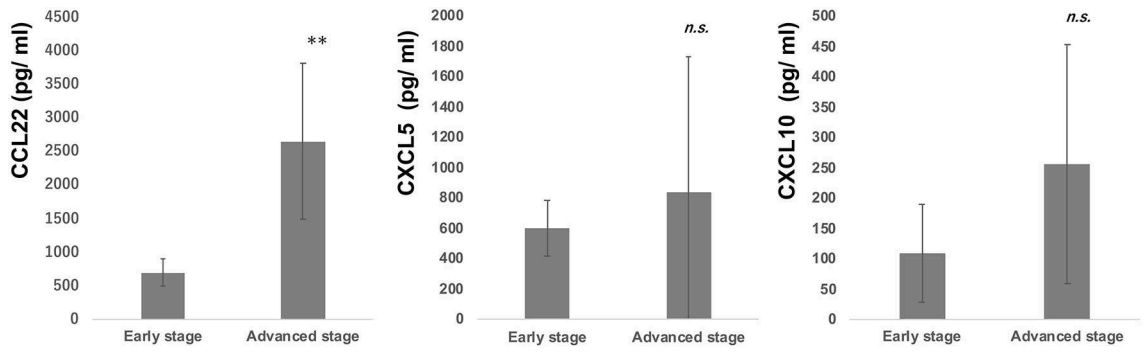


FIGURE 2 | Serum levels of CCL22, CXCL5, and CXCL10 in early and advanced CTCL patients. Serum levels of CCL22, CXCL5, and CXCL10 were examined by ELISA in 9 early CTCL patients and 16 advanced CTCL patients. $^{**}p < 0.01$, Student's *t*-test; *n.s.*, not significant. Error bar represents ± standard deviation.

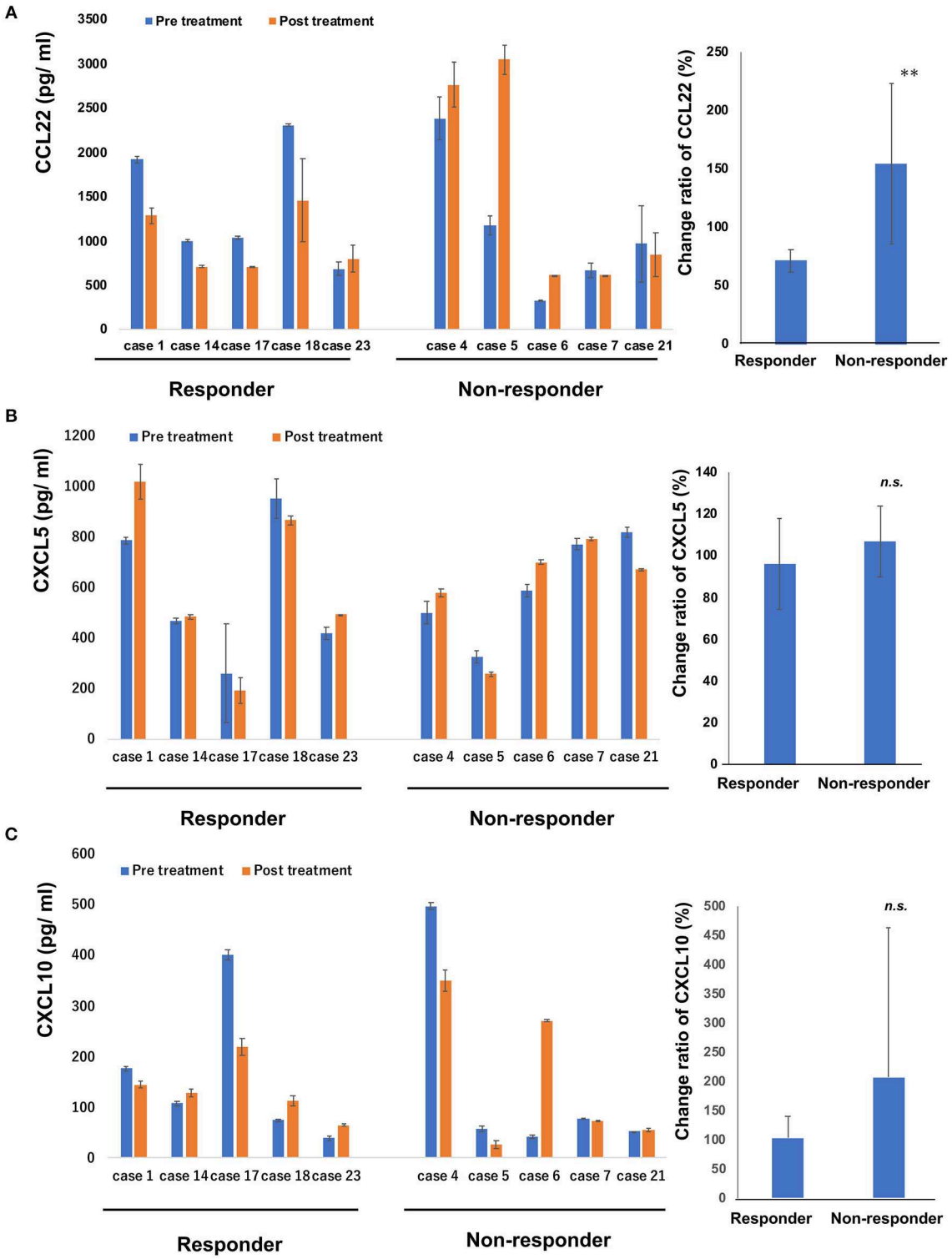


FIGURE 3 | Serum levels of CCL22, CXCL5 and CXCL10 in patients treated with bexarotene. Serum levels of CCL22 **(A)**, CXCL5 **(B)**, and CXCL10 **(C)** in responders ($n = 5$) and non-responders ($n = 5$) at day 0 and day 28 were measured by ELISA. Error bar represents ± standard deviation. Change ratio is calculated described below: (post-treatment serum chemokine)/(pre-treatment serum chemokine level) × 100 (%). Change ratios of serum CCL22 **(A)**, CXCL5 **(B)**, and CXCL10 **(C)** in CTCL are calculated in each sample and the average are shown. Error bar represents ± standard deviation. $^{**}p < 0.01$, Mann–Whitney *U*-test; *n.s.*, not significant.

responded to bexarotene therapy, but were increased in CTCL patients who showed progressive disease 4 weeks after starting bexarotene therapy (**Figure 3A**). The change ratio of serum CCL22 in CTCL patients who responded to bexarotene therapy was significantly lower than that in CTCL patients who showed progressive disease by 4 weeks after starting bexarotene therapy (**Figure 3A**). In contrast to serum CCL22, no differences were identified in the change ratios of serum CXCL5 (**Figure 3B**) or CXCL10 (**Figure 3C**) among responders and non-responders to bexarotene therapy.

CD163$^+$ TAMs Produce CCL22, but Not CXCL5 and CXCL10 in Patients With MF

Since oral intake of bexarotene decreased serum CCL22 in CTCL patients, we next investigated source cells of CCL22, CXCL5, and CXCL10 in the lesional skin of MF, as the largest subtype of CTCL. The number of CCL22-producing cells significantly increased in advanced stages compared to that in the early stage (**Figure 4A**). In contrast, the number of CXCL5-producing cells significantly decreased in parallel with MF stage (**Figure 4A**). CXCL10-producing cells were not detected in the lesional skin of MF (data not shown). IF staining revealed that CD163$^+$ M2 macrophages mainly produced CCL22 (**Figure 4B**), but a few CD163$^+$ M2 macrophages produced CXCL5 in the lesional skin of MF (**Figure 4B**).

Bexarotene Decreased Production of CCL22 From Monocyte-Derived M2 Macrophages *in vitro*

Since CD163$^+$ TAMs produced CCL22 in the lesional skin of MF, we hypothesized that bexarotene might decrease the production of CCL22 from CD163$^+$ M2 macrophages. To test this, we

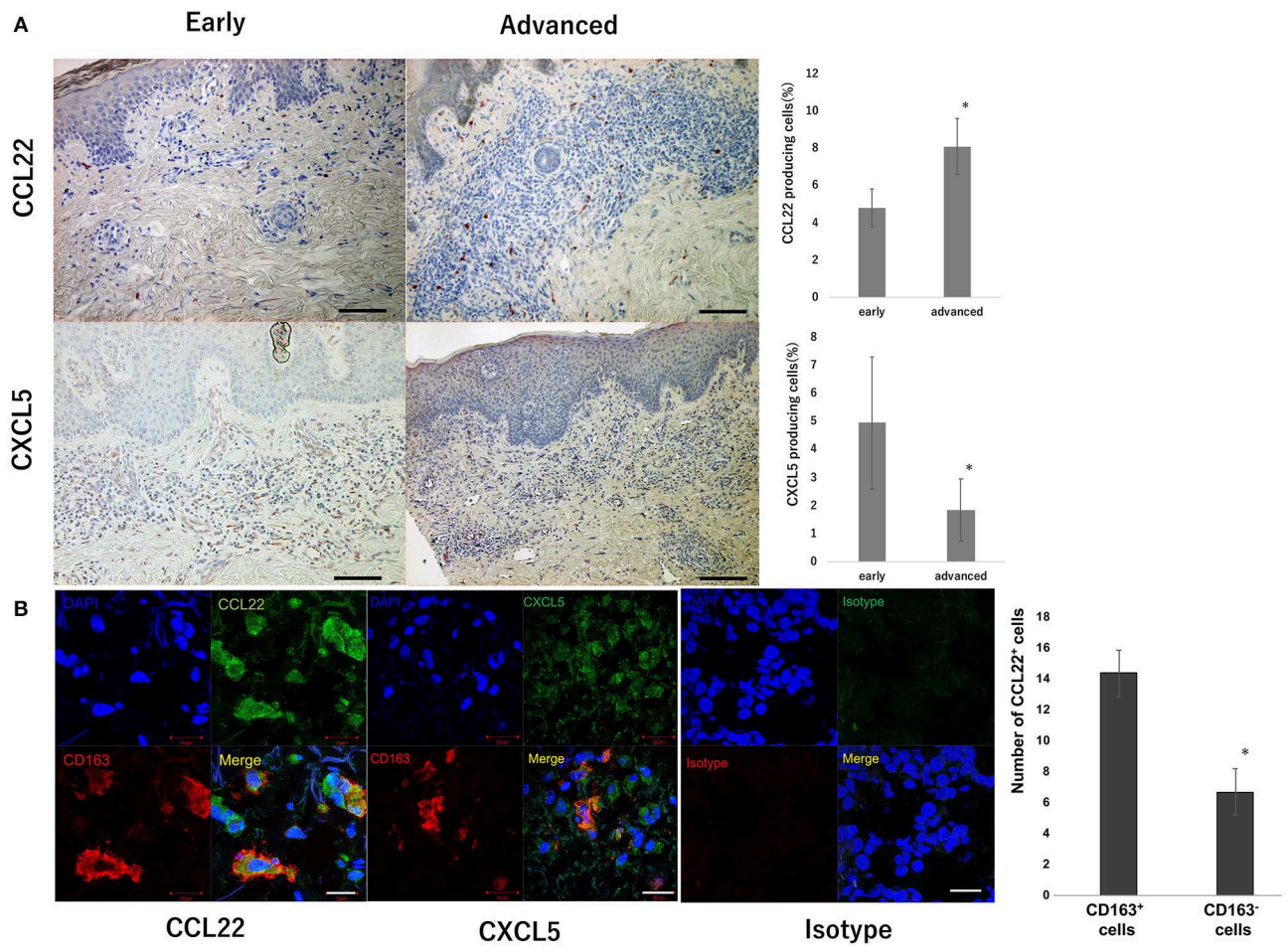


FIGURE 4 | CCL22 producing cells in the lesional skin of MF. Representative paraffin-embedded tissue samples from the lesional skin at each stage of MF. Sections were deparaffinized and stained using anti-CCL22 or anti-CXCL5 antibodies. Three representative high-power fields of each section were selected from dermis associated with a dense dermal lymphoid infiltrate. Sections were developed with 3-Amino-9-ethylcarbazole. The percentage of CCL22$^+$ and CXCL5$^+$ cells was calculated as follows: CCL22$^+$ cells or CXCL5$^+$ cells/hematoxylin-positive cells ×100. Quantification of percentages of CCL22$^+$ cells and CXCL5$^+$ cells from 9 early CTCL patients and 16 advanced CTCL patients is shown. Scale bar, 100 μm. Error bar represents ± standard deviation **(A)**. IF staining of CTCL for CCL22 (green), CD163 (red), and DAPI (blue, nuclei), and CXCL5 (green), CD163 (red), and DAPI (blue, nuclei). A merged image is also shown, with green and red combining into yellow. The isotype control IgG1 stains as red or green. Scale bar, 20 μm **(B)**. Representative specimens from three cases are shown. The number of immunoreactive cells was counted at a magnification of ×400. The average of three independent fields in each sample was calculated (*$p < 0.05$). Error bar represents ± standard deviation.

evaluated the production of chemokines from CD163$^+$ M2 macrophages using M2 macrophages generated from peripheral blood mononuclear cells (PBMCs) in healthy donors (15). Production of CCL22 was significantly decreased by bexarotene in a dose-dependent manner (**Figure 5A**). The purity of cultured CD163+ M2 macrophages is >90% as assessed by FACS analysis (**Figure 5B**).

DISCUSSION

Since most CTCL treatment requires subsequent additional therapy (2), evaluation of the immunological background of CTCL is important. For example, Richardson et al. reported that bexarotene reduced the expression of chemokine receptors to suppress the chemotaxis of CTCL cells to CCL17 *in vitro* (12). They concluded that bexarotene might inhibit migration of CTCL cells to the skin by suppressing CCR4 expression

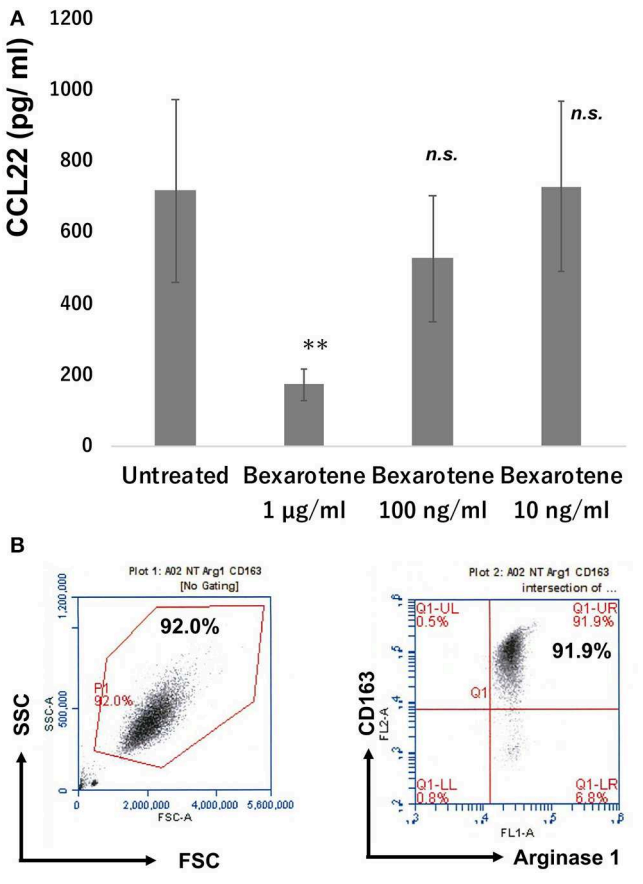


FIGURE 5 | Production of CCL22 from M2 macrophages treated with bexarotene. Culture supernatant from M2 macrophages was harvested as described in section Materials and Methods and measured by ELISA ($n = 3$). Data from each donor were obtained from triplicate assays, and mean ± SD was calculated. Representative data from at least three independent experiments are shown. $^{**}p < 0.01$, Mann–Whitney *U*-test; *n.s.*, not significant. Error bar represents ± standard deviation **(A)**. M-CSF-induced M2 macrophages from PBMCs: the expression of CD163 and arginase 1 was examined by flow cytometry **(B)**.

(12). In other reports, bexarotene selectively induced CTCL lineages to increase integrin β7 expression and function prior to growth arrest and apoptosis *in vitro* (11). Although those reports partially explain the efficacy of bexarotene for CTCL patients, direct evidence for the immunomodulatory effects of bexarotene is lacking. Since most CTCL treatment requires subsequent additional therapy (2), evaluation of the immunological background of CTCL is important.

TAMs create an immunosuppressive tumor microenvironment by producing various chemokines that attract other immunosuppressive cells such as Tregs, myeloid-derived suppressor cells (MDSCs), or even attract CTCL cells to establish the tumor microenvironment (12, 16, 19). In CTCL, at least, periostin and IL-4 could determine the functional maturation of TAMs, leading to development of the characteristic microenvironment of CTCL in each stage (16). Notably, administration of IFN-α or IFN-γ, both of which have been approved for use in the treatment of CTCL, has been shown to modulate the chemokine profiles of TAMs in the lesional skin of CTCL (20), suggesting that re-polarization of TAMs into anti-tumor macrophages might be one of the possible mechanisms of anti-CTCL drugs. In the present study, IF staining suggested the possible source of CCL22 is CD163$^+$ TAMs, and bexarotene decreased CCL22 production from CD163$^+$ M2 macrophages *in vitro*, suggesting that bexarotene induces anti-CTCL effects by suppressing CCL22 production from TAMs in CTCL patients.

CCL22 diverts Tregs and controls B16F10 melanoma growth (21, 22). Indeed, intratumoral administration of anti-CCL22 antibody inhibited B16F10 melanoma growth by decreasing Treg recruitment at the tumor site (21), suggesting that a reduction in tumor-derived CCL22 could suppress melanoma growth. In CTCL, Chang et al. reported that anti-CCR4 antibody significantly suppressed MAC-1 mouse CTCL growth *in vivo* by inhibiting CCR4/CCL22 pathways and antibody-dependent cellular cytotoxicity activities (22). Since CCL22 attracts CCR4$^+$ lymphocytes, such as CTCL cells, Tregs, and Th2 cells (23), the decrease in CCL22 might suppress the development of tumor mass *in vivo*. Indeed, in this study, bexarotene decreased serum CCL22 in 80% of CTCL patients who responded clinically to bexarotene, but did not decrease serum CCL22 in any of the CTCL patients who were not clinically responsive to bexarotene. These data suggested that CCL22 could provide a biomarker to evaluate the efficacy of bexarotene in patients with CTCL.

Since intraperitoneal injection of bexarotene decreased CXCL5 and CXCL10 mRNA expression in EL4 mouse T-cell lymphoma *in vivo*, we evaluated serum levels of these chemokines in patients with early and advanced CTCL. Unlike CCL22, no differences in serum levels of CXCL5 and CXCL10 were identified between early and advanced CTCL. Moreover, no differences in serum levels of CXCL5 and CXCL10 were identified between responder and non-responder patients. Although both CXCL5 and CXCL10 are important for immunosuppression in the tumor microenvironment by recruiting polymorphonuclear MDSCs, neutrophils, Tregs, and effector T cells in solid tumor such as melanoma, renal cell carcinoma, esophageal carcinoma, and

pancreatic cancer (23–27), bexarotene did not affect serum levels of CXCL5 and CXCL10 in the present study. Since no significant difference was identified in serum levels of CXCL5 and CXCL10 between early and advanced CTCL, these chemokines might not directly affect CTCL progression.

This study investigated the immunomodulatory effects of bexarotene *in vivo* using an EL4 mouse T-cell lymphoma model, followed by CTCL patients treated with bexarotene. Our findings suggested the clinical benefit of bexarotene is partially explained by the suppressive effects on the production of CCL22 from M2-polarized TAMs, which should contribute to the recruitment of CTCL cells, Tregs, and Th2 cells in the lesional skin of CTCL (**Supplementary Figure 1**).

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The protocol for the animal study was approved by the ethics committee at Tohoku University Graduate School of Medicine for Animal Experimentation, Sendai, Japan (permit number: 2017MdLMO-216). The research complied with the Tohoku University Graduate School of Medicine's Animal Experimentation Ethics guidelines and policies. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol for the human study was approved by the ethics committee at Tohoku University Graduate School of Medicine, Sendai, Japan (permit number: 2018-1-772). All patients provided written informed consent to participate.

AUTHOR CONTRIBUTIONS

TF designed the research study. KT, TF, YS, and CL performed experiments with the mouse model. KT, TF, YS, CL, and YK gathered and analyzed the human data. TF, YK, DO, SF, AMi, HN, MN, and AMo treated the patients and acquired the clinical data and samples. TF wrote the manuscript. TF and SA supervised the study.

FUNDING

This study was supported in part by the Japan Agency for Medical Research and Development (18cm0106434h0001 and 19cm0106434h0002).

ACKNOWLEDGMENTS

We wish to thank Minophagen Pharmaceutical Co. Ltd. for partially funding the mouse study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00907/full#supplementary-material

Supplementary Figure 1 | Bexarotene suppresses the recruitment of CTCL as well as Tregs and Th2 by the reduction of CCL22 from TAMs.

REFERENCES

1. Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH, et al. WHO-EORTC classification for cutaneous lymphomas. *Blood*. (2005) 105:3768–85. doi: 10.1182/blood-2004-09-3502
2. Quaglino P, Maule M, Prince HM, Porcu P, Horwitz S, Duvic M, et al. Global patterns of care in advanced stage mycosis fungoides/Sezary syndrome: a multicenter retrospective follow-up study from the Cutaneous Lymphoma International Consortium. *Ann Oncol*. (2017) 28:2517–25. doi: 10.1093/annonc/mdx352
3. Kim YH, Bagot M, Pinter-Brown L, Rook AH, Porcu P, Horwitz SM, et al. Mogamulizumab versus vorinostat in previously treated cutaneous T-cell lymphoma (MAVORIC): an international, open-label, randomised, controlled phase 3 trial. *Lancet Oncol*. (2018) 19:1192–204. doi: 10.1016/S1470-2045(18)30379-6
4. Shono Y, Suga H, Kamijo H, Fujii H, Oka T, Miyagaki T, et al. Expression of CCR3 and CCR4 suggests a poor prognosis in mycosis fungoides and Sézary syndrome. *Acta Derm Venereol*. (2019) 99:809–12. doi: 10.2340/00015555-3207
5. Rubio Gonzalez B, Zain J, Rosen ST, Querfeld C. Tumor microenvironment in mycosis fungoides and Sézary syndrome. *Curr Opin Oncol*. (2016) 28:88–96. doi: 10.1097/CCO.0000000000000243
6. Ehrentraut S, Schneider B, Nagel S, Pommerenke C, Quentmeier H, Geffers R, et al. Th17 cytokine differentiation and loss of plasticity after SOCS1 inactivation in a cutaneous T-cell lymphoma. *Oncotarget*. (2016) 7:34201–16. doi: 10.18632/oncotarget.9077
7. Zhang C, Hazarika P, Ni X, Weidner DA, Duvic M. Induction of apoptosis by bexarotene in cutaneous T-cell lymphoma cells: relevance to mechanism of therapeutic action. *Clin Cancer Res*. (2002) 8:1234–40.
8. Duvic M, Hymes K, Heald P, Breneman D, Martin AG, Myskowski P, et al. Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous T-cell lymphoma: multinational phase II-III trial results. *J Clin Oncol*. (2001) 19:2456–71. doi: 10.1200/JCO.2001.19.9.2456
9. Scarisbrick JJ, Morris S, Azurdia R, Illidge T, Parry E, Graham-Brown R, et al. U.K. consensus statement on safe clinical prescribing of bexarotene for patients with cutaneous T-cell lymphoma. *Br J Dermatol*. (2013) 168:192–200. doi: 10.1111/bjd.12042
10. Chou CF, Hsieh YH, Grubbs CJ, Atigadda VR, Mobley JA, Dummer R, et al. The retinoid X receptor agonist, 9-cis UAB30, inhibits cutaneous T-cell lymphoma proliferation through the SKP2-p27kip1 axis. *J Dermatol Sci*. (2018) 90:343–56. doi: 10.1016/j.jdermsci.2018.03.006
11. Wang L, DeMarco SS, Chen J, Phillips CM, Bridges LC. retinoids bias integrin expression and function in cutaneous T-cell lymphoma. *J Invest Dermatol*. (2015) 135:2102–8. doi: 10.1038/jid.2015.122
12. Richardson SK, Newton SB, Bach TL, Budgin JB, Benoit BM, Lin JH, et al. Bexarotene blunts malignant T-cell chemotaxis in Sezary syndrome: reduction of chemokine receptor 4-positive lymphocytes and decreased chemotaxis to thymus and activation-regulated chemokine. *Am J Hematol*. (2007) 82:792–7. doi: 10.1002/ajh.20952
13. Fujimura T, Ring S, Umansky V, Mahnke K, Enk AH. Regulatory T cells (Treg) stimulate B7-H1 expression in myeloid derived suppressor cells (MDSC) in *ret* melanomas. *J Invest Dermatol*. (2012) 132:1239–46. doi: 10.1038/jid.2011.416

14. Kakizaki A, Fujimura T, Furudate S, Kambayashi Y, Yamauchi T, Yagita H, et al. Immunomodulatory effect of peritumoral administration of interferon-beta on melanoma through tumor-associated macrophages. *Oncoimmunology*. (2015) 4:e1047584. doi: 10.1080/2162402X.2015.1047584
15. Fujimura T, Kambayashi Y, Furudate S, Asano M, Kakizaki A, Aiba S. Receptor activator of nuclear factor k-B ligand (RANKL) promotes the production of CCL17 from RANK+ M2 macrophages. *J Invest Dermatol*. (2015) 135:2884–7. doi: 10.1038/jid.2015.209
16. Furudate S, Fujimura T, Kakizaki A, Kambayashi Y, Asano M, Watabe A, et al. The possible interaction between periostin expressed by cancer stroma and tumor-associated macrophages in developing mycosis fungoides. *Exp Dermatol*. (2016) 25:107–12. doi: 10.1111/exd.12873
17. Masuoka M, Shiraishi H, Ohta S, Suzuki S, Arima K, Aoki S, et al. Periostin promotes chronic allergic inflammation in response to Th2 cytokines. *J Clin Invest*. (2012) 122:2590–600. doi: 10.1172/JCI58978
18. Ando T, Xiao W, Gao P, Namiranian S, Matsumoto K, Tomimori Y, et al. Critical role for mast cell Stat5 activity in skin inflammation. *Cell Rep*. (2014) 6:366–76. doi: 10.1016/j.celrep.2013.12.029
19. Fujimura T, Kambayashi Y, Fujisawa Y, Hidaka T, Aiba S. Tumor-associated macrophages: therapeutic targets for skin cancer. *Front Oncol*. (2018) 8:3. doi: 10.3389/fonc.2018.00003
20. Furudate S, Fujimura T, Kakizaki A, Hidaka T, Asano M, Aiba S. Tumor-associated M2 macrophages in mycosis fungoides acquired immunomodulatory function by interferon alpha and interferon gamma. *J Dermatol Sci*. (2016) 83:182–9. doi: 10.1016/j.jdermsci.2016.05.004
21. Furudate S, Fujimura T, Kambayashi Y, Kakizaki A, Hidaka T, Aiba S. Immunomodulatory effect of imiquimod through CCL22 produced by tumor-associated macrophages in B16F10 melanomas. *Anticancer Res*. (2017) 37:3461–71. doi: 10.21873/anticanres.11714
22. Chang DK, Sui J, Geng S, Muvaffak A, Bai M, Fuhlbrigge RC, et al. Humanization of an anti-CCR4 antibody that kills cutaneous T-cell lymphoma cells and abrogates suppression by T-regulatory cells. *Mol Cancer Ther*. (2012) 11:2451–61. doi: 10.1158/1535-7163.MCT-12-0278
23. Forsthuber A, Lipp K, Andersen L, Ebersberger S, Graña-Castro, Ellmeier W, et al. CXCL5 as regulator of neutrophil function in cutaneous melanoma. *J Invest Dermatol*. (2019) 139:186–94. doi: 10.1016/j.jid.2018.07.006
24. Soler-Cardona A, Forsthuber A, Lipp K, Ebersberger S, Heinz M, Schossleitner K, et al. CXCL5 facilitates melanoma cell-neutrophil interaction and lymph node metastasis. *J Invest Dermatol*. (2018) 138:1627–35. doi: 10.1016/j.jid.2018.01.035
25. Najjar YG, Rayman P, Jia X, Pavicic PG Jr, Rini BI, Tannenbaum C, et al. Myeloid-derived suppressor cell subset accumulation in renal cell carcinoma parenchyma is associated with intratumoral expression of IL1β, IL8, CXCL5, and Mip-1α. *Clin Cancer Res*. (2017) 23:2346–55. doi: 10.1158/1078-0432.CCR-15-1823
26. Lunardi S, Lim SY, Muschel RJ, Brunner TB. IP-10/CXCL10 attracts regulatory T cells: implication for pancreatic cancer. *Oncoimmunology*. (2015) 4:e1027473. doi: 10.1080/2162402X.2015.1027473
27. Lu L, Pan K, Zheng HX, Li JJ, Qiu HJ, Zhao JJ, et al. IL-17A promotes immune cell recruitment in human esophageal cancers and the infiltrating dendritic cells represent a positive prognostic marker for patient survival. *J Immunother*. (2013) 36:451–8. doi: 10.1097/CJI.0b013e3182a802cf

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

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership