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Perspective

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SUMMARY

The role of myeloid cells in tumor immunity is multifaceted. While dendritic cells support T cell-mediated tumor control, the highly heterogenous populations of macrophages, neutrophils, and immature myeloid cells were generally considered immunosuppressive. This view has led to effective therapies reinvigorating tumor-reactive T cells; however, targeting the immunosuppressive effects of macrophages and neutrophils to boost the cancer immunity cycle was clinically less successful. Recent studies interrogating the role of immune cells in the context of successful immunotherapy affirm the key role of T cells, but simultaneously challenge the idea that the cytotoxic function of T cells is the main contributor to therapy-driven tumor regression. Rather, therapy-activated intra-tumoral T cells recruit and activate or reprogram several myeloid effector cell types, the presence of which is necessary for tumor rejection. Here, we reappreciate the key role of myeloid effector cells in tumor rejection as this may help to shape future successful immunotherapies.

THE ROLE OF MYELOID CELLS IN TUMOR GROWTH IS PHASE AND CONTEXT DEPENDENT

Tumors are composed of cancer cells in close contact with different types of stromal and immune cells. The immune composition of the tumor microenvironment (TME) is highly heterogeneous across tumors, even between tumors of the same type or stage, and this was shown to impact clinical outcome.[1] A series of seminal studies on immunosurveillance of cancer in mice has strongly pointed to the importance of cytotoxic $CD8^+$ T cells in the prevention of tumor development and outgrowth.[2–4] An enormous effort has been made to define the prognostic contribution of the most abundant immune cell populations in tumors from patients treated in the pre-immunotherapeutic era. Collectively, these studies illustrated the importance of effector T cells and dendritic cells (DCs) for a good prognosis and highlighted the negative contribution of Tregs and myeloid cell populations (macrophages, neutrophils, and their immature counterparts, including myeloid derived suppressor cells) as suppressors of T cell immunity.[1] Consequently, the development of treatments to start, sustain, and boost the activation of tumor-specific T cells became a priority. This approach was guided by a framework that pinpoints important steps in the induction of an effective anticancer T cell response—also known as the cancer immunity cycle[5]—and has successfully spear-headed the development and clinical implementation of T cell based immunotherapy as a cancer treatment modality. In recent years, checkpoint inhibition,[6,7] chimeric antigen receptor (CAR) T cell administration,[8] bispecific T cell engagers,[9] and therapeutic vaccination against tumor antigens[10] have yielded clinical benefits for many patients. Moreover, many studies focused on increasing and activating DCs in the TME while preventing tumor infiltration by the negatively contributing immune cells,[11,12] the latter to alleviate extrinsic immune escape or improve the clinical response rate to immunotherapy.

Blocking the recruitment, migration, or function of myeloid cells has met varying clinical benefit (reviewed in studies by Barry et al.,[11] Lasser et al.,[13] and Ordentlich[14]). For instance, depletion of intra-tumoral monocyte or macrophage populations through inhibition of the tyrosine kinase receptor colony stimulating factor 1 receptor (CSF-1R) has been widely explored as monotherapy or in combination with checkpoint blockade.[11,13,14] While successfully depleting tumor associated monocytes/macrophages, these therapies have generally failed to provide clinical benefit, except in the case of tumors driven by the overexpression of CSF-1.[13–15] In fact, inhibition of CSF-1R-signaling was shown to even suppress the efficacy of otherwise effective immunotherapy.[16–18] Another clinically applied strategy was to inhibit chemokine signaling leading to the attraction of myeloid cells into the TME. While CCR2 antagonists reduced intra-tumoral myeloid cells, these strategies failed to elicit an overt clinical response.[11,19] Similarly, inhibition of CXCR2 to reduce intra-tumoral neutrophils displayed limited clinical efficacy.[13] CCR5 blockade increased the ratio of intra-tumoral immune-supportive to immunosuppressive macrophages, and while no direct clinical responses were observed, re-exposure to previous unsuccessful treatments resulted in higher median survival in these patients.[20,21] Targeting of specific suppressive myeloid cell populations was clinically successful in some cases. Treatment with the chemotherapeutic agent paclitaxel has been shown to specifically decrease immunosuppressive myeloid cells and to provide clinical benefit.[22,23] Clever-1-expression by macrophages is associated with impaired antigen presentation and immunotherapy resistance.[24] The use of an antibody to block Clever-1 resulted in disease control in 25%–40% of patients and this was associated with macrophage and T cell activation.[25] Targeting of IDO-expressing immunosuppressive myeloid cells, via a peptide vaccine-induced IDO-specific $CD8^+$ T cell response, resulted in a significant increase in overall

survival of metastatic lung cancer patients.[26] Finally, PI3K-γ signaling in macrophages promotes an immunosuppressive phenotype,[27] and PI3K-γ inhibition was shown to reprogram immunosuppressive tumor-associated myeloid cells into a more immunogenic phenotype, resulting in a reshaped, more inflammatory TME in mice and humans.[28,29] Although limited clinical benefit was observed, there was a relationship between strong changes in the TME and longer progression free survival or disease control.[28,29] However, aside from this handful of studies, most clinical studies that aimed to block suppressive myeloid cell infiltration and function failed to show clear clinical benefit.

Several reasons may account for this apparent failure to generate clinical benefit. First, researchers that designed clinical studies aimed at targeting suppressive myeloid cell function may have been guided by encouraging results from preclinical studies conducted in mice. Phenotypical and biological differences between human and murine myeloid cells may underly inefficient translation.[30] A second reason is the presence of compensatory mechanisms upon targeting one immunosuppressive myeloid cell population.[30] In patients with pancreatic ductal adenocarcinoma and in mouse models, enhanced numbers of $CXCR2^+$ tumor-associated neutrophils (TANs) have been reported following targeting of $CCR2^+$ macrophages.[19,31] Furthermore, a series of mouse studies demonstrated that CSF-1R inhibition augmented the attraction of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) by cancer-associated fibroblasts (CAFs).[32] Additionally, CSF-1R depletion resulted in an increase of regulatory T cells (T_{reg}s),[33] and signaling through CSF-2R on a subset of tumor-associated macrophages (TAMs), blunted the impact of CSF-1R inhibition.[33,34] An additional reason for the limited clinical benefit of suppressive myeloid cell targeting is that the role of myeloid populations during immunotherapy remains incompletely understood. Depending on several factors, including tissue environment, time, origin, metabolism, and the expression of transcription factors, myeloid cells rapidly adopt a tissue-specific signature and display phenotypic and functional differences. While under resting conditions myeloid cell heterogeneity is relatively steady, the composition of myeloid cells changes and becomes considerably more complicated during tumor progression. Inflammatory stimuli not only regulate and shape myeloid cell function locally, but also systemically. For example, they stimulate the highly responsive hematopoietic stem progenitor cells (HSPCs) in the bone marrow to engage in the emergency myelopoiesis (EM) pathway, aimed to increase the output of myeloid cells into the bloodstream. Simultaneously, reprogramming of HPSCs may occur during EM to influence myeloid cell maturation or to entrain specific functions in mature myeloid cells that are already active before recruitment into the tumor,[35,36] thereby further contributing to the heterogeneity of myeloid cells in the TME. Thus, while intra-tumoral myeloid cell composition is a highly dynamic process where myeloid cell production, maturation and functional state are rapidly adapted in response to local and systemic cues, first attempts to modulate the role of myeloid cells in cancer has largely been skewed by the context in which they have been studied, namely (untreated or unsuccessfully treated) progressively growing tumors. In this phase, the intra-tumoral immune response generally is shaped by chronic inflammation that favors the presence of immune cells that enhance rather than inhibit tumor growth, consistent with processes observed in wound healing.[37,38] In contrast, the contribution of myeloid cells during the phase of tumor destruction, a context associated with acute inflammation, is less well studied. DCs are an exception and their capacity to stimulate T cells locally is well explored.[38–40]

It has long been argued that during the destruction phase of a tumor, the immune response converges into a single mechanism that includes the activation of adaptive and innate cytotoxic mechanisms.[41] Indeed, a study examining the cytotoxic role of T cells in tumor control showed that this still occurred when T cells were deficient for multiple lytic pathways, and reported that they previously observed an interferon (IFN)γ-dependent co-infiltration with innate effector cells.[42] This suggests that during this acute destruction phase innate effector cells cooperate with T cells or even function as the sole effector cells mediating tumor regression, as discussed in the following. It was also recognized that the prognostic value of tumor-infiltrating T cells is influenced not only in a negative but also in a positive manner by other immune cells in the TME, and that the coordinated presence of several adaptive and innate immune cell types was beneficial for tumor control. It was not possible to pinpoint which cells exactly were required to colocalize in the tumor, as this was dependent on the tumor type and context studied. However, it was evident that not only the presence of T cells but that the entire immune contexture of a tumor determines clinical outcome.[1,43–45] To grasp why this is the case, one should consider the whole process of tumor cell killing. First, T cells may induce apoptosis, leading to programmed cell death of tumor cells. Subsequently, apoptotic cell removal by canonical phagocytosis (efferocytosis) is a crucial next step that prevents necrosis-induced chronic inflammation and subsequent local immune suppression.[46,47] Alternatively, T cells may induce ferroptosis of cancer cells via the release of IFNγ.[48] Consequently, these cancer cells express 1-steaoryl-2-15-HpETE-*sn*-glycero-3-phosphatidylethanolamine (SAPE-OOH) allowing their phagocytosis via binding to toll-like receptor (TLR)2 on macrophages.[49] Programmed cell removal is an orchestrated process in which damaged tumor cells and debris are actively recognized and cleared by phagocytic innate immune cells. However, cancer cells are known to interfere with pathways involved in apoptosis, ferroptosis and programmed cell removal. This suggests that the latter is another tumor surveillance mechanism, acting independently from direct T cell recognition, that evolving cancer cells need to overcome.[47,50,51] Indeed, cancer cells regularly express several different pro-phagocytic "eat-me" and anti-phagocytic "don't eat-me" signals, the expression of which varies depending on tumor and other features. Common to most tumors is the modulation of either type thereby affecting phagocytosis by macrophages or neutrophils[47,50,52–55] and blockade of trogocytosis/trogoptosis by neutrophils.[56] The existence of such alterations also indicates the importance of myeloid cells in immune mediated control of tumor growth.

Thus, for a long time the role of myeloid cells has been studied in the context of tumors that already escaped immune control and during a phase where they were progressively growing or when metastases occurred. Here, myeloid cells mainly take on a functional state that promotes tumor growth. The mechanisms

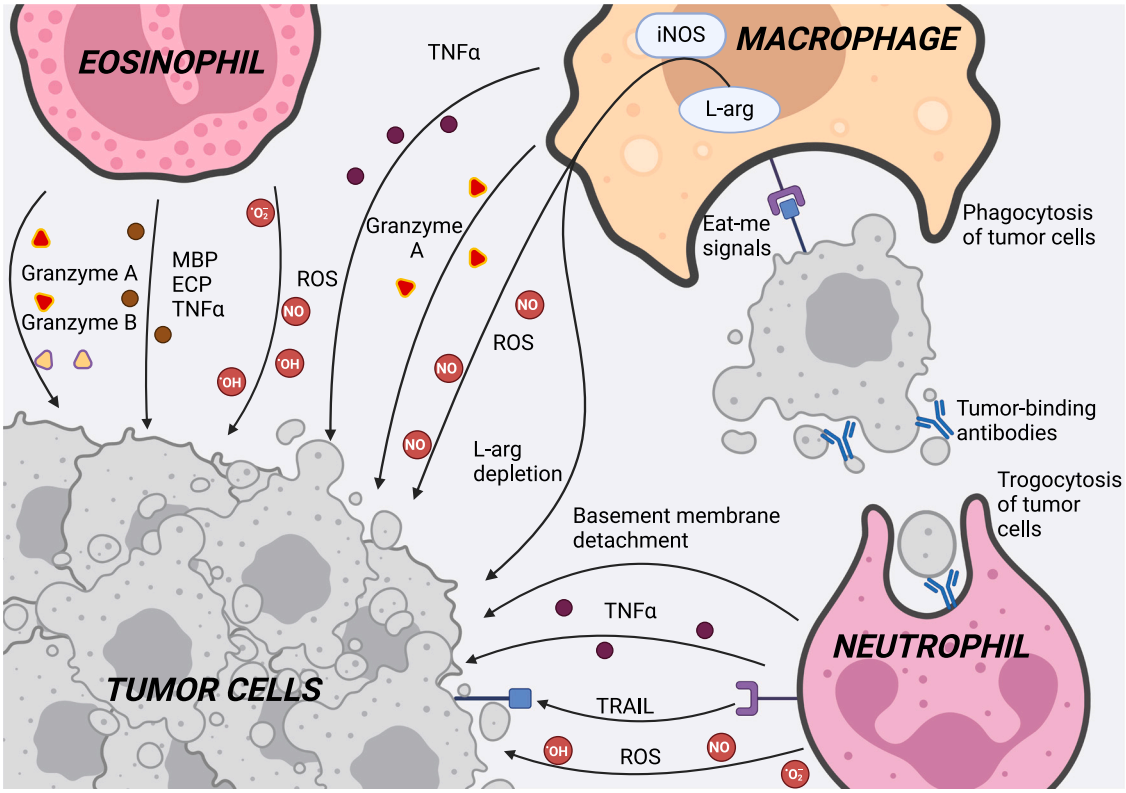

Figure 1. Mechanisms employed by myeloid effector cells for killing tumor cells
Macrophages secrete TNFα, granzyme A, and reactive oxygen species (ROS), the production of which also depletes L-arginine in the TME. In addition, macrophages phagocytose tumor cells. Neutrophils destroy epithelial basement membranes, perform trogocytosis of tumor cells, utilize TRAIL to engage the TRAIL receptor on tumor cells, and secrete TNFα and ROS. Eosinophils directly kill tumor cells through the secretion of GzmA, granzyme B, MBP, ECP, EPX, EPO, and TNFα, in addition to producing ROS. Created in BioRender. van der Burg, S. (2024) https://BioRender.com/f69l216.

underlying their tumor-supporting role have been eloquently discussed by others.[11,57,58] However, in the context of successful immunotherapy the immune system's destruction modus is reinvigorated. This phase is characterized by a different TME, now involving myeloid cells with an anti-tumor directed functional state. In this perspective, we reappreciate this role of myeloid effector cells as this has important implications for our efforts to overcome resistance to T cell therapy, and how to shape future immunotherapies.

HOW DO EFFECTOR MYELOID CELLS MEDIATE TUMOR REGRESSION?

Out of all immune cell types, macrophages are the most prominent intra-tumoral immune population and serve multiple homeostatic roles depending on tissue localization. When activated under acute inflammatory conditions, macrophages may take on a functional cell state that was historically referred to as "M1." To acknowledge that macrophages form a continuum of phenotypes between classically and alternatively ("M2") differentiated, this state has recently been pragmatically re-coined as "M1-like." The presence of M1-like macrophages is associated with a better prognosis in several cancer types.[1] Several tumor-derived chemokines, including CCL2, CCL3, CCL4, CCL5, CCL18, CCL20, and CSF-1 actively recruit monocytes into the TME.[59–61] Once in the TME, these monocytes may differentiate into suppressive myeloid cells[62,63] or—provided that appropriate local innate and adaptive inflammatory signals are present—will differentiate into activated, cytotoxic M1-like macrophages. Signals that drive this M1-like differentiation include IL-12[64] and TLR triggering,[18,65,66] but also CD40-CD40L interactions,[67] tumor necrosis factor alpha (TNFα),[68] and IFNγ.[18,66,69,70] The fact that under certain conditions TLR4 agonists may activate immune suppressive macrophages[71,72] indicates that additional signaling in the TME determines the final macrophage phenotype. Here, Notch signaling may come into play as it has extensively been implicated in cell-fate decisions. While Notch1 signaling has been shown to amplify the M1-like polarization properties of IFNγ, Notch4 signaling interferes with IFNγ and TLR4 signaling by reprogramming the macrophage's response by favoring STAT3 over STAT1 phosphorylation.[73,74]

Following activation, macrophages may utilize several pathways to kill tumor cells (Figure 1). First, they produce reactive oxygen species (ROS), predominantly superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), that cause oxidative stress and apoptosis in target cells and act in concert with secreted serine proteases, such as granzyme A (GzmA).[75,76] In tumor cells, H_2O_2 was shown to trigger an intracellular signaling pathway that leads to the activation and opening of the non-selective cation channel transient receptor potential cation channel, subfamily

M, member 2 (TRPM2), and a lethal influx of calcium (Ca^{2+}) into cancer cells.[77] Besides ROS, macrophages also produce nitric oxide (NO), the diffusion of which into surrounding tumor cells results in intracellular accumulation of toxic secondary oxidants and cellular apoptosis through activation of the mitochondrial pathway.[78,79] Interestingly, the production of NO by macrophages requires the conversion of L-arginine by inducible NO synthase (iNOS), resulting in the depletion of this critical T cell nutrient from the TME, an additional mechanism through which macrophages were shown to control tumor growth in a mouse lymphoma model.[18] The requirement of arginine catabolism for the cytotoxic function of macrophages and expression of iNOS itself, suggests that either intense iNOS activity in M1-like macrophages, the often-found co-expression of arginase, or the presence of suppressive macrophages expressing arginase may ultimately limit the cytotoxic function of macrophages via arginine depletion. This, however, is circumvented in two ways. During the generation of NO by iNOS, an intermediate subproduct is generated that inhibits arginase activity. Furthermore, when arginine is catabolized citrulline is also produced. M1-polarizing stimuli such as IFNγ and TLR signaling increase the expression of arginosuccinate synthase 1 that allows the recycling of citrulline to replenish the intracellular arginine pool (reviewed elsewhere[80]). Potentially, this mechanism also allows M1-like macrophages to keep the negative effects on tumor-specific T cells to a minimum, as suggested by experiments in which the activation of intra-tumoral T cells by a therapeutic vaccine was reduced in wild-type mice when compared to iNOS knockout mice, while still being strong enough to convey a therapeutic effect.[18]

Macrophages may also produce TNFα to induce necrosis, pyroptosis, or apoptosis of tumor cells.[17,68,81] Additionally, they kill tumor cells through phagocytosis, a process that was found to be influenced by the expression of several ligands on living cells. Macrophages are geared to phagocytose cells that express "eat-me-signals" such as calreticulin, opsonizing antibodies, SLAM family member 7 (SLAMF7) and phosphatidylserine,[82,83] but lack the expression of ligands that function as "don't-eat-me" signals. For example, CD47 is expressed by several types of cancer and functions as the ligand for the phagocytic inhibitory receptor signal-regulatory protein alpha (SIRPα), expressed by macrophages. CD47 blockade enhances tumor cell phagocytosis and elimination by macrophages.[82] The expression of SIRPα on macrophages is downregulated in an IL-6 and suppressor of cytokine signaling 3 (SOCS3)-mediated process, and this is not only required for effective M1-like activation and function,[84] but also for their capacity to induce tumor regression.[85] Several other don't-eat-me ligands expressed by tumor cells have been identified, including leukocyte immunoglobulin-like receptor subfamily B member 1[52] and CD24[86] that can bind to the complex of major histocompatibility class I (MHCI) and beta-2 microglobulin (B2m) and Siglec10, respectively. Interestingly, tumor-expressed PD-L1 may act as a don't-eat-me signal and interferes with phagocytosis by macrophages. A study of tumor-associated macrophages revealed that the expression of PD-1 by macrophages increased during cancer progression and negatively influenced their capacity to phagocytose tumor cells, and as such their capacity to reduce tumor growth.[54,87] The expression of don't-eat-me signals does not necessarily hamper phagocytosis of tumor cells, as was demonstrated for CpG-stimulated macrophages that retain the capacity to phagocytose tumor cells, irrespective of CD47 expression.[88] Potentially, the CpG-mediated changes in their carbon metabolism allow macrophages to resist the metabolic stress and associated loss of M1-like function induced by phagocytosis.[83] Finally, M1-like macrophages may also be guided by the adaptive immune system to kill antibody-opsonized tumor cells through antibody-dependent cellular cytotoxicity (ADCC), since they express the high-affinity Fc receptor FcGR1 (CD64).[57,89] The killing mechanisms applied by macrophages most likely depend on the signals by which they are activated, and the susceptibility of the tumor cells to these mechanisms.[17,65,66,68,79,88]

Neutrophils are a hallmark of the acute inflammatory response and key defenders against infections. They form the predominant immune population in the blood and are poised to be rapidly recruited to sites of acute inflammation. Their attraction to the TME mainly occurs via the chemokine receptors CXCR1 and CXCR2[90,91] whose cognate ligands (e.g., CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8) can be produced by tumor-infiltrating leukocytes, fibroblasts, endothelial cells or tumor cells.[92] Some inflammatory cytokines (e.g., IL-17, TNFα, and IFNγ) have been implicated in neutrophil mobilization, recruitment and activation in cancer.[93–96] For instance, TGF-β is associated with pro-tumor neutrophil phenotype characterized by expression of arginase.[97,98] Whereas a more tumor-rejecting neutrophil, characterized by the production of TNF and ROS, can be induced by type I IFN.[99] There are several mechanisms through which neutrophils eliminate cancer cells (Figure 1).[95,100,101] In the oxidative burst, the primary ROS H_2O_2 and superoxide are produced and released, forming the most important weapon of neutrophils.[100,102,103] Alternatively, NO is produced, which together with ROS causes oxidative damage and cell death. Neutrophils can promote tumor cell apoptosis by secretion of TNFα, acting via TNF-related apoptosis inducing ligand (TRAIL).[104,105] TRAIL signaling in tumor cells was shown to upregulate PD-L1, hence blockade of this inhibitory receptor may also synergize with neutrophil mediated killing.[106] TNFα was also found to stimulate the expression of *MET*, encoding the hepatocyte growth factor (HGF) receptor, in murine and human neutrophils.[105] In one study, *MET* was instrumental for neutrophils to leave the bloodstream, for iNOS production upon HGF stimulation, for NO release and for subsequent cancer cell killing.[105] However, this mechanism is probably context dependent as the MET-signaling pathway in neutrophils has also been shown to dampen the efficacy of immunotherapy.[107] Neutrophils also express the Fc receptors FcγR and FcαR, and can kill tumor cells through ADCC.[108] Cells opsonized by antibodies, because of a type 2 immune response or after targeted antibody immunotherapy, are recognized by neutrophils and killed through trogocytosis.[56] Neutrophil trogocytosis is inhibited by the don't-eat-me signals of the SIRPa-CD47 axis, and blocking this axis enhances neutrophil ADCC.[109,110] Similarly, the expression of PD-L1 by tumor cells may inhibit killing by PD-1^+ neutrophils.[55] Neutrophils have been described to express leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) and Siglec-10, but the role of these receptors in tumor cell phagocytosis by neutrophils has not been reported yet.[111,112]

IFNγ in the TME enhances the capacity of neutrophils to kill tumor cells in an iNOS dependent manner. IFNγ directly induces the expression of iNOS on neutrophils and when its gene product colocalizes with myeloperoxidase, this catalyzes the reaction of H_2O_2 into more toxic intermediates.[95,96] The colocalization of these two enzymes therefore increases the formation of the highly reactive and toxic peroxynitrite, suggesting that iNOS mediates the enhanced oxidative burst and cytotoxicity of IFNγ stimulated neutrophils.[95,96] The activation of iNOS may also result in L-arginine deprivation to impair the growth of T cell lymphoma.[18] Another mechanism through which neutrophils may control growth of epithelial tumors is by promoting tumor cell detachment from the basement membrane by disassembly of the integrin α6β4 adhesion complex. This was shown in a genetic model for endometrial cancer, where hypoxia drove the production of CXCL5, allowing the recruitment of neutrophils via CXCR2. In this model, neutrophils were the principal effector cells that opposed tumor growth in the early phase of progression.[113,114]

Eosinophils are granulocytes classically known for their role in allergy and anti-helminth responses, but recently a solid basis has been established with respect to their anti-tumoral functions (Figure 1). IL-5 signaling via IL-5Rα is crucial for eosinophil differentiation, maturation, expansion, survival, and release from the bone marrow.[115] The chemokines involved in the attraction of eosinophils into the TME are eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26), which mainly act via CCR3, a chemokine receptor highly expressed by eosinophils.[116] This is sustained by studies showing a spatial association between the presence of CCL11 or CCL24 and tumor-infiltrating eosinophils. RAGE and TLR4, the receptors for high-mobility group box 1 (HMGB1), may also be involved in their recruitment and activation, as well as ST2 and IL-1RAcP that can bind IL-33.[115,117] *In vivo*, IL-33 induced substantial accumulation of degranulating eosinophils and tumor control in the CT26 model for colorectal cancer.[118] IL-33 also led to eosinophil accumulation within necrotic areas of B16F10 melanoma, mediated through the induction of canonical chemokine production by the tumor cells.[118,119]

Several studies have shown direct eosinophil-mediated cytotoxicity in co-cultures of mouse or human eosinophils with different types of hematological and solid tumor cells.[115] The main cytotoxic mediators shown to be directly involved in tumor cell killing are major basic proteins (MBPs),[120] eosinophil cationic protein (ECP),[121,122] TNFα and GzmA.[123] The use of GzmB has also been reported.[119] MBPs exert their toxic effect[120] through disruption of the cell membrane[124] by increasing its permeability via surface charge interactions.[125] ECP induces functional tunnels in the membranes of target cells, thereby facilitating the entry of the other cytotoxic molecules.[126] GzmA activates a pathway that starts in mitochondria, leading to the generation of ROS and subsequently to the translocation of the endoplasmic reticulum-associated SET complex to the nucleus, where it induces single-stranded DNA damage.[127] GzmA also targets histones, lamins and several DNA damage repair proteins.[75,128] Interestingly, cells resisting caspase or GzmB induced cell death, by overexpression of the anti-apoptotic BCL-2 family members are still sensitive to GzmA.[75] In addition to MBP, ECP, TNFα and GzmA, eosinophils can also secrete Eosinophil peroxidase (EPX/EPO) to kill tumor cells directly[129] or via stimulation of macrophage TNFα and H_2O_2 release.[130,131] Finally, EPX/EPO catalyzes the formation of potentially toxic reactive oxygen and nitrogen species.[124,132] IFNγ in the TME may also contribute to eosinophil-mediated cytotoxicity. In a genetic model for inflammation-induced colorectal cancer in $Apc^{min/+}$ mice, degranulating eosinophils were essential for tumor rejection, independently of $CD8^+$ T cells, by inducing apoptotic tumor cell death via MBP secretion. These eosinophils displayed an interferon-dependent signaling signature and stimulation of human and mouse eosinophils with IFNγ (but not TLR ligands) consistently potentiated eosinophil-mediated killing of several tumor cell lines.[133] Importantly, the ability of eosinophils to recognize and bind tumor cells appears to be important for their cytotoxic effects. IL-18 and IL-33 enhance the expression of lymphocyte function-associated antigen 1 (LFA1) and CD11b on eosinophils, and the expression of intracellular adhesion molecule 1 (ICAM1) on tumor cells. This facilitates cell-cell recognition and adhesion, required for the formation of an immune synapse to deliver their cytotoxic proteins (ECP, EPX, and GzmB) into the tumor cell, ultimately resulting in tumor control by eosinophils.[118,119,123,134]

TUMOR CONTROL IS NOT ALWAYS MEDIATED VIA DIRECT CANCER CELL KILLING BY T CELLS

The key role found for tumor-specific $CD8^+$ T cells in immunosurveillance and thus the prevention of tumor outgrowth, spurred the development of T cell based immunotherapy. Simultaneously, this finding led to the negligence of several important older studies on (treatment-induced) regression of established tumors, describing the role of other immune cells that act in concert with $CD8^+$ T cells. In hindsight, this inattention for other immune effector cells may explain why T cell-based therapies are only effective in a subset of patients.

Studies in the early 70s–80s already suggested that the immune mechanisms underlying the prevention of cancer cell outgrowth may differ from those involved in the therapy of established tumors. Immune-mediated regression of tumors did not always necessitate T cells to exert direct cancer cell cytotoxicity.[135–137] More specifically, while $CD8^+$ T cells were proven imperative for tumor immune surveillance and shown to play an important role in tumor regression, it was also shown that not their cytotoxic capacity, but their ability to produce IFNγ was key to tumor control. As such, not only $CD8^+$, but also non-cytotoxic IFNγ-producing $CD4^+$ T cells are important for tumor regression.[42,138–140] This is supported by real-time imaging of $CD8^+$ T cell mediated cancer cell apoptosis *in vivo*, following adoptive cell transfer of activated $CD8^+$ cytotoxic T cells. These analyses revealed the slow rate at which cytotoxic T cells kill tumor cells *in situ*.[141] In combination with the actual limited numbers of $CD8^+$ T cells that reach solid tumors *in vivo*, it is hard to reconcile how T cell mediated killing on its own is efficient against rapidly dividing cancer cells.[42] This suggests that other mechanisms may be required to aid the observed destruction of a rapidly growing tumor. For instance, tumor-reactive $CD8^+$ T cells and $CD4^+$ T cells may utilize IFNγ and TNFα to instigate a state of permanent cancer cell growth arrest by inducing senescence,[142] a process recently shown to be key for the sustained clinical success of checkpoint inhibition.[143] Alternatively,

other effector cells may also contribute to tumor control. Isolation of tumor infiltrating lymphocytes (TILs) and macrophages from tumors at the start of regression revealed more efficient killing of cancer cells by macrophages than by T cells,[144] implying that most of the workload can be delegated to the myeloid cells present in the tumor. This would fit with the observation that a lack of myeloid effector cell infiltration is associated with lack of tumor regression despite a strong influx of tumor-specific $CD8^+$ T cells.[145] Furthermore, several studies have shown that MHC class II negative tumors are controlled by $CD4^+$ T cells when they activate previously recruited macrophages in the neighborhood of cancer cells.[146] During this process, the tumor-infiltrating macrophages ingest and present the tumor antigen on MHC class II locally to the $CD4^+$ T cells, and subsequently become endowed with the capacity to kill the cancer cells in their vicinity.[147]

There are several studies showing that immune therapy mediated tumor control may not even require the response of T cells at all. Systemic injection of polyI:C to Lewis lung carcinoma bearing mice activated tumor-infiltrated macrophages to rapidly produce inflammatory cytokines and to become M1-like macrophages in a Toll-Interleukin-1 Receptor (TIR) domain containing adaptor molecule 1 (TICAM-1)-dependent manner. Treated tumors showed TNFα-dependent hemorrhagic necrosis and growth suppression.[65] In another study, local administration of poly(I:C)+R848 not only reduced the growth of the treated murine fibrosarcoma or lung cancers but also reduced the growth of non-injected distant tumors and suppressed metastasis, while simultaneously inducing resistance to tumor rechallenge.[148] This effect was associated with increased infiltration of T cells and M1-like ($iNOS^+$) macrophages, which were able to produce T cell-attracting chemokines (CXCL10 and CCL5) and cytolytic molecules (TNFα and NO).[148] Interestingly, αCD40 therapy in combination with TNFα and a tumor-binding antibody also activated neutrophils to kill and control the growth of multiple types of cancer cells.[149] In addition, neutrophils recruited upon the tumor's intrinsic inflammatory response to hypoxia, were shown to impede early-stage tumor growth in a mouse model of PTEN-deficient uterine cancer, by inducing the detachment of tumor cell from the basement membrane. This was independent of T cells and cellular senescence.[114] Direct activation of macrophages, either via ligation of CD40 on their cell surface[150] or through metabolic rewiring using a TLR9 agonist,[88] resulted in tumor regression and growth control in models of pancreatic ductal adenocarcinoma, independently of T cells. Moreover, the adoptive transfer of macrophages and neutrophils isolated from spontaneous regression/complete resistance mice controls tumor outgrowth and induces regression independently of T cells,[151–153] provided that the tumors secrete the right chemokines to attract these immune cells.[154] Lastly, treatment of different types of human cancer cells xenografted in NOD scid gamma mouse (NSG) mice (lacking T, B, and natural killer [NK] cells) using an antibody to block CD47 to block this don't eat-me signal also resulted in tumor regression and control.[82,155]

In conclusion, the cytotoxic capacity of $CD8^+$ T cells during therapy may not display the bandwidth needed to kill enough cancer cells of a progressively growing tumor, nor may this cytotoxic capacity always be necessary to control tumor growth. $CD8^+$ T cells may also exploit the cytostatic effect of IFNγ and TNFα (rather than perforin/GzmB) to control tumor growth directly, or indirectly by activating myeloid effector cells. In fact, tumor control may even be achieved in the absence of adaptive immunity when the appropriate activation signals are provided to myeloid effector cells.

THE INTERPLAY BETWEEN T CELLS AND MYELOID CELLS IN EFFECTIVE IMMUNOTHERAPY

The introduction of the cancer immunity cycle paradigm by Chen and Mellman[156] proposes that a series of steps—linked in a cycle—is required for the generation of an effective T cell mediated immune response to tumors. The cancer immunity cycle is strongly focused on the initiation, adaptation and maintenance of tumor-specific T cells as central players in an effective anti-tumor response and provides a framework for additional key players in the response to the tumor threat. Indeed, the most recent version integrated the presence of tertiary lymphoid structures in tumors as a place where mature DCs may reinvigorate tumor-infiltrating T cells.[5] Furthermore, it highlighted the contribution of innate immune cells (monocytes, macrophages, neutrophils) and non-immune cells (e.g., CAFs) as elements that suppress T cell immunity and restrict the migration of T cells into the cancer cell nests. However as postulated previously, the function of myeloid cells is phase and context dependent. Therefore, this section first discusses several recent studies indicating that the presence of several myeloid subpopulations is associated with good clinical outcome after immunotherapy. Additionally, it discusses how these cell subpopulations support the recruitment and re-activation of T cells, to end with studies reporting that myeloid cells are recruited and activated by T cells to act as supporting tumoricidal effector cells in response to immunotherapy (Figure 2).

That effective immune mediated tumor control is associated with the presence of specific myeloid cells is well demonstrated in the context of checkpoint therapy or T cell therapy by the clear correlation between their tumor infiltration and that of T cells.[16,157,158] For instance, immunotherapy-driven expansion of neutrophils is observed in small cell lung cancer patients treated with CTLA-4 plus PD-1 checkpoint blockers. In these patients the therapy-induced increased neutrophils over lymphocytes ratio is associated with significantly better progression-free survival than in patients with a decreased ratio.[159] In addition, treatment of mouse CT26 colon cancer with αPD-L1 shows that the presence of a proinflammatory $CXCL9^+$ macrophage population is associated with therapeutic outcome.[160] Importantly, The Cancer Genome Atlas (TCGA) analysis revealed a strong positive correlation between *CXCL9* expression and treatment response in patients.[160] Moreover, an unbiased assessment of tumor-infiltrating cells by single-cell RNA sequencing and longitudinal assessment of cellular protein expression by mass cytometry revealed significant remodeling of the lymphoid and myeloid compartments after successful therapy of murine T3 sarcomas using αPD-1 and αCTLA-4 antibodies. During checkpoint therapy, multiple subpopulations of monocytes and macrophages changed over time in a manner that was partially dependent on IFNγ, with a specific increase in $iNOS^+$ macrophages in responding tumors.[158] The gene signature of these $iNOS^+$ macrophages strongly correlated to

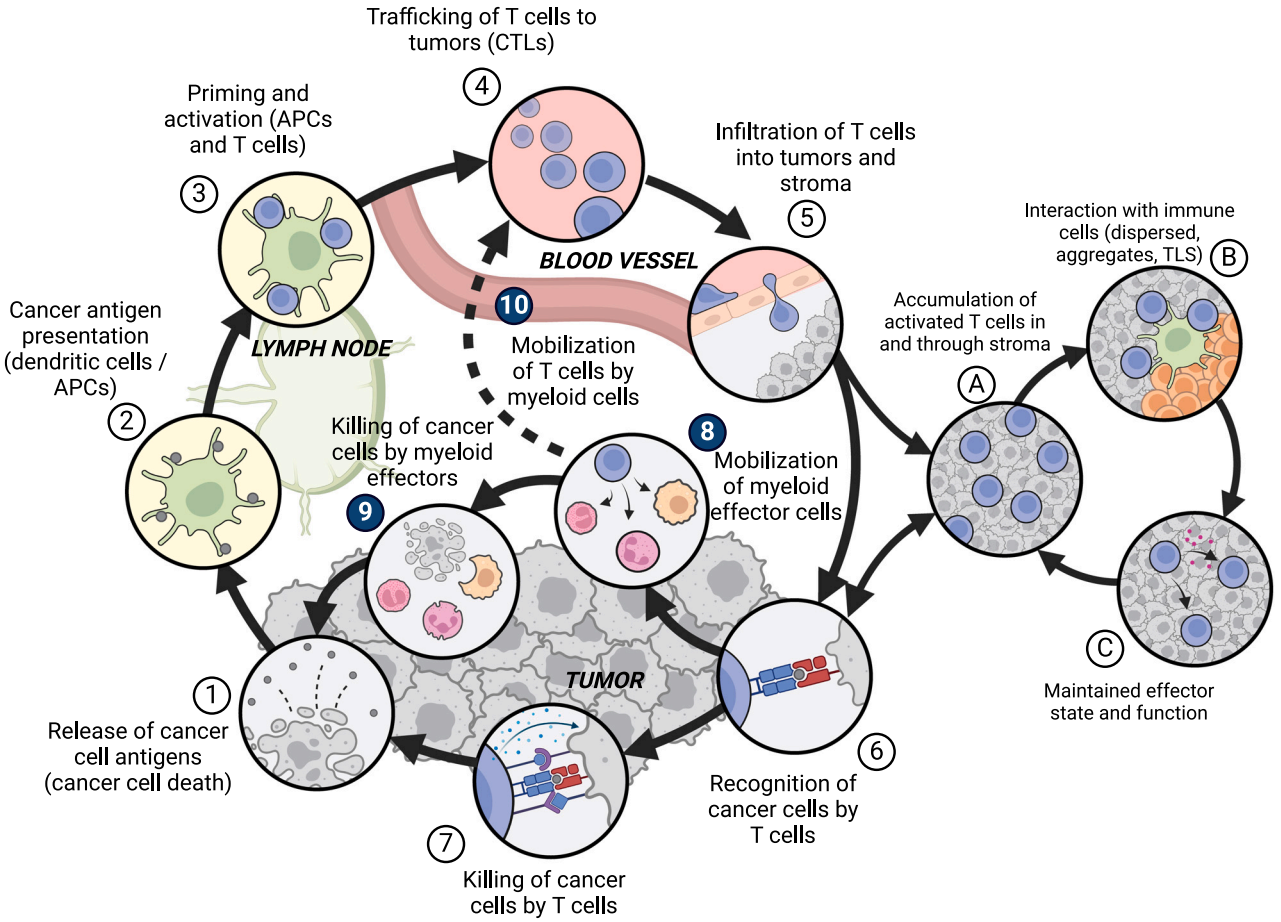

Figure 2. An updated cancer immunity cycle

The immunologic rejection of cancer cells is a self-amplifying process that is guided by an adaptive T cell response against tumor antigens (1–7) and promoted in part by generating peri-tumoral lymphoid aggregates or tertiary lymphoid structures (A-C), but is also dependent on the mobilization of innate myeloid effector cells (8) that kill cancer cells (9). These myeloid cells also amplify the rejection process by attracting additional T cells into the tumor (10). Adapted from Mellman 2023.[5] Created in BioRender. van der Burg, S. (2024) https://BioRender.com/w44c914.

that of human macrophages that were stimulated with IFNγ, indicating that they have a human counterpart.[25] Notably, in non-small cell lung cancer patients the presence of "M1 macrophage" and "peripheral T cell" signatures predicted durable clinical benefit and progression free survival when treated with αPD-1.[161] Similar observations were made for adoptive T cell therapy. For instance, the treatment of human tumor xenografts in *rag*−/− mice (lacking T and B cells) revealed that CAR T cell therapy mediated tumor rejection required IFNγR expression by host cells and was associated with a marked increase in intra-tumoral M1-like macrophages.[162] Furthermore, TIL therapy of melanoma patients showed that the tumors responding to this therapy already displayed more activated T cells, macrophages, and DCs at baseline. In these patients, successful adoptive cell transfer (ACT) not only restored the TIL repertoire and reprogrammed the tumor macrophage population toward CXCL9$^+$, CXCL10$^+$ M1-like macrophages, but also stimulated the interaction between CD8$^+$ T cells and CD68$^+$ macrophages, as determined by spatial analysis.[163] An extensive meta-analysis involving more than 3,000 checkpoint blockade treated patients across 14 types of solid tumors, sustained the notion that enhanced cross-talk between macrophages and T cells contributes to the efficacy of immunotherapy.[164] Thus, in several studies of successful T cell based immunotherapy, the clinical outcome is positively associated with the presence of myeloid effector cells. This may just reflect the capacity of the ongoing immune response to successfully reset the TME toward acute inflammation but may just as well imply that the effector function of these cells itself is a requirement for optimal tumor control.

A series of studies has delved into the contribution of these myeloid effector cells to recruit and support T cells at several steps in the cancer immunity cycle. In the murine B16-F10 melanoma model, αCTLA-4 in conjunction with ICOS activation or with αPD-1 resulted in an increased macrophage infiltrate with M1-like properties. Depletion of these macrophages was associated with a strong reduction of tumor-infiltrating CD4$^+$ and CD8$^+$T cells and an abrogation of the clinical effect, suggesting that M1-like macrophages contribute to the anti-tumor response by facilitating T cell attraction.[157] In line with this, the combination of αCTLA-4 and αPD-1 was associated with increased CD8$^+$ T cell infiltration, the production of the T cell attracting chemokines CXCL9 and CXCL10 as well as a strong influx with

CXCL9$^+$ macrophages in the AT-3ova model of triple-negative breast cancer.[165] In this model, the depletion of macrophages or blockade of the CXCL9/CXCL10 receptor CXCR3 expressed on T cells, reduced T cell infiltration and abrogated checkpoint blockade efficacy.[166] Importantly, high expression of *CXCL9* and *CXCL10*, predominantly produced by macrophages, was also shown to be associated with increased T cell influx and better responses to immune checkpoint inhibition across multiple patient cohorts and cancer types.[165,167,168] Not only macrophages but also intra-tumoral eosinophils contribute to an effective response to checkpoint blockade by attracting T cells into the TME. Lung metastases of 4T1 or PyMT breast cancer cells display specific infiltration with eosinophils that have a high expression of Siglec-F. These eosinophils were activated by IFNγ and TNFα, most likely produced by local NK, NKT and CD4$^+$ T cells, which resulted in an increased secretion of T cell recruiting chemokines CCL5, CXCL9, and CXCL16. Experiments in which these eosinophils were depleted or transfused revealed their positive impact on T cell infiltration and on tumor growth control, the latter only in mice with a functional T cell compartment.[169] Eosinophils also play an important role in the syngeneic (MC38 and CT26) and genetic ($Apc^{Min/+}$) tumor models of colorectal cancer. These tumors are spontaneously infiltrated by eosinophils, resulting in a reduced tumor growth when compared to conditions in which eosinophils are ablated. Whereas MC38-infiltrating eosinophils predominantly supported the activation and function, but not numbers of the tumor-infiltrating T cells, $Apc^{Min/+}$ tumor-infiltrating eosinophils were responsible for the recruitment and activation of tumor-controlling type 1 CD4$^+$ T cells.[170] The same study revealed that while tumors with many eosinophils were also characterized by high CD8$^+$ T cell infiltration, the reverse was not true, eluding to the eosinophil-mediated attraction of T cells in human cancers.[170] Similarly, eosinophil-mediated activation (but not recruitment) of tumor-rejecting CD8$^+$T cells was found in the *Keratin14-Cre*;*Cdh1*$^{f/f}$;*Trp53*$^{f/f}$ (KEP) mouse model for *de novo* mammary tumorigenesis after treatment with a combination of αCTLA-4, αPD-1, and cisplatin. Combination treatment resulted in an increased IL-5 production by CD4$^+$ T cells. This stimulated the release of eosinophils from the bone marrow, leading to the systemic expansion of eosinophils and their subsequent attraction into the tumor via treatment induced local production of the alarmin IL-33.[171] Part of this may be explained by the release of EDN and/or MBP by eosinophils as this is known to activate DCs,[172,173] a cell type known to activate CD8$^+$ T cells. Although in-depth analyses of eosinophil-driven attraction and activation of T cells are lacking in patients, increased eosinophil levels were shown to be associated with improved responses to immune checkpoint inhibitors across human cancer types and with an increased T cell (inflamed) signature.[171] Finally, neutrophils were also shown to foster the attraction and activation of tumor-rejecting T cells. Bacillus Calmette-Guerin (BCG) treatment of bladder cancer drove the recruitment and activation of neutrophils, their cross-talk to attracted monocytes, ultimately resulting in increased T cell trafficking to the tumor.[174] Furthermore, tumor cell-derived IFNα/γ induced the activation of Ly6Ehi neutrophils that produce IL-12b to support the tumor-infiltration, proliferation, activation, and killing capacity of CD8$^+$ T cells during PD-1 blockade in the mutagenized 4T1 breast cancer model.[175] The presence of these activated neutrophils was cross validated in several models reactive to PD-1 blockade.[175] Importantly, resections of early lung cancer revealed that also in human cancers neutrophils can stimulate T cell proliferation and effector functions.[176]

The effector myeloid cell-mediated increased infiltration of T cells is not only attributed to the production of chemokines but is also likely to be the result of vascular normalization and activation, allowing T cells to extravasate from the blood vessels in tumors. One example of this was found for the highly vascularized insulinomas that develop in the RIP1-Tag5 (RT5) mouse model of spontaneous pancreatic islet carcinogenesis. In this model, low dose local irradiation resulted in the differentiation of macrophages toward an iNOS$^+$ M1-like phenotype. Apart from chemokine-mediated cytotoxic T cell recruitment, these macrophages also boosted the attraction and function of these T cells by inducing vascular normalization and activation and by suppressing the production of angiogenic, immunosuppressive, and tumor growth factors. The effects of low dose irradiation, the accumulation of M1-like macrophages and their role in vascular normalization and T cell attraction were confirmed in patients with pancreatic cancer.[177] iNOS$^+$ macrophage derived NO was shown to induce the expression of adhesion molecules on blood vessels in the tumor that were required for T cell extravasation. Interestingly, iNOS$^+$ macrophages in tumors appear to tailor the amount of NO needed to promote endothelial activation and T cell infiltration, rather than the higher NO dose associated with immune suppression.[178] Similarly, in the MO-4 and HCmel1274 melanoma models, the depletion of regulatory T cells resulted in an increased eosinophilic tumor infiltration and tumor rejection. This was shown to rely on the eosinophil mediated production of several T cell attracting chemokines and subsequent tumor-infiltration and spatial co-localization with CD8$^+$ T cells.[179] Interestingly, the cooperation between eosinophils and CD8$^+$ T cells resulted in M1-like macrophage polarization, decreased expression of angiogenic factors and vascular normalization in tumors.[179] This eosinophil-mediated effect on tumor vasculature was shown to depend on the attraction of IFNγ-producing T cells, during αCTLA-4 mediated inhibition of tumor growth of orthotopically implanted EO771 and spontaneous MMTV-PyVT breast cancers.[180] These findings confirmed earlier observations in these models as well as in MC38 and CT26 checkpoint blockade sensitive models.[181]

Finally, there is ample evidence to support a role for M1-like macrophages in the intra-tumoral restimulation of tumor-reactive T cells, important for optimal T cell-mediated tumor control.[83] This is likely to depend on the context in which these tumor-antigen presenting macrophages interact with CD8$^+$ T cells, as they may also drive T cell exhaustion, especially under hypoxic conditions.[182] Recent work showed that treatment of MHC class I deficient murine tumors with mRNA encoded IL-2 resulted in the restoration of an inflamed TME. When combined with a tumor-targeting monoclonal antibody, this therapy restored tumor control. This effect was driven by M1-like macrophages residing in the TME and cross-presenting neoantigens to CD8$^+$ T cells that released IFNγ upon cognate interaction.[183] Thus, in the context of successful immunotherapy myeloid effector cells were shown to boost the cancer immunity cycle by increasing T cell attraction via chemokines, by facilitating

T cell extravasation from blood vessels, by optimizing the tumor's microenvironment and/or by cross-presenting tumor antigens to stimulate the effector functions of T cells (Figure 2).

Although the aforementioned text may suggest a singular direction of events, it is the interplay between T cells and myeloid cells that drives successful immunotherapy. It turns out that effector T cells also actively recruit and activate myeloid effector cells in the TME. These associations have also been made for human cancers, but mechanistic data underpinning this dual interaction is mostly limited to a preclinical setting. In the MC38 tumor model PD-1-blockade mobilized $CD8^+$ T cells from the invasive margin into the tumor cell bed, which was followed by an increase of the macrophage population with a predominant M1-like signature in responding tumors. Tumor shrinkage was particularly evident in tumors containing both $CD8^+$ T cells and myeloid cells in their centers, indicating that the attraction and functionality of both cell types was required for tumor control.[184] Direct evidence that macrophages are attracted into the tumor by checkpoint blockade activated tumor-infiltrating T cells comes from studies where the TME was analyzed after T cell depletion. In two models of glioblastoma, dual checkpoint blockade (αPD-1 and αCTLA-4) in combination with an oncolytic herpes simplex virus encoding IL-12 resulted in the complete rejection of tumors. This was associated with an increased presence of $CD4^+$ and $CD8^+$ T cells as well as M1-like macrophages. Depletion studies revealed all three cell types to be necessary for tumor control. However, $CD4^+$ T cells were key as their depletion not only resulted in failure to increase the number of M1-like macrophages but also in a lack of clinical reactivity, indicating the critical role for $CD4^+$ T cells to recruit M1-like macrophages into tumors.[185] Similar observations were made for $CD8^+$ T cells during the successful treatment of muscle invasive bladder cancer induced in *Pten*$^{fl/fl}$; *Trp53* $^{fl/fl}$ mice using a combination of αPD-1 and αCD40 antibodies. Treatment resulted in the influx of IFNγ/TNFα producing $CD8^+$ T cells and M1-like macrophages. Depletion of these cell types revealed that both were required for tumor control, albeit that depletion of $CD8^+$ T cells had the strongest negative impact. Yet while depletion of $CD8^+$ T cells led to a reduction in the number of recruited intra-tumoral M1-like macrophages, the reverse did not affect $CD8^+$ T cell infiltration or function. This indicated that the M1-like macrophages acted as effector cells downstream of the $CD8^+$ T cells and was dependent on IFNγ.[186] The cooperation of $CD8^+$ T cells with other immune effector cells was also apparent from a study in which three murine models for large transplantable tumors (B16F10, DD2-Her-2/neu, and TC-1) and the (BRAF/PTEN) inducible melanoma tumor model were used. Mice were treated with a combination of tumor-binding antibodies, IL-2, PD-1 blockade and vaccination with $CD8^+$ T cell epitopes. Tumor control critically depended on $CD8^+$ T cells but also required downstream activated macrophages, neutrophils, and NK cells for optimal protection.[187] $CD8^+$ T cell mediated orchestration of myeloid cells to become innate effector cells within the TME is also observed in models where tumor control was observed following therapeutic vaccination as single therapy or in combination with a CD3-bispecific antibody. In the RMA-Qa1b$^{-/-}$ model, vaccination resulted in an increased influx of $iNOS^+$ neutrophils and macrophages, both of which were important for the therapeutic efficacy as their depletion impaired survival.[18,188] Depletion of the two types of myeloid cells did not change the numbers or phenotype of tumor infiltrating $CD8^+$ T cells, indicating that these myeloid cells acted downstream of $CD8^+$ T cells as well as that $CD8^+$ T cells need to cooperate with the myeloid effector cells to gain control over tumor cell growth.[18] Interestingly, the neutrophils present in RMA-Qa1b$^{-/-}$ tumors lost their activated phenotype after macrophage depletion, suggesting their activation is downstream of M1-like macrophages.[18] In the B16F10 melanoma model, the clinical effect of combined vaccination and CD3 bispecific antibody treatment also required M1-like macrophages whereas the neutrophils played no role. In both models the recruitment and full activation of the myeloid effector cells in tumors was shown to be fully dependent on activated intra-tumoral T cells. Spatial analyses revealed that they recruited macrophages into the tumor and to their close vicinity via the CCR5 axis in order to enable ($iNOS^+$) M1-like macrophage differentiation and activation via T cell-derived IFNγ and endogenous TLR ligands that are released in the TME upon tumor cell death.[18] IFNγ most likely acted through the downregulation of Dicer1, thereby unleashing the M1-like state activation of macrophages.[189] In a cohort of patients with breast cancer, a similar observation was made as the response to immune checkpoint inhibition was associated with the presence of both CCR5-ligand producing ($CD8^+$ and $CD4^+$) T cells and M1-like macrophages, with a clear positive correlation between the presence of these T cells and macrophages.[18]

Myeloid effector cells may also be recruited and locally activated by tumor-specific T cells to function as the dominant effector cells in the TME. Therapeutic vaccination of TC-1 tumors was shown to result in intra-tumoral co-localization of $CD8^+$ T cells and macrophages. However, while inhibition of T cell infiltration using CXCR3-KO mice did not prevent therapy-induced regressions, depletion of macrophages did, suggesting their requirement for the observed regression.[17] Alternatively, murine KP lung and MC38 colon tumors can be successfully treated with αCD40 agonistic antibodies. Following treatment, neutrophils expanded rapidly in the tumor, and predominantly displayed a SiglecF^{lo}CD14$^+$ phenotype with an IFNγ-stimulated gene signature, rather than SiglecF^{hi} neutrophils that are known to accelerate tumor progression.[159] While the development of these tumoricidal neutrophils clearly required the CD40-mediated activation of cDCs and IFNγ-producing $CD8^+$ T cells in the tumor, neutrophil-specific knock out of IRF1, to prevent specific IFNγ-signaling and as such the capacity of neutrophils to differentiate into the SiglecF^{lo}CD14$^+$ phenotype, fully abrogated tumor control.[159] Furthermore, this IFNγ-responsive gene signature was also found in human neutrophils and an immunotherapy-driven increased neutrophil-to-lymphocyte ratio was shown to correlate with better outcome. In a separate study, the rejection of wild-type B16 melanoma after B16-GMCSF tumor cell vaccination depended on both $CD4^+$ Th1 and Th2 cells. In this setting, Th2-derived IL-4 was shown to mediate the recruitment of eosinophils and Th1-derived IFNγ was required for the activation of $iNOS^+$ macrophages. Together, these myeloid cells were able to kill tumor cells in a manner that involved their production of NO and superoxide.[190] In the regressive (REG) colon cancer tumor model, tumor cell vaccination induced a response by $CD4^+$ and $CD8^+$ T cells in rats. These

T cells could not effectively kill tumor cells but, via their production of IFNγ, enabled macrophages to efficiently kill tumor cells.[144] In two different studies involving the adoptive transfer of tumor-specific CD4+ T cells for the control of tumor escape variants, either iNOS+ neutrophils or macrophages were required. In the first, adoptive transfer of CD4+ T cells was combined with agonistic OX40 binding antibodies and tumor cells were killed by neutrophils in an iNOS dependent manner, but it required the presence of tumor-specific CD4+ T cells, especially in the early phase of the antitumor response.[191] This αOX40-derived anti-tumor neutrophil gene signature was associated with better outcome in αCTLA-4 treated patients as well. The second study involved a combination of adoptive transfer of melanoma-specific CD4+ T cells with chemotherapy, vaccination and stimulation with polyI:C and CpG. Here, the transferred CD4+ T cells were shown to interact with MHC class II + myeloid cells at the invasive margin, resulting in the recruitment of monocytes into the TME. Importantly, these spatial interactions were also found in melanoma patients.[138] In the animal model these monocytes subsequently required IFNγ and polyI:C and CpG for full iNOS induction, adaptation to a tumoricidal phenotype and subsequent eradication of tumors in a macrophage-derived NO dependent fashion even in the absence of MHC class II and CD8+ T cells.[138] A potential explanation for why the killing of cells by myeloid effector cells remains restricted to the tumor site comes from a study in the MOPC315 murine model of multiple myeloma, in which the T cells recognize a secreted tumor neoantigen. Here, macrophages present the antigen to the T cells in the tumor and in return are activated to become M1-like macrophages.[192] This triggers the production and secretion of NO that then diffuses toward surrounding tumor cells. As a result, toxic secondary oxidants (predominantly peroxynitrite) are intracellularly accumulated, leading to apoptosis through activation of the mitochondrial pathway.[79] No killing was observed in areas with tumor antigen negative cells, and M1-like macrophages were shown to preferentially localize into antigen-rich tumor areas, together with CD4+ T cells.[193] Thus, immunotherapy-induced tumor regression can be mediated by tumoricidal myeloid effector cells, but this process is initiated and sustained locally by tumor-reactive T cells, stressing the antigen-specific nature in this process where cancer cells are killed by non-antigen specific effector cells. This view is in line with the very early observations of Evans and Alexander[135] describing that cooperation between T cells and macrophages is required for tumor control, but that macrophages need to be periodically reactivated by the T cells to retain their tumoricidal properties.

In summary, there is abundant evidence to support the notion that in the context of effective immunotherapy, macrophages, neutrophils, and eosinophils do not only positively contribute to the attraction and stimulation of tumor-specific T cells in the TME, but also cooperate with the intra-tumoral T cells or even act as the dominant contractors in the killing of tumor cells. This places T cells and myeloid effectors side-by-side at the execution phase in the cancer immunity cycle (Figure 2).

PERSPECTIVE

A series of recent studies clearly point at a key role for macrophages, neutrophils and eosinophils for immunotherapy to be successful. A profound insight on the role of these myeloid cells during the whole process of therapy-induced destruction phase of tumors will lead to a better understanding of the mechanisms underlying successful immunotherapy. Consequently, they will point at the hurdles that need to be addressed when patients do not respond to clinically active therapies. As such, it will be important to focus our future research not only on the expansion and function of T cells but also on proper examination of the presence, role and functional state of myeloid cells in clinical studies or cohorts of patients receiving immunotherapy.

Current endeavors to improve immunotherapy via the modulation of myeloid cells, are mostly addressing the immune suppressive role of myeloid cells. This approach remains valid as long as myeloid cell targeting focuses toward specifically (timed) depletion of defined suppressive cell states or prevention of their actions rather than generic targeting of total myeloid cell populations. In view of the compensatory influx of tumors by other immune suppressive cells, this may require dual targeting of the macrophages and the corresponding compensatory mechanism(s).[31–34] Also the observation that highly selective depletion of immunosuppressive macrophages, leaving other macrophage populations untouched, results in more favorable clinical outcomes during immunotherapy supports the idea to preferentially target myeloid cells in a highly specific manner.[194–196] In parallel, one should aim to recruit, activate, and/or unleash myeloid effector cell states with a positive contribution to the cancer immunity cycle. In some patients this may occur spontaneously, or is induced after modulation of immunosuppressive mechanisms, but it is likely to require active stimulation of myeloid cells, especially in tumors resistant to T cell-based therapies.[197–200] Several studies on the activation of myeloid cell effectors have been performed, generally tested as single cancer therapy with limited success,[66] but more is to be expected when such approaches are optimally positioned within an immunotherapeutic strategy that unleashes the full potential of the immune system. One potential way to give a head start to the local generation of myeloid effector cells is to interfere in the process of trained immunity. Under non-acute inflammatory conditions EM is associated with output from the bone marrow of myeloid cells poised to become suppressor cells.[35,87] Interference with systemic inflammatory signals, for instance by vaccination-induced IFNγ or by ligation of CD40, may reprogram HSPCs and their output in the blood to myeloid cells that have already acquired traits of effector cells before they enter the TME.[159,201] Another, non-mutually exclusive approach is the metabolic reprogramming of myeloid cells. One of the hallmarks of cancer is the metabolic reprogramming of the TME to support tumor growth. For the tumor-infiltrating myeloid cells these local metabolic conditions, with a lack of oxygen, glucose and amino acids while being enriched with fatty acids, are quite different from normal tissue, to which they must adapt to survive. This process changes the differentiation and function of the myeloid cells displaying enhanced glutamine (macrophages) and fatty-acid metabolism (macrophages and neutrophils) coupled to immune suppression (reviewed in a study by Li et al.[202]). Interference with glutamine metabolism resulted in the reprogramming of tumor associated macrophages toward a more inflammatory phagocytic profile.[203] In another study targeting IL-4/IL-13 induced cholesterol-25-hydroxylase in macrophages abrogated

their immunosuppressive function and resulted in the enhanced infiltration and activation of T cells, synergizing with checkpoint therapy.[204] Finally, the injection of SLIT2 protein enhanced glycolysis and reduced fatty acid oxidation in the bone marrow, with as result the production of more M1-like tumor-rejecting macrophages.[205] An exciting alternative avenue in cancer immunotherapy that could be applied to immunologically colder tumors is the exploitation of the strong anti-tumor potential of macrophages, via so-called CAR macrophages or CAR M. These are engineered macrophages that express a modified CAR, which, upon binding to its specific tumor antigen, triggers the activation of a pro-inflammatory (or M1-like) phenotype and its effector functions.[206] Currently, the therapeutic efficacy of CAR M therapy for solid cancers is being evaluated in two clinical trials (NCT04660929 and NCT03608618).

In conclusion, the response rate to T cell based immunotherapies is likely to be improved by combining them with strategies that aim to leverage the T cell supporting and tumoricidal functions of myeloid cells simultaneously. New strategies to recruit, activate or unleash such myeloid effector cells are being developed.

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DECLARATION OF INTERESTS

M.J.v.E. and J.M. are currently employed by the Peter MacCallum Cancer Center, Australia.

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