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Macrophages are critical effectors of antibody therapies for cancer

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Macrophages are innate immune cells that derive from circulating monocytes, reside in all tissues, and participate in many states of pathology. Macrophages play a dichotomous role in cancer, where they promote tumor growth but also serve as critical immune effectors of therapeutic antibodies. Macrophages express all classes of Fcγ receptors, and they have immense potential to destroy tumors via the process of antibody-dependent phagocytosis. A number of studies have demonstrated that macrophage phagocytosis is a major mechanism of action of many antibodies approved to treat cancer. Consequently, a number of approaches to augment macrophage responses to therapeutic antibodies are under investigation, including the exploration of new targets and development of antibodies with enhanced functions. For example, the interaction of CD47 with signal-regulatory protein α (SIRPα) serves as a myeloid-specific immune checkpoint that limits the response of macrophages to antibody therapies, and CD47-blocking agents overcome this barrier to augment phagocytosis. The response of macrophages to antibody therapies can also be enhanced with engineered Fc variants, bispecific antibodies, or antibody-drug conjugates. Macrophages have demonstrated success as effectors of cancer immunotherapy, and further investigation will unlock their full potential for the benefit of patients.

Keywords: ADCP, antibodies, cancer, CD47, Fc receptor, immune checkpoint, immunotherapy, macrophages, phagocytosis, SIRPα

Abbreviations: Fc, fragment crystallizable; FcγR, Fcγ receptors; CD, cluster of differentiation; SIRPα, signal-regulatory protein α; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; NK, natural killer; M-CSF, macrophage colony stimulating factor; IgG, immunoglobulin G; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; GM-CSF, granulocyte-macrophage colony stimulating factor; HSC, haematopoietic stem cell; AML, acute myelogenous leukemia; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHP, Src homology 2 domain-containing phosphatase; ITAM, immunoreceptor tyrosine-based activation motif; CLL, chronic lymphocytic leukemia; BTK, Bruton's tyrosine kinase; ADC, antibody-drug conjugate

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Macrophages and cancer immunotherapy

Cancer immunotherapy is emerging as one of the most promising areas of cancer research and treatment.[1,2] Overall, the goal of cancer immunotherapy is to stimulate a patient's immune system to recognize cancer cells as foreign and attack them. A number of recent advances have sparked an unprecedented interest in the field. In particular, breakthroughs have been made using therapies that augment T cell responses to tumors. These include chimeric antigen receptor (CAR) T cells and immune checkpoint inhibitors, such as antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the programmed death (PD)-1/PD-ligand 1 axis.[3,4] Three immune checkpoint inhibitors (ipilimumab, pembrolizumab, nivolumab) have recently been approved for melanoma, and studies applying them to other cancers are advancing rapidly.

In contrast to efforts targeting the adaptive immune system, few therapies have been aimed at stimulating the myeloid arm of the immune system to attack cancer. The myeloid immune lineage consists primarily of granulocytes and monocytes, the latter of which can differentiate into macrophages or dendritic cells. Macrophages in particular are poised to be tremendous effectors of cancer immunotherapy. These innate immune cells reside in tissues throughout the body,[5] and specialized tissue-specific macrophage populations exist, e.g., Kupffer cells in the liver, microglia in the brain, osteoclasts in bone, and alveolar macrophages in the lungs. Macrophages are capable of performing phagocytosis, a process that involves the engulfment and degradation of material such as debris, dead cells, or pathogens. They recognize material for engulfment by pattern recognition receptors, scavenger receptors, and antibody fragment crystallizable (Fc)

receptors.[6] Macrophages participate in many states of pathology, including infection, inflammatory disease, wound healing, and cancer.[7]

The complex relationship between macrophages and tumors obscures the potential that macrophages have to act as immune effectors. Macrophages are often found in high numbers within tumors, and a number of studies have found the degree of macrophage infiltration correlates with poor prognosis across many different types of cancer.[8-13] At baseline, macrophages may promote tumor growth and dissemination by supporting angiogenesis, performing matrix remodeling, and secreting growth factors and immunosuppressive cytokines.[14] These "tumor-associated macrophages" have been contrasted with pro-inflammatory or "classically activated" macrophages that attack pathogens.[15] As a result, some therapies have been designed to deplete macrophages in tumors.[16]

Natural killer (NK) cells have classically been described as the primary immune effectors of antibodies therapies due to their involvement in the process of antibody-dependent cell-mediated cytotoxicity (ADCC). However, macrophages are crucial to the efficacy of many antibodies because they perform antibody-dependent cellular phagocytosis (ADCP) (**Fig. 1A**). Macrophages express all classes of Fcγ receptors (FcγR), in contrast to NK cells which primarily express FcγRIIIa.[17,18] The contribution of macrophages has been marginalized in the past because they are more difficult to study compared to NK cells or other peripheral blood leukocytes. Macrophages do not circulate in the bloodstream; hence they cannot be purified expediently in large quantities. Instead, macrophages must be differentiated ex vivo from circulating monocytes by culturing for a week or longer in the presence of human serum or growth factors such as macrophage colony stimulating factor (M-CSF).[19,20] Moreover, macrophage-mediated cytotoxicity occurs primarily via phagocytosis,[21,22] which is technically challenging to assay and requires microscopic visualization or flow cytometry to quantify cellular engulfment. Chromium release assays, the gold standard for measuring ADCC, are insufficient for evaluating cytotoxicity by macrophages because macrophages retain the radioactive probe after phagocytosis.[21] For these reasons, macrophages are underappreciated as effector cells that can target cancer.

Evidence supporting macrophages as effectors of therapeutic antibodies for cancer

Nonetheless, macrophage phagocytosis has been found to contribute to the efficacy of monoclonal antibodies for as long as they have been investigated as therapeutics. In studies published in the early 1980s, monoclonal antibodies against tumor antigens were found to stimulate phagocytosis of cancer cells in vitro, induce macrophage infiltration into tumors, and elicit macrophage-mediated destruction of tumors in mice.[23-25] More

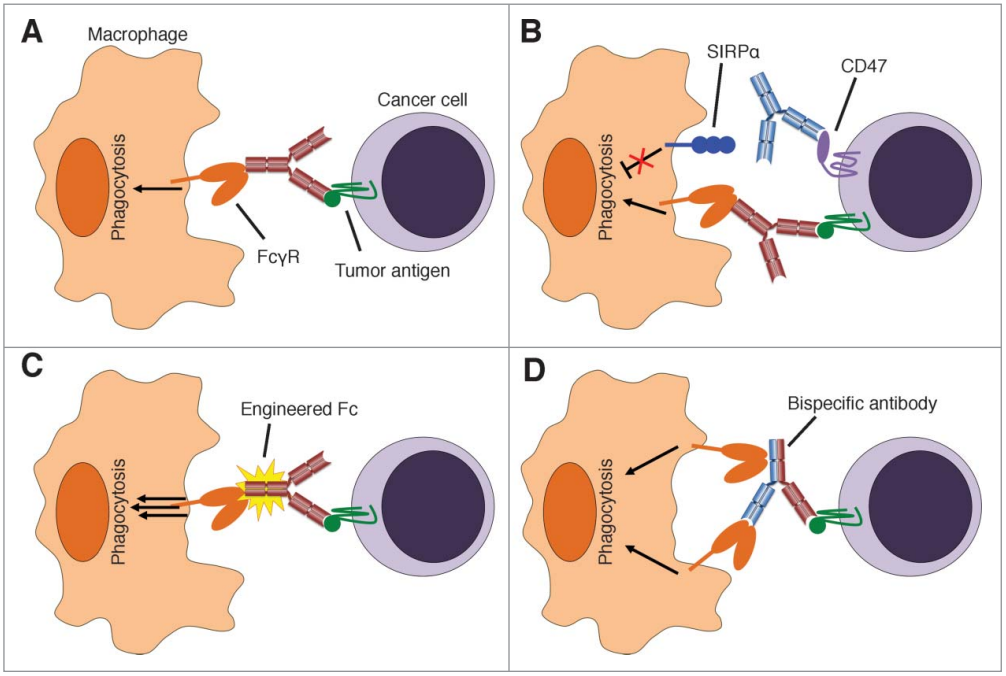

Figure 1. Augmenting macrophage responses to therapeutic antibodies. (**A**) Tumor-binding antibodies stimulate macrophage phagocytosis via Fcγ receptors (FcγR), which is a major mechanism of action of many therapeutic antibodies. (**B**) The CD47-SIRPα interaction inhibits maximal antibody-dependent phagocytosis. CD47-blocking therapies (blue antibody) prevent inhibitory signaling from SIRPα to augment macrophage activation. (**C**) Tumor-binding antibodies with engineered Fc fragments exhibit enhanced binding to Fc receptors and potently stimulate phagocytosis. (**D**) Bispecific antibodies that have dual specificity for tumor antigens and receptors on macrophages can augment phagocytosis and direct macrophage responses against tumors. "Trifunctional" antibodies have intact Fc fragments that can engage additional Fc receptors as depicted. Antibody-drug conjugates with immunostimulatory properties (not depicted) also deliver activating stimuli to macrophages.

recent studies have examined phagocytosis in response to therapeutic antibodies, such as the anti-CD20 antibody rituximab. In vitro, macrophage phagocytosis of lymphoma and leukemia cells in response to rituximab has been demonstrated in a number of studies using human macrophages.[26-30] Interestingly, macrophages polarized toward a tumor-associated state with M-CSF and IL-10 exhibited greater phagocytosis of rituximab-opsonized lymphoma cells than those polarized toward a pro-inflammatory state.[31] Polarization resulted in upregulation of multiple Fcγ receptors on macrophages, correlating with their phagocytic response. Furthermore, all subclasses of human immunoglobulin G (IgG) are able to induce human macrophage phagocytosis, as demonstrated using a panel of rituximab variants with identical variable regions but differing heavy chain isotypes.[32] Even human IgG4, which exhibits less ADCC,[33] has the potential to stimulate macrophage phagocytosis. This is likely mediated by its ability to engage Fc receptors that are present on macrophages but not NK cells. This finding suggests the majority of tumor-binding antibodies approved for therapy have the ability to stimulate macrophage phagocytosis. Antibody-dependent phagocytosis of solid tumors has also been demonstrated in vitro using anti-human epidermal growth factor receptor (HER) 2 antibodies against breast cancer and anti-epidermal growth factor receptor (EGFR) antibodies for colon cancer.[32,34] As described by Overdijk et al. in this issue of *mAbs*, daratumumab, an anti-CD38 antibody, was found to induce macrophage phagocytosis of multiple myeloma cells. Phagocytosis in response to a number of other investigational antibodies, such as anti-KIT antibodies for gastrointestinal stromal tumors,[35] has also been observed.

In vivo findings have also demonstrated a crucial role for macrophages as effectors of antibodies therapies. In studies using anti-CD20 antibodies, macrophage depletion with liposomal clodronate abrogated the ability of the antibodies to deplete normal and malignant B cells.[36,37] Similarly, CSF-1^{op} mice, which have defects in macrophage number and development, also had impaired responses to anti-CD20 antibodies.[36] In contrast, the antibodies remained effective in mice deficient in T and B cells or NK cells, suggesting macrophages are the main effectors of the antibodies in vivo.[36] Studies with transgenic mice expressing human CD20 have demonstrated that depletion of circulating cells opsonized by anti-CD20 antibodies occurs rapidly in the liver.[37] New efforts using intravital imaging have elegantly demonstrated that these effects are mediated by Kupffer cells, which immobilize and engulf the opsonized cells soon after administration of the antibodies.[38] Similarly, Kupffer cells eliminated circulating tumor cells and prevented liver metastases when antibodies were used in models of colon cancer and melanoma.[22,39] Investigations of anti-CD142 antibodies for breast cancer showed that although macrophages supported tumor growth, they were also essential for the anti-tumor effects of the antibodies.[40] Therefore, macrophages are key effectors to the efficacy of antibodies in vivo, and the reticuloendothelial system likely plays a major role in elimination of circulating tumor cells that are bound by therapeutic antibodies.

In clinical investigations, macrophages are commonly found in tumors in high numbers.[8-13] Studies on Fc receptor polymorphisms suggest antibodies have Fc-dependent mechanisms of action in patients. In particular, lymphoma patients with polymorphisms in FcγRIIIa that confer high affinity binding to antibodies exhibited greater therapeutic responses to rituximab.[41] While this receptor is expressed on both NK cells and macrophages, polymorphisms in FcγRIIa, a major mediator of phagocytosis,[42] also correlated with the therapeutic efficacy of rituximab for lymphoma, as well as cetuximab for colon cancer and trastuzumab for breast cancer.[41,43,44] Moreover, in lymphoma patients treated with conventional therapy, the degree of macrophage infiltration correlates with poor prognosis;[11] however, macrophage infiltration appears to be a favorable prognostic indicator when rituximab is added to conventional therapy.[45] These studies further implicate macrophages as important effectors for the therapeutic benefit of antibodies in patients. Other studies have examined combinations of antibody therapies with cytokines. Treatment with granulocyte-macrophage colony stimulating factor (GM-CSF), which activates macrophages and other myeloid cells, enhanced the efficacy of rituximab for follicular lymphoma and anti-GD2 antibodies for neuroblastoma.[46,47] As further evidence of the anti-tumor potential of macrophages in response to antibody therapies, a Phase 1 clinical trial of agonistic anti-CD40 antibodies demonstrated efficacy against pancreatic cancer primarily by macrophage effector functions.[48]

The CD47- signal-regulatory protein α axis: The myeloid-specific immune checkpoint

A key molecule that governs macrophage phagocytosis is CD47, a transmembrane protein that is widely expressed on the surface of many cell types throughout the body. Oldenborg et al. first identified a role for CD47 in regulating phagocytosis.[49] When the authors purified red blood cells from CD47$^{-/-}$ mice and transfused them into wild-type mice, they found that the CD47$^{-/-}$ red blood cells were rapidly cleared from the circulation.[49] The method of red blood cell removal was determined to be phagocytosis by macrophages in the spleen. This study demonstrated that CD47 serves as a "marker of self" to prevent macrophage phagocytosis. A role for CD47 in cancer was first identified from studies of haematopoietic stem cells (HSCs) and leukemia. HSCs occasionally exit their niches in the bone marrow and circulate through the peripheral blood. To avoid phagocytosis by macrophages in the spleen, these circulating HSCs upregulate CD47 on the cell surface.[50] Similarly, acute myeloid leukemia (AML) stem cells also upregulate CD47, presumably to avoid phagocytosis by splenic macrophages similar to normal HSCs.[51] CD47 was evaluated as a putative therapeutic target on AML using anti-CD47 antibodies that block the interaction between CD47 and signal-regulatory protein (SIRP) α, an inhibitory receptor on macrophages. These antibodies were able to stimulate macrophage phagocytosis of AML cells in vitro and exhibit therapeutic efficacy against AML in mouse models.[51] A broader role for CD47 in

cancer was appreciated when CD47 expression was examined on solid tumors such as ovarian cancer, bladder cancer, breast cancer, and leiomyosarcoma.[52,53] Again, CD47 was highly expressed on these cancers and treatment with anti-CD47 antibodies induced macrophage phagocytosis and stimulated anti-tumor responses in vivo, showing the broad promise of targeting the CD47/SIRPα interaction in cancer. Based on these results, a humanized anti-CD47 antibody (Hu5F9-G4) was developed at Stanford University, and it is now undergoing evaluation in a Phase 1 clinical study of patients with solid tumors (www.clinicaltrials.gov identifier: NCT02216409).

CD47 inhibits macrophage responses to therapeutic antibodies

CD47 acts by sending inhibitory signals through SIRPα, a transmembrane receptor that is expressed on macrophages and other myeloid cells.[54-56] SIRPα contains immunoreceptor tyrosine-based inhibition motifs (ITIMs) in its cytoplasmic tail. When CD47 binds to SIRPα, it causes phosphorylation of the ITIMs that activate the Src homology 2 domain-containing phosphatases SHP-1 and SHP-2.[57] The SHP phosphatases in turn cleave phosphate groups from proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs) and myosin light chains, thereby inhibiting pro-phagocytic signaling and preventing rearrangements to the cytoskeleton that are necessary for phagocytosis to occur.[57-59] Fc receptors are transmembrane proteins with extracellular domains that bind the Fc region of antibodies and cytoplasmic tails that contain ITAMs.[17] Upon binding to target-bound antibodies, conformational changes induce phosphorylation of the Fc receptor ITAMs, thereby initiating a signaling cascade that promotes phagocytosis. Phosphorylation of Fc receptor ITAMs is balanced by inhibitory signaling from the CD47-SIRPα axis.[60,61] The balance is likely mediated by SHP-1 and SHP-2 phosphatases that cleave phosphate groups from ITAMs of the Fc receptors as described above. In this manner, the CD47-SIRPα axis serves as a barrier to antibody-dependent phagocytosis. Based on these findings, CD47-blocking therapies were hypothesized to synergize with anticancer antibodies (**Fig. 1B**). Indeed, the combination of CD47-blocking antibodies with rituximab exhibited synergy in vitro and in vivo against lymphoma.[29] Furthermore, engineered SIRPα variants, 14 kDa proteins that potently block CD47 but lack the pro-phagocytic stimulus of an Fc were evaluated against cancer.[32] They synergized with rituximab, cetuximab, trastuzumab, and alemtuzumab by augmenting macrophage activity. Therefore, CD47 is a key regulator of macrophage phagocytosis, particularly when induced by therapeutic antibodies, and reagents that target the CD47-SIRPα axis may act as universal adjuvants to anticancer antibodies.

Conventional therapies and macrophage effector functions

Antibody therapies are typically used in unison with chemotherapeutic agents, and the effects of chemotherapy on macrophage effector functions are not fully understood. Agents that kill cancer cells with limited specificity may interfere with the ability of macrophages and other immune cells to act as therapeutic effectors. For example, vinca alkaloids may inhibit phagocytosis due to their effects on cytoskeletal rearrangement.[62] Even targeted therapies can have unanticipated effects on immune cell functions. Ibrutinib, a small molecule inhibitor used for the treatment of chronic lymphocytic leukemia (CLL) and mantle cell lymphoma, acts by disabling signals from Bruton's tyrosine kinase (BTK). While BTK promotes growth of B cell malignancies, it also transduces signals downstream of Fc receptors. As a consequence, ibrutinib inhibits ADCC and phagocytosis.[63,64] Although the addition of ibrutinib to rituximab regimens seems promising in clinical trials,[65] the inhibition of Fc receptor signaling suggests additional mechanisms to increase NK cell or macrophage functions may be beneficial. When combining these types of therapies with antibodies, it may be best to optimize the timing of treatments to avoid unfavorable interactions.

On the other hand, chemotherapeutic agents may stimulate inflammatory responses that enable the immune system to respond more effectively to anticancer antibodies. For example, the efficacy of doxorubicin was reduced when macrophages were inhibited, suggesting this agent acts in part by stimulating macrophage effector functions.[66] More recently, one study examined human leukemia xenografts that were refractory to treatment with alemtuzumab, a humanized anti-CD52 antibody.[67] The authors found that cyclophosphamide, a nitrogen mustard chemotherapeutic, stimulated secretion of inflammatory cytokines within the tumor microenvironment and produced synergy by increasing antibody-dependent phagocytosis. It will be important to evaluate which chemotherapeutic agents aid or hinder macrophage phagocytosis in order to tailor treatment regimens to maximize efficacy and specificity against tumors.

Engineering antibodies to engage macrophages

Based on the importance of macrophages as effector cells, additional efforts to enhance macrophage responses to antibodies are warranted. One approach is to alter the binding of antibody Fc fragments to Fc receptors via molecular engineering (**Fig. 1C**). Antibodies have been glycoengineered to lack fucosylation, which results in greater binding to Fcγ receptors. Consequently, these antibodies exhibit greater ADCC and phagocytosis, as evidenced by studies on obinutuzumab, a glycoengineered anti-CD20 antibody approved for the treatment of CLL.[68] Other protein engineering efforts have been aimed at developing Fc variants with enhanced binding to Fc receptors. Lazar et al. generated variants of human IgG1 with increased affinity for FcγRIIIa.[69] They found that these variants improved ADCC and macrophage phagocytosis in response to trastuzumab and rituximab. In another study, an anti-CD19 antibody with the same modifications improved ADCC and phagocytosis in vitro and enhanced efficacy in xenograft models of B cell malignancies.[70,71] This approach demonstrated safety and efficacy in a Phase 1 clinical study.[72] Additional engineering efforts identified variants of human IgG1 with increased binding to FcγRIIa, a major mediator of

phagocytosis, leading to increased macrophage-mediated destruction.[42] Another interesting approach created hybrids of IgG and IgA Fc chains, termed "cross-isotype" antibodies, that engage both FcαR and Fcγ receptors for enhanced myeloid effector functions including phagocytosis.[73] Conversely, when developing therapies for which immune effector functions are not desired, the response of macrophages must also be considered since they express all classes of Fcγ receptors and respond to all subclasses of human IgG. Mutant Fc variants that abolish binding to all Fcγ receptors have been described.[74,75]

An alternative strategy to engaging macrophages has focused on engineering bispecific antibodies that simultaneously bind antigens on tumor cells and receptors on macrophages (**Fig. 1D**). In this sense, they cross-link macrophages to cancer cells for enhanced efficacy and anti-tumor specificity. Many of these agents have targeted FcγRIIIa, expressed on NK cells as well as macrophages. An early attempt at this approach tested an antibody with dual specificity for HER2 and FcγRIIIa in a clinical study of patients with HER2+ adenocarcinoma.[76] Some signs of efficacy were observed, but the development of cytokine storm reactions with low dose administration precluded further investigation. New bispecifics targeting FcγRIIIa and CD30 are currently under development for Hodgkin lymphoma.[77] Chemically linked bispecific Fab fragments targeting FcγRI and HER2 have also been evaluated. This type of therapeutic was able to induce phagocytosis by macrophages in vitro and exhibited mild benefit in clinical trials.[34,78] A similar bispecific antibody targeting FcγRI and EGFR was also tested in clinical trials for solid tumors with minimal success.[79] The limited success in these studies targeting FcγRI may be due to the lack of an appropriate Fc to stimulate macrophages fully. Although bispecific antibodies targeting macrophages and tumors have not yet demonstrated sufficient efficacy in clinical trials, this approach holds much promise. Additional receptors on macrophages should be tested to determine the safest and most effective way to engage these immune cells for the benefit of patients.

Macrophage responses to antibody-drug conjugates

Antibody-drug conjugates (ADCs), which are tumor-binding antibodies conjugated to small molecules, are also emerging as novel anticancer agents. These therapeutics function by binding to tumor antigens and delivering a cytotoxic payload upon antigen internalization. However, since the antibodies can engage macrophages and other immune cells via Fc receptors, the collateral effects on immune cells must also be considered. In particular, ADCs that result in phagocytosis may in fact deliver their cytoxic payload to macrophages attacking tumors. The anti-CD30 antibody brentuximab, when tested as a naked antibody, was capable of stimulating phagocytosis and macrophage functions in vivo.[37] When brentuximab is conjugated to the cytotoxin vedotin, the resulting ADC could incapacitate macrophages and limit their function. Alternatively, ADCs could be designed to augment macrophage phagocytosis. These could include conjugates to immunostimulatory agents such as Toll-like receptor agonists or scavenger receptor ligands. Antibody conjugation to cytokines or chemokines that increase macrophage infiltration or activity could also be conceived. In one example, an anti-HER2 antibody fused with GM-CSF exhibited greater in vivo efficacy than the unmodified antibody.[80]

Conclusions

Macrophages are important mediators of the efficacy of many therapeutic antibodies for cancer. Macrophages are often present in high numbers within the tumor microenvironment, and tumor-associated macrophages may promote tumor growth in the absence of therapeutic intervention. Nonetheless, these macrophages can mount robust responses against cancer when given the appropriate antibody stimulus.[31,40,48] Macrophages fail to recognize tumor cells as foreign due at least in part to the CD47-SIRPα interaction, a myeloid-specific immune checkpoint. Studies with CD47-blocking therapies demonstrate the potential of macrophages in tumors, particularly in combination with tumor-binding antibodies. CD47-blockade lowers the threshold for macrophage phagocytosis, while tumor-binding antibodies direct macrophage attack against tumors for greater specificity. Furthermore, macrophage phagocytosis in response to antibodies may lead to antigen presentation that initiates long-lasting adaptive immune responses against tumors.[81] Additional approaches to engage macrophages in tumors include engineering Fc fragments for greater binding to Fc receptors, and the use of either bispecific antibodies that cross link macrophages and cancer cells or ADCs conjugated with immunostimulatory agents. By designing therapies that better engage macrophages, the full potential of the innate immune system can be realized for the benefit of patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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