Review

Cancer Research

Antibody-Dependent Phagocytosis of Tumor Cells by Macrophages: A Potent Effector Mechanism of Monoclonal Antibody Therapy of Cancer

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Abstract

Nowadays, it is impossible to imagine modern cancer treatment without targeted therapies, such as mAbs, that bind to tumor-associated antigens. Subsequently, mAbs can use a wide range of effector functions that mostly engage the immune system. mAbs can bridge immune effector cells with tumor cells, which can result in antibody-dependent cytotoxicity. Increasing evidence, however, identified macrophages as prominent effector cells and induction of antibody-dependent cell phagocytosis as one of the primary mechanisms of action mediated by mAbs. Macrophages are extremely effective in eliminating tumor cells from the circulation. Several immunosuppressive mechanisms may, however, hamper their function, particularly in solid malignancies. In this review, we discuss the evolving insight of macrophages as effector cells in mAb therapy and address novel (co)therapeutic strategies that may be used to fully unleash their cytotoxic capacity for the treatment of cancer. *Cancer Res; 75(23); 5008–13. ©2015 AACR.*

Introduction

mAbs represent a promising class of cancer therapeutics (1). One of the first mAbs that was approved for clinical application is the genetically engineered chimeric murine–human anti-CD20 mAb rituximab. This mAb is widely used in the treatment of B-cell malignancies and has significantly improved clinical outcome of patients. The success of rituximab sparked the development of multiple mAbs against a variety of targets for the treatment of hematologic or solid malignancies (2).

Anticancer mAbs can be directed against the tumor environment. For instance, anti- VEGF mAbs inhibit new blood vessel formation, whereas the checkpoint inhibitors anti-cytotoxic T-lymphocyte–associated protein (CTLA)-4 or anti–programmed death (PD)-1 mAbs target the immune system. However, most mAbs, like anti–HER-2 or anti-EGFR mAbs, are directed against tumor cells.

Antitumor cell mAbs use a wide range of mechanisms to induce tumor elimination (1). It is not yet resolved which of these modes of action play the most important role(s) in therapeutic success in patients. Direct effects include the induction of apoptosis or inhibition of cell growth by blocking the binding of a ligand to its growth factor receptor (Fig. 1A, I). The latter mechanism plays, for example, an important role in anti-EGFR mAb therapy, which is effective in patients with wild-type RAS, but because of various mutations in EGFR signaling routes intracellular signaling sustains even in the absence of ligand binding (3). Indirect effects require involvement of the immune system. Most clinically available mAbs are of the IgG isotype, which activates the complement cascade via binding to C1q, resulting in complement-dependent cytotoxicity (CDC; Fig. 1A, II). Furthermore, through their Fc tail, mAbs bind to Fc receptors, and thereby bridge tumor and effector immune cells.

The human Fc IgG receptor family includes several members (FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb). FcγRI, FcγRIIa, and FcγRIIIa are activating receptors, whereas FcγRIIb is inhibitory (4). FcγRIIIb is the most abundant Fc receptor that is exclusively expressed on neutrophils. This is a glycosyl phosphatidylinositol (GPI)–linked Fc receptor, and potential signaling pathways via this receptor have not yet been elucidated. Treatment with antitumor mAbs was ineffective in mice lacking one or more activating Fcγ receptors, whereas more antibody-dependent killing of tumor cells was observed in FcγRIIb$^{-/-}$ mice (5–7). Of note, expression of Fc receptors, including FcγRII, differs in mice compared with humans. Mice do not express the activating FcγRIIa, but only the inhibitory FcγRIIb. Although the FcγRIIb expression pattern on different cell types is comparable between mice and human, the balance between activating and inhibiting receptors may therefore differ. Nonetheless, it is clear that Fc receptor–mediated mechanisms of action are required for *in vivo* therapeutic efficacy. This is supported by clinical trials in which it was demonstrated that Fc receptor polymorphisms that affect affinity for IgG correlate with clinical efficacy of anti-CD20, anti-EGFR, or anti–HER-2 mAbs in cancer patients (3, 8).

Fc receptor–expressing immune cells with cytotoxic ability consist of neutrophils, natural killer (NK) cells, monocytes, and macrophages. Neutrophils efficiently kill tumor cells in the presence of IgA mAbs, but evidence that they play a major role in current IgG-based therapies is limited (9). NK cells effectively

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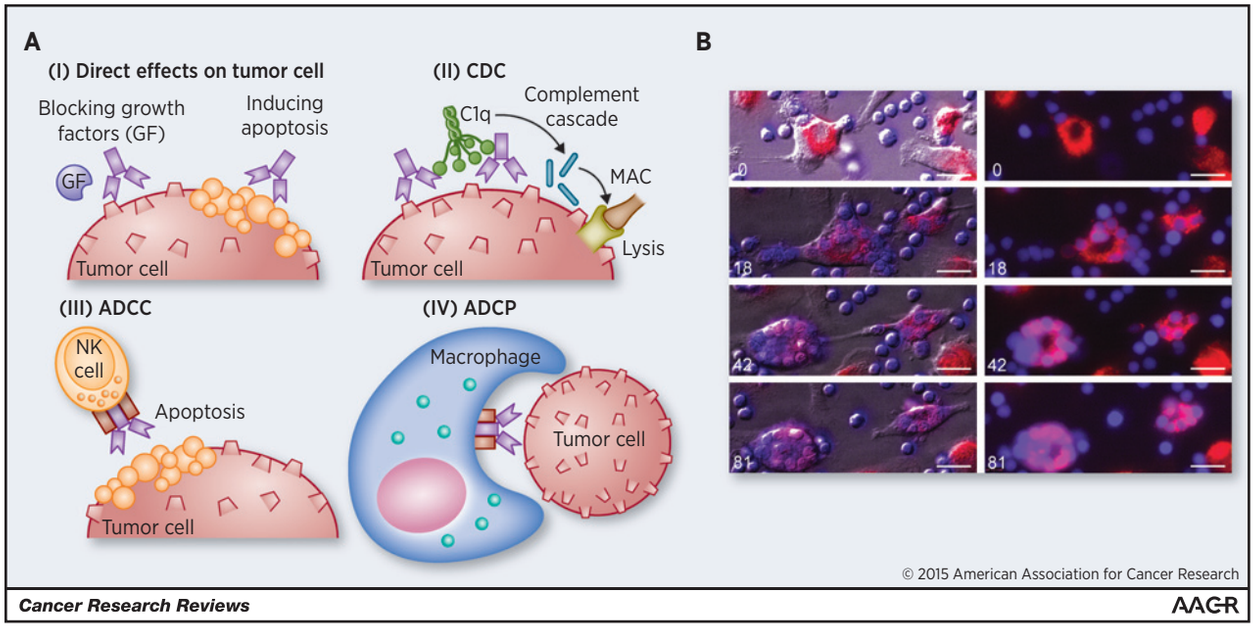

Figure 1.
A, simplified schematic overview of mAb-induced effector mechanisms. I and II, direct effects include induction of apoptosis or blockage of growth receptors (I), whereas CDC is induced after binding of C1q to mAb-opsonized tumor cells (II). III and IV, mAbs bridge immune effector cells with tumor cells, which leads to ADCC (III) or ADCP (IV). B, phagocytosis of primary CLL cells (blue) by human macrophages (red) in the presence of rituximab over time (indicated in minutes; see also Supplementary Movies). Left, overlay of bright field and fluorescence. Right, fluorescence only. Scale bar, 20 μm.

induce apoptosis in target cells via antibody-dependent cell cytotoxicity (ADCC; Fig. 1A, III) and have generally been considered as the main effector cells in mAb therapy (10). However, increasing evidence supports a major role for macrophages in the elimination of tumor cells.

Macrophages as Effector Cells in mAb Therapy

Human macrophages express the activating receptors FcγRI, FcγRIIa, and FcγRIIIa as well as the inhibitory FcγRIIb, whereas NK cells express only FcγIIIa (4). Consequently, the correlation between the clinical success of mAb therapy and the FcγRIIIa-**V**158 allotype versus FcγRIIIa-**F**158 allotype may be attributed to cytotoxicity of either macrophages or NK cells. However, it was also reported that a polymorphism in human FcγRIIa (FcγRIIa-131H/R) was associated with clinical responses to rituximab therapy, which supports a role for macrophages in B-cell lymphoma depletion (8). Similarly, the efficacy of treatment with antitumor mAbs was improved in mice that did not express the inhibitory receptor FcγRIIb (5), which cannot be a consequence of enhanced ADCC, as NK cells do not express this receptor. Instead, murine macrophages that express the activating FcγRI, FcγRIII, and FcγRIV, as well as FcγRIIb, were likely involved as effector cells in mAb-mediated tumor elimination *in vivo*. We previously demonstrated that anti-gp75 mAb therapy prevented liver metastases outgrowth of B16F10 melanoma (7). FcγRI and FcγRIV were involved in therapeutic efficacy, suggesting redundancy in function. As such, an important role for monocytes and macrophages was supported, as cells of the mononuclear phagocyte network are the only cells in mice that express both FcγRI and FcγRIV (4). Depletion of NK cells did not significantly influence therapeutic efficacy when Hodgkin lymphoma–bearing SCID mice were treated with anti-CD30 mAbs, whereas elimination of macrophages decreased survival (11). Similarly, depletion of macrophages, but not of either NK cells or neutrophils, rendered anti-CD40 mAb therapy ineffective in a xenograft model of non-Hodgkin lymphoma. Removal of B cells or B lymphoma cells after anti-CD20 mAb treatment was also dependent on the mononuclear phagocyte network and required expression of activating Fcγ receptors (6).

It was recently demonstrated that liver macrophages (Kupffer cells) are key effector cells for eliminating target cells that are present in the circulation. Kupffer cells mediated arrest and removal of B or T lymphoma cells after anti-CD20 or anti-fibronectin receptor mAb therapy, respectively (11, 12), which was in part responsible for prolonged survival of mice. This mechanism may be particularly important for patients undergoing resection of primary colorectal cancer. The presence of circulating tumor cells was detected in peripheral blood of patients. The numbers of circulating tumor cells were additionally increased during or after surgery, especially in portal blood, suggesting the dissemination of tumor cells by surgical manipulation (13). The presence of circulating tumor cells moreover correlates with poor prognosis of colorectal cancer patients (14). As the blood vessels from the intestines drain directly into the portal circulation, the liver is the first organ where circulating colorectal cancer cells enter. We previously demonstrated that the inflammatory reaction as a result of abdominal surgery alters the microenvironment of the liver, creating a niche in which tumor cells can adhere and grow out into metastases (15). Kupffer cells were unable to halt the development of liver metastases without mAb therapy. However, treatment with antitumor mAbs enabled rapid removal of circulating tumor cells by Kupffer cells, thereby preventing outgrowth (16). Thus, patients undergoing resection of colorectal carcinoma may greatly benefit from preoperative

mAb adjuvant therapy. This may also hold true for other malignancies. Circulating tumor cells have been detected in patients with breast cancer, head and neck cancer, non–small cell lung carcinoma, pancreatic cancer, and renal cell carcinoma, and are generally associated with poor survival (17).

The role of macrophages in solid tumors after mAb therapy is less clear. Kupffer cells were unable to eliminate established micrometastases (16). However, anti-CD142 mAb therapy was less effective in preventing breast cancer outgrowth and metastasis development when macrophages had been depleted, suggesting a contribution of macrophages in mAb-dependent killing of tumor cells (18).

Antibody-dependent phagocytosis

ADCC is most commonly used to describe mAb-induced cell death. This process involves degranulation of effector cells, thereby inducing apoptosis or lysis of target cells. ADCC is predominantly attributed to NK cells, although it was proposed that monocytes and macrophages may induce ADCC. Synapse formation between tumor cells and macrophages was observed in peritoneal lavages of mAb-treated mice (19), suggesting the occurrence of ADCC. However, recent studies with intravital microscopy showed that antibody-dependent cell phagocytosis (ADCP) was the main mechanism of action by macrophages (Fig. 1A, IV; refs. 12, 16). The liver was the main organ where B cells or B lymphoma cells were removed after anti-CD20 treatment, as Kupffer cells mediated arrest and subsequent engulfment of circulating cells in the liver sinusoids (12). We demonstrated that Kupffer cells were able to sample circulating tumor cells in the absence of mAbs, which was, however, not sufficient for removal. After mAb therapy, tumor cells were rapidly phagocytosed, which was dependent on FcγRI and FcγRIV (16). Kupffer cells were the most prominent effector cells in a rat colon carcinoma model (CC531s) when a low dose of mAb was given, as macrophage depletion rendered mAb therapy ineffective (20). When a higher mAb dose was given, monocytes were able to partly overcome the absence of macrophages, in which case ADCC may have contributed to therapeutic efficacy. A role for ADCP in *in vivo* clearance of leukemic xenografts in SCID-BEIGE mice after treatment with the anti-CD38 mAb daratumumab was indicated as well (21). A critical residue for C1q binding and complement activation was mutated (DARA-K322A), thereby excluding a role for CDC. In addition, SCID-BEIGE mice lack NK cells. *In vitro* ADCP of multiple myeloma cells of 11 of 12 patients was observed, whereas no extracellular lysis was seen after incubation of Daudi cells and macrophages in the presence of daratumumab for 24 hours.

Enhanced *in vitro* ADCP has been described for multiple tumor-associated antigens on malignant epithelial cells, including carcinoembryonic antigen (CEA), EGFR, HER-2, epithelial cell adhesion molecule (EpCAM), and human epithelial mucin-1 (11). Similarly, many molecules on hematologic cancers are targeted, such as CD20, CD30, CD38, CD40, and CD52. The uptake of tumor cells culminates in the establishment of vacuoles that are referred to as phagosomes (22). During maturation, late endosomes and lysosomes fuse with the phagosome to form phagolysosomes. The pH is lowered (∼4.5), and the phagosome becomes highly oxidative with generation of reactive oxygen species (ROS). It is also enriched with digestive enzymes in order to degrade the contents of the phagolysosome. With live-cell imaging, we observed fast acidification of phagolysosomes within macrophages after ADCP (16). Both *in vitro* and *in vivo* degradation was a slow process as tumor material was still present after 24 hours. ROS were produced as well, but neither ADCP nor acidification of phagolysosomes and breakdown of tumor cells was hampered in the presence of a ROS scavenger, indicating that ROS were not involved in these processes. In line with our findings, it was shown that ADCP of tumor cells by $p47^{-/-}$ macrophages, which lack the ability to produce ROS, was unaffected (23).

Induction of adaptive immune responses

Macrophages are antigen-presenting cells. Exogenous antigens that have been phagocytosed are presented via the MHC class II route, which leads to activation of CD4 helper T cells (T_h cells). In addition, macrophages were shown to cross-present exogenous antigens via the MHC class I route, thereby inducing cytotoxic $CD8^+$ cell responses (24). It was demonstrated that treatment with anti-CD20 mAb induced a cellular immune response (involving both CD4 and CD8 cells) *in vivo*, which was required for long-term survival (25). Induction of adaptive immune responses in cancer patients that have been treated with antitumor mAbs has, however, not been extensively investigated.

Peripheral macrophages are sessile cells with limited capacity to migrate, and therefore likely do not play a prominent role in activating naïve T cells. Nonetheless, macrophages may play a role in restimulation of effector T cells. Furthermore, macrophages in secondary lymphoid organs may contribute to T-cell activation when they ingest tumor cells that enter the lymph node. Intravital two-photon microscopy revealed that subcapsular sinus macrophages of tumor-draining lymph nodes captured tumor-derived antigens, resulting in the accumulation of these antigens on follicular dendritic cells, which were dynamically scanned by circulating B cells (26). It was furthermore demonstrated that dead tumor cells were phagocytosed by $CD169^+$ macrophages in tumor-draining lymph nodes, leading to cross-presentation of tumor antigens to $CD8^+$ T cells and antitumor immunity (27). Although uptake of tumor cells or antigens was antibody independent in these cases, these findings support the possibility that long-term adaptive immune responses may be induced in cancer patients, when mAb therapy is optimized, for example, by targeting to $CD169^+$ macrophages.

Strategies to Enhance ADCP

Overcoming the anti-inflammatory milieu of the tumor

A tumor does not only consist of malignant cells, but also contains local stromal cells and a diverse immune cell infiltrate, which together compose the tumor microenvironment. In general, the composition of immune cells favors an immunosuppressive milieu, as regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages (TAM) are abundantly present (28). Especially, alternatively activated (also referred to as "M2") macrophages, which have tumor-promoting properties, can dominate the immune cell infiltrate. The presence of TAMs has been correlated with clinical outcome in multiple malignancies. High density of macrophages infiltrates in breast, head and neck, mesothelium, thyroid, liver, pancreas, kidney, bladder, ovarian, uterus, and cervix cancer as well as in glioma, melanoma, and non-Hodgkin has been associated with poor prognosis (28). In contrast, high macrophage density in colorectal cancer was correlated with increased patient survival, which supports that TAMs in colorectal cancer have a more classically activated (or

"M1-like") phenotype. Conflicting data exist with respect to lung, prostate, bone, or esophagus cancer. The role of macrophages in tumor development has been extensively reviewed recently (28).

The clinical success of immunotherapeutic approaches is severely hampered by the immunosuppressive environment (29). The cytotoxic ability of effector cells, including after mAb therapy, is limited by the presence of anti-inflammatory mediators, such as prostaglandin E2 (PGE_2), IL10, and TGFβ. For example, PEG_2 was shown to inhibit CD52-mediated killing of tumor cells by macrophages (23). Interestingly, it was reported that TAMs, which had been isolated from murine breast cancer, promoted tumor cell invasion in *in vitro* 3D assays (18). Nonetheless, TAMs expressed Fcγ receptors and phagocytosed breast cancer cells in the presence of anti-CD142 mAbs *in vitro*. Depletion of macrophages resulted in decreased efficacy of anti-CD142 mAb therapy *in vivo*, supporting that TAMs contributed to tumor cell elimination. In contrast, a recent study showed that removal of M2-like TAMs significantly stimulated both influx of $CD8^+$ cytotoxic T cells and tumor shrinkage after anti-HER2 mAb therapy in a HER2-dependent breast cancer model (30). In addition, local delivery of IL21 into the tumor environment skewed the M2 phenotype of TAMs into more classically activated cytotoxic M1 macrophages.

Thus, manipulation of TAMs may be a promising therapeutic approach for the treatment of cancer. It was shown that inhibition of CSF-1, which is an important survival factor for macrophages, led to regression of established tumors and enhanced survival in xenograft models of glioma (31). Interestingly, treatment with CSF-1R inhibitors did not lead to depletion of TAMs, but decreased the expression of M2 markers, which suggests that repolarization into cytotoxic classically activated or M1 macrophages. Monocyte/macrophage activation was inhibited by an IL10-producing B cell subset (B10 cells), which reduced efficacy of anti-CD20 mAb therapy in a murine lymphoma model (32). Cotreatment with a Toll-like receptor 3 agonist overcame inactivation of monocytes and macrophages. In addition, blocking the IL10 receptor proved similarly effective in suppressing tumor growth in mice compared with CSF-1 receptor inhibition (33). Treatment with IFNγ and a calcineurin B subunit resulted in synergistic repolarization of macrophages and prolonged survival of mice bearing B16F10 melanoma (34). Thus, skewing the tumor micromilieu from immunosuppressive into proinflammatory may repolarize TAMs into macrophages with a cytotoxic M1-like phenotype, thereby potentially enhancing ADCP after mAb therapy.

Expression of CD47, the "don't eat me" signal

ADCP is also inhibited by the interaction of CD47 on tumor cells with the inhibitory receptor signal regulatory protein-α (SIRPα; CD172a) that is expressed on macrophages. CD47 is upregulated in various types of solid human cancers, and high expression was shown to correlate with poor survival of patients with ovarian cancer, glioma, or glioblastoma (35). Tumor cells that expressed CD47 were less sensitive to mAb-induced killing (36). In addition, anti-gp75 mAb therapy prevented the development of melanoma lung metastases more effectively in mice, in which the intracellular tail of SIRPα was mutated (36). Anti-CD47 mAb or high-affinity SIRPα monomers that were used as CD47 antagonists increased *in vitro* ADCP and *in vivo* efficacy of mAb therapy significantly, by interrupting the interaction between CD47 and SIRPα on macrophages (11, 37). SIRPα monomers synergistically enhanced the therapeutic effect of rituximab and the anti-CD52 mAb alemtuzumab in a B-cell lymphoma model or trastuzumab in a breast cancer model, respectively. Thus, antitumor mAb therapy is more effective when the CD47–SIRPα pathway is additionally blocked. The safety and efficacy of anti-CD47 mAbs are currently tested in phase I clinical trials.

The inhibitory Fcγ receptor FcγRIIb

To improve Fc receptor–mediated effector mechanisms, many second- and third-generation mAbs have been developed with specific mutations in their Fc tails to enhance binding to activating Fcγ receptors, or decrease binding to the inhibitory FcγRIIb (11). For example, antibodies against EpCAM with higher affinity for the activatory receptor FcγRIIa increased ADCP of LS180 adenocarcinoma cells by human macrophages. Similarly, enhanced phagocytosis of B-cell lymphoma, leukemia, and multiple myeloma cell lines was observed with engineered anti-CD19, anti-CD40, or anti-HM1.24 mAbs. An aglycosylated mutant of the anti-HER2 mAb trastuzumab resulted in a 75% enhancement of ADCP of tumor cells with low- to medium-expression levels of HER-2. To overcome binding of IgG to the inhibitory FcγRIIb receptor, it was furthermore investigated whether bispecific antibodies (BsAb) that target specific Fc receptors improved ADCP. A BsAb recognizing the high-affinity FcγRI on human macrophages and CD30 on lymphoma cells was able to effectively induce ADCP. However, the use of FcγRI BsAb in clinical applications was disappointing, presumably due to their short half-life (9). Alternatively, the possibility to use mAbs of the IgA isotype has been investigated. IgA anti-EpCAM was able to induce ADCP by macrophages, but less effectively compared than an IgG anti-EpCAM counterpart (11). However, IgA2 anti-EGFR mAbs were more effective compared with cetuximab in mediating ADCP in a short-term syngeneic peritoneal model in human FcαRI transgenic mice (38). Outgrowth of lung and peritoneal metastases was prevented by IgA2-EGFR mAb therapy. Thus, IgA mAbs may represent an interesting additional option for anticancer treatment, although its shorter half-life needs to be addressed in order for IgA to reach its full potential.

A cotherapy in which anti-FcγRIIb is blocked with mAbs also represents a promising approach. Not only may this limit induction of inhibitory signals in effector cells that decrease capacity, for example, ADCP, but it was also recently demonstrated that anti-FcγRIIb mAbs had additional modes of action in lymphoma models (39). FcγRIIb on (malignant) B cells promotes internalization of rituximab, thereby effectively abrogating CDC, ADCC, and ADCP, and leading to therapy resistance. Blocking FcγRIIb prevented internalization, which maximized cell surface accessibility of rituximab, and restored *in vitro* ADCC and ADCP as well as *in vivo* therapeutic efficacy of rituximab. Panitumumab, which is an IgG2 mAb, furthermore effectively induced ADCC by neutrophils and monocytes (albeit not by NK cells; ref. 40). Because IgG2 has very low affinity for FcγRIIb, it might be particularly suitable to recruit myeloid cells, including macrophages, as effector cells, as it will only induce activating signalling.

Conclusions and Future Directions

Macrophages are crucial effector cells in mAb therapy of cancer. They are particularly effective in eliminating circulating tumor cells, and as such are likely prominent cytotoxic cells for the removal of malignant hematopoietic cells after treatment with,

for example, anti-CD20 mAbs (Fig. 1B and Supplementary Movies S1 and S2). However, their potent ability to mediate ADCP of single target cells could also be utilized to remove minimal residual disease in patients with solid malignancies. For example, patients undergoing surgery to remove colorectal carcinoma may greatly benefit from preoperative mAb therapy to prevent adherence and outgrowth of circulating tumor cells in the liver. Moreover, the presence of circulating cancer cells is correlated with poor prognosis of patients with other malignancies, including breast cancer, head and neck cancer, pancreatic cancer, and renal cell carcinoma. Although surgery can remove the bulk of the tumor, adjuvant mAb therapy may induce ADCP of remaining tumor cells.

Most solid tumors contain a major population of macrophages, and as TAMs play an important role in tumor development, they represent ideal candidates for therapeutic strategies. However, to unleash their full cytotoxic capacity, it will be necessary to overcome several immunosuppressive mechanisms. The combination of therapeutic strategies that aim to reeducate M2-like macrophages, change the immunosuppressive tumor environment, or block the interaction with inhibitory receptors such as SIRPα or FcγRIIb may therefore significantly enhance the therapeutic success of mAb therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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