

The dual role of complement in cancer and its implication in anti-tumor therapy

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Abstract: Chronic inflammation has been linked to the initiation of carcinogenesis, as well as the advancement of established tumors. The polarization of the tumor inflammatory microenvironment can contribute to either the control, or the progression of the disease. The emerging participation of members of the complement cascade in several hallmarks of cancer, renders it a potential target for anti-tumor treatment. Moreover, the presence of complement regulatory proteins (CRPs) in most types of tumor cells is known to impede anti-tumor therapies. This review focuses on our current knowledge of complement's potential involvement in shaping the inflammatory tumor microenvironment and its role on the regulation of angiogenesis and hypoxia. Furthermore, we discuss approaches using complement-based therapies as an adjuvant in tumor immunotherapy.

Keywords: Anaphylatoxins; antibody therapy; cancer immunotherapy; complement dependent cytotoxicity

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Introduction

The contribution of inflammation in carcinogenesis has been observed over a century ago with opposing findings as to whether its outcome counters or promotes neoplasia (1,2). Presently, it is believed that acute inflammation participates in mechanisms of immune surveillance directly targeting tumor cells, at the early stages of tumor development or by containing metastatic cells. Chronic inflammation though, has been implicated in both the development and progression of the disease (1,3), where long-term exposure of healthy tissues to pro-inflammatory molecules can induce DNA damage, while inflammatory mediators can maintain and facilitate further evolvement of neoplastic cells (1-3). Complement is a fundamental component of innate immunity, which has been known for many years to be involved in the recognition and assistance in the elimination of invading

pathogens (4). However, our current view of complement's role extends beyond the simple targeting of intruders, since the powerful inflammatory molecules it contains contribute both to acute and chronic inflammation, orchestrating thus immunological and inflammatory processes (5). Moreover, complement participates in largely diverse processes, such as clearance of immune complexes, angiogenesis, mobilization of hematopoietic stem-progenitor cells and tissue regeneration (5-7). A disruption of complement homeostasis can lead to 'self-attack' and consequently members of the complement cascade have been implicated in immune-related pathogenesis, including neurodegenerative, ischemic (8) and age-related diseases (9,10). Interestingly, complement is also considered a functional bridge between innate and adaptive immune responses, allowing an integrated host defense to pathogenic challenges (11).

These multifunctional properties of the complement system implicate it in opposing roles in cancer (12). First, as a key player in tumor immunoediting, it helps target cancer cells and orchestrates an immune response against the progress of the disease. However, it can also be a part of the long lasting inflammatory status that can lead to malignant transformation and tumor development (13,14).

This review will discuss the role of inflammation in cancer development and progression and will address the involvement of complement in the shaping of the tumor microenvironment. Finally, we will focus on issues confronting the manipulation of complement activation in anti-tumor therapies.

Cancer and inflammation

The role of inflammation in targeting non-self cells is well known, however, most cancer types are highly non-immunogenic thus escaping immune surveillance. Moreover, it has been postulated for many years that chronic inflammation is associated with tumor formation, progression and transformation (15). On the other hand, for several types of tumors, there is no direct link between chronic inflammation and tumor progression, while in some cases, mild chronic inflammation can also lead to reduced tumor risk (asthma, eczema) (16). Of note, the presence of inflammatory cells in certain tumors has been associated with both favorable (17,18) and unfavorable clinical outcome (19-21). This paradox makes apparent the fact that the inflammatory response may differ substantially in different types of tissues. The growth of malignant tumors was often been defined as an autonomous process, but soaring evidence indicates that its dynamics depend greatly on the interaction between malignant cells and the host-derived tumor microenvironment (22). Although cancer cells exhibit a distinct molecular pathway activation signature that promotes their survival, proliferation and transformation (intrinsic pathway), the role of the inflammatory microenvironment (extrinsic pathway) plays an important role in either maintaining or impeding the progress of the disease. The elucidation of the interplay between these two pathways and its modulation is becoming the holy grail for modern anti-tumor approaches.

Hanahan and Weinberg (23) have re-visited their current view regarding the hallmarks of cancer that characterize the biological processes involved in the progress of the disease. The extended list of hallmarks includes (I) sustaining of proliferative signaling; (II) evading growth suppressors; (III)

resisting cell death; (IV) enabling replicative immortality; (V) the induction of angiogenesis; (VI) activating invasion and metastasis; (VII) reprogramming of energy metabolism; (VIII) evading immune destruction. In all these steps the way tumor cells interact, depends on their inflammatory microenvironment (23). The role of the tumor microenvironment is thus critical for the progress of the disease, and complement, as an essential part of the inflammatory response, is emerging as an orchestrator of the modulation of immune cells.

One of the multiple roles of the members of the complement cascade is to attract and control the activation of cells involved in both the innate and adaptive immunity. The anaphylatoxins C3a and C5a are powerful chemoattractants that guide neutrophils, monocytes and macrophages and other immune cells towards the sites of inflammation (5). For many years complement was thought to be involved in the targeting of tumor cells, since deposition of complement components has been shown in tissues of various cancer origins (24-26). Current studies are now challenging this dogma, since complement components have been implicated in many of the hallmarks of cancer but with opposing effects (12).

Inflammatory cells and cancer

T cells

CD8⁺ T cells are a major population of the anti-tumor immune response. These cytotoxic effector T cells (CTL) can recognize MHC class I restricted antigens on cancer cells and initiate cytolytic killing. In most cases, the higher the number of CTLs present at the tumor site, the better the prognosis for a patient is (27). However, tumors are able to escape immune clearance and several reasons for that have been proposed (28). The effects of CTLs are mediated by T-helper (Th) cells; these are CD4⁺ effector cells that normally lack any direct cytotoxic activity, yet they play a central orchestrating role in adaptive immunity. Th1 response has effective anti-tumor properties by promoting antigen presentation to CTL, but activated Th cells can also mediate tumor clearance independent of antigen presenting cells (APCs). Th cells are quite plastic and can undergo differentiation to regulatory T cells (Treg) that exert immunosuppressive properties, thus hindering effective tumor clearance (29) (*Figure 1*), or to Th17 cells, whose role in tumors remains highly debated (30,31). Apart from Th-derived Tregs, another population that is CD8⁺ can also have an immunosuppressive effect in tumors (32,33). Although the mechanism of Treg function

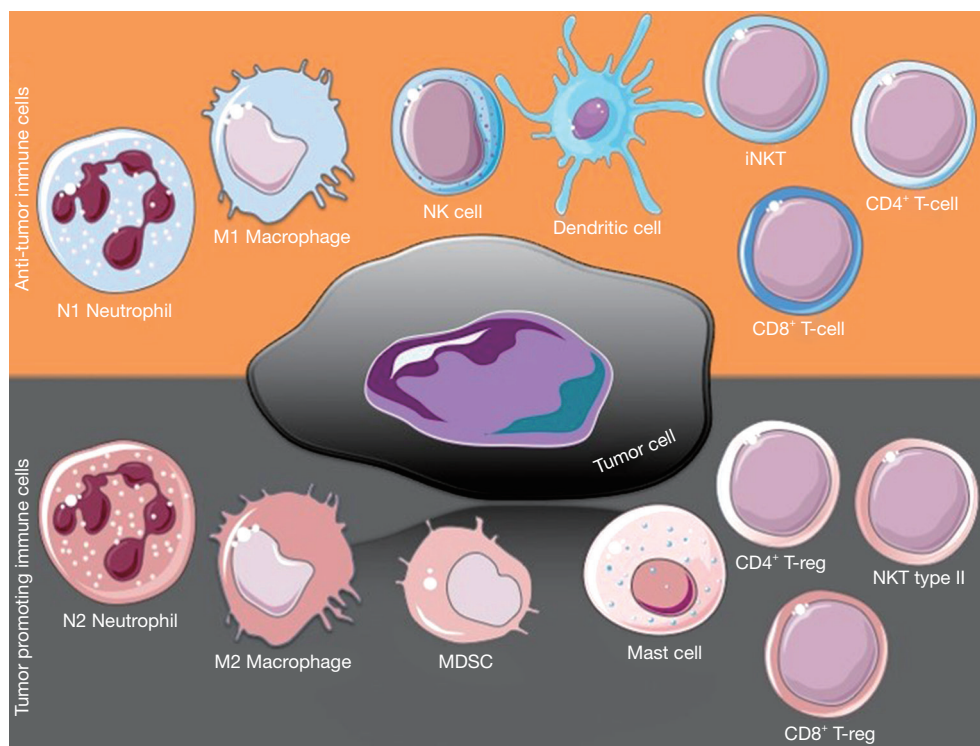


Figure 1 The role of inflammatory cells in the modulation of tumor growth. Most inflammatory cells can exhibit opposing properties when they interact with tumor cells. This behavior is tumor-type and stage specific and also depends on the overall tumor microenvironment. iNKT, invariant natural killer T cells (NKT type I); MDSC, myeloid-derived suppressor cells.

is complicated, their depletion can cause decreased tumor burden.

The complement system has been shown to control CD4⁺ T-cell activation and differentiation (34). C3 mediates Th1/Th17 polarization in human T-cell activation and skin graft versus host disease patients, while blocking of C3 activation with compstatin significantly inhibits Th1/Th17 polarization in activated human CD4⁺ T cells (35).

Invariant natural killer T cells (iNKT), or type I NKT cells, express a canonical TCR-V α -chain that recognizes glycolipid antigens presented by the monomorphic CD1d molecule (36). iNKTs are believed to recognize endogenous ligands presented by CD1d on tumor cells, damaged epithelial cells or APCs and result in increased antitumor responses through IFN- γ production (37). Type II NKT cells have diverse repertoires of TCRs and suppress the antitumor response through several mechanisms, including TGF- β production (37). Moreover, they have opposing and counter-acting effects on iNKTs (38), while themselves are being controlled by tumor-controlling iNKTs (39).

Interestingly, engagement of the membrane complement regulatory protein (mCRP) CD46 differentially affects

CD8⁺ T cell cytotoxicity, CD4⁺ T cell proliferation and IL-2 and IL-10 production (40-42). In addition, complement promotes Th17 differentiation with the participation of TLRs through C5aR signaling (43).

Macrophages

Tumor-associated macrophages (TAMs) comprise one of the largest immune populations in the tumor (44). Their role in tumor immunity can be complicated since they constitute a heterogeneous population that can be either classically activated M1 polarized, or alternatively M2 activated (44). This variation leads to opposing functions, with M1 polarized TAMs having anti-tumor pro-inflammatory properties and M2 TAMs having immune regulatory properties that promote tumor progression. TAMs participate in all stages of cancer progression, from contribution to genetic alterations and instability, regulation of senescence, interaction with and remodeling of the extracellular matrix, to promotion of invasion and metastasis (45). However, the tumor phenotype of TAMs is not always clear, because these cells can share both M1 and M2 characteristics. This variation in the macrophage

repertoire and function is tumor type and stage specific and is extensively reviewed by Biswas and Mantovani (46). Activated macrophages produce C3 and participate in complement-initiated phagocytosis of intruding entities, while they are also involved in the clearance of apoptotic and necrotic cells (47). Moreover, C5aR signaling in TLR-activated macrophages selectively inhibits the transcription of genes that encode the IL-12 cytokine family, that in turn drive the polarization and recruitment of Th1 cells (48).

Neutrophils

Polymorphonuclear neutrophils (PMNs) are professional phagocytes with potent anti-microbial and anti-inflammatory capacities. Their role in tumor progression can be opposing, since they can be either pro- or anti-tumorigenic (49). Tumor associated neutrophils (TANs) are believed to employ their dual role according to their polarization. The shift towards N1 polarization promotes tumor cell death, activates CTLs and inhibits tumor growth (50). On the other hand, N2 PMNs are thought to promote tumor angiogenesis and metastasis (51). Their role in tumor promotion is highlighted by Pekrek *et al.*, where depletion of PMNs in an *in vivo* model resulted in tumor growth inhibition (52).

Dendritic cells (DCs)

DCs are APCs found in tumors and present tumor-associated antigens (TAA) to T cells and NK cells. Large clinical studies have shown that the density of DCs correlates to the number of effector T cells in the tumor and both were associated with improved cell survival (53). However, the local tumor microenvironment was shown to have an effect on the maturation of DCs, thus impeding their anti-tumor activity by affecting CTL response (54). Chronic inflammation can suppress the immunogenicity of DCs and induce a tolerogenic phenotype. In spleen-derived DCs (sDCs), C5aR activation plays an important role for naive CD4⁺ Th cells to differentiate into either Th1 or Th17 effector cells, while blockade of the receptor in sDCs results in the expansion of Treg, as shown in murine models (55). Additionally, C1q has been shown to regulate the development of DCs from monocytes while affecting T cell stimulation (56).

Natural killer (NK) cells

NK cells are cytotoxic lymphocytes that recognize MHC-I molecules on target cells and can act directly without the need for prior sensitization (57). Apart from direct killing of cancer cells (58), NK cells produce IFN γ , which

is important for Th cell activation that leads to tumor clearance. In a murine melanoma model, C3^{-/-} mice had smaller tumors than wild-type animals, while this effect was abolished after NK depletion in the knockout animals, suggesting increased NK activity in the absence of C3.

Myeloid-derived suppressor cells (MDSCs)

MDSCs are found in the tumors of most cancer patients and experimental animal models. They can be categorized as monocytic and granulocytic MDSCs (59). MDSCs accumulate in response to pro-inflammatory mediators and suppress the activation of CD4⁺ and CD8⁺ T cells (60), as well as M1 macrophages and NK cells, thus blocking both innate and adaptive antitumor immunity. Moreover, they facilitate the activation and the anti-inflammatory action of Treg. Of interest, Markiewski *et al.* have shown the involvement of complement in MDSCs regulation in a murine cancer model (more on section “Modulation of infiltration and activation of immune cells by complement”).

Mast cells

Mast cells are APCs that can promote migration, and maturation of DCs, as well as lymphocyte recruitment (61). Their sentinel presence in epithelial tissues makes them one of the first immune cell populations to come in contact with neoplastic cells. They orchestrate inflammatory reactions and angiogenesis that shape the tumor microenvironment and promote tumor cell proliferation and invasion. Mast cells can affect Treg long-term repercussions (62). However, their presence in tumors has been correlated with both favorable and poor prognosis (61). They express C5aR and C5a has been shown to activate them and to induce degranulation (63), while both C5a and C3a induce chemotaxis (64).

It is becoming apparent that the participation of each immune cell type can have opposing results on tumor pathophysiology. The interplay between these populations depends on the type and stage of tumor. Complement is a known orchestrator of immune responses and is responsible for modulating the functions of most immune cells.

Role of complement in cancer

Modulation of infiltration and activation of immune cells by complement

Despite the multifactorial role of complement in several disease models, little is known regarding its direct

implication in the regulation at the tumor-specific setting.

The role of complement in orchestrating the inflammatory state in cancer

Markiewski *et al.* have shown that complement cascade can regulate inflammatory cells to suppress the immune response and promote tumor growth (14). More specifically, using a murine model of cervical cancer and mice deficient in various complement components (C3, C4, factor B and C5aR) the authors showed that C5a presence in the tumor microenvironment regulates the accumulation and migration of MDSCs, which express receptors for C5a, and boosts the effectiveness of these cells by increasing their content of reactive oxygen and nitrogen species, as well as arginase, all of which contribute to MDSC-mediated immunosuppression. Moreover, this was taken a step further, since the blockade of C5a with either treatment with a peptide antagonist of the C5a receptor, or using C5aR knockout animals, resulted in an increased number of CD8⁺ CTL in the tumor site. Finally, the importance of C5a involvement in this model was further highlighted when treatment with an established chemotherapeutic agent, paclitaxel (Taxol), showed similar results regarding the retardation of tumor growth to those caused by the pharmaceutical blockade of C5aR (14). The role of C5aR on MDSC modulation was also confirmed in the model Lewis lung carcinoma (3LL) (65). In addition, in a mouse model of breast cancer, C5aR was shown to promote metastasis by suppressing CD8⁺ T cell function and by enhancing Treg generation (66). In the same model, alveolar macrophages were shown to contribute to the metastatic potential in a C5aR dependent manner (67).

The dual role of complement in angiogenesis

In order for tumors to be able to survive and progress, they require an adequate supply of oxygen and nutrients and an effective way to remove waste products. This is attained by neo-angiogenesis, through a complicated balance of pro- and anti-angiogenic molecules (68). This phenomenon has motivated several researchers to look into therapeutically inhibiting angiogenesis in an attempt to restrain tumor growth. Furthermore, the activation of both angiogenesis and immunosuppressive responses, often occur in the same cell types or are mediated by the same soluble factors (69). Several reports have examined the

role of complement in neovascularization in the context of cancer and other models. Nozaki *et al.* showed that the absence of receptors for C3a or C5a, after laser injury, is associated with decreased VEGF expression and neo-angiogenesis and that antibody-mediated neutralization of C3a or C5a or pharmacological blockade of their receptors also reduces neovascularization (70). However, Langer *et al.* showed, both *in vitro* and in a model of retinopathy of prematurity, that the deficiency in C3 or C5aR resulted in more neovascularization and angiogenesis (71). Furthermore, in a mouse model of ovarian cancer, Nunez-Cruz *et al.* showed that C3 and C5aR deficiency resulted in decreased vascularization and tumor growth (72). The proangiogenic effect of complement was further supported by the finding that C5a induced vessel formation in 3LL cells (65). A recent report has addressed the importance of the complement component of the classical pathway C1q in the modulation of tumor growth. More specifically, C1q was shown to be present at the vascular compartment of several types of cancer and its deficiency was associated with decreased vascularization and tumor growth in a murine model of melanoma (73).

The role of complement in cancer-mediated hypoxia

Another role of complement in tumor progression is through the modulation of hypoxia. Hypoxia is characterized by altered cellular metabolism and increased resistance to radiotherapy (74), while it is mostly noted in rapidly expanding tumors, where the oxygen consumption is higher, and in large tumors with no nearby blood vessels. Presence of hypoxia has been correlated with poor prognosis for cancer patients (75). Okroj *et al.* reported that hypoxia increases susceptibility of non-small cell lung cancer (NSCLC) cells to complement attack. They showed, in an *in vitro* model, that hypoxic cells had reduced expression of membrane-bound complement inhibitors CD46, CD55 and CD59, as well as decreased secretion of factor I and H. This was followed by increased C3b and C9 deposition on the cells. The authors concluded that hypoxia induced the activation of all three pathways of the complement cascade (76). However, other reports in HUVEC cells (human umbilical vein endothelial cells) have shown that hypoxia causes an increase in complement regulator expression, which might down-regulate complement activation especially after reoxygenation (77), thus highlighting once again the multiple mechanisms in

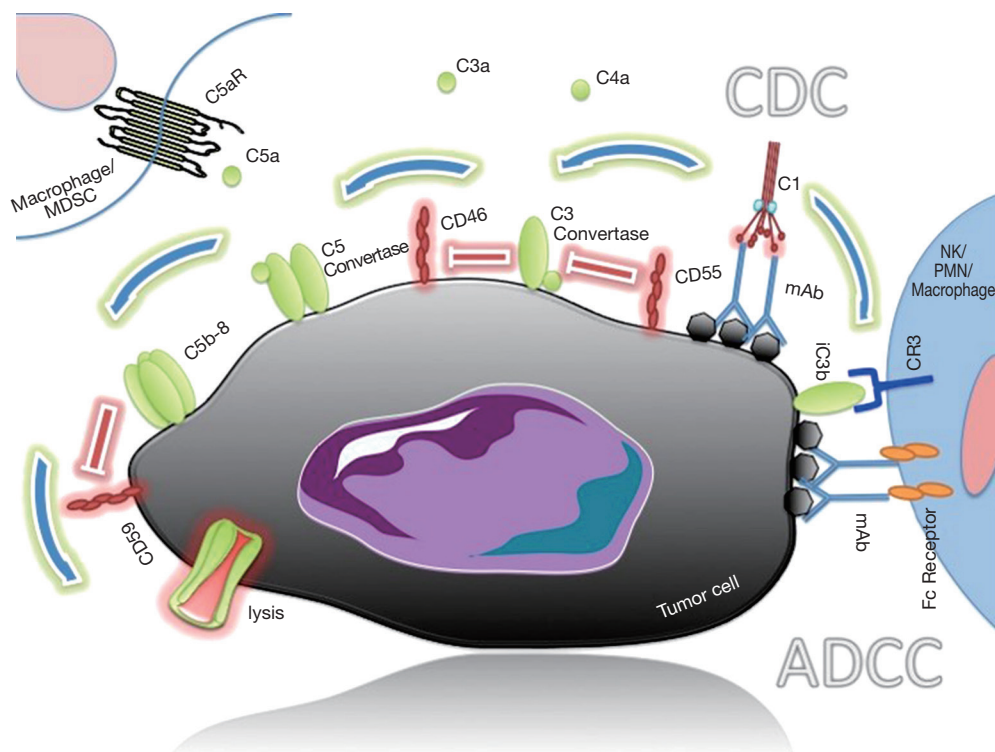


Figure 2 Complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) of tumor cells. Complement regulators (complement regulatory proteins, CRPs) expressed on the surface of tumor cells impede the effectiveness of therapeutic monoclonal antibodies (mAb) at several levels. Selective blockade of individual CRPs leads to increased CDC indicating that the regulatory process is a synergistic effect.

complement's functions.

Tumor therapy and complement

Complement dependent cytotoxicity and antibody therapy

The development of monoclonal antibody (mAb) therapy has been a hopeful anti-cancer treatment that targets directly antigens uniquely (or mostly) expressed in tumor cells, thus improving the specificity of the therapy (78). mAbs directed against TAA can initiate the natural anti-tumor responses, including complement-dependent cytotoxicity (CDC) (79). CDC is mediated by the C1 complex-initiated complement activation that results in anaphylatoxin production and deposition of C3b fragments on the target cells. C3b, together with C4b and C2a, forms the C5 convertase (C4b2a3b) that in turn cleaves C5 to C5a and C5b. C5b in turn participates in the formation of the C5b-C6-C7-C8-C9 complex that is the subunit component of the membrane attack complex (MAC). MAC formation

on the target cell's surface leads to subsequent cellular lysis (Figure 2). When cancer therapeutic Abs activate the classical complement pathway, they trigger the formation of MAC on cancer cells, leading to the killing of cancer cells through CDC (80). In antibody-dependent cellular cytotoxicity (ADCC), the Fc portion of the antibody is recognized by cells expressing the Fc-receptor (monocytes/macrophages, NK cells and granulocytes) and results in the activation of phagocytic or lytic properties of the effector cells. C3 membrane bound fragments (iC3b) can enhance ADCC by the recognition of iC3b by the complement receptor 3 (CR3) in conjunction with the antibody-Fc-receptor complex. Many clinical trials indicate that although mAb therapy causes no serious side effects, it has most often unsatisfactory results. So far, the most successful immunotherapy is the treatment of B-cell non-Hodgkin's lymphoma with rituximab, an antibody which recognizes and binds to CD20, with several others also currently being used in the clinic after successfully passing clinical trials, while more are still being evaluated.

On the other hand, there are cases where CDC is an unwanted side-effect of antibody-based immunotherapies. Immune checkpoint inhibition, such as the blockade of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 or its ligand (PD-1/L1), is gaining momentum in several types of cancer. PD-1 is expressed by activated T cells and down-modulates T cell effector functions upon binding to its ligands, PD-L1 and PD-L2, which are present on antigen-presenting cells. In patients with cancer, the expression of PD-1 on tumor-infiltrating lymphocytes and its interaction with its ligands on tumor and immune cells in the tumor microenvironment undermine antitumor immunity and support the rationale for PD-1 blockade in cancer immunotherapy. Several antibodies are currently in advanced clinical trials or have gained regulatory approval, however it is important that such antibodies do not elicit CDC, since the ultimate goal of this treatment is to enhance the function of the T cells and not their elimination (81).

In most cases, the efficiency of antibody-based immunotherapies is compromised by the endogenous inhibitors of the complement system that are uniformly called complement regulatory proteins (CRPs).

CRPs

CRPs are the natural rheostats that protect an organism from undesirable or nonspecific complement activation. They are present in most cells and are categorized in three major classes: those that are (I) in the fluid phase; (II) at the surface of host cell; (III) membrane-integral complement clearance receptors (82). During complement activation, normal cells in the local environment can be susceptible to complement-mediated damage. In order to protect the host cells from unwanted casualties and as a mechanism of regulation of complement cells possess CRPs associated with their cell membranes called mCRPs. The utilization of mAb treatment in cancer is failing to deliver the expected results, since several patients do not respond, while others develop resistance to such treatment. The efficiency of anti-tumor mAb-therapy can be impeded by CRPs (83). Therefore, current studies are looking at the effect of CRP expression by cancer cells in inhibiting CDC (83,84).

mCRPs

Protectin, or CD59, inhibits MAC formation by binding to the C8 and C9 and, while it is universally expressed in normal cells, it is highly expressed in many kinds of cancer

cells (85,86). It is very effective in protecting these cells from CDC, thus impairing therapeutic mAb treatment. Several groups have attempted to improve the therapeutic efficiency of mAbs by trying to inhibit the function of CD59. Moreover, a recombinant inhibitor (rILYd4) was utilized against CD59 in a CDC resistant lymphoma cell line and showed a synergistic effect together with the treatment with rituximab both *in vitro* (85) and *in vivo* (87). Other groups (88,89) have successfully blocked CD59 using mAbs in a model of NSCLC that led to a similar synergistic effect. Donev *et al.* (90) used a different approach and identified neural-restrictive silencer factor (REST), as an important regulatory component of the transcriptional machinery of the *CD59* gene. They inhibited REST using peptides against the promoter region of CD59 where REST binds. This resulted in a reduced CD59 expression that in turn led to sensitized tumor cells by complement-mediated killing triggered using anti-GD2, a mAb used in neuroblastoma immunotherapy.

Decay-accelerating factor (DAF), or CD55, restricts the action of complement by binding to and dissociating from both the classical (C4b2a) and the alternative (C3bBb) pathway C3/C5 convertases. Several tumor cells express CD55, resulting in increased CDC resistance (91,92), while the expression of CD55 has been demonstrated in hematological malignancies (93), colon, breast, gastric, ovarian, thyroid, prostate, pancreatic, melanoma, glioma, esophageal and cervical cancer (94,95). A decrease in susceptibility to CDC with rituximab was dependent on DAF expression in experiments utilizing an anti-CD55 mAb (96), strengthening the notion that pharmaceutical exploitation of mCRPs can lead to better treatment efficiencies. Moreover, tumor size was positively correlated with DAF expression levels in clinical samples (97,98). In an effort to further explore this mechanism, Zell *et al.* (99) applied anti-sense phosphorothioate oligonucleotides (S-ODNs) to knock down the expression of CD55 in a number of tumor cell lines and found increased C3 opsonization of the cells together with enhanced CDC. In another study, inhibition of CD55 with siRNA in a human cervical cancer cell line demonstrated increased CDC (100), as was the case in a prostate cancer *in vivo* model (101). Similarly, overexpression of both CD55 and CD59 was found on NSCLC cells and contributed to the acquisition of resistance to trastuzumab treatment (an anti-HER-2 mAb); in this model, the anticancer efficacy of trastuzumab could be enhanced when these mCRPs were neutralized with blocking mAbs (89).

Membrane cofactor protein (MCP), or CD46, has a cofactor activity for the inactivation of C3b and C4b by factor I. Like most mCRPs, it is expressed on various tumors including head and neck squamous cell carcinoma (HNSCC) (102), hematological malignancies (93,103), breast, prostate (104), ovarian (105,106), glioma (107), liver (108), renal (25) and cervical cancer (109) among others. Together with CD55, downregulation of CD46 with siRNA resulted in increased CDC in a number of cell lines (99,110). Moreover, incubation of lymphoma cells with a recombinant adenovirus-derived protein resulted in transient removal of CD46 from the cell surface, and induced the sensitization of the cells to CDC (111). Buettner *et al.* have shown that CD46 expression is mediated through signal transducer and activator 3 (STAT3) (104). STAT3 is shown to be overactivated in several tumors (112) and its inhibition led to diminished CD46 expression combined with increased CDC susceptibility in a human prostate cancer cell line (104). Interestingly, Hakulinen *et al.* have shown that tumor cells produce also a soluble form of CD46 that is fully functional, leading to C3b cleavage by factor I (113). Apart from its mCRP function, CD46 is also a receptor for several pathogens, including measles virus, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, group A *Streptococcus*, and human herpes virus 6. This additional property has been exploited by turning CD46 into a CDC-independent tumor target. The oncolytic measles virus can bind to CD46-high expressing multiple myeloma cells, leading to tumor cell death (103). Moreover, human adenoviruses are being utilized for the delivery of gene transfer therapy. Species B adenovirus uses CD46 as their primary cellular-attachment protein, thus making it a good candidate for tumor specific gene targeting (114,115).

Complement receptor-related gene y (Crry) is a murine mCRP that is a functional analog of human DAF and MCP (116). Although many *in vivo* studies utilize xenograft cells in immunocompromised animals, the use of murine models with animals that are immune intact provides a better insight into the immune response of the organism against the disease. Thus, several studies have used syngeneic models to examine the role of mCRPs in CDC tumor protection. RNAi mediated knock down of Crry in a metastatic cell model has shown enhanced mAb anti-tumor activity *in vitro*. However, although Crry knock down *in vivo* led to a decreased tumor burden and higher survival rates, these results were not affected by mAb treatment. The authors concluded that Crry can suppress T cell response, while enhancing complement activation on a tumor cell surface,

thus promoting protective T cell immunity (78). Similar results were produced by other groups (117) where mAb inhibition of Crry led to reduced tumorigenicity of murine cell lines *in vivo* without the need for anti-tumor mAb treatment. On the other hand, older studies had shown that mAb inhibition of Crry in a syngeneic colorectal cancer metastatic model led to increased complement activation and decreased lung tumor formation when combined with an anti-tumor mAb (118,119).

Fluid phase CRPs

Factor H is an essential regulatory protein that plays a critical role in the homeostasis of the complement system. It binds to C3b, thereby preventing subsequent formation of the lytic components at the cell surface. Resistance to factor H-mediated complement attack was found to be significant in ovarian cancer cells, lung cancer and glioblastoma cells (106,120-122). Moreover, the monoclonal antibody cetuximab had significantly higher activity on A549 cells, in which factor H was genetically downregulated with siRNA (122,123).

Factor I is a plasma serine protease that regulates complement activation by cleaving and thus inactivating C3b and C4b. Its activity depends on the presence of cofactors C4BP, MCP and CR1, which can support both C3b and C4b cleavage. Although the primary site of factor I production is the liver, Okroj *et al.* (88) have shown that functionally active factor I is produced and secreted by some NSCLCs and that, together with C4BP, leads to decreased C3b deposition thus limiting complement-dependent lysis. Moreover, in a recent report, factor I was detected in cutaneous squamous cell carcinomas and its expression was positively associated with tumor aggressiveness (124).

Despite the inhibitory role of CRPs in treatment with monoclonal antibodies, once again complement's role is dual. Wang *et al.* have shown, both *in vitro* (125) and *in vivo* (126), that complement components can impede the efficiency of rituximab by inhibiting the ability of rituximab-coated targets to induce NK cell activation. They concluded that C1q and C3 presence in the serum was required for these inhibitory effects, while C5 was not involved.

Therapeutic remarks

The high abundance of CRPs in many types of tumors, together with the promising results of the studies conducted so far, has led many to pursue the use of CRP inhibitors as an adjuvant to tumor therapy. Pharmaceutical blockade of selected CRPs, or a combination of them, could indeed

result in improved treatment efficiency, treatment of a greater variety of tumors, as well as higher survival rates and better prognosis. However, complement regulators are widely expressed in normal tissues, and random inhibition of CRP function can induce unwanted effects. The development of appropriate tumor-targeting strategies is vital if one wants to use CRP inhibition as an adjuvant treatment. The number of available CRP inhibitors is currently very limited, since the research interest of many groups is mostly focused in developing methods that harness complement activation rather than inducing it. Although several monoclonal antibodies against CRPs exist and newer are emerging (127), the non-specific nature of the treatment makes them hard to utilize. Several steps are taken towards this area, with research groups choosing different approaches.

One option is the use of bi-specific monoclonal antibodies (bs-mAbs). Bs-mAbs are antibodies that consist of fragments from two different mAbs, thus making a unique antibody that can simultaneously recognize two different epitopes. This approach is aiming at combining the tissue-specificity of an anti-tumor mAb together with the CDC effects of an anti-CRP mAb. Such antibodies against Crry, CD59 and CD55 have shown promising results (118,128-131).

A similar approach was followed by Macor *et al.* (132), using biotin labeled miniantibodies against CD55 and CD59 together with biotin-rituximab. The three-step biotin-avidin system resulted in a specific binding of the anti-CRP miniantibodies to cells where rituximab had specificity (132) and this was associated with increased animal survival.

In an attempt to maximize CDC efficiency in mAb therapy, Sato *et al.* (133) developed a unique approach, by generating a CDC-enhancing version of rituximab by converting the Fc portion of the human IgG1 heavy chain into IgG3 to create a novel chimeric constant region of mixed IgG1/IgG3 isotype, which possesses enhanced C1q binding properties and increased CDC (133). In their *in vivo* model they noticed that the potency of their antibody was much higher than rituximab alone.

Although blocking of the mCRP with mAbs enhances complement-mediated immunoclearance of tumors, their high molecular mass and the ubiquitous expression of their targets can pose serious limitations for their application in humans. An alternative approach, down-modulation of mCRPs, has been successfully achieved *in vitro* by RNA interference (134); however, there are numerous problems (e.g., *in vivo* stability, tissue specific targeting and unwanted

immune system activation) that are currently preventing the use of RNA interference *in vivo*.

Other approaches to control mCRP expression have involved the use of phosphatidylinositol-phospholipase C (PI-PLC) to down-regulate CD59 in a melanoma cell line that lead to sensitization to mAb treatment (135) or the use of cytokines such as IL1- β and IL-4 in human renal tumor cell lines that lead to down-regulation of mCRPs and increased C3 deposition in the cells (136). However, none of these approaches had a follow-up or were shown to be effective in *in vivo* models.

Conclusions

For many years the anti-tumor treatment approach focused on targeting the cancer cells themselves. However, current studies reveal that the control of the tumor microenvironment can play a complementary role to this approach. The elucidation of the complex mechanisms involved in the anti-tumor immune response in different types of cancer is required to improve cancer-related treatment and bring fresh weaponry to the bedside. Complement modulation is a promising candidate to supplement existing and design new anti-tumor therapeutics.

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Footnote

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