THE ROLE OF COMPLEMENT IN TUMORS

EDITED BY: Barbara Rolfe, Ruben Pio, Trent M. Woodruff, Maciej M. Markiewski and Helga D. Manthey

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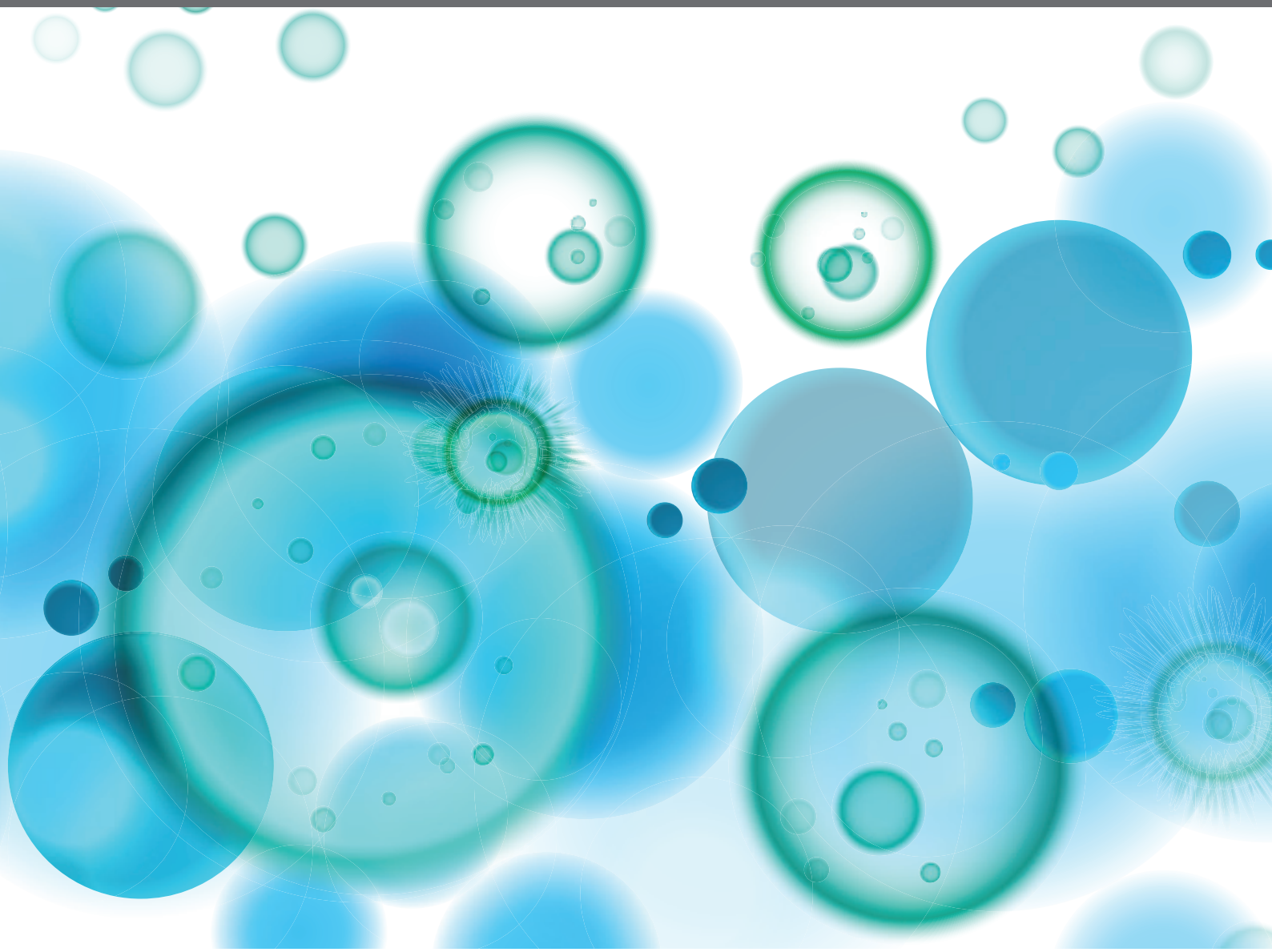

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THE ROLE OF COMPLEMENT IN TUMORS

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EDITORIAL
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Editorial: The Role of Complement in Tumors

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Editorial on the Research Topic

The Role of Complement in Tumors

Activation of the complement system is one of the earliest responses to invading pathogens and tissue damage (1). Complement activation leads to production of a range of effectors including the opsonin C3b, the anaphylatoxins C3a and C5a, and the C5b-9 complex (membrane attack complex; MAC) (2, 3). In addition to potent innate immune activities, complement effector systems also contribute to efficient adaptive immune responses (4). While critical to proper immune function, inappropriate or excessive complement activation contributes to many pathological inflammatory conditions (5), including cancer. As described in this issue, the complement system is increasingly recognized as a double-edged sword: on the one hand contributing to the anti-tumor response, but on the other protecting the tumor against immune attack and promoting metastasis.

COMPLEMENT-DEPENDENT CYTOTOXICITY AND C5b-9 (MEMBRANE ATTACK COMPLEX)

As described by Macor et al., the complement system has long been recognized to contribute to anti-tumor defense mechanisms via complement dependent cytotoxicity (CDC) (6) and antibody-dependent cell mediated cytotoxicity (ADCC) (7). The introduction of recombinant antibodies for cancer treatment has led to renewed interest in complement as an anti-tumor defense system. The protective role of complement in cancer is discussed, with focus on the beneficial effect of complement-fixing antibodies which initiate cancer cell killing via CDC.

The cytotoxic activities of C5b-9, and the mechanisms by which it damages cancer cells, are further discussed by Fishelson and Kirschfink, along with the multiple mechanisms that tumor cells employ to resist C5b-9-induced death. They discuss the potential for therapeutic approaches to counter tumor escape mechanisms and potentiate antibody-based immunotherapies, but caution that intervention strategies to augment complement activation could also worsen outcomes.

Although C5b-9 has traditionally been attributed an anti-tumoral role through CDC, Vlaicu et al. review the evidence that C5b-9 at a sub-lytic dose stimulates tumor growth. Hence strategies to counteract the tumor-promoting traits of C5b-9 and potentiate anti-tumoral actions (including enhanced efficacy of antibody-based immunotherapy) may be the next major direction for immuno-oncology.

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COMPLEMENT REGULATORY PROTEINS

As described by Geller and Yan, membrane and soluble complement regulatory proteins (CRPs) prevent excessive complement activation. Therefore, over-expression of CRPs by tumor cells protects them against complement-mediated attack, interferes with anti-tumor therapies, and enhances metastatic potential. The application of CRPs as prognostic biomarkers and therapeutic targets is discussed, along with the potential for combinatorial approaches with other anti-tumor therapies.

COMPLEMENT C1q

The first subcomponent of the classical complement pathway, C1q is a pattern recognition molecule locally synthesized by macrophages and dendritic cells (8). Bioinformatics analysis by Mangogna et al. suggests C1q as a new prognostic biomarker for several cancers.

ANAPHYLATOXINS C3a AND C5a

Since the seminal paper of Markiewski et al. (9) identifying a role for C5a in promoting tumor progression, similar effects have been demonstrated in a range of murine cancer models. Wang et al. propose C3aR and C5aR1 as a new class of immune checkpoints. They discuss findings suggesting that C3aR/C5aR signaling regulates T cell mediated antitumor immunity via transcriptional suppression of interleukin (IL)-10. Given resistance of the majority of patients to the current forms of immunotherapy, and adverse reactions associated with these approaches, the authors suggest the manipulation of the C3aR/C5aR/IL-10 pathway as an alternative strategy for cancer.

Lenkiewicz et al. discovered that C3 and C5 cleavage fragments enhance trafficking, motility and, therefore, dissemination of malignant cells in hematologic malignancies through a p38 MAPK and inducible heme oxygenase 1 (HO-1) manner. They propose that activation of the complement cascade in patients with these malignancies (e.g., triggered by infection) can contribute to faster dissemination of disease. Thus, targeting this pathway may ameliorate dissemination of leukemic cells and improve clinical outcomes in these patients.

Kleczko et al. discuss the potential for complement targeting therapies for the treatment of intractable cancers, in particular lung cancer. They review the mechanisms by which the anaphylatoxins C3a and C5a influence tumor growth and promote epithelial-mesenchymal transition and tumor metastasis. Since complement proteins can regulate both pro- and anti-tumorigenic pathways, the authors stress the need to better understand the effects of complement activation within tumor tissue, and how this may be influenced by different oncogenic drivers.

Cancer metastasis is estimated to be responsible for greater than 90% of cancer deaths (10). As discussed by Ajona et al., distorted complement homeostasis not only remodels the tumor microenvironment by inhibiting anti-tumor immune responses, but is also crucial to metastasis, endowing tumor cells with properties required for metastatic dissemination and establishment. Complement activation products (primarily C3a and C5a) induce a range of mediators which promote epithelial-mesenchymal transition, tumor growth, invasion, dissemination via lymphatic and circulatory systems, and also protect cells within the metastatic niche. The authors highlight the potential of complement-targeting drugs to augment the clinical efficacy of current immunotherapies and effectively eradicate both primary tumors and distant metastases.

CONCLUSIONS

Despite recent clinical advances in cancer immunotherapy, the estimated percentage of patients responding to checkpoint inhibitors in the United States in 2018 was only 12.46% (11). Hence there remains an urgent need for novel therapeutic strategies to boost response rates. As a critical link between the innate and adaptive immune systems, the complement system is a promising therapeutic target. However, knowledge is the key to realizing the clinical potential of complement-targeting therapeutics. Only a thorough understanding of the role of complement pathways in the tumor microenvironment will enable development of strategies to selectively (and safely) target the pro-tumor effects of complement, while simultaneously augmenting the anti-tumor effects. To quote Fishelson and Kirschfink, "currently we perceive only the tip of the ice-berg of … interactions between cancer cells and complement components." Indeed, given the diversity of responses, therapeutic protocols will likely need to be optimized for each cancer type and stage, and possibly for individual cancer patients, depending on their "immune history."

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of this work. BR drafted the manuscript, the other editors RP, TW, MM, and HM provided critical revisions and approved the submitted manuscript.

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REVIEW
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Complement as a Biological Tool to Control Tumor Growth

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Deposits of complement components have been documented in several human tumors suggesting a potential involvement of the complement system in tumor immune surveillance. *In vitro* and *in vivo* studies have revealed a double role played by this system in tumor progression. Complement activation in the cancer microenvironment has been shown to promote cancer growth through the release of the chemotactic peptide C5a recruiting myeloid suppressor cells. There is also evidence that tumor progression can be controlled by complement activated on the surface of cancer cells through one of the three pathways of complement activation. The aim of this review is to discuss the protective role of complement in cancer with special focus on the beneficial effect of complement-fixing antibodies that are efficient activators of the classical pathway and contribute to inhibit tumor expansion as a result of MAC-mediated cancer cell killing and complement-mediated inflammatory process. Cancer cells are heterogeneous in their susceptibility to complement-induced killing that generally depends on stable and relatively high expression of the antigen and the ability of therapeutic antibodies to activate complement. A new generation of monoclonal antibodies are being developed with structural modification leading to hexamer formation and enhanced complement activation. An important progress in cancer immunotherapy has been made with the generation of bispecific antibodies targeting tumor antigens and able to neutralize complement regulators overexpressed on cancer cells. A great effort is being devoted to implementing combined therapy of traditional approaches based on surgery, chemotherapy and radiotherapy and complement-fixing therapeutic antibodies. An effective control of tumor growth by complement is likely to be obtained on residual cancer cells following conventional therapy to reduce the tumor mass, prevent recurrences and avoid disabilities.

Keywords: complement system activation, tumor control, antibody-based immunotherapy, combination therapies, antibody

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INTRODUCTION

Cancer development is a complex biological process that starts with the malignant transformation of normal cells caused by genetic alterations and somatic mutations leading to unrestricted cell proliferation (1). The local microenvironment plays an important role in this process providing favorable conditions for the seeding of cancer cells in a protective niche that allows the growth and expansion of the tumor mass (2). Changes in the structural and organizational properties of

extracellular matrix favor adhesion and migration of cancer cells from the initial tumor site (3). Active angiogenesis equally contributes to these environmental changes with the formation of new leaky vessels that supply growing cancer cells with nutrients and promote their metastatic spread to distant organs (4, 5).

Tumor development is constantly controlled by the immune system that recognizes cancer cells as potential threats to body homeostasis and mounts a response leading to local recruitment of effector cells of both innate and adaptive immunity (6). Although cell-mediated immunity has long been recognized to play a critical role in tumor eradication through the action of natural killer cells and cytotoxic T lymphocytes (7, 8), studies reported in recent years have shown that the complement (C) system is also an important player in cancer immune surveillance and these studies have revealed the complex interaction of C with cancer cells. C components are synthesized by resident and recruited cells including fibroblasts, endothelial cells, tissue specific cells, and macrophages (9, 10) and are released in the tumor microenvironment. Biologically active products generated as a result of C activation may directly kill cancer cells or favor their eradication by promoting an inflammatory process. However, it is important to emphasize that C does not always provide an effective protection against tumor growth since its damaging effect on cancer cells can be prevented by C regulatory proteins (CRPs) over-expressed on the cell surface or by other mechanisms of cell resistance to C attack. These evasion strategies are more likely to be operating under conditions of fast tumor growth.

Recent studies have elucidated a novel aspect of C interaction with cancer cells showing that it is able to promote rather than to inhibit tumor development. Markiewski et al. (11) made the original observation that C5a, released in the microenvironment as a result of C activation, recruits and activates myeloid derived suppressor cells that suppress anti-tumor T-cell responses against HPV-induced cancer. Similar findings have been reported in other syngeneic models of mouse tumors invariably associated with C activation (12, 13). Importantly, C5aR1-deficiency and pharmacological blockade of C5aR1 by selective C5aR1 antagonists have been shown to impair tumor growth, pointing to the C5a/C5aR1 signaling axis as an effector mechanism of C-mediated tumor-promoting functions (14).

More recently, C1q secreted in the tumor microenvironment was reported to favor tumor progression by enhancing adhesion, proliferation, and migration of cancer cells and promoting angiogenesis independently on C activation (15).

Given these restraints in C-dependent tumor control, the system has apparently limited chances to provide an effective defense barrier against cancer development unless the C protective functions are made more efficient by optimizing the conditions of its activation and effector activities. In this review, we shall discuss the strategies that may turn the C system into a more efficient therapeutic tool by enhancing its activation on the surface of cancer cells and overwhelming the mechanisms adopted by tumor cells to evade C attack.

COMPLEMENT ACTIVATION AT TUMOR SITE

Immunohistochemical analysis of tumor tissue has provided useful information on the contribution of the C system to the immune response to cancer revealing the presence of C components in several solid tumors of different tissues and organs. Various cell types in tumor tissue including cancer cells represent the main source of these components which may also derive, at least in part, from the circulation as a result of the increased permeability of the tumor vessels. C deposits have been observed in a number of tumors (16) and in one study that examined the deposition of various C components in glioblastoma, C1q was found to be the most highly expressed component (17). We have recently shown that C1q is present in various tumors in the absence of other C components and exerts functions unrelated to C activation (15). However, this does not exclude C activation at tumor sites as suggested by tissue deposition of known markers of C activation including C4d, C3d, and SC5b-9 (18, 19). Local changes in tumor tissue due to necrosis and apoptosis and, more importantly, inflammation are responsible for C activation to a degree related to the extent of these changes, in particular of the inflammatory process. This suggests that C deposits are likely to be negligible in the initial stages of tumor growth when the inflammatory reaction is hardly detectable and is probably more evident at a later stage of tumor expansion associated with an overt inflammatory reaction. Tumor cells may partly contribute to C activation using cell-bound proteases exposed on their surface to cleave C5 and to generate C5a, which in turn enhances cancer cell invasion (20).

It is not easy to evaluate the impact of C activation at tissue sites on tumor development because the immunohistochemical data have mostly been obtained from well-established cancers. Importantly, C activation products are mainly localized in the tumor microenvironment and found to be weakly or moderately bound to some but not all cancer cells, suggesting that they have limited effect in reducing cell survival. However, it is possible that C exerts a protective effect in the early phase of cancer growth, contributing to induce tumor regression, although this is difficult to ascertain in patients. One way to address this issue is to utilize mice that develop spontaneous tumors and analyze the effect of C activation on its progression at tumor sites. Using BALB/c females expressing the activated rat Her2/neu oncogene, Bandini et al. (21) have shown that the mammary carcinoma developing in $C3^{-/-}$ mice manifests faster growth rate and earlier lung metastasis than the tumor in wild type animals, suggesting that C activated by antibodies (Abs) directed against Her2/neu oncogene and/or other tumor-associated antigens may control tumor growth. Different results were obtained using a syngeneic mouse model of ovarian cancer which showed similar growth in wild type and $C3^{-/-}$ mice due to secretion of C3 by tumor cells that exerts a stimulating effect on cell proliferation (22). Overall, the available data support a dual role of C in tumor immune surveillance and its ability to either prevent or promote tumor progression depends on the characteristics of cancer cells and the anti-tumor efficiency of the C system.

TABLE 1 | FDA-approved complement-fixing antibodies.

Target antigen	INN	Company	Source	Year of first US FDA (EU EMA) approval	Therapeutic indication(s)
CD20	Ibritumomab tiuxetan	Biogen Idec	Murine IgG1 (type I)	2002 (2004)	NHL
	Ofatumumab	Genmab and GSK	Human IgG1 (type I)	2009 (2010)	CLL
	Rituximab	Biogen Idec, Genentech (Roche)	Chimeric IgG1 (type I)	1997 (1998)	NHL; CLL
CD38	Daratumumab	Janssen-Cilag, Genmab	Human IgG1/κ	2015 (2016)	MM
CD52	Alemtuzumab	Millennium Pharmaceuticals and Genzyme	Humanized IgG1	2001	CLL
EGFR	Cetuximab	ImClone (Eli Lilly), Merck Serono and BMS	Chimeric IgG1	2004	Head and neck cancer; colorectal cancer
GD2	Dinutuximab	United Therapeutics Europe	Human IgG1/κ	2015	Neuroblastoma
HER2	Pertuzumab	Roche	Humanized IgG1	2012 (2013)	Breast cancer

INN, International Non-proprietary Name; NHL, Non Hodgkin Lymphoma; CLL, Chronic Lymphocytic Leukemia; MM, Multiple Myeloma.

CANCER CELLS AS POTENTIAL TARGET OF COMPLEMENT ATTACK

Expression of tumor-associated molecules on cells undergoing malignant transformation can lead to C activation on the cell surface by all three activation pathways. The lectin pathway has been implicated in C activation on glioma cells which express, like many other malignant cells, high mannose glycopeptides that bind MBL and trigger consumption of C4 and C3, but this reaction fails to induce cell lysis (23). Virus transformed cells express novel antigens that are able to activate the alternative pathway, as is the case of EBV-infected B lymphoblastic cell lines (24–26) and T and monocytic cell lines infected by HIV (27). The classical pathway of C can be activated on cancer cells by natural Abs, preferentially of IgM isotype, that recognize carbohydrate moieties on cell surfaces (28, 29). Cytotoxic Abs reacting with carbohydrate epitopes of gangliosides GD2 and GD3 on neuroblastoma and melanoma cell lines have been detected in a small number of sera from normal individuals (30). Unfortunately, besides the low frequency, the natural Abs are not efficient in promoting C-mediated cell killing due to their low titer and affinity.

Attempts have been made to vaccinate cancer patients with the aim to induce production of therapeutic Abs. The anti-tumor response has not always been satisfactory, although a novel vaccination procedure has recently been developed in rabbits to stimulate the generation of IgG Abs that cause strong C-mediated lysis of myeloma cells carrying the CD38 antigen (31).

Despite this improvement, the development of recombinant Abs against tumor antigens remains the preferential approach to stimulate selective C activation on cancer cells, although the identification of specific tumor-associated antigens able to discriminate cancer cells from healthy tissue still represents a major limitation in Ab-mediated cancer therapy. A major progress has been made in the immunotherapy of hematologic malignancies, in particular those derived from B cells, with the generation of monoclonal Abs directed against target antigens, such as CD19 and CD20, present only on B-cells at late stages of development, and not on hematopoietic stem cells that are therefore unaffected by the treatment. Conversely, the development of therapeutic Abs against solid tumors has been limited by the difficulty to identify specific target antigens on cancer cells, whether overexpressed self-antigens, or neoantigens due to tumor-specific mutations or oncogenic viruses.

Only 15 monoclonal Abs have been approved by FDA for the treatment of all different solid tumors (32), and only 3 of them are C-fixing molecules, as described in **Table 1**.

FACTORS AFFECTING THE EFFICIENCY OF THE ANTIBODIES TO ACTIVATE COMPLEMENT: THE ANTIBODIES STRUCTURE

Among the molecular characteristics of recombinant Abs responsible for C activation, the Ig class is critically important since human IgM, IgG1, and IgG3 are known to be the most effective C activators whereas IgG4 fails to bind C1q (33). The structure of the Fc region of these Abs has been extensively investigated to improve their therapeutic efficiency and changes of some amino acids in this region were found to enhance the Ab activity (34). In particular, computational design followed by high-throughput screening techniques has allowed the identification, production, and characterization of Fc variants with increased ability to bind C1q and to promote C-dependent cytotoxicity (CDC) (35).

Glycosylation is an important secondary modification of immunoglobulins that has a significant impact on their capacity to activate different arms of the immune system. The addition of conserved glycans, in particular α (1,6)-linked core fucose, to the Fc region, was shown to be critical for the interaction of the Ab with the C system (36). This observation has raised strong research interest in several biotech companies, resulting in the commercialization of the anti-CD20 Ab obinutuzumab (Genentech, San Francisco, CA, USA).

Terminal mannosylation is another important post-translational modification that prolongs the half-life of the Abs in the circulation and favors binding of mannose-binding lectin (MBL) (36). Importantly, the terminal glycosylation of IgG has been shown to influence CDC without affecting Ab-dependent cell cytotoxicity (ADCC). In addition, an increased content of terminal galactose potentiates CDC activity by enhancing the binding of C1q to the modified Ab (37).

The discovery that hexamerization of Abs after binding to target antigens leads to a successful activation of the classical pathway represents a major advance in the development of new strategies to enhance C activation by IgG (38, 39). The critical role of this process in C activation is supported by the finding that some mutations of Fc amino acid sequence of anti-CD20 IgG result in impaired hexamer formation and reduced cell lysis. Conversely, other mutations have the opposite effect and similar results were obtained introducing the same mutations in the IgG4 isotype. A certain degree of flexibility of antigen-bound Abs allows a conformational change required for hexamerization. The ability of anti-CD20 Abs to exhibit a more efficient CDC after hexamer formation is shared also by anti-CD52 and anti-HLA Abs (38, 39).

FACTORS AFFECTING THE EFFICIENCY OF THE ANTIBODIES TO ACTIVATE COMPLEMENT: THE ANTIGEN

Irrespective of the C-activating capacity of anti-tumor Abs, the characteristics of the target antigen remain of pivotal importance for a successful tumor cell lysis. The beneficial effects of Abs in cancer immunotherapy depend on the expression pattern and the tissue specificity of tumor antigen that should be present exclusively or predominantly on cancer cells to allow selective or almost exclusive targeting of tumor cells. It is equally important that the tumor antigens are expressed also on metastatic cells which represent the main target of Ab-based immunotherapy since other therapeutic approaches including surgery, radiotherapy, and chemotherapy can be used to obtain an effective control of primary tumor. Moreover, the tumor antigens should be stably expressed on the cell surface to serve as useful targets for immunotherapy, whereas intracellular antigens, though specific for tumor cells, can only be used for diagnostic purposes. It is important to point out that tumor cells are often heterogeneous in the expression level of tumor antigens that may influence their susceptibility to CDC. We have observed that cells expressing low levels of CD20 isolated by cell sorting from a population of either chronic lymphocytic leukemia (CLL) or cancer B-cell lines and kept in culture for over a week give rise to cells expressing higher level of CD20 that more easily undergo C-mediated lysis (40, 41). This observation suggests that repeated injections of Abs administered at appropriate time intervals can be used to allow the emergence of cell clones expressing higher levels of CD20 and more susceptible to CDC. Finally, the release of antigen in the tumor microenvironment and in the circulation may lead to blockade of therapeutic Ab and contributes to reduce its expression level on the cell surface, making cancer cells less susceptible to Ab-mediated C-dependent killing (9).

The number of antigenic sites does not always account for the capacity of a monoclonal Ab to cause CDC. In this regard, the impact of the different distribution of two tumor antigens, the alpha isoform of folate receptor (42) and CD20 (43), on Ab binding and C activation has been compared. The folate receptor is associated with epithelial ovarian carcinoma cells and is expressed on several cell lines at a concentration of about 1×10^6 molecules/cells (44). CD20 is present on cancer B-cells at a substantially lower expression level of around 40,000–70,000 molecules/cell (45). Despite the marked difference in the number of cell-associated antigenic sites, the chimeric anti-CD20 Rituximab is able to activate C (46) and to kill B cells whereas a chimeric anti-folate receptor Ab fails to do so (42).

Additional factors may play a relevant role in promoting a more efficient C activation by recombinant Abs, including the proximity of the target epitopes to the cell surface (47), the density of target antigen (48), and the Ab-induced movement of the antigens across the cell membrane (49).

A recent study by Cleary and colleagues provided convincing evidences that the efficacy of C-mediated killing of cancer cells induced by Ab is largely influenced by the distance of the target epitope from the cell membrane and the greater the distance from the cell surface, the lower the efficiency of cell lysis (47). They used target cells transfected with fusion proteins containing either CD20 or CD52 epitopes attached to various CD137 scaffolds and showed that the cells displaying the target epitopes closer to the membrane were more susceptible to CDC than those expressing the epitopes furthest away from the cell surface. These data clearly suggest that the position of the epitope in the target antigen is an important factor to consider in the selection of therapeutic Abs. The surface expression level of the antigen has been shown to be equally important for an efficient C activation on both hematological and solid tumors. Golay et al. (45) analyzed freshly isolated cells from patients with B-CLL and prolymphocytic leukemia for CDC induced by Rituximab and found that the C sensitivity of these cells correlated with the surface expression of CD20. Derer et al. (48) reached similar conclusions using a fibroblast cell line expressing different levels of Epithelial Growth Factor Receptor (EGFR) and reported data indicating that the cell susceptibility to CDC progressively increased at higher expression level of EGFR. An increased antigen density resulting from Ab-induced movement of the tumor-associated antigen across the cell membrane can also contribute to enhance Ab-dependent C activation. CD20 is an example of a membrane antigen, that is induced by the type 1 Abs rituximab and ofatumumab to translocate to the lipid rafts (49). As a consequence, the immune complexes reach a critical concentration required for hexamer formation and C1q binding (50).

FACTORS AFFECTING THE EFFICIENCY OF THE ANTIBODIES TO ACTIVATE COMPLEMENT: THE COMPLEMENT SYSTEM

C is an important player in Ab-induced tumor cell death and has therefore a major impact on the efficacy of therapeutic Abs.

A clinical observation in patients with CLL treated with Abs is that the depletion of cancer and normal cells in the blood of patients is impaired in the presence of reduced levels of C components (51). Clearance of CLL cells induced by Abs to CD20 has been shown to be associated with C consumption, particularly of the early components, which persist for several days to weeks (52). This would cause a reduced therapeutic effect of subsequent infusions of the same Abs to control the malignant cells that circulate in blood in increasing number due to migration from bone marrow or lymph node. Using an *in vitro* model to evaluate the CDC of Burkitt's lymphoma cell lines induced by ofatumumab and rituximab, Beurskens et al. (51) have investigated the effect of different concentrations of anti-CD20 Abs on cell killing in two consecutive steps. They found that the dose of anti-CD20 Abs tested in the first step was critical for the degree of cell killing in the second step. In particular, using the maximal dose of anti-CD20 Abs in the first step, the cell lysis did not exceed 30% in the second step, while the percentage of cell killing increased to over 80% using a lower Ab concentration in the first step. These data suggest that the best therapeutic option would be to use the minimal concentration of Ab to trigger C-mediated killing of a relatively high number of cells leaving a C level sufficient to clear newly emerging malignant cells treated with an additional administration of Ab.

The critical role of C in CDC induced by recombinant Abs is supported by other uncontrolled studies suggesting that the killing of cancer B cells could be enhanced based on supplementation with purified C components or fresh frozen plasma (53, 54).

The response to immunotherapy of tumors that develop extravascularly is likely to be different from that of circulating cells. Unfortunately, it is difficult to evaluate the concentration of the Ab at cancer site, nor is it easy to measure the activity of the C system in the tumor microenvironment. However, the amount of Abs that reaches tumor sites (55) should be sufficient to activate C if the Abs tend to form hexamers that require limited amount of C components to activate the system (39).

Evidence supporting local C deposition was obtained by our group using a mouse xenograft model of B-cell lymphoma established in SCID mice with the intraperitoneal injection of a lymphoma cell line (56). This model is characterized by the development of peritoneal tumor masses and formation of foci of lymphoid cells in the spleen, liver, and bone marrow. Injection of rituximab into tumor-bearing mice resulted in the deposition of the Ab, C3, and C9 on tumor cells and in prolonged survival of these animals.

COMPLEMENT-MEDIATED CANCER CELL DAMAGE AND REGULATION

The importance of late C components in tumor development has recently been investigated by Verma et al. (57) in a xenogenic mouse model of B-cell lymphoma. They showed that tumor-bearing C5 deficient animals treated with rituximab died within the 52 days period of observation whereas all C5 sufficient mice survived. Although the tumor tissue was not examined for complement deposition, the membrane attack complex (MAC) is likely to have contributed to the C protective effect in this model.

MAC assembly on the cell membrane is the final step of C activation. Tumor cell killing caused by Ab-mediated C activation takes a few minutes to complete under standard *in vitro* conditions (52) and is largely mediated by increased Ca^{2+} influx and rapid activation of a large variety of enzymes as a result of MAC insertion (58, 59). C5a and other C activation products can also contribute to tumor control by recruiting to the tumor microenvironment inflammatory cells that cause cell death via C-dependent cell cytotoxicity and phagocytosis (60).

A large body of evidence has been collected showing that cancer cells can resist CDC by several different mechanisms acting either on the cell surface or intracellularly.

Removal of MAC from the cell surface is one of these mechanisms observed in different tumor cell types after the activation of the C system by mAbs (61–64). This removal is usually mediated through membrane vesiculation, directed both to the inner and the outer sides of cell surface (65).

Overexpression of the membrane-associated C regulatory proteins (mCRPs) CD46, CD55, and CD59 is another mechanism by which cancer cells can evade undesired C attack due to spontaneous or Ab-induced C activation. The mCRPs act at different steps of the C sequence by favoring the decay of the C3 convertases (CD55), promoting the degradation of C3b and C4b (CD46), and preventing the assembly of MAC (CD59) (66, 67). Because of their high expression level on several tumors, mCRPs are considered promising targets for cancer immunotherapy. CD46 has been shown to be highly expressed on colorectal, breast (68), prostate, lung, liver, and ovarian carcinoma (69) cancer cells. Elevated levels of CD55 have been documented in a wide range of cancers including lung, colorectal, gastric, breast, and cervical cancers as well as in leukemia (66). CD59 is also overexpressed on different types of carcinoma and sarcoma and on melanoma cells (70).

An important point to emphasize is that hyper-expression of mCRPs on the surface of tumor cells does not necessarily mean that they are equally involved in cell protection from C attack. Almost two decades ago, we analyzed various B-lymphoma cell lines for their susceptibility to CDC and found that all expressed increased levels of CD55, CD46, and CD59 and were variably resistant to C lysis (46). However, using neutralizing Abs to mCRPs, we were able to show that the resistance to C-dependent cell lysis was abrogated by blocking the inhibitory activity of CD55 and CD59 whereas inhibition of CD46 was totally ineffective (46). In contrast, CD46 appears to play a more prominent role in protecting ovarian cell lines from C attack as suggested from the substantial increase in C-mediated cell lysis observed inhibiting CD46 activity with anti-CD46 neutralizing Abs (42). These findings have important clinical implication for the selection of mCRP to inhibit in the immunotherapy of different tumors.

THERAPEUTIC STRATEGIES

Antibodies and Complement Activation

Over the past 20 years, therapeutic Abs have rapidly become the leading product in the biopharmaceutical market. Currently, there are more than 30 FDA-approved therapeutic Abs for cancer treatment and some of them are C-fixing Abs that mediate CDC (**Table 1**).

Rituximab was the first C-fixing Ab to receive FDA approval and has been used successfully to treat a large number of patients with CD20-expressing B-cell malignancies. Because CD20 is expressed on several B cell-derived cancer cells and also on normal cells from the late pro-B cell through memory cells, while absent on plasma cells and precursors hematopoietic stem cells, it is understandable why treatment with anti-CD20 Abs induces depletion of cancer cells but does not interfere with the repopulation of the B-cell compartment (71). Analysis of the binding mode of anti-CD20 Abs and their epitope specificity has led to the identification of two types of Abs that differ in their ability to form distinct complexes with CD20. Type I Abs stabilize CD20 in lipid rafts leading to stronger C1q binding and increased C activation whereas type II Abs exhibit reduced C1q binding that results in lower levels of cell death mediated by CDC (72).

Rituximab, ofatumumab and ibritumomab tiuxetan are examples of type I Abs known to be efficient activator of the C cascade (71). On the contrary, type II Abs like tositumumab performed poorly in CDC (49) and the same was observed for the optimized type II Ab obinutuzumab which fails to induce CDC (72, 73). However, Bologna et al. have reported that C plays a role in cell killing induced by high dose of the type II glycoengineered anti-CD20 mAb obinutuzumab on B-CLL expressing high levels of CD20, as suggested by the ability of the anti-C5 Ab eculizumab to totally prevent cell lysis (74).

In addition to anti-CD20 Abs, other approved C-fixing Abs directed against various tumor antigens have been recognized to activate C both *in vitro* and *in vivo* including anti-CD52 alemtuzumab (75), anti-CD38 daratumumab (76, 77), anti-EGFR cetuximab (78, 79), anti-GD2 dinutuximab (80) and anti-HER2 pertuzumab (81).

Strategies to Improve Complement Activation by Therapeutic Antibodies

Combination of Different Antibodies

The efficiency of C activation on the cell surface is largely influenced by the epitope density of the antigen recognized by recombinant Abs which can affect the formation of an adequate number of immune complexes capable of binding C1q. Two different strategies have been reported to increase the formation of C1q-fixing dimers. One approach is to use a combination of two Abs recognizing different epitopes on the same antigen sufficiently close to allow juxtaposition of the IgG Abs which is critical for C1q binding. Spiridon et al. (82) were the first to analyze C-mediated lysis of Her-2^+ human breast cancer cell lines induced by several mAbs and showed an enhanced killing using a mixture rather than individual mAbs. Our group has investigated the C-fixing ability of two Abs, cMOV18, and cMOV19, that bind to distinct epitopes of the alpha isoform of folate receptor, highly expressed on epithelial ovarian cancer cells. Interestingly, the mixture of these two Abs was able to activate C and to cause death of ovarian cancer cells while individual Abs were totally ineffective (42). A similar pattern of C activation was obtained using combination of the anti-EGFR Abs cetuximab and matuzumab, which recognize different nonoverlapping epitopes of EGFR (79). Cetuximab was reported to induce some degree of CDC in lung cancer cell lines only at high concentrations (40 μg/mL) (79), whereas lower amount (10 μg/mL) of either cetuximab or matuzumab failed to trigger C activation. Interestingly, the mixture of the two Abs was able to induce C1q and C4c fixation leading to strong activation of CDC (50 and 80% of lysis of squamous cell carcinoma and glioblastoma cells, respectively) (78).

Although this approach has not yet been introduced in clinical practice, it represents a promising future development in immunotherapy with C-fixing Abs.

Neutralization of membrane Complement Regulatory Proteins

Different approaches based on anti-mCRP Abs or silencing mCRP expression in combination with therapeutic Abs have been evaluated *in vitro* and *in vivo* by various groups to prevent the C-inhibitory effect of mCRP. We initially reported an increased susceptibility of follicular and Burkitt's lymphoma cell lines to CDC induced by Rituximab in the presence of Abs to CD55 and CD59 (46). These findings were later confirmed using an *in vivo* model of human CD20+ B-lymphoma established in severe combined immunodeficient mice treated with rituximab in combination with anti-CD55 and anti-CD59 Abs that resulted in a significant animal survival (56). Similar enhancing effect of anti-CD55 and anti-CD59 Abs was reported on C-mediated killing of two human lung carcinoma cell lines induced by Herceptin (trastuzumab) (83). Neutralizing Abs to CD46 and CD59 were instead required to enhance CDC of ovarian carcinoma cells induced by the mixture of cMOV18 and cMOV19 (42). Down-regulation of all three mCRPs obtained with cationic liposomes (AtuPLEXes) loaded with siRNAs proved effective in inducing substantial increase of CDC of HER2 positive breast, lung and ovarian adenocarcinoma cell lines stimulated by trastuzumab and pertuzumab (84).

Although lysis of C-resistant tumor cells can be restored by the addition of Abs neutralizing mCRPs, their use is limited by the ubiquitous expression of mCRPs on both normal and tumor cells. One way to avoid undesired side effects that may derived from the binding of Abs to normal cells resulting in decreased expression of mCRPs is to selectively deliver the Abs to tumor cells. To this end, our group has generated two bispecific Abs containing binding specificity to CD20 and either CD55 or CD59. These Abs were able to recognize CD20 expressed on Burkitt's lymphoma cell lines and to neutralize membrane-bound CD55 and CD59 enhancing cell susceptibility to C-mediated lysis. An *in vivo* model of Burkitt's lymphoma developed in SCID mice was used to investigate the tissue distribution of bispecific Abs that were found to target selectively the tumor mass due to the high affinity of the anti-CD20 portion as opposed to

the lower affinity of the anti-CD55 or anti-CD59 arms and to prevent tumor development (60). The therapeutic effect of the Abs was largely dependent on C activation, as revealed by the increased deposition of C3 and C9 in tumor masses, and also by local recruitment of macrophages and NK cells. Importantly, the combination of these two bispecific Abs resulted in the survival of 100% of treated mice whereas treatment with a single bispecific recombinant Ab (MB20/55 or MB20/59) induced the survival of only 20% of animals (60).

An interesting approach aimed at inhibiting mortalin, an heat shock protein over-expressed in many cancer types, has been proposed by Fishelson and his group (85) to interfere with MAC formation and its release from cell surface. The level of mortalin is inversely related to MAC deposition and its over-expression in erythroleukemic cells protects from C activation through the classical pathway, while protein down-regulation using specific siRNA increases the level of cell-bound C9.

COMPLEMENT ACTIVATION POTENTIATES STANDARD THERAPIES

Complement and Radiotherapy

Together with surgery and chemotherapy, radiotherapy is a clinical mainstay of treatment for many malignancies especially for aggressive tumors with poor prognosis.

Recent studies support an association between radiation therapy for both human and murine cancers and C activation. An elegant study by Surace et al. (86) showed that local irradiation of melanoma and colon carcinoma developing in mice with a single dose of 20 or 5 Gy resulted in rapid and transient C activation triggered by tumor cells undergoing necrosis, apoptosis, and mitotic catastrophe with the possible contribution of natural IgM Abs bound to necrotic cells. In addition, they documented tumor deposition of C3 activation products and local increases in C3a and C5a, which induce maturation and activation of tumor-associated dendritic cells expressing the receptors for these anaphylatoxins and in turn promoting the anti-tumor activity of $CD8^+$ T lymphocytes. The important role of C activation in the control of tumor growth was supported by the finding that radiotherapy failed to exert a protective effect against the tumor in mice deficient in either C3, or in C3a or C5a receptors, suggesting the critical contribution of locally released C3a and C5a.

Somewhat different results were obtained by Elvington et al. (87) who used a lymphoma model in mice receiving low dose radiotherapy fractionated over a period of approximately 5 months. They found that C inhibition induced by the administration of CR2-Crry resulted in longer survival and reduced tumor mass in tumor-bearing mice. A possible explanation for these contrasting results is that a radiation treatment administered over a prolonged period of time induces a C-independent inflammatory response that contributes to promote tumor growth. Overall, these data indicate that dose and fractionation in the radiation therapy need to be further investigated to find optimal conditions that combine the beneficial anti-tumor effects of radiotherapy and C activation.

Complement and Chemotherapy

Limited information is available on the interplay between the C system and chemotherapeutic agents and the effect of this interaction on tumor control.

Levels of C3 and C4 were measured in patients with breast cancer treated with epirubicin/docetaxel-based neoadjuvant chemotherapy and found to be substantially reduced (88). This finding cannot be explained by C consumption because the low concentrations of C3 and C4 were not associated with a parallel increase in the level of the C activation product C4d. The relevance of this observation is unclear since the levels were equally reduced in responders and non-responders to chemotherapy. A similar conclusion was reached in another study that examined the changes in C activity in patients with various types of cancer and revealed a significant reduction in C activity which was not accompanied by a corresponding increase in the level of C3d (89). More direct evidence supporting the beneficial effect of a combination therapy with a chemotherapeutic agent and a recombinant Ab was obtained from a multicenter clinical trial conducted in patients with advanced non-small-cell lung cancer (90). The patients receiving the monoclonal Ab cetuximab directed against epidermal growth factor receptor (EGFR) in combination with cisplatin/vinorelbine survived longer than those treated with the chemotherapeutic agents alone. In a subsequent study, cetuximab was found to bind to a lung cancer cell line expressing EGFR and to activate C resulting in the assembly of

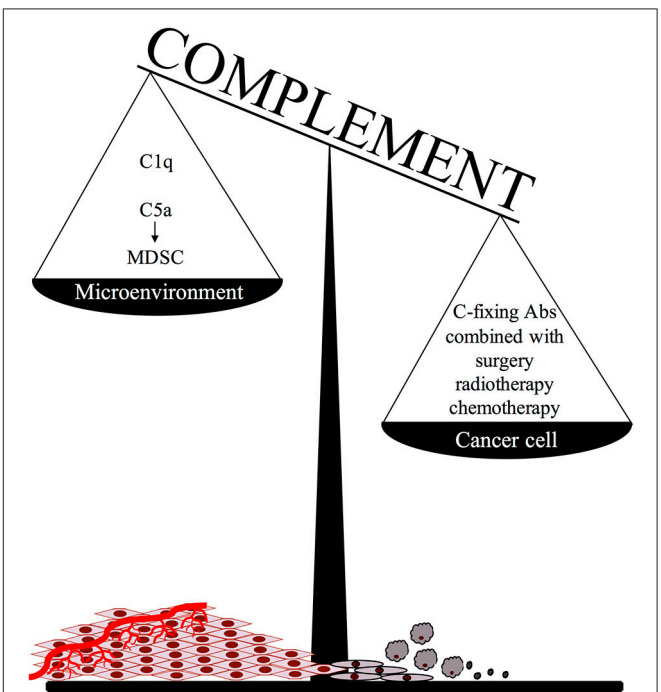

FIGURE 1 | The ambivalent role of complement in tumor growth.

the membrane attack complex and cell death. C involvement in the killing of tumor cells was further documented by the finding that the inhibitory effect of cetuximab on tumor growth in an *in vivo* xenogeneic model of A549 lung cancer cells in nude mice was abolished in tumor-bearing mice treated with cobra venom factor to deplete C (79). These are promising results that need to be confirmed using a similar approach in the study of other tumors because the effect of chemotherapy on C activation and the consequent impact of these treatments on cancer cell killing may be different in various tumors.

CONCLUSION

The introduction of recombinant Abs into the clinic to control tumor growth has fostered the interest in C as an anti-tumor defense system acting in close collaboration with other components of both innate and acquired immunity. This has prompted the development of various strategies to optimize their therapeutic efficiency including structural modifications of the Abs to promote C activation and also control C inhibitors expressed on the tumor cell surface to enhance Ab-induced C-mediated cell killing. Major efforts are being made to selectively deliver mCRPs neutralizing agents to tumor cells and the recently generated bispecific Abs that target cancer cells and inhibit mCRP appear to move in this direction.

An important point to consider when adopting this therapeutic approach is that C activated in the tumor microenvironment, particularly in the case of slow growing tumors associated with an inflammatory process developing in the surrounding tissue, may promote cancer expansion due to recruitment of suppressor cells by locally released C5a (**Figure 1**). We believe that this undesired effect may be prevented or markedly reduced by focusing Ab dependent C activation on residual tumor cells after surgical removal or substantial reduction of tumor mass after radio and/or chemotherapy. It is important, though, that the protocols for radiation therapy and chemotherapeutic treatment are selected to be highly effective in the control of tumor growth with limited pro-inflammatory side effects and negligible C activation in the tumor microenvironment.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Complement in Metastasis: A Comp in the Camp

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The complement system represents a pillar of the innate immune response. This system, critical for host defense against pathogens, encompasses more than 50 soluble, and membrane-bound proteins. Emerging evidence underscores its clinical relevance in tumor progression and its role in metastasis, one of the hallmarks of cancer. The multistep process of metastasis entails the acquisition of advantageous functions required for the formation of secondary tumors. Thus, targeting components of the complement system could impact not only on tumor initiation but also on several crucial steps along tumor dissemination. This novel vulnerability could be concomitantly exploited with current strategies overcoming tumor-mediated immunosuppression to provide a substantial clinical benefit in the treatment of metastatic disease. In this review, we offer a *tour d'horizon* on recent advances in this area and their prospective potential for cancer treatment.

Keywords: cancer, metastasis, complement, tumor microenvironment, anaphylatoxin, bone colonization

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INTRODUCTION

The complement system represents a master component effector of innate immunity. Complement activation and regulation encompasses more than 50 soluble and membrane-bound proteins.

The function of complement, which entails the recognition and removal of pathogens and harmful entities, is accomplished by a multistep and sequential serine proteases-mediated cascade. The release of proteolytic fragments mediates key homeostatic and effector functions including: opsonization, inflammation, adaptive immune regulation, coagulation, tissue repair, neural development, bone homeostasis, angiogenesis, and host–microbiota symbiosis (1). Owing to the potentially deleterious effects of the complement system, its activity is tightly regulated at different levels by a number of soluble and membrane-bound proteins (2). Inappropriate complement activation underlies a variety of physiopathological conditions including inflammatory diseases and cancer (3).

Because many of the complement functions modulate tumor progression, their preeminent roles in promoting tumor cell dissemination are not surprising. This review focuses on recent findings on the major role of the complement system in tumor progression and highlights its key contribution to the different steps of the metastatic cascade.

COMPLEMENT ACTIVATION

Complement is mainly activated via three different recognition pathways: the classical, the lectin, and the alternative pathways. These three modes of complement activation converge into the

generation of C3 convertases, which cleave C3 into C3a and C3b. C3a is an anaphylatoxin displaying an inflammatory regulation role. C3b can act in the opsonization process and as a component of the C5 convertase (4).

The classical pathway is triggered by the binding of C1q to antigen-antibody complexes, dying cells, extracellular matrix proteins, pentraxins, amyloid deposits, prions, or DNA (5).

The lectin pathway starts through binding of proteins homologous to C1q (mannose-binding lectin and H-, L-, or M-ficolins) to carbohydrate structures on pathogens (6). Both the classical and the lectin pathways then sequentially cleave C4 and C2 for the generation of the classical/lectin C3 convertase (C4bC2b) (4).

Finally, the alternative pathway is initiated by the spontaneous hydrolysis of C3, also known as the "tickover" of C3, which after the formation of C3(H_2O) can bind to factor B. Cleavage of factor B by factor D forms the initial alternative pathway C3 convertase, C3(H_2O)Bb (7).

Although these three routes of activation differ in their mechanisms of target recognition and initiation, they converge at C3 cleavage, yielding the active fragments C3a and C3b. C3b binding to C3 convertases assembles the C5 convertase that cleaves C5 into the anaphylatoxin C5a, and C5b. The latter fragment is indispensable to assemble the membrane attack complex which mediates targeted lysis (8).

Additional pathways of complement activation include C3 and C5 extrinsic protease cleavage (9–11), the C2-bypass pathway (12), and the properdin-mediated direct convertase formation on microbial surfaces (13).

Among the complement-derived downstream effectors, C3a and C5a play diverse roles in both homeostasis and disease. These molecules bind to their cognate seven-transmembrane domain receptors C5a receptor 1 (C5aR1; CD88) and C3a receptor (C3aR), respectively. C5a can also bind to C5aR2 (14). The role of C5aR2 remains poorly understood. Recently, it has been reported that the binding of C5a to C5aR2 in carcinoma-associated fibroblasts promotes tumor formation and chemoresistance by providing a survival niche for cancer stem cells (15).

Recent discoveries have also revealed that complement activation is not only restricted to the extracellular space, as originally thought, but also occurs in the cytoplasm. The intracellular components of complement (the so-called complosome) modulate metabolic processes during T cell effector differentiation (16, 17) but so far, their intracellular functions remain largely unexplored.

COMPLEMENT IN CANCER PATIENTS

Neoplastic transformation involves complex genomic and epigenomic alterations perturbing normal cell homeostasis. Local or distant dissemination of tumor cells, one of the hallmarks of cancer, represents a multistep process that entails the gain of novel cellular functions which include invasion, increased cell locomotion, intravasation, survival in the circulation, overcoming immune attack, and colonization in foreign cellular niches to form secondary tumors (18).

Overcoming immune attack is a key step in tumor progression. Altered immune recognition is achieved by a variety of mechanisms (19), including the modulation of the complement system. Complement activation has been described in cancer patients with hematological malignancies such as lymphomas (20), and in a plethora of solid tumors (21–23). Furthermore, intact complement proteins were found increased in blood of patients with lung cancer (24, 25), neuroblastoma (26), and digestive tract tumors (27). However, complement-mediated cytotoxicity is circumvented by different mechanisms, most of which include the upregulation of complement regulatory proteins (28–30). These regulators normally protect tumor cells from complement-mediated destruction, and can be grouped into two categories: membrane-bound complement regulatory proteins (mCRPs) and soluble regulators. High expression of the mCRPs membrane cofactor protein (CD46), decay-accelerating factor (CD55), and CD59 (protectin) on tumor cells is associated with increased metastatic potential, and poor prognosis in a range of tumors (31–34). Similarly, the soluble regulators factor H and FHL-1 have been found elevated in biological fluids from ovarian (35), bladder (36) and lung cancer patients (37), and are also associated with poor prognosis (38). Other soluble regulators as clusterin (39), C1 inhibitor (40), factor I and C4b-binding protein (C4BP) (41) are secreted by tumor cells into the tumor milieu and could also be detected in the circulation.

Activation of the complement system by tumor cells was long believed to only benefit the patient. Preclinical data suggest that complement can evoke potent complement-dependent cytotoxicity against tumor cells, and a range of therapeutic strategies have been designed to potentiate complement activation and overcome the protection mediated by complement inhibitors. This approach has been specially tailored to enhance the therapeutic efficacy of monoclonal antibodies (42). However, recent findings have challenged this view, providing evidence of the cancer-promoting potential of complement activation and the utility of complement inhibition as an anticancer therapy (43). Complement components coopted by tumor cells can lead to the acquisition of self-advantageous functions tilting the balance toward tumor progression. For instance, lung cancer cells are recognized by the complement system more efficiently than their normal counterparts. This effect is mediated by the direct binding of C1q and leads to the subsequent activation of the classical complement pathway (44). This activation is compensated by the expression of factor H/FHL-1 and CD59 (45, 46). This equilibrium in complement activity would explain the elevated levels of complement fragments found in biological fluids from these patients. Thus, C4d, a split product of the classical complement pathway, is increased in biological fluids of lung cancer patients. Detection of C4d is associated with poor prognosis, and has been proposed as a potential biomarker of clinical value in the management of lung cancer patients (44, 47, 48). Similar results were obtained in oropharyngeal tumors by detecting C4d in saliva (49). Moreover, other complement factors have been associated with cancer. Anaphylatoxin C5a is increased in plasma from lung cancer patients (50, 51), and is associated with metastatic potential in

lung and gastric cancer patients (52, 53). Similarly, C1QB is one of the top-scoring genes associated with lung metastases in osteosarcoma patients (54).

Taken together, these studies indicate an association between complement activation and malignant progression.

COMPLEMENT IN THE TUMOR MICROENVIRONMENT

Of all complement proteolytic fragments derived from complement activation, anaphylatoxins are by far, the best described in cancer. Anaphylatoxins C5a and C3a trigger spurious tumor intracellular signaling pathways by binding to their cognate receptors expressed in tumor and immune cells. These signaling events deeply perturb the tumor milieu by inducing the recruitment and/or tumor-promoting abilities of myeloid-derived suppressor cells (MDSC), macrophages, neutrophils, and mast cells, preventing efficient T cell-mediated responses (55).

Elevation of C5a or C5aR1 levels has been observed in solid tumors including lung (50, 53), gastric (56), ovarian (57), breast (58), urothelial (59), and clear cell renal cancers (60).

C5a induces the recruitment of MDSCs into the tumor microenvironment, and markedly dampens anti-tumor T-cell responses. C5aR1 mediates these effects on two subpopulations of MDSCs. On one side, C5a is a potent chemoattractant for granulocytic MDSCs (a neutrophil-like subpopulation) and on the other, C5a stimulates the monocytic MDSC subpopulation with the concomitant production of reactive oxygen and nitrogen species (61).

C5aR1 expressed on MDSCs is also able to bind ribosomal protein S19 (RPS19), which is released from apoptotic tumor cells into the tumor microenvironment, leading to a shift toward Th2 cell responses with increased levels of immunosuppressive TGF-β (62). Accordingly, pharmacological blockade of C5aR1 in a syngeneic model of lung cancer impaired tumor growth, decreased the percentage of splenic MDSCs, and downregulated immunosuppression-related genes including ARG1, IL6, IL10, CTLA4, LAG3, and PDL1 within the tumor milieu (50).

Besides MDSCs, C5a affects the biology of other leukocytes present in the tumor microenvironment. C5a elicits a strong pro-inflammatory infiltration with secretion of MCP-1, responsible for the recruitment of immunosuppressive macrophages, and increase of arginase-1 and IL-10 (63). Similarly, fibrinolytic enzyme-mediated generation of C5a regulates the protumorogenic properties of $C5aR1^{+}$ mast cells and macrophages, leading to hampered antitumor CD8 T-cell responses in a model of squamous carcinogenesis. Interestingly, the combined treatment based on cytotoxic chemotherapy and the blockade of C5aR1 synergistically increased the recruitment and the cytotoxic properties of $CXCR3^{+}$ effector memory CD8 T cells by IFNγ-dependent mechanisms (64). Ablation of PTX3, an important negative regulator of inflammation and complement activation, resulted in amplification of complement activation, MCP-1 production, and tumor-promoting macrophage recruitment. Consistently, pharmacological blockade of C5aR1 reversed these pro-tumorogenic effects (65).

Although far less studied than C5a, the anaphylatoxin C3a also preconditions a tumor-promoting microenvironment. Signaling mediated by C3a binding to C3aR contributes to melanoma tumorigenesis by inhibiting neutrophil and CD4 T-cell responses (66). Autocrine complement C3 inhibits IL-10-mediated cytotoxic properties of tumor-infiltrating CD8 T lymphocytes through complement receptors C3aR and C5aR1, and enhances melanoma and breast cancer growth (67).

Moreover, complement activation may underlie the ability of tumors to evolve and adapt to different cues of the microenvironment increasing tumor progression. Thus, under hypoxic conditions, lung cancer cells downregulate complement inhibitors, factor H and factor I, to increase their susceptibility to complement activation (68). This phenomenon may fuel the generation of C5a which in turns may contribute to hypoxic stress in the tumor milieu to promote tumor progression through the inhibition of cell-mediated immunity. Indeed, in a syngeneic lymphoma model the impact of C5a in tumor microenvironment is dose-dependent (69).

Complement effectors can also affect tumor progression independently of complement activation. Factor B and factor I promote squamous cell tumor growth upon the activation of ERK1/2 (70, 71). C1q promote angiogenesis and lung metastasis in a syngeneic model of murine melanoma (72). In malignant pleural mesothelioma, C1q binds to hyaluronic acid in the tumor microenvironment and enhances tumor proliferation (73). C1q secreted by mesenchymal stromal cells mediates the activation of β-catenin in chronic lymphocytic leukemia and enhances malignant progression (74). On the other hand, properdin, a positive regulator of complement activity, induces endoplasmic reticulum-stress response and exerts a tumor suppressive role in breast cancer (75).

In summary, tumors are able to perturb complement-related immune effectors favoring tumor progression. Distorted complement homeostasis remodels the tumor microenvironment by inhibiting the anti-tumor immune responses and contributes to the metastatic dissemination of cancer cells (**Figure 1**).

EARLY METASTASIS STEPS: COMPLEMENT EFFECTS ON TUMOR CELLS

Epithelial–mesenchymal transition (EMT), loss of cell-cell adhesion, and increase of motility, invasiveness, and intravasation of tumor cells are at the core of the early metastatic events (18). Tumor-associated complement-activation modifies the tumor cell behavior endowing early metastatic traits. Perturbed complement activation leads to the generation of growth factors, proangiogenic factors, and other mediators that promote tumor growth and dissemination. These acquired pro-metastatic functions are mediated by the C3a and C5a stimulation of C3aR and C5aR1 in tumor cells, respectively, which triggers spurious intracellular signaling pathways. For instance, C5aR1 in lung tumor cells activates the p44/42 MAPK and NF-κB signaling pathways leading to the secretion of IL-8, VEGF, and MCP-1 to the tumor milieu (53). Complement

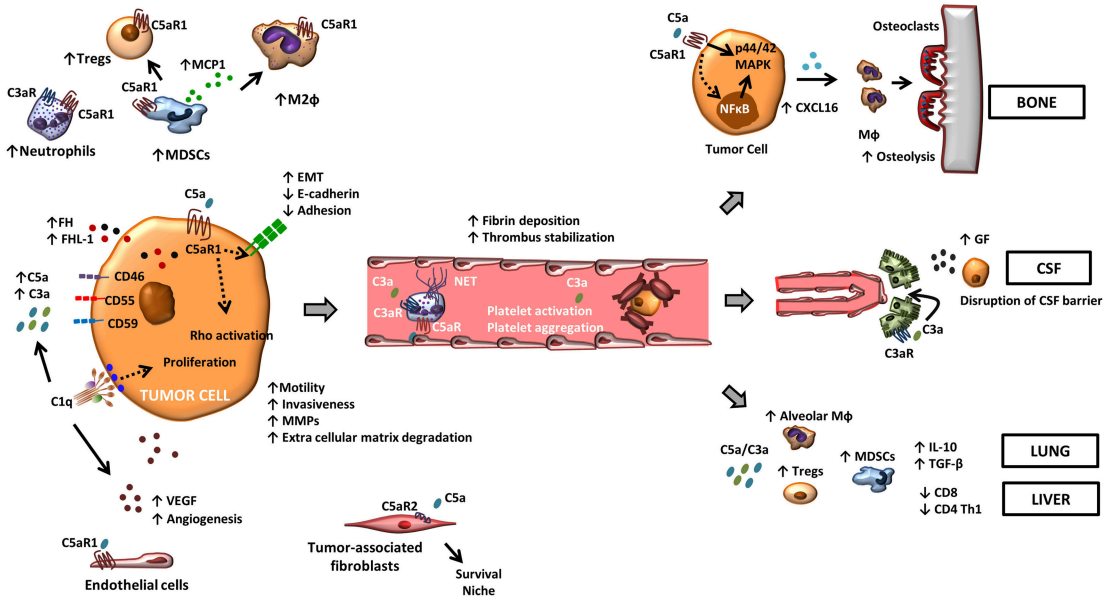

FIGURE 1 | The role of complement in metastasis. Tumor-associated complement activation generates anaphylatoxins C5a and C3a in the tumor microenvironment. Binding of these molecules to their cognate receptors promote a range of tumor-promoting functions. C5a, through its receptor C5aR1, facilitates the recruitment and the activity of suppressive leukocyte subsets such as MDSCs, neutrophils, and Tregs in the tumor microenvironment. C3a also contributes to a suppressive tumor microenvironment by recruiting neutrophils. C5aR1 signaling affects endothelial function and tumor-associated angiogenesis, and the binding of C5a to C5aR2 in carcinoma-associated fibroblasts promotes tumor formation by providing a survival niche for cancer stem cells. In tumor cells, C5a/C5aR1 axis modulates tumor-induced MMP expression, increases tumor cell migration and invasiveness, enhances the release of pro-angiogenic factors, and induces EMT. Binding of C1q to tumor cells enhances tumor cell proliferation and favors angiogenesis in a complement activation-independent manner. Complement anaphylatoxins also facilitate tumor dissemination by stimulating a hyper-coagulation state and NETs, and adapt specific organ environments to the metastatic spread. This includes the disruption of the CSF barrier, the induction of CXCL16-mediated osteoclastogenesis, and the generation of an immunosuppressive microenvironment.

components facilitate tumor dissemination by inducing an EMT in tumor cells which leads to the acquisition of a motile and less adherent phenotype. C5a/C5aR1 axis mediates the upregulation of transcription factor Snail and a concomitant decrease in E-cadherin and claudin-1 gene expression levels with increased invasiveness in hepatocellular carcinoma (76). In ovarian cells, TWIST1 enhances C3 expression and mediates EMT (77). According to these findings C5aR1-tumor expression was associated with tumor invasiveness, vascular and lymphatic invasion, liver metastasis, and poor outcome in patients with gastric tumors (78). Furthermore, C5aR1 inhibition hampers lung cancer cell migration, and up-regulates the expression of E-cadherin, suppressing EMT and invasiveness. Consistently, a negative correlation between the expression of C5aR1 and E-cadherin was found in lung primary tumors (79).

Initial steps for the acquisition of a metastatic phenotype also involves the secretion of stromelysins and other matrix metalloproteinases (MMPs) able to degrade different extracellular matrix components, especially the basal membrane, allowing for tumor cell intravasation and dissemination to local or distant sites (33). C5a markedly enhances cancer-mediated MMP activities and migratory and invasive tumor cell activities (80). C5a stimulation also decreases tumor adhesion to extracellular matrix proteins including collagens I and IV (53). Aberrantly expressed C5aR1 increases cell locomotion, cytoskeletal rearrangements with the formation of lamellipodia and membrane ruffling in liver bile duct malignant cells (80). C5aR1 signaling promotes motility and invasiveness through the activation of RhoA, and leads to enhanced invasion and vascular invasion in gastric cancer cells (56). ERK and PI3K, downstream C5aR1 activation, mediate an increase in cell invasiveness in renal cancer cells (81).

C3a-mediated stimulation elicits an increase in p42/44, p38 MAPK, and PKB/AKT activation and downregulates inducible hemeoxygenase-1 (HO-1) in leukemic cells (82). Autocrine stimulation of C5aR1 and C3aR upon C5a and C3a binding leads to PI3K/AKT signaling and regulates the proliferation and invasiveness of ovarian tumor cells (57).

In summary, complement-mediated effects are crucial in the early stages of metastasis, involving changes in tumor cell adherence to surrounding stroma and neighboring cells, increasing local invasiveness and promoting lymphatic and hematogenous dissemination.

COMPLEMENT EFFECTS ON DISSEMINATION

The host microenvironment at local or distant sites provides signals permissive for tumor promotion. Critical pathways triggered in the surrounding stroma and/or endothelial or lymphatic cells are required for proper cell-cell and cell-matrix engagement and for the secretion of a panoply of protumorogenic factors (83, 84). In addition, vascular or lymphatic vessels provide

a major route by which tumor cells exit the primary tumor site, enter the circulation and establish metastasis (85). Furthermore, tumor vascular density is a prognostic indicator of metastatic dissemination. In cancer, complement may be involved in the modulation of the angiogenic program in the tumor microenvironment, although the specific role of complement in angiogenesis is highly dependent on the tumor type. For instance, C5aR1 blockade does not affect tumor angiogenesis in murine models of lung or cervical cancer (50, 61). In contrast, genetic inhibition of C3 and C5aR1 impairs endothelial cell function in an ovarian cancer model (86). C5a also supports an angiogenic program displayed by infiltrating macrophages in squamous cell carcinoma (64). C1q deposition on melanoma cells increases tumor vascular density and facilitates tumor progression (72). The evidence that complement has a role in endothelial homeostasis might have implications also at secondary metastatic sites, a possibility which remains largely unexplored.

Once in the circulation, tumor cells have to overcome the mechanical constraints imposed by sheer-stress, anoikis induced by cell anchorage-independency, and the immune attack. A role of platelets, together with fibrin and thrombin, has been invoked for the establishment of distant metastasis by protecting circulating tumor cells from mechanical stress and facilitating engraftment at target sites (87).

Complement components contribute to a hyper-coagulation state allowing tumor cell survival in the circulation. C3a induces platelet activation and aggregation favoring a pro-thrombogenic state (88). Similarly, C5a stimulates neutrophils to release Tissue Factor, inducing a prothrombotic phenotype (89). C3aR in neutrophils stimulates neutrophils extracellular traps (NETs) (90), extracellular structures composed of chromatin and degrading enzymes (myeloperoxidase, cathepsin G, and elastase) that contribute to form a three-dimensional scaffold that supports fibrin deposition and thrombus stabilization and entraps platelets, erythrocytes and tumor cells, driving a protumorogenic state (91).

This pro-tumorogenic milieu also favors the subsequent dissemination of tumor cells to neighboring or distant sites. Homing of tumor cells to target sites could also be actively mediated by factors released by target organs that act as potent tumor cell chemoattractants (92). But tumors also precondition target organs creating a hospitable niche by the mobilization of bone marrow-derived myeloid cells, tumor secreted factors such as VEGF, TGF, TNF (93–95), and tumor released-exosomes which also modulate the tumor microenvironment (96, 97). These nanometer-sized vesicles, which contain a complex cargo of membrane receptors, nucleic acids, cytoskeletal components, and intracellular proteins, act as unique vehicles for transport to local or distant organs. Tumor derived exosomes, which are more abundantly released in inflammation, represent another mechanism of immunosuppression. As observed for tumor cells, exosomes display CD55 and CD59, conferring resistance against complement-mediated lysis (98), and potentially regulating the exosome-mediated cross-talk associated with the metastatic program.

These events largely studied in murine models collectively contribute to prepare the "fertile soil" invoked by the Paget's hypothesis (99), and crystallize the concept of "premetastatic niche" (100). The premetastatic niche consists in the accumulation of aberrant immune cells and extracellular matrix proteins in target organs (101). Emerging data demonstrate that C5a contributes to the lung premetastatic niche by regulating the expression of TGF-β and IL-10 by immature myeloid cells and the subsequent accumulation of regulatory T cells, the proliferation of resident alveolar macrophages in the premetastatic lungs, and a decrease in the number and the maturation status of lung dendritic cells. As a consequence, effector CD4 T-cell responses skew toward Th1 responses (102, 103).

LATE STEPS OF METASTASIS

A similar paradigm to which occurs in the primary tumor could also influence metastatic behavior in the target organ. Tumor cells need to overcome the constraints imposed by the "foreign soil" and require compatibility with the hosting milieu. Each organ provides unique opportunities which could be exploited in the benefit of tumor cells by propelling the growth of micro to macrometastases (104). An increasing body of evidence indicates that complement is involved in this process, resulting in tumor outgrowths at secondary sites.

Genetic abrogation of C5aR1 in the host dampens M2-polarized tumor associated macrophages, leading to a decrease of liver and lung metastases in a syngeneic colon cancer model (52). Pharmacological inhibition of C5aR1 increases the infiltration of CD8 cytotoxic T cells in metastatic nodules, and impairs lung and liver metastatic processes with no effect detected in primary tumors. Thus, genetic or pharmacological inhibition of C5aR1 results in impaired metastasis (103).

Moreover, activation of C5aR1 in tumor cells leads to an increased prometastatic activity. For instance, in a lung cancer model of bone metastasis, C5a/C5aR1 axis induced the production of pro-osteoclastogenic factors favoring skeletal metastases. Among these factors, CXCL16 released upon C5aR1 signaling led to osteoclastogenic activation and osteolytic lesions. These effects were blocked by C5a inhibition or genetic silencing of C5aR1 in tumor cells, suggesting its implication in skeletal metastases (53). Indeed, complement is involved in bone homeostasis and turnover (105). Bone-forming osteoblasts and bone-resorbing osteoclasts are tightly regulated to ensure a balanced bone mass. Receptor activator of nuclear factor k-B ligand (RANKL), which is secreted by osteoblasts, binds to its receptor on the membrane of committed monocytes to differentiate into osteoclasts (106). Complement modulates osteoclasts differentiation *in vitro* and *in vivo* through C5aR1, but no effects were exerted in osteoblast differentiation (107). However, C3aR and C5aR1 signaling by C3a and C5a in osteoblasts modulates the release of pro-inflammatory pro-osteoclastogenic cytokines IL-6 and IL-8, and C5a increases RANKL in osteoblasts, overall favoring a pro-osteoclastogenic milieu (108). Because of these bone-specific mechanisms, the complement system might be specially relevant in skeletal metastases (53). Indeed, lung primary tumors that metastasize to bone show higher C5aR1 levels than those that metastasize to other locations, suggesting its major role in the tumor-induced skeletal lesions. Nevertheless, this axis also mediates lung

metastases, since lung tumor colonization was decreased when lung cancer cells were devoid of C5aR1 (53).

In brain metastases, an elegant study by Massagué et al. unveiled a different prometastatic mechanism. C3 was upregulated in four leptomeningeal metastatic models and proved necessary for tumor growth within the leptomeningeal space. C3a, generated after C3 cleavage and bound to the C3aR expressed on the choroid plexus, was able to disrupt the blood-cerebrospinal fluid barrier. This effect was critical since blockade of this step provided a survival benefit in these models. However, C3 did not mediate cancer cell entry into the cerebrospinal fluid but other determinants were required for full tumor cell colonization (109).

Inhibition of complement-related proteins, and specially anaphylatoxins (14), has been proposed as a therapeutic option for maximizing the clinical efficacy of current immunotherapies. Recent studies have provided support of this idea after combined inhibition of anaphylatoxins and PD-1 signaling for the treatment of metastatic cancer. Administration of PD-1/PD-L1 blocking antibodies resulted in intratumoral complement activation and the subsequent accumulation of C5a within the tumor milieu (110). Importantly, the combination of C5a and PD-1 blockade reversed CD8 T-cell exhaustion, and markedly reduced lung cancer metastasis in two syngeneic animal models (111).

CONCLUSIONS

The complement system represents an important player in tumorigenesis and metastasis. Its relevance stems from its ability to foster a protumorogenic milieu by modulating tumor-immune responses. It also endows tumor cells with cell functions required for metastatic dissemination. Preclinical studies support the idea that the therapeutic blockade of complement has potential in combinatorial immunotherapy to effectively eradicate primary tumors and distant metastases. A better understanding of the mechanisms of interaction of the complement system with tumor cells and their microenvironment is required for designing combined novel immunotherapeutic regimens able to effectively target established tumors.

AUTHOR CONTRIBUTIONS

DA, RP, and FL designed the concept. DA and FL wrote the manuscript. SO-E prepared the figure. All authors read and approved the final version of the manuscript.

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REVIEW
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Complement C5b-9 and Cancer: Mechanisms of Cell Damage, Cancer Counteractions, and Approaches for Intervention

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The interactions of cancer cells with components of the complement system are highly complex, leading to an outcome that is either favorable or detrimental to cancer cells. Currently, we perceive only the "tip of the iceberg" of these interactions. In this review, we focus on the complement terminal C5b-9 complex, known also as the complement membrane attack complex (MAC) and discuss the complexity of its interaction with cancer cells, starting with a discussion of its proposed mode of action in mediating cell death, and continuing with a portrayal of the strategies of evasion exhibited by cancer cells, and closing with a proposal of treatment approaches targeted at evasion strategies. Upon intense complement activation and membrane insertion of sufficient C5b-9 complexes, the afflicted cells undergo regulated necrotic cell death with characteristic damage to intracellular organelles, including mitochondria, and perforation of the plasma membrane. Several pro-lytic factors have been proposed, including elevated intracellular calcium ion concentrations and activated JNK, Bid, RIPK1, RIPK3, and MLKL; however, further research is required to fully characterize the effective cell death signals activated by the C5b-9 complexes. Cancer cells over-express a multitude of protective measures which either block complement activation, thus reducing the number of membrane-inserted C5b-9 complexes, or facilitate the elimination of C5b-9 from the cell surface. Concomitantly, cancer cells activate several protective pathways that counteract the death signals. Blockage of complement activation is mediated by the complement membrane regulatory proteins CD46, CD55, and CD59 and by soluble complement regulators, by proteases that cleave complement proteins and by protein kinases, like CK2, which phosphorylate complement proteins. C5b-9 elimination and inhibition of cell death signals are mediated by caveolin and dynamin, by Hsp70 and Hsp90, by the mitochondrial stress protein mortalin, and by the protein kinases PKC and ERK. It is conceivable that various cancers and cancers at different stages of development will utilize distinct patterns of these and other MAC resistance strategies.

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In order to enhance the impact of antibody-based therapy on cancer, novel precise reagents that block the most effective protective strategies will have to be designed and applied as adjuvants to the therapeutic antibodies.

Keywords: complement, C5b-9, complement-dependent cytotoxicity, regulated necrosis, cancer immune resistance

PREFACE

The complement system may affect cancer in several forms, ranging from promotion of cancer growth and metastasis, on the one hand, to antibody-based cancer eradication, on the other. Upon encounter of the cancer cells with the complement system, activation may proceed via the classical, alternative, and/or lectin pathways (1) (**Figure 1**). This initiation step leads to formation of a C3 convertase (C4b2a or C3bBb) that deposits C3b molecules on the cells, followed by formation of a C5 convertase (C4b2a3b or C3bBb3b) that cleaves C5 and initiates formation of the C5b-9 complexes, termed the membrane attack complexes (MAC). Here, we will focus on the anti-cancer cytotoxic activity of complement, with an emphasis on the mode of action of the MAC. Reviews on the cancer-promoting activities of complement (2–4) and on complement activation by clinical anti-cancer antibodies (5–7) have been published recently; therefore, these topics will not be covered in this review. Another topic recently reviewed is the insights into the fine structural details of the complement MAC (8–11). MAC expresses a plethora of non-lytic and sublytic activities that have been reviewed elsewhere (12–15) and are thus excluded from this review. Here we will describe the current status of research on the cytotoxic effects of MAC, emphasizing the findings, dogmas, and open questions in our quest to better understand the fine mechanistic details of MAC-induced cancer cell death. Next, we will present the currently recognized counter-mechanisms utilized by cancer cells to resist complement-dependent cytotoxicity (CDC). Finally, we will discuss several potential therapeutic approaches for the intervention and potentiation of antibody-based anti-cancer immunotherapy that have been proposed and tested.

MECHANISMS UNDERLYING COMPLEMENT-MEDIATED CANCER CELL DAMAGE

Perspective: The Early Studies on Osmotic Cell Death

Studies on cancer cell killing by complement have been conducted long before the identification of the complement terminal pathway responsible for mediating cell damage and death. As early as 1950s, Kalfayan et al. (16), Ellem (17), and Green et al. (18) investigated the action of antibody and complement on rabbit Brown-Pearce carcinoma cells, rat Ehrlich and mouse Krebs ascites tumor cells, respectively. They observed cell swelling and increased plasma membrane leakiness. They proposed that complement impairs cell membrane integrity, increases cell permeability to anions, cations, and water, and causes osmotic cell swelling up to the point that the membrane collapses, culminating in osmotic cell lysis (19). The leakage from the cells was proposed to occur through functional, stretching, and possibly reversible "holes" in the swelling cells, which could be blocked, to some extent, by increasing the osmotic pressure of the extracellular medium (20). The concept of complement-induced osmotic lysis of target cells is still popular today but, as discussed later, it must be viewed with a grain of salt. Kim et al. (21) subjected Ehrlich ascites tumor cells to CDC and demonstrated that osmotic protection effectively prevented cell swelling but did not rescue the cells from death. They hypothesized that the cells died following activation of metabolic events that were detrimental to cell survival or through activation of a "suicidal" mechanism of programmed cell death. In conclusion, osmotic burst of inflated complement-damaged cells may occur, but these bursts are most likely a consequence of metabolic collapse of the cell rather than the cause of cell death.

The Complement Cell Death Mediator: A Concerted Action of Toxic Moieties

Membrane pores caused by complement were first visualized by electron microscopy on red blood cell membranes as large ring structures (22). Similar lesions were viewed on *E. coli* cell walls (23). Over the years, ample information on the fine ultrastructure of the MAC that can activate cell death has been gathered (24) and has been recently further examined (8–11, 25–27). For a complete updated view of the MAC structure, the reader is referred to those publications. The observed ring structure apparently corresponds to the structure of polymerized C9 molecules attached to their polymerization accelerator, the C5b-8 complex (28). However, even today we have only a partial view of the fine details of the cytotoxic mechanisms activated by MAC, eventually leading to the point of no return and cell death. Besides the paucity of investigations on the subject, several reasons account for that. First, the early dogmas were based on investigations with complement-targeted artificial membranes and red blood cells, which are clearly different, largely passive targets, compared with nucleated cells (29–34). Second, very large variation exists in refractoriness to the MAC, even among closely related cancer cell lines and even within a supposedly homogenous population of cultured cancer cell lines. Third, in target cells MAC activates concomitantly several signaling pathways and biochemical events, some cytotoxic and others pro-survival, and it is the particular balance among them that dictates cell fate, survival, or death. Finally, activation of the terminal complement pathway may result in generating, in the target cell membrane, a cocktail of membrane-inserted protein complexes: C5b-8, C5b-9_1, C5b-9_2, C5b-9_3, and so on, up to C5b-9 with 12-18 polymerized C9 molecules (28, 35). Each of these complexes

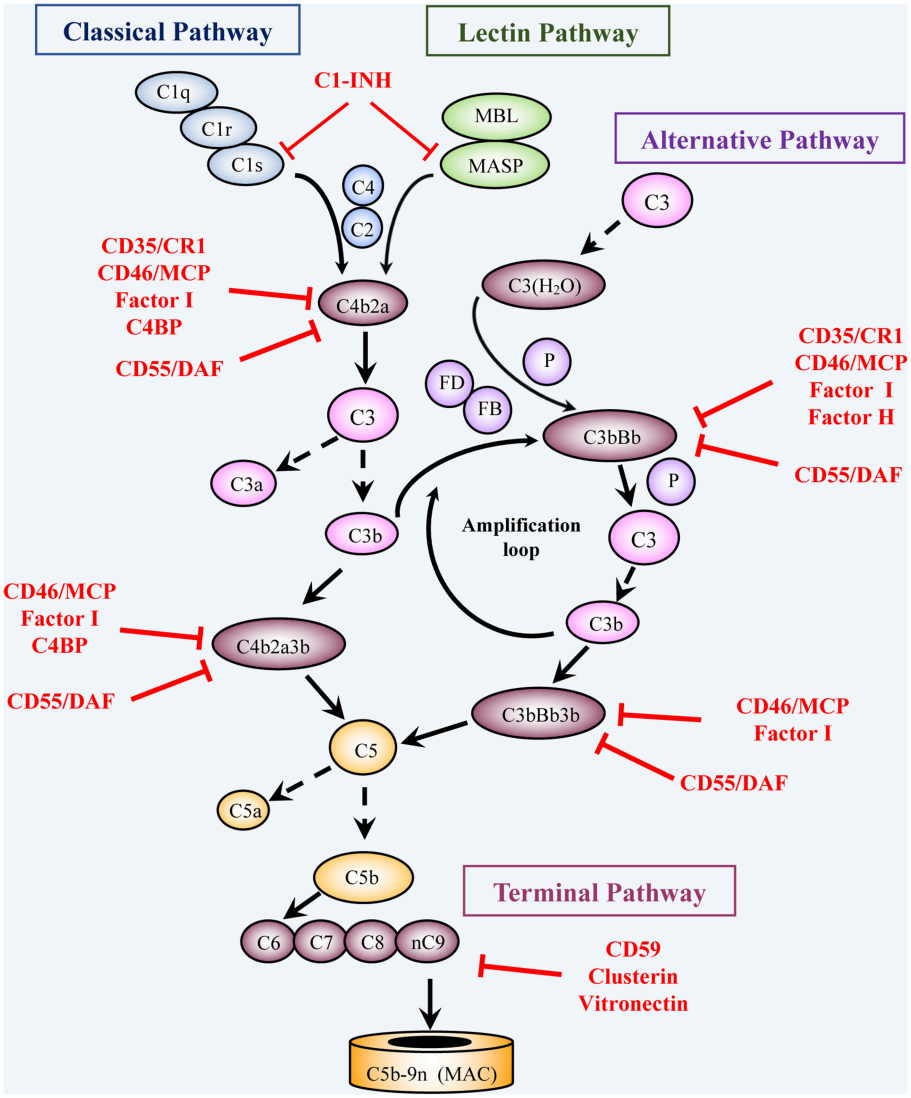

FIGURE 1 | Activation and regulation of the complement pathways. Activation: Complement activation proceeds through four converging pathways shown in this simplified scheme, i.e., the classical (CP), lectin (LP), alternative (AP), and terminal (TP) pathways. Activation of the CP and LP can be potentiated by components of the AP (Amplification loop). Binding of C1 (a complex of C1q, 2C1r, 2C1s) via C1q to antigen-bound antibodies initiates the CP, whereas binding of MBL or ficolin (in complex with MBL-associated serine proteases, MASP) to carbohydrates (e.g., microbial) initiates activation of the LP. The AP is initiated by C3 spontaneously hydrolyzed at a low rate into $C3(H_2O)$ or following another C3-tickover event. The three pathways generate a C3 converting enzyme, a C3 convertase (that cleaves C3 into C3a and C3b), by activation of C4 and C2 (CP and LP: C4b2a), or of factors B and D (AP: C3bBb). AP activation is facilitated by properdin (P). The resulting C3b not only opsonizes target cells but also joins the C3 convertases and turns them into C5 convertases, which convert C5 into C5a and C5b. Subsequent TP activation by assembly of C5b with C6, C7, C8 and multiple C9 molecules, generates the membrane attack complex, (C5b-9, MAC). By binding to specific receptors, C3a and C5a exert multiple cell stimulatory activities, ranging from allergy and anaphylaxis to promotion of acquired immunity by stimulation of lymphocytes and antigen presenting cells. Regulation: Complement activation is tightly regulated by multiple soluble and membrane proteins. Soluble inhibitors include: C1 inhibitor (C1-INH), C4 binding protein (C4BP), factor H (FH), factor I (FI), Clusterin and Vitronectin. The membrane regulatory proteins are: Decay Accelerating Factor (DAF, CD55), Membrane Cofactor Protein (MCP, CD46), Complement Receptor 1 (CR1, CD35), and CD59. As shown in the figure, C1-INH interferes with activation of C1r, C1s, and MASP. C4BP, FH, CD55, and CD35 restrict formation and stability of the CP and AP C3/C5 convertases or promote FI-mediated inactivation of C4b (CD35/CR1, CD46/MCP, C4BP) or C3b (CD35/CR1, CD46/MCP, FH). Clusterin and vitronectin prevent the association of the forming C5b-9 complexes with the membrane, whereas CD59 limits cell damage by preventing MAC complex formation.

may induce in the target cell slightly different signals that have not yet been discretely characterized. Detailed analysis of the effect of the terminal complement complex size on the lysis of rat Ehrlich ascites tumor cells by human complement indicated that complexes containing more C9 per C5b-8 are cytolytically more potent. Nevertheless, the kinetics of cell death appeared similar in cells bearing C5b-9 complexes that have either 1 or 4 C9 molecules per C5b-8 (36). Moreover, some human cancer

cells, such as U938, HL60, and B-CLL cells, could be lysed by C5b-8 alone, in the absence of C9, when a sufficient number of complexes were deposited on them (37, 38). Hemolysis of sheep red blood cells could be efficiently activated by C5b-9 complexes generated with thrombin-cleaved C9, which cannot undergo classic ring-like polymerization, but forms apparently, string-like oligomeric structures that may lead to leakage of membranes (39, 40). Hence, it is improbable that MAC, with its various intermediary complexes, activates a unified mechanism of cell death in all cell types. An additional level of complexity has been introduced by reports of apoptotic cell death induced by MAC (41), but this has not been observed so far with cancer cells undergoing CDC.

Calcium Ions Influx: Dose-Dependent Dichotomy

At non-toxic or sublytic doses, MAC has been shown to trigger numerous signals in many types of cells, normal and malignant. This topic has been extensively discussed recently and will not be covered here (12–15). Initially, measurements with pigeon erythrocyte sealed "ghosts" revealed an increase in intracellular calcium ions, which begins within seconds after binding of MAC and supposedly precedes the cell death process activated by lytic doses of MAC (42). This transient rise of intracellular free Ca^{2+} in target cells was thought to be required for cell death. However, later it became apparent that the rise in the level of intracellular calcium ions is essential for cell survival and recovery (43). Reduction of the extracellular Ca^{2+} concentration by chelation delays the onset of cell death, as measured by LDH release, but the cells eventually die like control cells (44, 45). Similarly, increasing the concentration of Ca^{2+} around the cells accelerates the rate of cell death without affecting the final percentage of dead cells (36). An intriguing question is: can CDC be blocked by intracellular chelation of the calcium ions? Intracellular Ca^{2+} chelation with BAPTA-AM was shown to efficiently block mitochondrial distress in human lung epithelial cells responding to a non-lytic dose of MAC, cells that do not undergo cell death (46). Furthermore, calcium ionophores that pump Ca^{2+} into the cell induce in K562, human erythroleukemia cells, a state of resistance to CDC (47). Can BAPTA-AM block CDC when cells are exposed to lytic MAC doses? BAPTA-AM reduced the release of LDH from rat hepatocytes subjected to lytic antibody and complement by ∼40% without affecting the rate of cell death (48). Clearly, MAC activates a surge of $[Ca^{2+}]_i$ in target cells but its exact impact on the process of cell death still awaits clarification. Furthermore, based on earlier findings, the involvement of calcium-independent processes in the critical events determining cell death cannot be ruled out.

Beyond Calcium Ions: The Cell Death Propagators in a Regulated Necrotic Process

The molecular checkpoints that tilt the balance within MAC-bearing cells between a protective state and cell collapse have not yet been identified. It is well-accepted that exposure of nucleated cells to multiple ("lytic") MAC hits (34) is needed to overcome the cells' innate resistance (described below) and to kill the cells by necrotic-type cell death. Intensive research on apoptosis, and more recently on necroptosis induced by numerous effector molecules, has clearly revealed that compound regulated molecular processes accompany and/or lead to cell death (49–52). Those findings have prompted adopting a similar research approach in the analysis of the mechanism underlying CDC. Recently, MAC was shown to activate RIPK1, RIPK3, and MLKL, known transducers of necroptotic cell death activated by several exogenous ligands of TNF receptor, Fas, TLR, and other membrane receptors (53). Necroptotic cell death, also termed regulated necrosis, is characterized by increased membrane permeabilization and mitochondrial damage (49, 50, 54, 55), much like CDC. Inhibitors of RIPK1, RIPK3, and MLKL reduce the extent of CDC, whereas overexpression of these proteins enhances cell sensitivity to CDC (53). Two additional intracellular proteins that may play a role in the multi-factorial cell death process activated by lytic MAC are the c-Jun kinase JNK (56) and the BH3-only protein Bid (57). Apparently, in some cells, the RIP kinases, MLKL, JNK, and Bid, act as components in one or more lined cascades of intracellular molecular interactions activated by sublytic and lytic MAC concentrations (53, 57). At lytic MAC concentrations, this cascade may promote a regulated necrotic cell death (**Figure 2**). Blocking any of these five proteins markedly lowers the extent of CDC but does not block it completely. Therefore, it appears that this cascade acts in concert with other death-promoting processes, calcium-dependent or -independent, which still await characterization. Of note, activated MLKL was shown in necroptotic cells to oligomerize at the plasma membrane, increase membrane permeabilization, and induce a Ca^{2+} influx (55, 58–60). Co-localization of MAC with MLKL at the plasma membrane (53) suggests that they may collaborate in mediating cell death. In general, cancer cells that express sufficient levels of the RIPKs, MLKL, and Bid might be sensitive to this necroptotic-like pathway once activated by MAC. In contrast, cancers that suppress the expression or function of any or all of these proteins are expected to be protected from this cytotoxic pathway even if triggered by MAC.

Lytic MAC: Mitotoxicity and Metabolic Depletion

Mitochondria play a pivotal active role in activating the intrinsic pathway of apoptotic cell death, mostly after mitochondrial outer membrane permeabilization (61). Mitochondrial swelling and damage were observed in cells undergoing necrotic cell death induced by complement (62, 63). The cellular ATP level drops rapidly in cells attacked by MAC in copy numbers that are above the lytic threshold, apparently after mitochondrial dysfunction, accompanied by leakage of cytosolic ATP from the cells through pores in the plasma membrane (64–66). In theory, mitochondrial damage and cellular metabolic depletion beyond a point of "no return" may induce cell collapse and death. However, to date, there is no strong evidence supporting an active role for mitochondria in CDC. Perhaps, they are mere innocent bystanders damaged by the necrosis executioners? Reactive oxygen species are generated throughout the necrotic

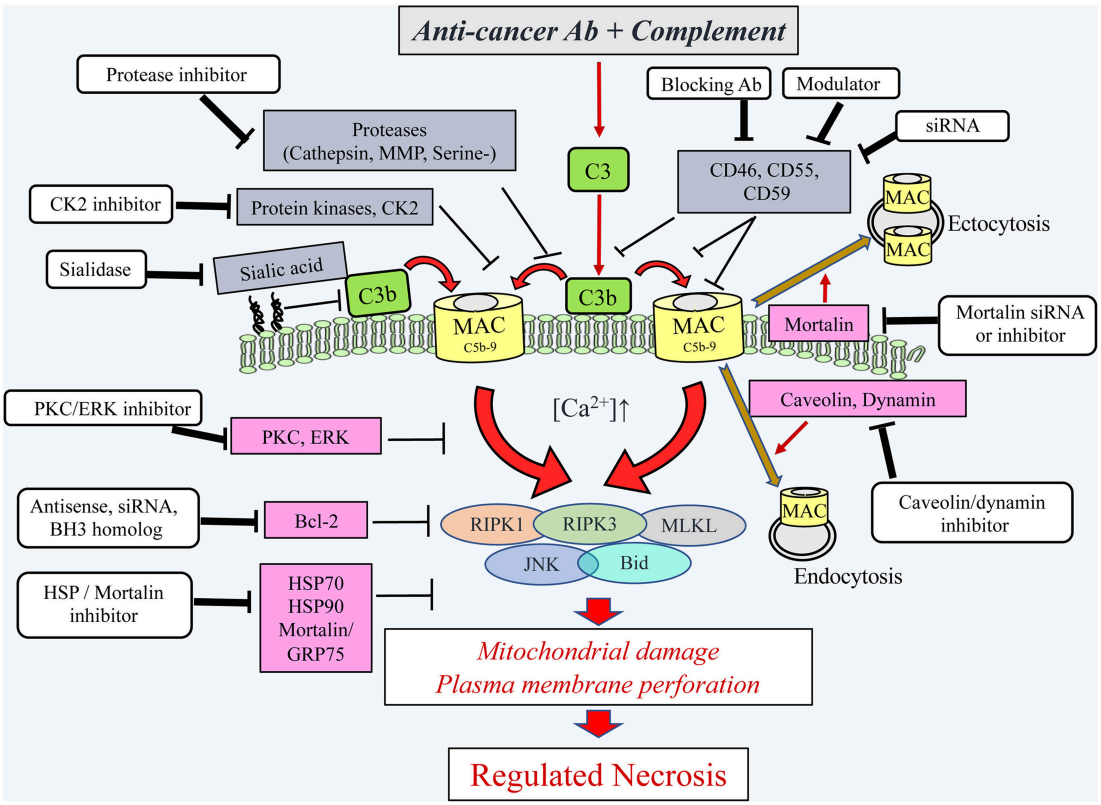

FIGURE 2 | Schematic presentation of the cytotoxic pathways, induced in cancer cells by the complement C5b-9, the counteractive cellular resistance mechanisms, and postulated approaches to overcome this cancer evasion. Following the binding of antibodies to cancer cells, the complement system is activated and deposits C4b and C3b molecules that serve as initiators of C3/C5 convertase activation. The C5 convertases initiate the activation of the terminal complement pathway and the formation of the C5b-9 complexes (24). Upon insertion of the C5b-9 complexes into the plasma membrane of cancer cells, they induce calcium ion influx and activate pro- and anti-lytic signals. This scheme depicts the proteins proposed to be involved in the ensuing cancer cell death (encircled) and the proteins protecting the cancer cells from the lytic processes. Extracellular (gray boxes) and intracellular (purple boxes) protective proteins are indicated. Several reagents (white boxes) that will block the protective proteins are indicated and proposed for adjuvant therapy to therapeutic antibodies. Ab, antibody; Bcl-2, B-cell lymphoma/leukemia-2; BH3, Bcl-2 homolog domain-3; Bid, BH3 interacting domain death agonist; CK2, casein kinase 2; ERK, extracellular signal-regulated kinase; HSP90, heat shock protein 90; HSP70, heat shock protein 70; JNK, c-jun N-terminal kinase; MAC, complement membrane attack complex; MLKL, mixed lineage kinase domain-like protein; MMP, matrix metalloproteinase; PKC, protein kinase C; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; serine-, serine protease; siRNA, small interfering RNA.

process (15). Still, whether they take part in the MAC-induced cell death process is also an open question. The involvement of mitochondria and mitochondrial ROS in necroptosis triggered by various necroptosis inducers was extensively investigated in several types of target cells (67). Ample earlier findings have supported a pivotal role for mitochondria in necroptosis, but more recently, several investigations casted doubt on that notion (67). Thus, for example, mitochondrially deficient cells were shown to be responsive to TNF/zVAD treatment and to undergo necroptosis (68). It will be of interest to examine the relative sensitivity of these cells to CDC. At present, we can conclude that whether mitochondria are dispensable or essential for MAC-induced necrotic cell death remains to be further investigated. Similar to necroptosis (67), it is likely that different cell types may mediate CDC by an array of distinct mitochondria-dependent and -independent strategies.

NORMAL CELLS AND EVEN MORE SO, CANCER CELLS CAN RESIST MAC-INDUCED CELL DEATH

The fate of a target cell attacked by MAC is dictated by two mutually exclusive processes: (a) the rate and extent of the formation of the C5b-9 complexes and their insertion into the target cell membrane, and (b) the capacity of the target cell to block C5b-9 complex formation and to resist cell damage inflicted by C5b-9. Cancer cells can resist CDC by using a plethora of extracellular and intracellular mechanisms (**Figure 2**). The number of membrane-inserted C5b-9 complexes may be restricted by inhibiting the complement activation cascade earlier at the C3/C5 activation stage, by blocking complex assembly and/or membrane insertion, and by facilitating complex removal from the cell surface. All these protective strategies have been

identified in cancer cells and are described below. It is generally accepted that cancer cells are more resistant to CDC than are normal cells due to their elevated expression of protective mechanisms. Apparently, during the tumorigenic process, the complement system reacts against the transformed cells by eliminating or modifying the complement-sensitive malignant cells, thus enriching the cancer cell population for complement-resistant cells. This process resembles the selection of antibiotic-resistant bacteria (69). This hypothesis remains to be supported by *in vivo* evidence; however, *in vitro* studies show that sensitive cancer cells may be transformed into cells expressing increased complement resistance, transiently, after a brief treatment with a sublytic dose of MAC (70) and more stably, following several cycles of exposures to cytolytic MAC (71). A MAC-resistant phenotype may be acquired upon the elevation or reduction in the expression level of microRNAs such as miR-200, miR-217 (72), and others that are currently under investigation.

Basal Physiological Cell Resistance to CDC

As shown already in 1974 (73, 74), nucleated cells can resist MAC-induced damage. Several inhibitors of protein synthesis were shown to increase the cell's susceptibility to CDC. Since the elevated sensitivity to CDC was acquired hours after the complete shutdown of protein synthesis (75), it is likely that the treated cells became sensitive only after catabolism of long-lived protective proteins. These earlier findings were followed by the development of the concept of multi-hit characteristics of nucleated cell death by MAC, implicating the cooperation of multiple MACs in cell death (34). Another interesting earlier finding was that damaged tumor and mast cells could be rescued by exogenous application of cAMP (76, 77). Consequently, activation of cAMP by sublytic MAC supports cell recovery from MAC damage (78). These findings were confirmed in leukemia cells treated with dibutyryl cAMP or with activators of intracellular cAMP (3-isobutyl 1-methyl xanthine and forskolin), which were shown to reduce cell death (79). In contrast, H-89, an inhibitor of the cAMP-dependent kinase PKA, enhanced carcinoma cell sensitivity to CDC. Apparently, phosphorylation events mediated by several protein kinases dictate the basal capacity of cells to resist MAC damage (79, 80). Protein phosphorylation events involving PKC, MEK, and ERK support the survival of cancer cells undergoing a complement attack (81–83). Protein phosphorylation may upregulate the expression of the complement membrane regulators on cancer cells (84–86) and facilitate MAC elimination from K562 cells (87, 88). The transcription factor NF-κB also plays a role in cell protection from CDC (89). One of its postulated functions is upregulation of a protein phosphatase that inactivates JNK, thus reducing cell death signaling. However, further investigation is required to fully identify the pro-survival phosphoproteins and phosphatases and their precise mode of action.

Proteins of the heat shock protein family (HSPs), well-known general house keepers, damage/ repair proteins and targets in cancer therapy (90–92), most probably also contribute to the basal resistance of cancer cells to CDC. Thus, far, a role for Hsc70/Hsp70 (93) and Hsp90 (94, 95) in cell protection from CDC has been shown. Pharmaceutical inhibitors of Hsp70 and Hsp90 sensitize cancer cells to CDC. Hsc70 relocates within minutes from the cytoplasm to the cell surface after exposing K562 cells to sublytic complement (93). Upon inhibition of Hsp90, Ramos cells become more sensitive to the action of Rituximab and complement (95). Additional thorough experimentation is required to fully comprehend how Hsc70/Hsp70, Hsp90, and other HSPs regulate CDC. The fact that Hsp90 can directly interact with C9 and that Hsp90 inhibitor enhances MAC deposition (95) suggest that Hsp90 down-regulates MAC deposition by blocking its assembly and/or facilitating its rate of removal from the cell surface. Hsp90 can potentially reduce CDC by suppressing mitochondria-initiated calcium-mediated stress responses (96).

Anticomplementary Response on the Cancer Cell Surface

Like all normal cells, cancer cells are protected from autologous complement attack by several specific cell-surface complement inhibitors: CD55 (decay accelerating factor, DAF), CD46 (membrane cofactor protein, MCP), CD59, and CD35 (complement receptor type 1, CR1) (1, 97–99) (**Figure 1**). In addition, certain proteolytic enzymes, protein kinases, and sialic acid residues (described below) confer on the cells elevated resistance to CDC (100).

Membrane Complement Regulatory Proteins

Immunohistochemical analysis revealed the expression of CD59, CD55, and CD46 on uveal melanoma (101), thyroid carcinoma (102), lung and kidney cancer (103, 104), colon adenocarcinoma (105), and prostate cancer (106). This was supported by analysis of human tumor cell lines derived from human malignant gastrointestinal tumors (107), melanoma (108), breast cancer (109, 110), renal tumor (110), Burkitt lymphoma (111), neuroblastoma (112), and ovarian (113), and prostate carcinoma (79). In primary uterine cervix tissue, the expression of CD46, but not of CD55, was found to increase during transition from normal to premalignant to malignant cells (114). CR1/CD35 was identified in malignant endometrial tissue (115) and on leukemic blasts (116). Increased membrane regulator expression relative to the corresponding normal tissue has been reported in many tumors (103, 105, 114, 115, 117–122). Colorectal and gastric carcinomas and osteosarcoma have increased the expression of CD55 (123), whereas gastric carcinoma exhibits high levels of both CD55 and CD59 (124). Overexpression of CD59 was also identified by expression profiling for pancreatic cancer (125). Upregulation of membrane regulator expression on tumor cells is often correlated with increased complement resistance (86, 111). In ovarian cancer, resistance to complement correlated with high levels of CD55 expression (113). In melanoma cell lines with variable CD59 expression, resistance to death by anti-ganglioside antibody and homologous complement positively correlated with the expression level of CD59 (126).

Several clinical studies support a postulated function of membrane complement regulatory proteins (and thus, the extent of complement resistance) in cancer progression. Poorer prognosis in colorectal carcinoma correlates with the expression level of CD59 (127). Local tumor progression and tissue

dedifferentiation of prostate cancer also correlate with CD59 expression (128). Analysis of 120 breast cancer patients revealed a worse prognosis associated with CD59 overexpression (129). In contrast, another report concluded that loss of CD59 correlated with poor survival in 520 breast cancer patients (130). In colorectal cancer patients, a 7-year survival was significantly reduced when the tumors expressed high levels of CD55 (131). CD55 overexpression was also reported as an independent risk factor for recurrence of breast cancer in patients receiving postoperative adjuvant therapy containing trastuzumab (132).

The expression level of the membrane complement regulators may also be shaped by cytokines, growth factors, or hormones, which are released into the tumor microenvironment (83, 133, 134). For example, TNFα and IL-1ß enhanced the expression of CD55 and CD59 in colon adenocarcinoma cells (135). TNFα, IL-1α, and INFγ enhanced CD55 expression in lung cancer cells (136). In hepatoma cells, TNFα, combined with IL-1ß and IL-6, enhanced CD55 and CD59 expression but decreased CD46 expression (134). Transcription abnormalities (137) and the microRNA level of expression (72) may also affect the expression level of the membrane complement regulators. Evidently, the factors and molecular mechanisms that determine the expression level of each of the membrane regulator proteins *in vivo* in each cancer type (and in normal cells) remain to be further investigated.

Exposure to chemotherapeutic drugs may also modify the level of the regulators′ expression. 5-azacytidine was shown to elevate the levels of CD55 and CD59 in Burkitt lymphoma cell lines (111) but only of CD59 in melanoma cells. In contrast, levamisole reduces CD59 levels in colon adenocarcinoma cell lines (138) and after pretreatment of breast carcinoma cells with tamoxifen, trastuzumab-induced CDC was enhanced due to CD55 down-regulation (132). Conversion of cancer cells from being drug-sensitive to drug-resistant is also associated with modification of their complement sensitivity. Doxorubicin-resistant human colon carcinoma cells are more sensitive to CDC than are doxorubicin-sensitive cells (139). KB-V1, a multidrug-resistant variant of KB-3-1, the human oral carcinoma cell line, exhibits a higher susceptibility to CDC than do its parental multidrug-sensitive cells (140). The increased complement sensitivity was associated with a reduced expression of CD55. Inversely, drug resistance was associated with CDC resistance in the HL60 myeloid leukemia cell lines (141). In ovarian carcinoma cells, drug resistance was associated with complement resistance and with membrane complement regulator overexpression (142). Hence, the impact of any drug on the expression of membrane regulators and on CDC resistance needs to be determined for each drug and cancer type.

Released or secreted membrane complement regulators in the cancer microenvironment may also support cell resistance to CDC. Soluble forms of membrane regulators have been identified in several body fluids, even under normal conditions. They are either produced by alternative splicing or released from the cell surface through enzymatic cleavage. Thus, sera of cancer patients contain active, soluble forms of CD46 (143). Elevated CD55 concentrations in stool specimens have been proposed to have diagnostic value for patients with colorectal cancer (144). A constitutive release of soluble CD59, which retains its activity as well as its GPI-anchor from human melanoma cells, was reported (145). In primary tumor sections, CD55 and/or CD59 were found in the stroma of breast, colorectal, lung, renal, and cervical carcinomas (103, 123, 146). *In vitro*, endothelial cells, HeLa cells (147) as well as osteosarcoma and colorectal cells (123, 148) release CD55 in a soluble form or deposit it into their extracellular matrix. K562 erythroleukemia cells (83) and breast, ovarian, and prostate carcinoma cell lines (79) secrete soluble CD59. Elevated plasma levels of soluble CR1 were found in leukemia patients (149).

The observed correlations between elevated expression or secretion of one or more of the membrane complement regulatory proteins on cancer cells and (a) enhanced resistance to CDC or (b) poor cancer prognosis, suggests that the membrane complement regulatory proteins have an effect on prognosis through their impact on complement resistance. Thus, by suppressing C3 deposition on the cancer cells, CD46 and CD55 can lower, on one hand, the extent of MAC generation and CDC, and on the other hand, reduce immune protection through complement-dependent cellular cytotoxicity. CD59 can down-regulate MAC generation and CDC. However, a direct correlation between cancer patients' prognosis and the complement resistance level of their cancer, still remains to be established. We cannot rule out non-complement-mediated effects of the membrane complement regulatory proteins of cancer cells on the patients' immune response. Thus, the membrane complement regulatory proteins on cancer cells, through intracellular signaling, or cooperation with other cell surface receptors may potentially modulate cell resistance to immune effector cells such as natural killer cells and cytotoxic T lymphocytes (150, 151).

Membrane Surface Proteases, Protein Kinases, and Sialic Acid

Cancer cells become increasingly protected from CDC by expression on their cell surface of proteases that proteolytically degrade the deposited complement proteins (152). Thus, degradation of bound C3b by a C3-cleaving serine (153) or cysteine protease (154), respectively, was demonstrated on human and murine melanoma cells. C3-cleaving serine protease activity was also identified on the surface of U937 cells (155). Membrane serine proteases on K562 erythroleukemia cells also appear to contribute to their complement resistance (156). Matrix metalloproteinases (MMP) membrane type-1 (MT1) can cleave bound C3b off breast cancer cells and protect *in vitro* breast carcinoma and melanoma cells from CDC (157). Transfection of B16F1 melanoma cells with MT1-MMP enhanced their capacity to form lung metastases in normal but not in C3-deficient C57BL/6 mice (157). The effect of those proteases on proteins of the terminal complement pathway has not been tested. However, it is conceivable that these and other membrane proteases have a similar degradative impact on C5-C9. This still awaits determination.

Ecto-protein kinases (ecto-PK) are extracellular protein kinases that can phosphorylate both cell-surface and external proteins. Serine/threonine and tyrosine ecto-PKs were found on the surfaces of K562, U937, and HL-60 cells (158), and an ecto-casein kinase 2 (CK2)-like activity was associated with breast and ovarian carcinoma cells (159). C9 phosphorylation by ecto-CK2 was shown to be protective in K562 cells against CDC, possibly by inhibiting MAC formation or by leading to the production of an inactive or unstable MAC (160). Further investigation of this strategy of CDC evasion is warranted.

Brief treatment with sialidase, which removes sialic acid from the cell surface, has been shown to confer on several cell types increased sensitivity to CDC. Thus, removal of sialic acid from red blood cells (161, 162), murine sarcoma cells (163), and human bladder carcinoma cells (164) sensitized them to lysis by complement. Human prostate, breast, and ovarian carcinoma cells also utilize surface sialylation for protection from complement (79). High sialic acid expression correlates with lower complement activation, probably because of inactivation of C3b by factors H and I, which is more efficient on surfaces rich in sialic acid (162). The sialic acid inhibitory activity on CDC of mouse erythrolukemia MEL cells is apparently abrogated by O-acetylation at its 9-hydroxyl group (165). α2-6 hypersialylation apparently lowers the response of CLL cells to Rituximab therapy through its action on complement (166). Thus, by limiting the extent of C3 deposition, sialic acid may also control the assembly of C5b-9 complexes on the cancer cells.

Soluble Complement Regulators in the Cancer Microenvironment

Soluble complement inhibitors such as C1 Inhibitor, factor H, and factor I are predominantly synthesized by hepatocytes and macrophages but can also be released from other tissues, although in considerably smaller amounts. In the cancer microenvironment, these secreted inhibitors may contribute to protection of cancer cells from complement attack by blocking complement activation at the C1 and C3 activation steps (99). In support of this, a growing number of reports indicate that cancer cells of various origins secrete one or more complement inhibitor. Synthesis of C1 Inhibitor has been described in astroglioma and neuroblastoma (112), breast cancer cell lines, and in a primary ovarian carcinoma cell line (156). Factor H is expressed both in lung adenocarcinoma and cutaneous squamous cell carcinoma (167, 168) and high levels of factor H and factor H-like protein-1 were shown to be secreted by ovarian tumor cells (83, 169). Additionally, factor H was found to be elevated in bronchoalveolar lavage fluids and the sputum of patients with lung cancer (170). Chronic lymphocytic leukemia (CLL) cells that bind factor H to their surface resisted Rituximab-mediated CDC (171). Factor H was coexpressed with factor I in glioma and rhabdomyosarcoma cells in its plasma form and in a truncated form (172). Tumor-associated factor I is postulated to promote the progression of cutaneous squamous cell carcinoma (173) and positively correlates with poor survival and recurrence of breast cancer (174).

Active Removal of the Membrane-Inserted MAC

An additional important defensive tactic used by cancer cells to resist CDC is rapid elimination of MAC from the cell surface. This was first shown with U937 histiocytic leukemia cells, Ehrlich ascites tumor cells (175, 176), and neutrophils (177). Neutrophils remove MAC both by endocytosis and exocytosis (178). Elimination of MAC by exo-vesiculation has been described in glomerular epithelial cells, platelets, and oligodendrocytes (179–181). The intracellular signals involved in MAC elimination include Gi proteins (182), PKC and ERK (88, 183). The process of MAC removal through outward and inward vesiculation was imaged in MAC-bearing K562 erythroleukemia cells (184). Membrane vesicles shed from MAC-bearing neutrophils contain MAC and have elevated levels of cholesterol and diacylglycerol, suggesting selective membrane protein and lipid sorting during the ectocytosis process (185). In support, the elimination of MAC by endocytosis is inhibited in K562 cells after cholesterol depletion (186). MAC endocytosis in K562 cells largely depends on caveolae and dynamin-dependent intracellular release of MAC-loaded endosomes (186). The process of MAC removal by exo-vesiculation was also partially characterized in K562 cells and was found to require the expression of the mitochondrial stress protein mortalin/GRP75 (87). Mortalin is over-expressed in many cancer types and is an essential survival stress protein (187). It was shown to be significantly protective from CDC (188). Its exact mode of action remains to be elucidated; however, evidently, mortalin inhibitors efficiently sensitize K562 cells and colorectal carcinoma HCT116 cells to CDC (189).

INTERVENTION STRATEGIES TO OVERCOME CANCER RESISTANCE TO CDC

As previously described, cancer cells escape CDC through amplification of an array of resistance strategies that block the formation of MAC, facilitate MAC elimination from the cell surface, or inhibit the cytotoxic consequences of MAC insertion into the plasma membrane (**Figure 2**). In order to overcome that resistance, more potent antibodies and polymeric antibodies have been engineered (5–7). Attachment of complement-activating proteins such as CVF, C3b, C7, or C9 directly to therapeutic antibodies represents an alternative means to strengthen complement attack and thereby to overcome complement resistance of cancer cells (190–193). Here, we will restrict our description to intervention strategies that may be or have been developed to augment the CDC of cancer cells by weakening their anti-MAC resistance mechanisms. These include the following: (1) blocking or silencing the membrane complement regulatory proteins, (2) inhibiting the extracellular enzymes that interfere with complement activation, and (3) inhibiting the intracellular pathways that support cell resistance and recovery (**Figure 2**). An additional, yet unexplored approach,

which is based on the earlier findings, is targeting a sialidase to the cancer microenvironment or blocking the sialylation of surface glycoconjugates in cancer cells, which is expected to sensitize them to CDC.

Antibody-Mediated Neutralization of Complement Regulator Expression

Specific inhibition of complement regulators' activity is best achieved with monoclonal antibodies that enhance the susceptibility of cancer cells to CDC (86, 194, 195). Thus, blocking antibodies markedly enhance the anti-tumor activity of Rituximab *in vitro* and *in vivo* (196). Neutralization of CD55 in Burkitt lymphoma cells (111), leukemia cells (196–199), melanoma cells (200), and breast cancer cells (86) increased their sensitivity to complement. Similarly, inhibition of CD59 with a monoclonal antibody led to efficient sensitization to CDC of neuroblastoma cells (112), leukemic cells (83, 199), breast (86), ovarian (113), renal (201), and prostate carcinoma cells (106). Mini-antibodies targeting both CD55 and CD59 were shown to enhance Rituximab-dependent CDC *in vitro* and to increase the survival of Rituximab-treated SCID mice in a xenograft model of human $CD20^+$ B-cell lymphoma (196). Bispecific antibodies targeting both CD20 and CD55 or CD20 and CD59 were also shown to potentiate the CDC of CD20-positive lymphoma cells *in vitro* and to prevent the growth of human lymphoma cells in SCID mice (202). Neutralization of the soluble complement regulators may also be applied to cancer immunotherapy. Thus, anti-factor H antibody increased antibody-dependent CDC of colorectal cancer treated with anti-CEA monoclonal antibody (203). Inhibition of factor H activity with a recombinant protein reflecting the factor H short-consensus repeat 18–20 improved the CDC of CLL cells in the presence of Rituximab and the blockage of CD55 and CD59 further enhanced CDC (171).

Silencing of Complement Regulators' Expression by RNAi

Another specific approach is to knock down the expression of the membrane complement regulators' expression by siRNAs. RNA interference (RNAi), mediated by small interfering RNA (siRNA), is the most efficient strategy for specific silencing of therapeutically relevant genes (204). In the last years numerous strategies have been developed for a better delivery of siRNAs in *vitro* and *in vivo* (205). We have shown that silencing of single or multiple complement regulators by anti-sense oligonucleotides or siRNAs results in a significant increase of opsonization and CDC of tumor cell lines of various histological origin (194, 206). Silencing of CD55 and CD59 in breast cancer cells with specific shRNA enhanced CDC (207). Using chemically stabilized anti-complement regulators, siRNAs and AtuPLEX, we observed a significant knockdown of regulator expression on HER2-positive carcinoma cells. Subsequently, treatment with a combination of two anti-HER2 antibodies, trastuzumab and pertuzumab, and normal human serum, augmented C3 binding and CDC could be recorded (208). Similar results were observed with lymphoma cells in which silencing of complement regulators enhanced antibody-dependent CDC (209). For specific delivery of liposomes or lipoplexes loaded with siRNA molecules into cancer cells, transferrin may be attached to them to facilitate their binding to cancer cells through transferrin receptor (TfR/CD71) and their active entry into the cells (210). By using this approach, delivery of siRNA molecules specific to CD46, CD55, and CD59 to transferrin receptor-positive carcinoma cells was achieved and promoted the knockdown of the complement regulators and enhanced CDC (211).

Neutralization of Extracellular Protective Enzymes

Considering the aforementioned anti-complement effects of certain proteases and protein kinases, it is likely that tailor-made protease and/or kinase inhibitors will promote antibody-based immunotherapy. *In vitro* and a few *in vivo* results support this hypothesis. Treatment of K562 cells with serine protease inhibitors markedly enhanced their sensitivity to CDC (156). CK2 inhibitors also augmented Raji cell killing by Rituximab and complement (160). Single-chain variable fragment (ScFv) directed to cathepsin L was used to inhibit the tumorigenic and metastatic phenotype of human melanoma cells in nude mice (212). In addition, injection of an anti-cathepsin L ScFv lentiviral vector into tumors already induced in nude mice inhibited tumor growth and associated angiogenesis (213). Whether or not the complement system is involved in the latter anti-tumor effects of the anti-cathepsin L treatment remains unresolved.

Inhibition of Intracellular Protective Pathways

As described above, the list of intracellular molecular pathways supporting cancer cell resistance to CDC is increasing. Currently, we can hypothesize that a coordinated inhibition of any of the following active molecules in the following cancer cells: cAMP, PKC, MEK/ERK, Hsp70, Hsc70, Hsp90, and mortalin, combined with complement-activating antibody, will amplify cancer cell death and increase the sensitivity of cancer to immunotherapy. For each of these molecules, this claim has been clearly supported *in vitro* by data and now awaits *in vivo* testing. Inhibition of PKC and MEK1, the ERK kinase, lowers the rate of MAC elimination from the cells and sensitizes them to CDC (81, 82, 88, 183). Inhibitors of MEK-ERK are in clinical use now in cancer therapy (214–216), and testing their impact on the therapeutic efficacy of anti-cancer antibodies is highly warranted. Heat shock proteins are over-expressed in cancer and play a significant role in resistance to various types of therapy (217). The list of inhibitors of heat shock proteins that have been developed for clinical use is growing and a few have entered clinical trials (90–92, 218, 219). The use of these heat shock protein inhibitors as adjuvants to antibody-based therapy may yield a superior clinical outcome. Mortalin belongs to the family of heat shock proteins and is also over-expressed in cancer (187). The mortalin expression level in colorectal adenocarcinoma cells correlates with poor patient survival (220). Mortalin inhibitors like MKT-077 could be considered as complementary treatment to anticancer antibody therapy. In support, pretreatment with MKT-077 sensitized K562

cells to CDC (87, 189). Unfortunately, thus far, testing of MKT-077 in patients has been stalled due to toxicity effects (221) and alternative inhibitors are being sought. Mortalin silencing with specific siRNA reduced MAC elimination and increased the sensitivity of K562 cells to CDC (189). Therefore, it is reasonable to predict that combining reagents that knockdown or inhibit mortalin with anti-cancer antibody therapy will be advantageous to cancer patients.

CONCLUDING REMARKS

Complement activation on and around cancer cells has been postulated to elicit several concomitant physiological and immunological responses that may act cooperatively to either mediate cancer cell death or promote cell survival, growth, and metastasis. In theory, these responses may also negate and annul each other. Multiple strategies to overcome complement resistance, as described here, open up new opportunities for improving antibody-based immunotherapy. Undoubtedly, applying any of the intervention treatments described above, together with a therapeutic antibody, will produce on and around the cancer cells/mass, besides C5b-9 complexes, additional complement activation products, such as cancer-bound iC3b, which promotes antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDCC) as well as C3a and C5a, which may suppress cellular anti-cancer immune response. Consequently, in the worst scenario, intervention strategies to augment complement activation may worsen the outcome of the anti-cancer antibody therapy. Hence, for each cancer type, therapeutic antibody, and intervention strategy, an optimal protocol will have to be developed that favors cancer destruction over cancer promotion.

AUTHOR CONTRIBUTIONS

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

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Is the Complement Protein C1q a Pro- or Anti-tumorigenic Factor? Bioinformatics Analysis Involving Human Carcinomas

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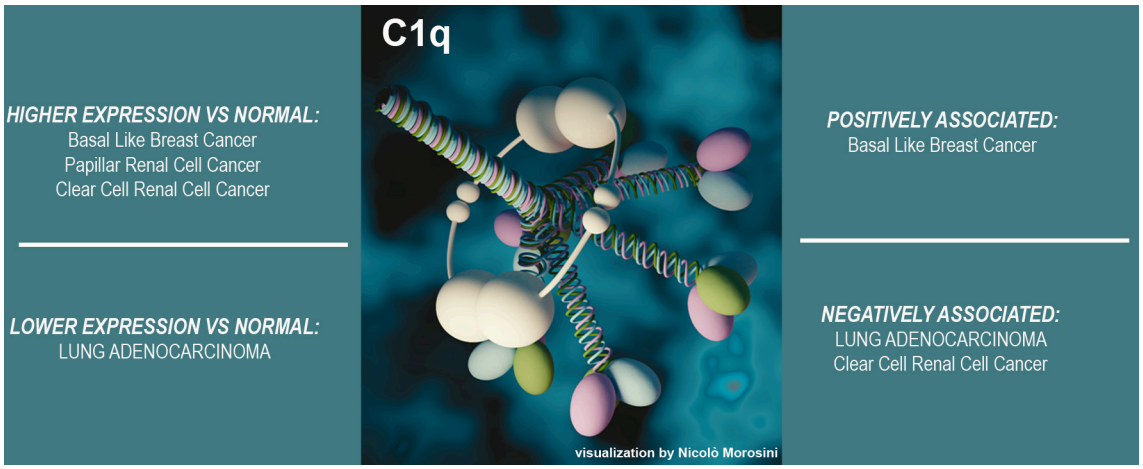
C1q is the first subcomponent of the classical pathway of the complement system and belongs to the C1q/Tumor Necrosis Factor superfamily. C1q can perform a diverse range of immune and non-immune functions in a complement-dependent as well as -independent manner. Being a pattern recognition molecule of the innate immunity, C1q can recognize a number of self, non-self and altered-self ligands and bring about effector mechanisms designed to clear pathogens via opsonisation and inflammatory response. C1q is locally synthesized by macrophages and dendritic cells, and thus, can get involved in a range of biological processes, such as angiogenesis and tissue remodeling, immune modulation, and immunologic tolerance. The notion of C1q involvement in the pathogenesis of cancer is still evolving. C1q appears to have a dual role in cancer: tumor promoting as well as tumor-protective, depending on the context of the disease. In the current study, we performed a bioinformatics analysis to investigate whether C1q can serve as a potential prognostic marker for human carcinoma. We used the Oncomine database and the survival analysis platforms Kaplan-Meier plotter. Our results showed that high levels of C1q have a favorable prognostic index in basal-like breast cancer for disease-free survival, and in HER2-positive breast cancer for overall survival, while it showed a pro-tumorigenic role of C1q in lung adenocarcinoma, and in clear cell renal cell carcinoma. This *in silico* study, if validated via a retrospective study, can be a step forward in establishing C1q as a new tool as a prognostic biomarker for various carcinoma.

Keywords: complement, classical pathway, C1q, tumor, microenvironment, prognosis



GRAPHICAL ABSTRACT | Summary of the conclusions of the study.

INTRODUCTION

C1q is the first recognition subcomponent of the complement classical pathway, which when associated with C1r and C1s, forms a C1 complex, allowing the activation of the complement cascade (1). By virtue of its ability to bind to IgG and IgM containing immune complexes and activating the classical pathway, C1q acts as prototypical link between innate and adaptive immune wings of the immune system (2). C1q can bind to a range of non-self-target ligands (pathogens), altered self (β-amyloid peptide, prion protein, apoptotic and necrotic cells via phosphatidylserine and DNA, respectively), and cell surface receptors (such as calreticulin and gC1qR) (3). Several features of the C1q render it a versatile molecular sensor of damage-modified self or non-self antigens (4). C1q, unlike most of the complement proteins which are exclusively produced by hepatocytes, can also be synthesized in a local environment by a wide range of cell types including macrophages and dendritic cells (5). Local synthesis, therefore, offers an additional avenue to C1q in order to exert specific functions *in situ* that are strictly connected to its site of production without involving complement activation (6).

C1q is an hexametric glycoprotein of about 460 kDa, resembling a "bouquet of tulips" being composed by three polypeptide chains: A (28 kDa), B (25 kDa), C (24 kDa), which are the product of three distinct genes clustered in the same orientation, and in the order A–C–B, on a 24 kb stretch of DNA on chromosome 1p (7). Each chain consists of a C-terminal globular head (gC1q) domain and an N-terminal triple-helical collagen-like (cC1q) domain (8). C1q associates with the Ca^{2+}-dependent $C1r_2$-$C1s_2$ tetramer, of about 360 kDa, to form the soluble pentameric C1 complex (9). The C-terminal ends of A, B and C chains assemble together to form a heterotrimeric gC1q domain, which by virtue of its modular organization, can work independently and engage with a diverse range of target ligands (3). While the gC1q domain latches on to the charge patterns on the ligands, the cC1q domain can interact with effector mechanism inducers, such as C1r, C1s, cell surface receptors, etc. Thus, a combination of a highly versatile and modular gC1q domain and a cell surface interacting cC1q domain, together with its local synthesis, makes C1q a potent orchestrator of molecular pathways. C1q is involved not only in innate and adaptive immune mechanisms, but also in a wide range of physiological and pathological processes, such as placental development (10, 11), pre-eclampsia (12, 13), wound healing (14) and cancer (15–18).

Markiewski et al. provided evidence that C1q is present in syngeneic mouse tumors. Indeed, they found that the activation of the classical pathway is the major contributor to complement-mediated tumor progression (19). Subsequently, we showed that locally expressed C1q had important effects in the tumor microenvironment (TME) (17). C1q expressed in the stroma and vascular endothelium of several human malignant tumors acted as a tumor-promoting factor by favoring adhesion, migration and proliferation of cancer cells as well as angiogenesis and metastasis. C1q-deficient ($C1qa^{-/-}$) mice, bearing a syngeneic B16 melanoma, exhibited slower tumor growth and prolonged survival, compared to C3 or C5 deficient mice although it has been shown that C3/C5 deficiency may also create microenvironment suboptimal for tumor growth (20, 21). Recently, we demonstrated that C1q is abundantly present in malignant pleural mesothelioma (MPM), where it can combine with hyaluronic acid (HA), which is a principal component of the TME, and enhance the tumor growth by promoting cell adhesion and proliferation (18). However, other have shown a pro-apoptotic effect of C1q on prostate (15) and ovarian cancer cells *in vitro* (16). These rather two set of contradicting studies warranted a systematic analysis of the context of the disease and TME that can render C1q protective or pathogenic in cancer.

In the current study, we performed a bioinformatics analysis, using Oncomine database and the survival analysis platforms

Abbreviations: TME, Tumor microenvironment; ECM, extracellular matrix; BLBC, basal-like breast cancer; CCRCC, clear cell renal cell carcinoma; PRCC, papillary renal cell carcinoma; OS, overall survival; DFS, disease-free survival; WOX1, WW-domain containing oxidoreductase.

Kaplan-Meier plotter, in order to investigate whether C1q can serve as a potential prognostic marker for human carcinoma, i.e., tumors of epithelial origin. Our results showed that high levels of C1q have a favorable prognostic index in basal-like breast cancer (BLBC) and in HER-2 positive breast cancer. However, we found a pro-tumorigenic role of C1q in lung adenocarcinoma, and in clear cell renal cell carcinoma (CCRCC). This study is an important step forward in highlighting C1q as a new prognostic candidate biomarker for a range of carcinomas.

METHODS

Oncomine Database Analysis

The expression levels of *C1QA*, *C1QB*, and *C1QC* genes in various carcinomas were analyzed using Oncomine (www.oncomine.org), a cancer microarray database and web-based data mining platform from genome-wide expression analyses (22, 23). We compared the differences in mRNA level between normal tissue and carcinoma. The mRNA expression levels in neoplastic tissues compared to the healthy tissues were obtained as the parameters of p-value < 0.05, fold change >2, and gene ranking in the top 10%. Information about the dataset used in this study is summarized in **Supplementary Table 1**.

Kaplan-Meier Plotter Database Analysis

A Kaplan-Meier plotter database can be used to assess the effect of 54,675 genes on survival using 10,461 carcinoma samples (5,143 breast, 1,816 ovarian, 2,437 lung, and 1,065 gastric cancer patients with a mean follow-up of 69/40/49/33 months) using probe sets on the HGU133 Plus 2.0 array from Gene Expression Omnibus (GEO). For other human carcinoma, a total of 3,439 patients with RNA HiSeq data from The Cancer Genome Atlas (TCGA) cohort were collected. The prognostic significance of *C1QA*, *C1QB*, and *C1QC* expression and survival in several carcinomas was analyzed by Kaplan-Meier plotter (www.kmplot.com/analysis/) (24). The hazard ratio with 95% confidence intervals and logrank p-value was also computed.

Immunohistochemical Analysis

Normal and neoplastic human tissues, including breast, kidney and lung, were selected from the archives of the Department of Pathology, University of Trieste. Immunohistochemistry (IHC) was performed using a polymer detection method. Briefly, tissue samples were fixed in 10% v/v buffered formalin and then paraffin embedded. Four μm-thick tissue sections were deparaffinized and rehydrated. The antigen unmasking technique was carried out using Novocastra Epitope Retrieval Solutions, pH 9 (Leica Biosystems) in a PT Link pre-treatment module (Dako) at 98°C for 30 min. Sections were then brought to RT and washed in PBS. After neutralization of the endogenous peroxidase with 3% v/v H_2O_2 and Fc blocking by a specific protein block (Novocastra, Leica Biosystems), samples were incubated overnight at 4°C with rabbit polyclonal anti-human C1q (dilution 1:200) antibodies (Dako). Staining was carried out via polymer detection kit (Novocastra, Leica Biosystems) and DAB (3,3′-Diaminobenzidine; Dako, Denmark) substrate-chromogen. Slides were counterstained with Harris Haematoxylin (Novocastra, Leica Biosystems). Sections were analyzed under the Axio Scope A1 optical microscope (Zeiss) and microphotographs were collected through the Axiocam 503 color digital camera (Zeiss) using the Zen2 software.

Statistical Analysis

Survival curves were generated by the Kaplan-Meier plotter. All results are displayed with p-values from a log-rank test. P-values < 0.05 were considered significant. Similarly, with Oncomine, the statistical significance of data (p-values) was provided by the program.

RESULTS

Bioinformatic Analysis of the Three Genes Encoding Human C1q A, B, and C Chains in Normal Epithelial Tissues and Carcinomas

The expression of *C1QA*, *C1QB*, and *C1QC* genes was analyzed between different carcinoma and normal tissue counterparts using the Oncomine database. The threshold was determined as the following values: p-value < 0.05, fold change >2, and gene ranking in the top 10%. Carcinomas included in this analysis were: bladder carcinoma, breast cancer, cervical squamous cell carcinoma, esophageal carcinoma, head-neck squamous cell carcinoma, clear cell renal cell carcinoma (CCRCC), papillary renal cell carcinoma (PRCC), liver hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, ovarian cancer, pancreatic ductal adenocarcinoma, rectum adenocarcinoma, gastric carcinoma, and uterine corpus endometrial carcinoma. We only investigated carcinomas in which all the three C1q chains showed a significant prognostic effect by Kaplan-Meier plotter analysis. The *C1QA*, *C1QB*, and *C1QC* genes were either over-expressed, or downregulated depending on the type of carcinoma investigated, as compared to their normal tissue counterparts. All the three C1q chains showed a differential prognostic significance. These data appear to suggest that C1q can have pro- or anti-tumorigenic implications, depending on the carcinoma types (**Table 1**). Thus, detailed analyses of the expression profiles of all three C1q chains were performed.

Significance of C1q Expression in Breast Carcinoma

Bioinformatics analysis of *C1QA*, *C1QB*, and *C1QC* mRNA expression was performed in the context of the breast cancer using Karnoub's, Finak's, Curtis's, and Perou's datasets. A higher expression level of the three chains of C1q was detected as compared to normal breast tissue (**Figure 1A**, $p<0.05$). When breast cancer was stratified into different histological subtypes, *C1QA*, *C1QB*, and *C1QC* mRNA expression achieved a statistical significance only in medullary carcinoma (**Figure 1B**, $p<0.05$). To evaluate the prognostic significance of C1q in all breast cancers, we considered their molecular classification, such as

TABLE 1 | Prognostic significance of C1q in patients with carcinomas.

CANCER	Cancer subtype	Gene symbol	DFS/PFS	OS
	***n* = number of patients**			
Breast[a]	Triple-negative *n* = 618 for DFS *n* = 241 for OS	C1QA	HR = 0.47 (0.34–0.66) *p*-value (4.7e-6)	HR = 0.52 (0.32–0.85) *p*-value (0.0079)
Breast[a]	Triple-negative *n* = 618 for DFS *n* = 241 for OS	C1QB	HR = 0.56 (0.43–0.72) *p*-value (5.6e-6)	HR = 0.46 (0.28–0.75) *p*-value (0.0014)
Breast[a]	Triple-negative *n* = 360 for DFS *n* = 153 for OS	C1QC	HR = 0.58 (0.42–0.8) *p*-value (0.0009)	HR = 0.38 (0.2–0.71) *p*-value (0.0019)
Breast[a]	Luminal A *n* = 1,933 for DFS *n* = 611 for OS	C1QA	HR = 1.31 (1.1–1.55) *p*-value (0.0021)	ns
Breast[a]	Luminal A *n* = 1933 for DFS *n* = 611 for OS	C1QB	HR = 1.54 (1.29–1.83) *p*-value (1.0e-6)	HR = 2.09 (1.47–2.97) *p*-value (2.6e-5)
Breast[a]	Luminal A *n* = 841 for DFS *n* = 271 for OS	C1QC	HR = 1.36 (1.07–1.74) *p*-value (0.0132)	ns
Breast[a]	Luminal B *n* = 1,149 for DFS *n* = 433 for OS	C1QA	ns	ns
Breast[a]	Luminal B *n* = 1149 for DFS *n* = 433 for OS	C1QB	ns	ns
Breast[a]	Luminal B *n* = 407 for DFS *n* = 129 for OS	C1QC	ns	ns
Breast[a]	HER2+ *n* = 251 for DFS *n* = 117 for OS	C1QA	HR = 0.49 (0.33–0.72) *p*-value (0.0002)	HR = 0.17 (0.08–0.39) *p*-value (2.1e-6)
Breast[a]	HER2+ *n* = 251 for DFS *n* = 117 for OS	C1QB	HR = 0.61 (0.37–0.99) *p*-value (0.0434)	HR = 0.26 (0.12–0.55) *p*-value (0.0001)
Breast[a]	HER2+ *n* = 156 for DFS *n* = 73 for OS	C1QC	ns	HR = 0.28 (0.13–0.63) *p*-value (0.001)
Kidney[b]	Clear cell renal cell carcinoma *n* = 530 for OS	C1QA		HR = 1.76 (1.3–2.38) *p*-value (0.0002)
Kidney[b]	Clear cell renal cell carcinoma *n* = 530 for OS	C1QB		HR = 1.55 (1.15–2.1) *p*-value (0.0035)
Kidney[b]	Clear cell renal cell carcinoma *n* = 530 for OS	C1QC		H = 1.65 (1.21–2.24) *p*-value (0.0012)
Kidney[b]	Papillary renal cell carcinoma *n* = 287 for OS	C1QA		ns
Kidney[b]	Papillary renal cell carcinoma *n* = 287 for OS	C1QB		ns
Kidney[b]	Papillary renal cell carcinoma *n* = 287 for OS	C1QC		ns

(Continued)

TABLE 1 | Continued

CANCER	Cancer subtype	Gene symbol	DFS/PFS	OS
	***n* = number of patients**			
Lung[c]	Adenocarcinoma	C1QA		HR = 2.11 (1.66–2.68)
	n = 720 for OS			*p*-value (6.4e-10)
Lung[c]	Adenocarcinoma	C1QB		HR = 1.83 (1.45–2.31)
	n = 720 for OS			*p*-value (2.0e-7)
Lung[c]	Adenocarcinoma	C1QC		HR = 3.29 (2.39–4.52)
	n = 673 for OS			*p*-value (9.9e-15)
Lung[c]	Squamous cell carcinoma	C1QA		ns
	n = 524 for OS			
Lung[c]	Squamous cell carcinoma	C1QB		ns
	n = 524 for OS			
Lung[c]	Squamous cell carcinoma	C1QC		HR = 0.64 (0.46–0.89)
	n = 271 for OS			*p*-value (0.0084)

[a] *Using 5,143 cancer samples on the HGU133 Plus 2.0 array from Gene Expression Omnibus, GEO.*
[b] *Using 817 cancer samples on the RNA HiSeq data from The Cancer Genome Atlas, TCGA.*
[c] *Using 2,437 cancer samples on the HGU133 Plus 2.0 array from GEO.*
DSF, disease-free survival; OS, overall survival.

luminal-A, luminal-B, HER-2 positive, and basal-like cancers (BLBC) (**Supplementary Table 2**).

According to Kaplan-Meir plotter data, *C1QA*, *C1QB*, and *C1QC* mRNA expression was positively associated with a disease-free survival (DFS) rate in patients with BLBC (**Figure 1C**, $p<0.05$) and with an overall survival (OS) rate with HER-2 positive cancers (**Table 1**). This correlation was not evident in luminal-A and luminal-B patients. Only *C1QB* mRNA expression was negatively associated with high DFS and OS rates in the breast cancer patients with luminal-A, and to a DFS rate with all breast cancer.

The IHC analysis within the BLBC microenvironment revealed that C1q was diffusely present in the tumor stroma and was expressed by macrophage-like cells, suggestive of tumor-infiltrating myeloid elements (**Figure 2A**).

C1q Expression in Kidney Carcinoma has a Negative Correlation

The *C1QA*, *C1QB*, and *C1QC* mRNA expression was evaluated in kidney cancer based on the results obtained from different datasets. In CCRCC, the expression of the three C1q chains was higher as compared to normal kidney (**Figure 3A**, $p<0.05$). However, in the case of PRCC, this trend was evident only for *C1QA* and *C1QB* mRNA expression (data not shown). The data obtained from Kaplan-Meier plotter showed a negative relationship between *C1QA*, *C1QB*, and *C1QC* mRNA expression and OS rate of patients with CCRCC (**Figure 3B**, $p < 0.05$). No correlation was observed between *C1QA*, *C1QB*, and *C1QC* mRNA expression and OS in the PRCC patients (**Table 1**).

Within the CCRCC microenvironment, C1q was found to be mainly expressed in the tumor stroma and in the small vessels, and it was associated with the cell membrane of tumor cells (**Figure 2B**).

Lower Level of C1q Expression in Lung Carcinoma

While examing *C1QA and C1QB* mRNA expression in lung cancer, using Selamat's, Wachi's and Bhattacharjee's datasets, we found a lower expression level in adenocarcinoma (**Figure 3C**, $p<0.05$) and in squamous cell carcinoma (data not shown, $p<0.05$) than in normal lung tissue; *C1QC* mRNA expression was significant only in lung adenocarcinoma. As shown in **Figure 3D**, *C1QA, C1QB*, and *C1QC* mRNA expression levels negatively correlated with an OS rate of the patients with lung adenocarcinoma ($p<0.05$); no correlation with OS was observed in squamous cell carcinoma (**Table 1**).

IHC in lung adenocarcinoma revealed C1q staining in the stroma and some macrophage-like positive cells into the tumor mass (**Figure 2C**).

DISCUSSION

In this paper, we performed bioinformatics analysis to explore if C1q level could act as a possible prognostic marker in various carcinomas, in view of its reported dichotomous effects on cancer cells (pro- and anti-tumorigenic). C1q is present in colon, lung, breast, pancreatic carcinoma, and melanoma. C1q can promote adhesion, proliferation and migration of melanoma cells (17). We found C1q in abundance in all histological variants (epithelioid, sarcomatoid, and biphasic) of asbestos-induced malignant pleural mesothelioma. C1q bound high and low molecular weight HA and acted as a tumor-promoting factor (18). In addition, C1q exerted a protective effect against apoptosis, suggesting an overall pro-tumorigenic activity (17). However, Hong et al. recently observed that C1q, expressed in normal prostate, was downregulated in benign prostatic hyperplasia and prostate cancer (15). C1q was able to induce apoptosis and growth suppression of human prostate DU145

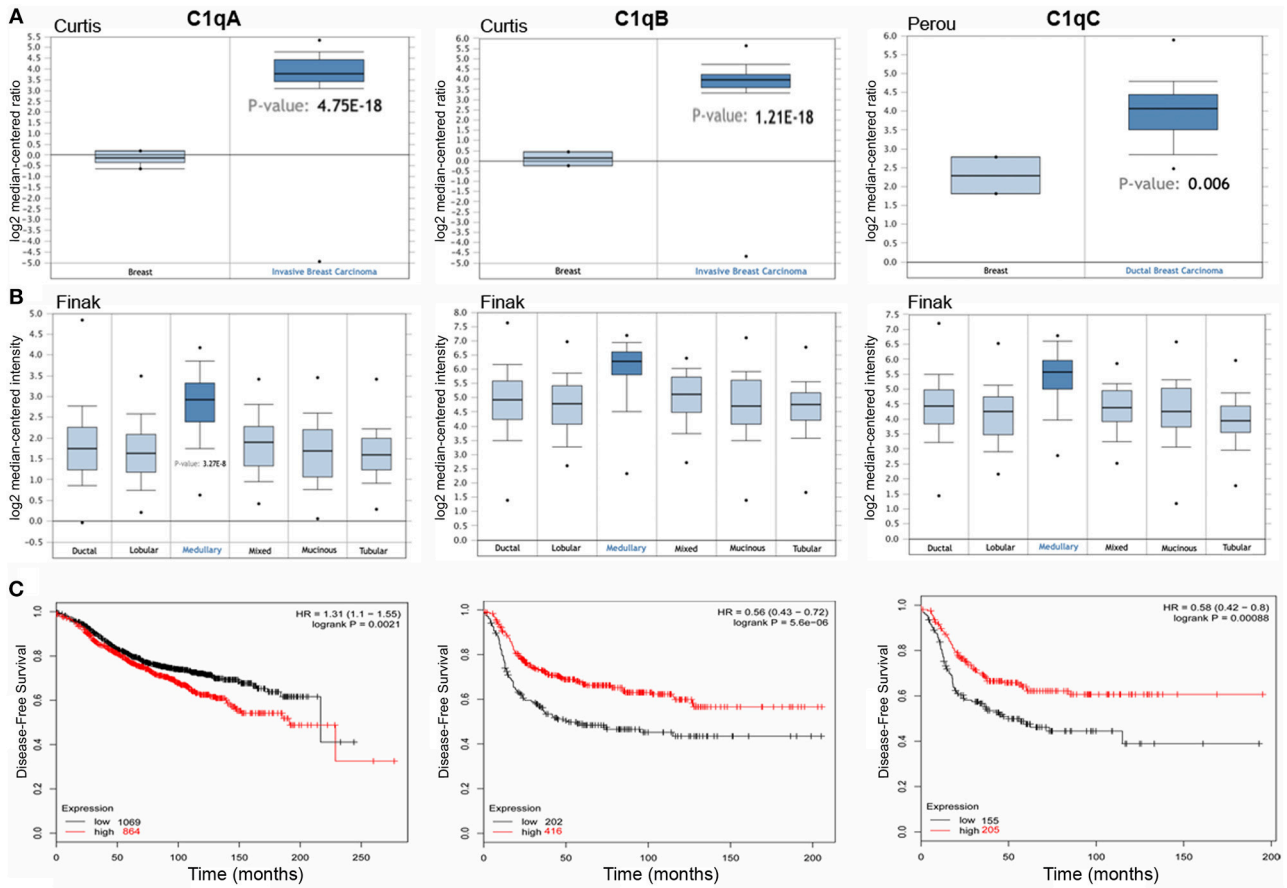


FIGURE 1 | *C1QA*, *C1QB*, and *C1QC* expression in invasive breast carcinoma. Curtis's datasets were used for bioinformatics analysis to explore *C1QA* and *C1QB* mRNAs expression in the breast cancer, whereas Perou's datasets was used for bioinformatics analysis to evaluate *C1QC* mRNA. A higher C1q mRNAs expression was detectable in invasive breast carcinoma compared to normal breast tissue **(A)**. The analysis of the different breast carcinoma histotypes by Finak's dataset revealed how the medullary breast cancer presented the major intensity of C1q mRNA expression **(B)**. According to the data Kaplan-Meir plotter, *C1QA, C1QB,* and *C1QC* mRNA expressions was positively linked to a disease-free survival (DFS) rate in patients with basal-like cancers **(C)** ($p<0.05$) and to an overall survival (OS) rate with HER-2 positive cancers (**Supplementary Table 2**). HR, hazard ratio.

cells, through direct activation of the tumor suppressor WW-domain containing oxidoreductase (WOX1). C1q also have a pro-apoptotic effect on an ovarian cell line, SKOV3, acting via a TNF-α induced apoptosis pathway that involves upregulation of Bax and Fas (16).

In a syngeneic murine model of melanoma in C57BL/6 strain, C1q-deficient mice showed prolonged survival and slower tumor growth, as compared to wild-type mice (17). However, Bandini et al. found that neuT mice, a genetically engineered mouse model for mammary carcinoma that was made deficient for the C1qA chain (neuT-C1KO mice), manifested an accelerated tumor growth associated with an increased number of intra-tumoral vessels, compared to wild-type neuT mice. These differences in tumor progression were attributed to a reduced activation of WW domain containing oxidoreductase (WWOX) in C1q–deficient mice (25).

In view of these rather contradicting roles of C1q in tumor progression, we performed a systematic bioinformatics analysis of the expression of C1q, and its correlation with the survival rate in different carcinoma histotypes, using Oncomine and Kaplan-Meier plotter tools. We selected the carcinomas that showed all the three chains of human C1q statistically significant for the prognosis; in several cases, the prognosis was differentially linked to the C1q chains, or limited to one or two C1q chains. We often noticed the mRNA encoding for only one or two C1q chains, something that would impede synthesis of a functional C1q molecule. Indeed, we have provided evidence in the past that the expression of C1qC chain is essential for the production of functional C1q by the endothelial cells of the decidua (26). Moreover, mesothelioma cells are impaired in C1q A chain synthesis (18).

Our bioinformatics analysis highlighted that high levels of C1q have a favorable prognostic index in BLBCs for DFS and $HER2^{+}$ breast cancer for OS, (**Graphical Abstract**) consistent with the *in vivo* studies by Bandini et al. using C1q-deficient mice (25). Inflammation is a major characteristic of these types of tumors. One possible explanation for the observed positive association between C1q expression and favorable

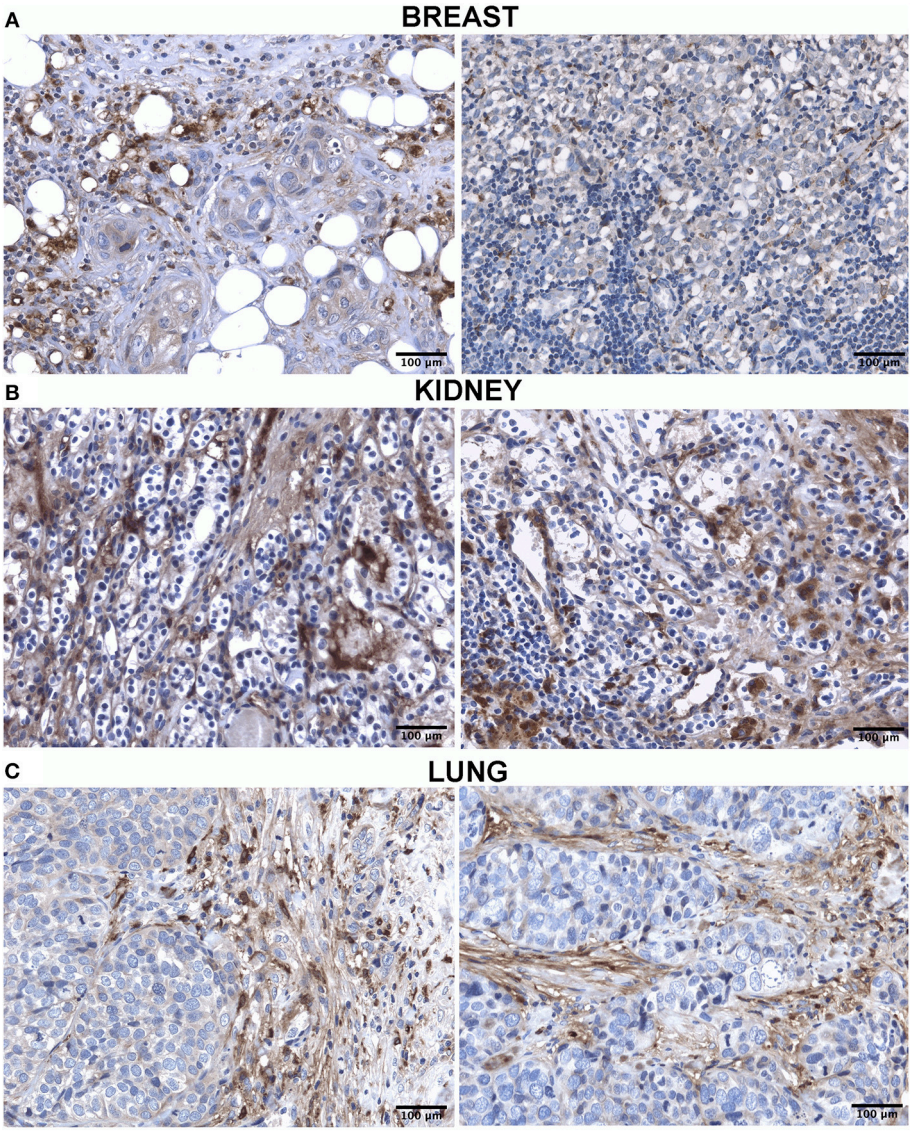


FIGURE 2 | Immunohistochemistry analysis for C1q in breast **(A)**, kidney **(B)** and lung carcinoma **(C)**. Representative microphotographs showing expression of C1q in different carcinoma. The expression of C1q in carcinoma was observed in all tissues with differential distribution in the TME, as described in the result section. DAB (brown) chromogen was used to visualize the binding of anti-human C1q antibodies; scale bars, 50µm.

prognostic index could be due to the correlation between the presence of C1q and dendritic cells (CD11c positive cells) in TME. High CD11c expression in BLBCs is associated with a significantly higher OS ($p = 0.047$) as compared to low CD11c expression (27). Dendritic cells themselves can be a potential source of C1q within the TME (28, 29). C1q, although present, is not able to bind BLBC cells (MDA-MB-231), and hence, not able to promote tumor progression (unpublished data), probably due to downregulation of putative C1q receptor(s). It is thus crucial to understand the differences in good prognosis survival between BLBCs and $HER2^{+}$ breast cancer, the role of inflammation, and that of C1q in determining such differences.

Wilson et al. (30) found that C1q chain genes were enriched in the stroma compartment of triple-negative breast cancers. The analysis of publicly available data sets revealed that the genes encoding for the C1q chains were associated with a poor prognosis in BLBC using the TCGA dataset (504 patients). In our analysis, using the GEO dataset that include 5,143 patients, we observed a positive prognostic effect for BLBCs in DFS and HER2-positive breast cancers in OS. The opposite results were obtained for CCRCCs and lung adenocarcinomas in OS.

A negative prognostic effect arose from the analysis of kidney and lung carcinomas (**Graphical Abstract**). The most frequent histological subtypes include CCRCC and PRCC (CCRCC ∼75%; PRCC ∼10%) (31). The expression of C1q in kidney

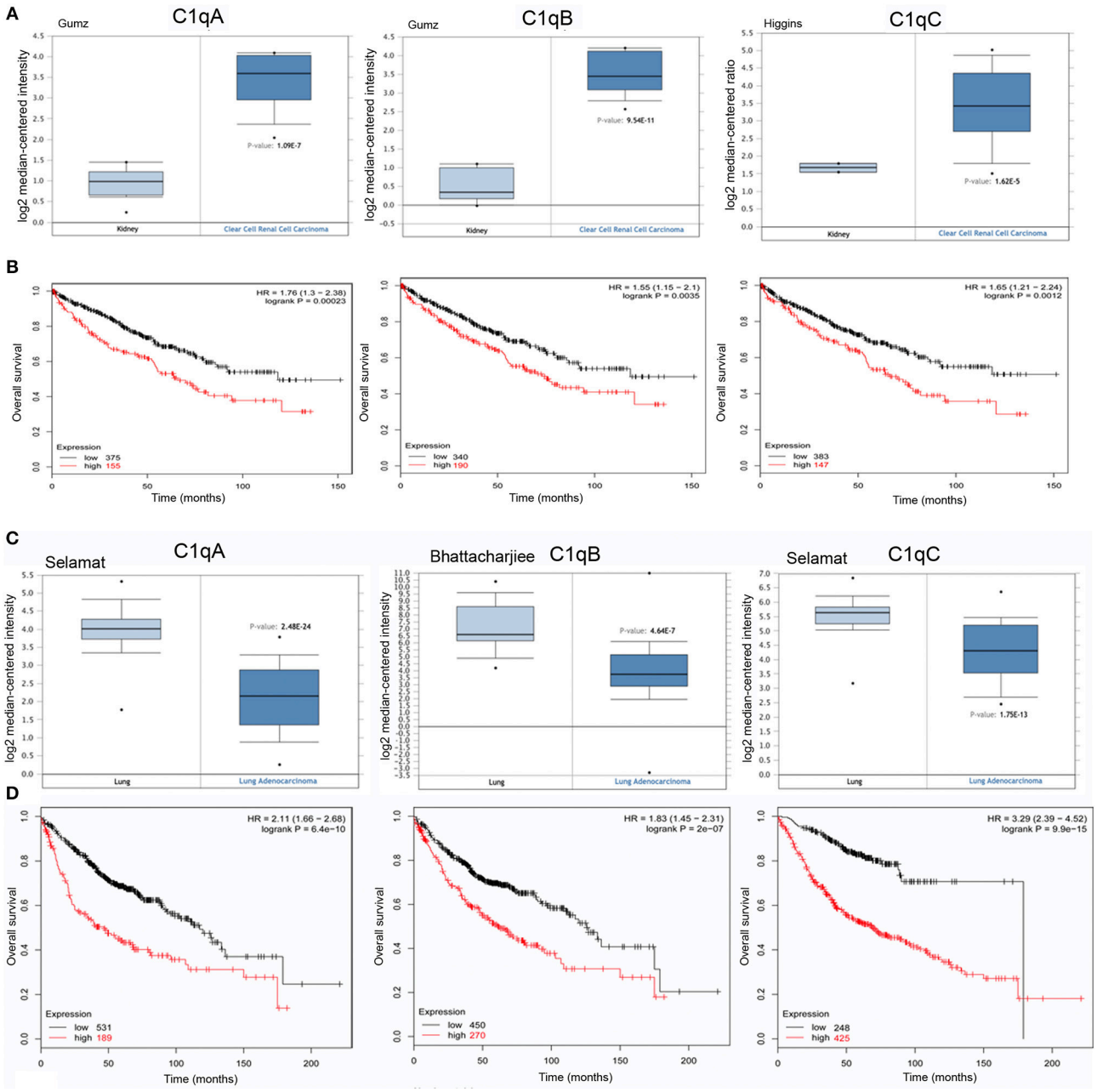


FIGURE 3 | Pathological significance of C1q expression kidney and in lung cancer. Gumz's dataset have explored *C1QA* and *C1QB* mRNA expression in kidney, whereas Higgins' dataset were used for *C1QC* mRNA expression. A higher C1q expression was detectable in CCRCC cancer than that in normal tissue **(A)**, $p < 0.05$. According to the data from Kaplan-Meier plotter, C1q mRNA expressions were negatively related to an overall survival rate of the patients with CCRCC **(B)**. HR, hazard ratio. Selamat's dataset have revealed a lower *C1QA* and *C1QC* mRNA expression in lung adenocarcinoma that in normal lung tissues **(C)**, whereas Bhattacharjiee's dataset was used for *C1QB*, but the results were in accordance with Selamat's one. There was a negative association between C1q mRNA expression and a favorable prognosis in patients with lung cancer, for Kaplan-Meir plotter **(D)**. HR, hazard ratio.

cancer is increased as compared to normal kidney tissue (**Figure 3A**) and C1q has a negative prognostic effect in the case of CCRCC (**Figure 3B**); no association was evident for PRCC. CCRCC tumor is characterized by an increased response to HIF that promotes blood vessel growth. Targeted therapies directed against VEGF, VEGF receptor, and mTOR play a crucial role in the management of metastatic CCRCC (32). We can hypothesize that C1q can also participate in promoting angiogenic processes in this particular tumor (14).

C1q has a negative prognostic value in lung tumors limited to adenocarcinomas, the most common form of lung cancer (**Figure 3D**). According to the WHO classification of lung

tumors, there are four major histological types: adenocarcinomas, squamous cell carcinomas, large cell carcinomas, and small cell carcinomas (33). It is worth noting that C1q expression is reduced in lung cancer compared to the normal lung as we observed for surfactant protein D (SP-D) (34). Although C1q expression in lung cancer is lower than in normal tissue, lung cancer cells bind C1q present in the tumor microenvironment and activate the classical complement pathway (35). Tumor transformation is also concomitant with the loss of key defense molecules entrusted with early recognition and removal of the altered self (36).

A number of factors can modulate the role of C1q in the TME. C1q interaction with the ECM components can adversely interrupt its putative functions, as is the case with HA. It is also possible that certain tumors downregulate the putative receptor for C1q in order to escape possible apoptosis induction. Proliferative and apoptotic responses to C1q can be dictated by distinct receptors that are yet to be discovered. Last but not the least, the orientation of the C1q molecule, while engaging with the tumor cells, can also define the C1q-mediated implications. Our study encompasses all the above-mentioned possibilities, including tumor heterogeneity.

ETHICS STATEMENT

This study was carried out as per the recommendations of governmental guidelines, and approved by the CEUR (Comitato Etico Unico Regionale, FVG, Italy; number 34/2016). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AM and RB: conception and design. CA, DB, and AR: development of methodology. BB, DB, CA, and GZ: acquisition of data. AM, DB, BB, FZ and CA: analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis). RB, UK, CA, PZ, and GR: writing, review, and/or revision of the manuscript. RB: study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00865/full#supplementary-material

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Role of C5b-9 and RGC-32 in Cancer

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The complement system represents an effective arsenal of innate immunity as well as an interface between innate and adaptive immunity. Activation of the complement system culminates with the assembly of the C5b-9 terminal complement complex on cell membranes, inducing target cell lysis. Translation of this sequence of events into a malignant setting has traditionally afforded C5b-9 a strict antitumoral role, in synergy with antibody-dependent tumor cytolysis. However, in recent decades, a plethora of evidence has revised this view, highlighting the tumor-promoting properties of C5b-9. Sublytic C5b-9 induces cell cycle progression by activating signal transduction pathways (e.g., Gi protein/ phosphatidylinositol 3-kinase (PI3K)/Akt kinase and Ras/Raf1/ERK1) and modulating the activation of cancer-related transcription factors, while shielding malignant cells from apoptosis. C5b-9 also induces Response Gene to Complement (RGC)-32, a gene that contributes to cell cycle regulation by activating the Akt and CDC2 kinases. RGC-32 is expressed by tumor cells and plays a dual role in cancer, functioning as either a tumor promoter by endorsing malignancy initiation, progression, invasion, metastasis, and angiogenesis, or as a tumor suppressor. In this review, we present recent data describing the versatile, multifaceted roles of C5b-9 and its effector, RGC-32, in cancer.

Keywords: C5b-9, cancer, RGC-32, cell proliferation, apoptosis

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INTRODUCTION

Carcinogenesis in human somatic cells involves a series of genetic and epigenetic alterations that culminate in the generation of a malignant tissue fully prepared to elude most anticancer defense strategies. To date, there are seven key alterations in cancerous cells: self-sufficiency in growth signals; insensitivity to growth suppressors; evasion of apoptosis, enabling replicative immortality; sustained angiogenesis; tissue invasion (metastasis) (1) and the presence of cancer-related inflammation (CRI) (2). Essential orchestrators of CRI are the tumor-associated inflammatory cells (macrophages, fibroblasts, T cells and myeloid-derived suppressor cells) and their secreted chemokines and cytokines, along with complement activation within the tumor microenvironment (2, 3).

The complement system represents an effective arsenal of innate immunity as well as an interface between innate and adaptive immunity. An ancestral instrument in fighting invasive pathogens and efficient clearance of debris, the complement system can be activated by the classical, alternative, or lectin pathway, all of which unite at the level of C3 activation. All three pathways lead to the membrane attack complex formation and to cell lysis. Activation of the terminal complement proteins C5 to C9 generates membrane-inserted complexes C5b-7, C5b-8, and finally C5b-9, the so-called membrane attack complex (MAC) (4, 5).

Evidence supporting complement activation, in association with C5b-9 deposits during the antitumoral response exists for a variety of human malignancies (6). Niculescu et al. provided the first immunohistochemical support for the presence of C5b-9 deposits (along with IgG, C3 and C4 deposits) in a human cancerous tissue, namely breast carcinoma (3). Thereafter, numerous studies demonstrated C5b-9 deposition indicating complement activation in human thyroid (7), ovarian (8), endometrial (6), gastric (6–10), liver (6), colon, renal, and lung carcinomas (11), as well as in human osteosarcoma (12), medulloblastoma (6), glioma (6, 13), and gastrointestinal stromal tumor (6) tissues. High levels of soluble C5b-9 were also detected in the ascitic fluid of ovarian cancer (14). Elevated circulating C9 protein levels have been reported in the serum or plasma of colon (15) and gastric (16) adenocarcinoma, oral squamous cell carcinoma (17) and squamous cell lung cancer (18) patients.

C5b-9 has been shown to possess antitumoral properties, acting in synergy with monoclonal antibody (mAb)-based immunotherapies, many of which use complement activation and C5b-9 as an effector to kill tumor cells (19, 20). In this context, the mAb triggers C5b-9 assembly on cells leading to tumor destruction. Nevertheless, in recent decades, a plethora of evidence has brought about a conceptual switch in this paradigm (21) and exposed the tumor-promoter properties of C5b-9.

Here, we summarize the available data concerning the complex and versatile role of C5b-9, and that of its pivotal effector RGC-32, in cancer.

EFFECTS OF LYTIC C5b-9 ON TUMOR CELLS

Successful achievement of cell lysis during complement-dependent cytotoxicity (CDC) requires the formation of multiple C5b-9 complexes on the cell surface (22). Once malignant Ehrlich ascites cells already bearing C5b-8 complexes are exposed to C9, a rapid and extensive ATP depletion, coupled with leakage of the adenine nucleotides ATP, ADP, and AMP, precedes cell death. Other prelytic events include the loss of mitochondrial membrane potential with consequent defective ATP synthesis and a vigorous Ca^{2+} influx, which initiate necrotic cell death (23).

The morphologic and biochemical changes induced by lytic MAC attack do share some features with those seen in apoptosis (nucleolar changes), although most features correspond more closely to necrotic changes (loss of volume control and defective mitochondria) (5, 24). The main biochemical changes include Bid cleavage, caspase activation, and activation of extracellular DNases (25–27). The impact of lytic C5b-9 on the malignant signaling pathways is multifaceted, since CDC has been documented to use several necrotic cell death pathways involving the receptor-interacting protein kinase 1 (RIPK1), receptor-interacting protein kinase 3 (RIPK3) and mixed-lineage kinase domain-like protein (MLKL) (28), in concert with the effectors JNK and Bid. The RIPK1/RIPK3/MLKL pathway closely resembles TNF-alpha-induced necroptosis (28).

Tumor cells have developed complement resistance through C5b-9 removal (29), expression of membrane complement-regulatory proteins (mCRPs) and other cell surface-protective molecules, and secretion of soluble complement inhibitors (30, 31). The ability of a cell to survive an initial complement-mediated membrane attack affords its resistance against future attacks (32).

The use of mAb-based immunotherapies that stimulate the destruction of tumor cells by CDC has received a lot of interest (19). The quest for optimal efficacy in CDC has incited many research teams. For instance, Diebolder et al. have shown that IgG hexamerization after antigen binding leads to more effective complement activation and fixation, and thus a more potent CDC (33). Narrow C5b-8 pores formed without C9 are sufficient for CDC due to efficient antibody-mediated hexamer formation (34). By neutralizing mCRP expression on leukemia cells, Mamidi et al. were the first to achieve both enhanced CDC and improved complement-dependent cellular cytotoxicity by monocyte-derived macrophages and macrophages induced by two anti-CD20 antibodies (rituximab and ofatumumab) and one anti-CD52 antibody (alemtuzumab) (35). Of late, the miR-200b, miR-200c, and miR-217 microRNAs have been recognized as potential regulators of mortalin as well as CD46 and CD55 expression in leukemia/ lymphoma and have been observed to coordinate the quantity of C5b-9 deposited on target cells (36).

SUBLYTIC C5b-9 INDUCES TUMOR CELL PROLIFERATION AND TRANSCRIPTIONAL ACTIVATION IN MALIGNANT CELLS

Sublytic levels of C5b-9 assembly in the membrane of malignant cells generate several different biological responses: activation of signal transduction pathways, proliferation, and modulation of apoptosis (5, 37) (**Figure 1**).

One of the first investigations of C5b-9 looked at the generation of signal messengers in Ehrlich carcinoma cells by the sublytic terminal complement complexes (TCC) C5b-9, C5b-8, and C5b-7 and identified the signal messengers involved in eliminating TCC from the cell surface (44). Exposure of Ehrlich carcinoma cells to C5b-9 caused an increase in cytosolic Ca^{2+}. In addition, sublytic C5b-9 and C5b-8 substantially increased PKC activity, and C5b-8 and C5b-7 induced an increase in cAMP (44). In another report, sublytic C5b-9 assembly in lymphoblastoid human B cells stimulated the production of diacylglycerol (DAG) and ceramide (mediators of inflammation and tissue repair), along with PKC activation (45). Rapid elimination of TCC

Abbreviations: AP-1, activator protein 1; CDC, complement-dependent cytotoxicity; CDK1, cyclin–dependent kinase 1; CyB1, cyclin B1; CyE, cyclin E; EBV, Epstein-Barr virus; EGF, epidermal growth factor; ERK1, extracellular signal-regulated kinase 1; FGF2, fibroblast growth factor 2; HIF-1α, hypoxia inducible factor 1 alpha; IKKα, inhibitor of nuclear factor kappa-B kinase subunit alpha; JAK1, Janus kinase 1; MAC, membrane attack complex; MEK1/2, mitogen activated protein kinase kinase 1; mTORC2, mammalian target of rapamycin complex 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PLK1, polo-like kinase 1; RGC-32, response gene to complement 32; RIPK, receptor-interacting protein kinase; TCC, terminal complement complex; VEGF, vascular endothelial growth factor; STAT3, signal transducer and activator of transcription 3.

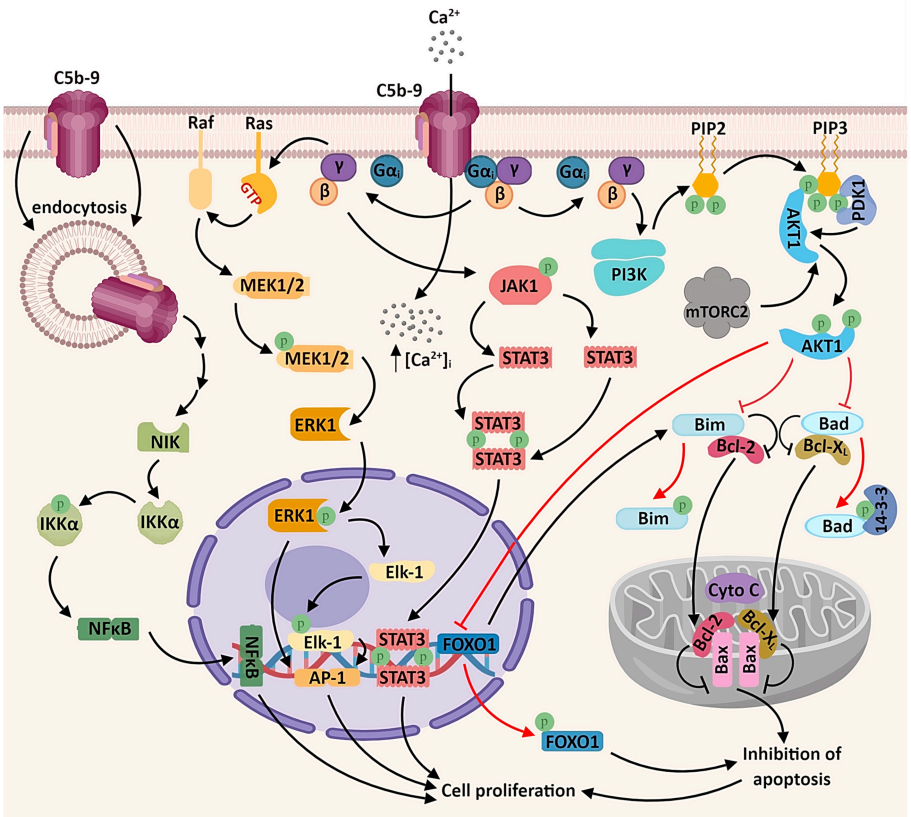

FIGURE 1 | Sublytic C5b-9 promotes tumor growth and survival by activating several signaling pathways. The assembly of C5b-9 complexes in the cellular membrane activates the heterotrimeric G proteins of the G_i subtype (38). The β-γ complex is thought to activate several intracellular signaling cascades, including: a) phosphatidylinositol 3 kinase (PI3K)-Akt signaling pathway. Activated Akt phosphorylates and inactivates the pro-apoptotic factors Bad and Bim, resulting in the release of the pro-survival factors Bcl-2 and Bcl-xL, which migrate into the mitochondrial matrix and inhibit the release of cytochrome C (cyto C), thus inhibiting apoptosis. Akt also phosphorylates the transcription factor FOXO1, promoting its nuclear exclusion and inhibiting FOXO1-mediated transcription of pro-apoptotic factors (6, 39); b) Ras-Raf-MEK1-ERK1 signaling pathway, resulting in the activation of transcription factors associated with cell proliferation (AP-1 and Elk1); c) JAK1-STAT3 signaling pathway, leading to the formation of STAT3 homodimers and their nuclear translocation (6, 39–41). Another recently described mechanism which may account for the activation of genes associated with cell proliferation is the activation of the non-canonical NF-κB signaling pathway by the endosomal C5b-9 complexes, involving the NF-κB-inducing kinase (NIK) (42). An important consequence of C5b-9 assembly is the increased concentration of cytosolic calcium ions (Ca^{2+}), either through direct entry of extracellular Ca^{2+} or by endoplasmic reticulum release triggered by intracellular second messengers (43) (not shown).

from the membrane surface was inhibited by pretreatment with pertussis toxin, suggesting the involvement of a G_i protein (38).

One of the pioneer studies on sublytic C5b-9-induced tumor cell proliferation through mitotic signaling (40) demonstrated that a significant increase in DNA synthesis over the C5b6 level is induced by C5b-9 in the human lymphoblastoid B cell line JY 25. This effect (but not the basal DNA synthesis activity) could be abrogated by pertussis toxin pretreatment, indicating the involvement of activated G_i proteins in DNA synthesis induced by C5b-9 in tumor cells (40) (**Figure 1**). Pretreatment of cells with PD98059 (specific inhibitor of MEK1 activation) was also effective in abolishing C5b-9-induced DNA synthesis (40). Both ERK1, a member of a potentially pro-oncogenic signal transduction pathway, and PI3K contribute to the transmission of downstream cellular effects prompted by sublytic C5b-9 (40, 46, 47). Indeed, as shown by Pilzer et al., C5b-9 deposition on the K562 leukemic cell membrane activates PKC and ERK protein kinases (48), which then induce the relocation of the mitochondrial chaperone mortalin from the mitochondria to the plasma membrane, where mortalin escorts exo-vesiculated C5b-9 complexes (49). Co-localization of mortalin and C5b-9 in distinct puncta at the leukemic cell plasma membrane region has also been well-documented (49). Mortalin is overexpressed in a multitude of malignancies, and a high level of circulating mortalin was recently demonstrated to correlate with high mortality in colorectal cancer patients (50). Mortalin supports the process of carcinogenesis by suppressing pathway-mediated growth-inhibitory signaling, inactivating tumor suppressor p53, and activating epithelial-to-mesenchymal transition (EMT) signaling (51). In addition, Rozenberg et al. has found that HSP90 binds to mortalin and protects cells from complement-mediated cytotoxicity by inhibiting, together with mortalin, C5b-9 assembly on the plasma membrane (52).

Among the signal transduction networks regulating cancer progression that have been found to function downstream of sublytic C5b-9 are p38/MAPK/JNK1 and JAK1/STAT3 (39, 41).

Cellular proliferation induced by membrane-inserted sublytic C5b-9 relies on the activation of the Gi protein/PI3K/Akt kinase and Ras/Raf-1/ERK1 pathways and regulation of cell cycle-specific genes and proto-oncogenes (5, 47) (**Figure 1**).

Of note, activation of activating protein 1 (AP-1) transcription factor has also been documented following C5b-9 treatment of lymphoblastoid B-cell lines (40). Consistent with this finding, stimulation with C5b-9 enhances the expression of the oncogenic proteins c-jun, JunD, and c-fos (53). AP-1 functions are dependent on the specific Fos and Jun subunits contributing to AP-1 dimers (54). AP-1 activity is crucial to oncogenesis, and there is evidence that it has an ambivalent role: while it can act as a tumor promoter in some cancer types, it also represses tumor formation in others (54). NF-κB is another major transcription factor known to be activated by sublytic C5b-9 (42, 47, 55). Sublytic C5b-9–induced, NF-κB–regulated proteins may further enhance cell survival (56) (**Figure 1**).

In smooth muscle cells (SMC), sublytic assault by MAC stimulates release of insulin-like growth factor-1 (57), whereas in glomerular epithelial cells it causes transactivation of the receptors for epidermal growth factor (EGF), human epidermal growth factor 2/Neu, fibroblast growth factor, and hepatocyte growth factor, all vital growth factors during tumor development (58). CT26 colon carcinoma cells exposed to sublytic C5b-9 exhibit significant changes in genes involved in Ca^{2+} and G-protein signal transduction, early response transcription factors (EGR1, EGR2) and four genes encoding proteins with extracellular localization: AREG, CXCL1, MMP3, and MMP13 (59). Network analysis has suggested an important role for the EGF receptor as the main canonical signaling cascade in the response to sublytic C5b-9 in the colon carcinoma cells (59). This connection is very pertinent to carcinogenesis, since alterations in EGF receptor signaling are common events in several human cancers (60).

Non-lethal C5b-9 activates cell cycle by directly influencing major cell cycle regulators: in aortic SMC, sublytic C5b-9 increases the activity of the cyclin-dependent kinases CDK4 and CDK2, whereas in endothelial cells it increases the levels of cell division cycle protein 2 (CDC2), cyclin D1, and proliferating cell nuclear antigen (PCNA) (39).

SUBLYTIC C5b-9 PROTECTS TUMOR CELLS FROM APOPTOSIS

Sublytic complement-induced protection against TNF-α-mediated apoptosis accompanies the induction of the anti-apoptotic proteins Bcl-2 and Bcl-xL, along with suppressing the TNF-α-induced decrease in the amount of Bcl-2 and Bcl-xL (61, 62). The anti-apoptotic effects of sublytic C5b-9 encompass events such as activation of NF-kB and inhibition of caspase-8 activation (61, 63, 64). Fascinating insight into the relationship of microvesicles to apoptosis has been provided by the work of Stratton et al. in prostate cancer cells (43). Sublytic C5b-9 deposition is among the positive signals that result in a high Ca^{2+} cellular influx and membrane depolarization; stimulation of microvesicle release then ensures shedding of excess intracellular calcium and export of damaging agents such as deposited C5b-9 and caspase-3. This circuit provides cells with an effective mechanism to thwart apoptosis (43).

It should be noted, however, that data also exist in support of the ability of sublytic C5b-9 to activate various molecules that potentially contribute to programed cellular death. In lung epithelial cells, MAC insertion has been observed to induce Ca^{2+} influx, leading to mitochondrial overload and loss of mitochondrial transmembrane potential. These changes prompt NLPR3 inflammasome activation, as well as IL-1β production, cytoplasmatic cytochrome c release and caspase activation (65). A similar chain of events has been described in macrophages in which "bystander" deposition of MAC on the plasma membranes of phagocytic macrophages incite NLRP3 inflammasome and caspase-1 activation, together with IL-1β and IL-18 release (66). Bystander C5b-9 deposition has also been found to modulate T-cell polarization and leucocyte recruitment to the phagocytic sites (66). Despite the analogy with apoptosis, the involvement of NLRP3 inflammasomes, caspase-1, IL-1β, and IL-18 rather evokes another form of programmed cellular death, pyroptosis (67). While activation of pyroptosis provides powerful ammunition against many types of cancers, other researchers have reported that the NLRP3 inflammasome and IL-1β pathway promote cancer progression in animal and human breast cancer models (68) and asbestos-induced malignant mesothelioma (69). In addition, sublytic C5b-9 has been shown to interact with effectors of TNFα-induced necroptosis, yet another type of programmed cell death: exposure of human erythroleukemia K562 cells to sublytic C5b-9 causes the activation of RIPK1, RIPK3, and MLKL, co-localization of RIPK3 with RIPK1 in the cytoplasm and co-localization of RIPK3 and MLKL with C5b-9 at the plasma membrane (28). The meaning of the association between C5b-9 and necroptotic effectors has many nuances: RIPK3 and MLKL are in fact seen as putative tumor suppressors (70), but *in vitro* work in breast cancer cells has recently highlighted the contributions of the necroptotic genes RIPK1, RIPK3, and MLKL in promoting anchorage-independent tumor growth and mediating tumor cell resistance to radiation (71).

C5b-9 AND ANGIOGENESIS

Although initiated by cellular destruction and hypoxia, the propagation of the vascular network in a malignant environment is sustained by upregulation of pro-angiogenic factors (e.g. vascular endothelial growth factor [VEGF], TGF-α, TGF-β, TNF-α, EGF, fibroblast growth factor [FGF]) and downregulation of negative angiogenic regulators (IL-10, IL-12, angiopoietin-2, angiotensin) (72).

Accelerated C5b-9 deposition, accompanied by VEGF, β-FGF, and TGF-β2 release is seen during laser-induced choroidal neovascularization in age-related macular degeneration in CD59-deficient mice (73). Likewise, exposure of retinal pigment epithelium cells to oxidative stress has been found to induce sublytic C5b-9 activation, triggering VEGF secretion via the Src and Ras-Erk pathways (74).

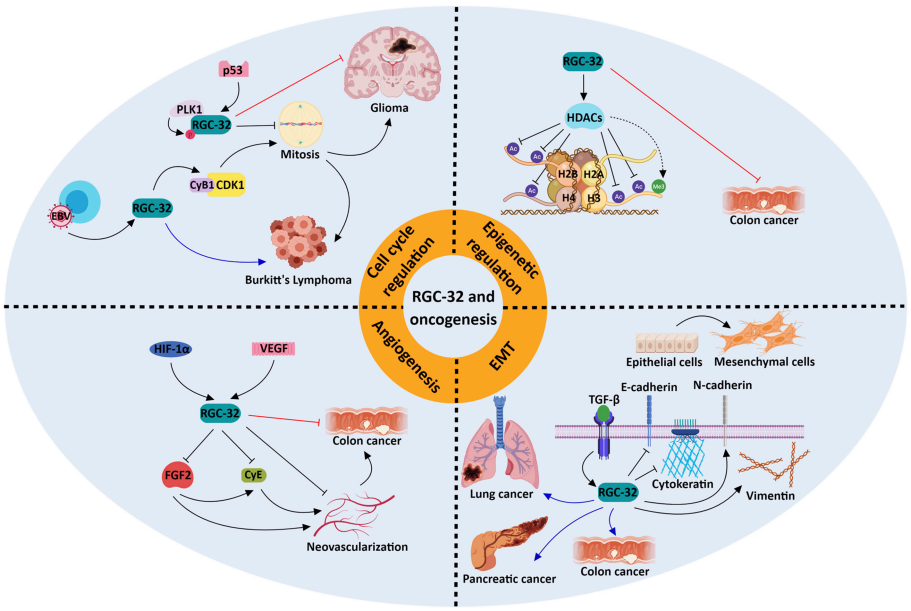

FIGURE 2 | Molecular mechanisms underlying the role of RGC-32 in oncogenesis. RGC-32 can act both as a tumor suppressor (red inhibitory lines) and a tumor promoter (blue arrows) in a variety of cancers by activating a plethora of molecular pathways. RGC-32 plays an important role in: (a) promoting the TGF-β-induced epithelial-to-mesenchymal transition (EMT), a process in which epithelial cells lose their adhesiveness and gain myofibroblast-like phenotypes, inducing metastasis and cancer progression (80, 91, 92); (b) epigenetic modifications, by inducing histone deacetylases (HDACs), which in turn deacetylate various histone targets such as H2B at lysine 5 (H2BK5), H2BK15, H3K9, and H4K8 and indirectly promote the tri-methylation of H3K27. This in turn may result in transcriptional repression of genes associated with cancer progression (77); (c) cell cycle regulation, in which RGC-32 can promote mitosis by enhancing the activity of kinases crucial for cell cycle progression (93), or induce cell cycle arrest in a p53-dependent manner (88); (d) inhibition of angiogenesis, in which it may behave as a negative feedback regulator of hypoxia-induced signaling pathways (94). The involvement of RGC-32 in these processes might explain its apparent dual role as a tumor suppressor/promoter in the same type of tumor, such as colon cancer.

The effects of C5b-9 were later corroborated in cancer cells. In an osteosarcoma epithelial cell line, sublytic C5b-9 activation (via the alternative pathway) instigated production of angiogenic growth factors FGF1 and VEGF-A via the ERK signaling pathway (12).

RGC-32 AND CANCER

The RGC-32 gene was first cloned from rat oligodendrocytes via differential display by Badea and coworkers, in their quest to identify the genes differentially expressed in response to sublytic complement activation (75, 76). RGC-32 fundamentally regulates cellular processes such as the cell cycle, differentiation, wound healing and tumorigenesis (75, 77). It directly binds to cyclin-dependent kinase CDC2 and Akt and stimulates their kinase activity (75, 78).

Various studies have described an aberrant RGC-32 mRNA expression in human cancers: up-regulation in colon (79, 80), ovarian (81, 82), breast (79, 83, 84) and prostate (79) cancers and lymphomas (85, 86) and downregulation in glioblastomas (87), astrocytomas (88), adrenocortical carcinomas (89), and multiple myelomas (90).

We have originally demonstrated a role for RGC-32 deregulation in colon adenocarcinoma, showing that the intensity of RGC-32 immunohistochemical staining corresponded to the increase in the TNM staging of the adenocarcinomas (77). Later, the expression of RGC-32 was shown to be up-regulated in pancreatic cancer tissues and to correlate with TNM stages (91).

Using a gene array and SW480 colon adenocarcinoma cells, we have identified groups of genes that are significantly changed by RGC-32 silencing (77), including genes implicated in chromatin assembly, cell cycle, and RNA processing. We have observed increased lysine acetylation at multiple sites on histones H2B, H3, and H4, and lessened expression of the histone deacetylase SIRT1 upon silencing of RGC-32 expression in SW480 cells (77) (**Figure 2**). Moreover, an absence of RGC-32 expression induces DNA synthesis and mitosis in colon cancer cells (77). Correspondingly, overexpression of RGC-32 in several cancer cell lines has been shown to delay G2/M cell cycle progression (88).

On the other hand, others have reported that RGC-32 promotes malignant cell proliferation in the colon adenocarcinoma cell line SW480 (80) and in lung adenocarcinoma LTE cells (92). Overexpression of RGC-32 protein in Epstein Barr virus (EBV)-immortalized B cells has been found to disrupt the G2/M checkpoint via CDK1 activation, and RGC-32 has been shown to be indispensable for the growth and survival of lymphoblastoid B cells (86, 93) (**Figure 2**).

The cooperation of RGC-32 with SMAD3, as TGF-β downstream effectors, in the regulation of EMT seen in renal tubular cells (95) indicates a possible involvement of RGC-32

in invasion and metastasis. RGC-32 was shown to influence expression of vimentin, cadherin, and the transcription factors Snail and Slug in pancreatic and colon cancer lines (80, 91) (**Figure 2**). Also, excessive RGC-32 expression in a colon cancer cell line prompts cytoskeleton reorganization and cell migration (96). Similarly, RGC-32 has been demonstrated to induce EMT and to promote cancer cell migration and invasion in lung adenocarcinoma cells via decreases in the protein level and activity of the matrix metalloproteinases MMP-2 and MMP-9 (92, 97).

Research focusing on the effects of RGC-32 in animal cancer models has yielded contrasting data. Colon cancer tumors lacking RGC-32 that were implanted into nude mice were observed to have a lower growth rate and significantly smaller tumor volumes than did the tumors with intact RGC-32 expression (80). In striking contrast, inoculating RGC-32 into colon cancer tumors placed subcutaneously in mice resulted in a significant tumor growth suppression and decline in angiogenesis (94) (**Figure 2**).

The thesis of RGC-32 as a functional dyad (tumor suppressor/promoter) accounts for its contradictory behavior during cancerogenesis: the protein acts in a pleiotropic manner in distinct malignant settings, dependent on the cellular lineage and on the various ligands. For instance, RGC-32 exerts a tumor-suppressive effect in lung adenocarcinomas with wild-type *TP53*, but a tumor-promoting effect in the tumors carrying *TP53* mutations (98). Targeting RGC-32 should be done in conjunction with the role played in specific tumors as well as by using biomarkers that can predict the efficacy of RGC-32 inhibitors in cancer patients. Future studies are needed in order to find effective RGC-32-based drugs.

CONCLUSIONS

Considering all the available data, the role of C5b-9 in cancer is indisputably versatile: while it is lethal to tumor cells in a lytic context, when C5b-9 becomes activated to a sublytic level, it instead stimulates tumor growth through several mechanisms. Counteracting these tumor-promoting traits of C5b-9 by therapeutically surmounting CDC resistance in cancer cells and potentiating the antitumoral actions of C5b-9 (and therefore the efficacy of mAb-based immunotherapy) constitutes the next major direction in the field of immuno-oncology.

AUTHOR CONTRIBUTIONS

HR designed the study. SV, AT, VR, and HR wrote the manuscript. All authors approved the manuscript.

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REVIEW
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Targeting the Complement Pathway as a Therapeutic Strategy in Lung Cancer

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Lung cancer is the leading cause of cancer death in men and women. Lung adenocarcinoma (LUAD), represents approximately 40% of all lung cancer cases. Advances in recent years, such as the identification of oncogenes and the use of immunotherapies, have changed the treatment of LUAD. Yet survival rates still remain low. Additionally, there is still a gap in understanding the molecular and cellular interactions between cancer cells and the immune tumor microenvironment (TME). Defining how cancer cells with distinct oncogenic drivers interact with the TME and new strategies for enhancing anti-tumor immunity are greatly needed. The complement cascade, a central part of the innate immune system, plays an important role in regulation of adaptive immunity. Initially it was proposed that complement activation on the surface of cancer cells would inhibit cancer progression via membrane attack complex (MAC)-dependent killing. However, data from several groups have shown that complement activation promotes cancer progression, probably through the actions of anaphylatoxins (C3a and C5a) on the TME and engagement of immunoevasive pathways. While originally shown to be produced in the liver, recent studies show localized complement production in numerous cell types including immune cells and tumor cells. These results suggest that complement inhibitory drugs may represent a powerful new approach for treatment of NSCLC, and numerous new anti-complement drugs are in clinical development. However, the mechanisms by which complement is activated and affects tumor progression are not well understood. Furthermore, the role of local complement production vs. systemic activation has not been carefully examined. This review will focus on our current understanding of complement action in LUAD, and describe gaps in our knowledge critical for advancing complement therapy into the clinic.

Keywords: lung cancer, complement-immunological terms, oncogene, immunotherapy, microenvironment

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INTRODUCTION

Lung cancer is the leading cause of cancer death in both men and women (1). While there is clearly an established risk for lung cancer associated with cigarette smoking, recent data indicate an increased risk of lung cancer in never smokers, especially in women (1). Thus, while decreased rates of cigarette smoking should lower the incidence of lung cancer, lung cancer will remain a major cause of cancer death. In spite of active research identifying new therapeutic targets, the overall survival rate for lung cancer still remains discouragingly low, underscoring the need for both new preventive and therapeutic approaches. Historically, lung cancer has been subdivided based

on histology into two major subtypes: non-small cell lung cancer and small cell lung cancer (see **Figure 1**). About 85% of lung cancers are non-small cell lung cancer (NSCLC) with small cell lung cancer (SCLC) making up the majority of the remainder. There are a few other minor types of lung cancer such as large cell carcinoma, adenosquamous cell carcinoma, and sarcomatoid carcinoma, but these are rare. SCLC typically express a range of neuroendocrine markers and transcription factors that play crucial roles in their differentiation (2, 3).

NSCLC has further been subdivided into adenocarcinoma and squamous cell carcinoma (see **Figure 1**). These classifications are based on cells of origin as well as histology. Squamous cell lung cancer (SCC) generally arises from the proximal airway while adenocarcinomas develop from more distal locations (4). SCC begins in the squamous cells that make up the alveolar-capillary membrane, the only barrier between the air in the lungs and the capillary blood. Tracheal basal cell progenitors have been speculated to be the origin in mouse lung SCC due to the fact that the gene expression and histopathology patterns of SCC frequently resemble these cells (5, 6). About 30% of all lung cancers are classified as squamous cell lung cancer. It is more strongly associated with smoking than any other type of NSCLC. While numerous oncogenic drivers have been identified for lung adenocarcinoma, it has been more challenging to identify drivers for SCC (7, 8). Adenocarcinoma (Greek: adenos, gland plus karkinos, cancer) is a cancer that begins in cells in the glands. Using genetic models it has been demonstrated that lung adenocarcinomas (LUAD) originate from either type II pneumocytes or Clara cells (9). In addition, earlier studies have identified a bronchioalveolar stem cell population as being the potential cell of origin (10). Adenocarcinoma accounts for approximately 40% of all lung cancers.

Two major advances have occurred during the past decade which hold promise for the treatment of lung cancer, particularly LUAD. The first of these is the identification of multiple oncogenic drivers and the recognition that subdividing LUAD based on these drivers will dictate therapy. This has resulted in the development of multiple targeted therapies which have been approved for treatment of subsets of LUAD. The second breakthrough is the advent of novel immunotherapy approaches, specifically the use of antibodies targeting immune checkpoint inhibitors. These have been shown to be effective in NSCLC and are approved for subsets of LUAD as well as for SCC (11–14). Nevertheless, in spite of these novel approaches, the overall survival rate for NSCLC has not significantly improved, underscoring the need for new therapeutic approaches. As discussed below, therapeutic approaches are particularly constrained by the oncogenic drivers. There are currently no approved agents targeting K-Ras dependent lung cancer; however, this subset of patients show a response to immune checkpoint inhibitors (15). In contrast, while numerous targeted therapies are approved for LUAD driven by mutations in tyrosine kinase receptors, these patients show a very poor response rate to immune checkpoint inhibitors (16). In going forward, it is therefore critical to integrate our preclinical knowledge to define how specific oncogenes engage the immune tumor microenvironment. This review will focus on the complement pathway, largely in LUAD. Once considered a pathway associated with inhibition of tumor initiation and progression, it has become clear from work of multiple groups that complement is in fact complex and can actually promote progression of multiple cancers, including LUAD through promoting inflammation and regulating immunosuppressive pathways. These studies suggest that targeting complement either as monotherapy, or in combination with other immunotherapies represents a novel strategy for treatment, and possibly prevention of lung cancer.

ONCOGENIC DRIVERS AND TARGETED THERAPIES

Studies performed during the past 15 years have subdivided lung adenocarcinoma according to the dominant oncogenic driver (17, 18). This has resulted in a paradigm shift in the treatment of this disease. Whereas, earlier clinical studies had tested potential therapeutic agents in an unselected group of patients, discovery of distinct oncogenic drivers has resulted in targeted therapies against that dominant oncogene, with the concept of patient selection becoming standard of care. This has led to a focus on criteria for patient selection, with the model being to seek strong responses in a subset of patients rather than a more modest response across all patients. LUAD can be defined at the molecular level by recurrent "driver" mutations or amplifications, including, but not limited to: *ALK*, *BRAF*, *EGFR*, *FGFR1*, *KRAS*, *MET*, *RET*, *NTRK1,* and *ROS1*. These have been extensively reviewed elsewhere (18). The National Comprehensive Cancer Network (NCCN) guidelines now recommend routine testing for *NTRK, ALK*, *ROS1*, *BRAF*, and *EGFR* for all new cases of advanced lung adenocarcinoma for which we have therapies. Currently, personalized therapies that identify and target specific biomarkers have resulted in substantial benefits for NSCLC patients with *EGFR* mutations, gene alterations involving the anaplastic lymphoma kinase (*ALK*) gene, *BRAF* V600E mutation, or the *ROS1* gene. The common genomic alterations, frequencies, and current FDA-approved therapies to target the known mutations in NSCLC are summarized in **Table 1** (19). In this review we will briefly discuss EGFR, ALK, and K-Ras gene alterations in lung adenocarcinoma.

The epidermal growth factor receptor (*EGFR*) belongs to the avian erythroblastic leukemia viral oncogene homolog (*ERBB*) family, or also known as the *Her* family, that includes 4 different receptors: EGFR, ErbB2, ErbB3, and ErbB4 (20). EGFR is overexpressed in many cancers, including NSCLC, and several somatic mutations have been detected in NSCLC. The most prevalent mutation in the EGFR kinase domain—accounting for approximately 45%—is the inframe deletion of exon 19 between residues 747–750 (21). Another recurrent mutation that compromises another 45% of EGFR mutations is the mutation in exon 21 at the position 858 of kinase domain from a leucine (L) to an arginine (R). Exon 18 substitution and exon 20 in-frame insertions account for the rest. These gain-of-function EGFR mutations lead to constitutive phosphorylation and activation of cell survival and proliferation pathways (22). Targeting the EGFR with "first-generation" tyrosine kinase inhibitors (TKIs)

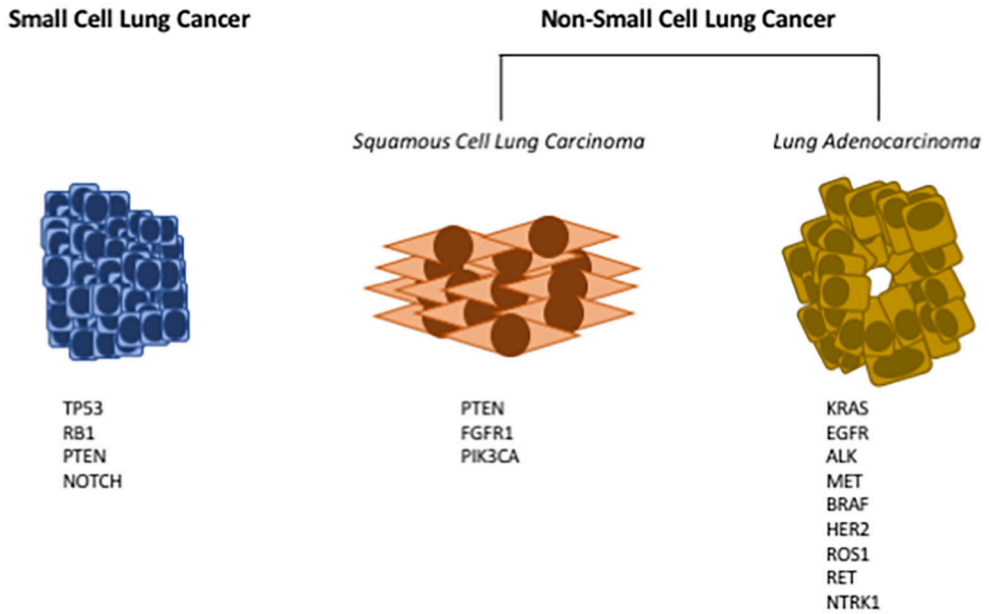

FIGURE 1 | Common driver mutations in lung cancer. Lung cancers have historically been subdivided into either small cell lung cancer (SCLC), or non-small cell lung cancer (NSCLC). Small cell lung cancer has few oncogenic driver mutations; here are listed the most frequently identified genetic mutations in SCLC **(Left)**. NSCLC can further be subdivided into squamous cell lung carcinoma (SCC), or lung adenocarcinoma (LUAD). Multiple oncogenic drivers have been identified in LUAD **(Right)**; for many of these targeted therapies have been developed. For SCC **(Middle)**, there are fewer identified oncogenic drivers, and no targeted therapies have been approved.

TABLE 1 | Genomic alterations of lung cancer.

	Type of alteration	Frequency (%)	FDA approved therapy
EGFR	Mutation	10–35	Yes
KRAS	Mutation	25–30	No
FGFR-1	Amplification	20	No
ALK	Rearrangement	5–7	Yes
MET	Amplification	2–4	Yes, but for a different mutation
ROS1	Rearrangement	1	Yes, but for a different mutation
RET	Rearrangement	1	Yes, but only for other cancers
BRAF	Mutation	1–3	Yes

EGFR, epidermal growth factor receptor; KRAS, Kristen RAt Sarcoma; FGFR-1, fibroblast growth factor receptor-1; ALK, anaplastic lymphoma kinase; MET, hepatocyte growth factor receptor. ALK, MET, ROS1, and RET are proto-oncogenes that arise from chromosomal rearrangements that generate a fusion gene, resulting in the constitutive activation of kinase domain.

such as gefitinib and erlotinib has been approved since 2003 for NSCLC. These TKIs compete with ATP in a reversible manner to bind the kinase domain of the receptor. Although initial responses in patients to these TKI agents can be dramatic, most patients will eventually relapse due to the acquisition of drug resistance, a common observation among many targeted therapies. Multiple mechanisms of acquired resistance to targeted EGFR therapy have been discovered in patients. Patients who became resistant to first generation EGFR TKIs often acquire a T790M somatic mutation, which has been designated a "gatekeeper" mutation (23) that increases affinity for ATP (24). Additional resistance mechanisms include amplification of hepatocyte growth factor receptor (*MET*), observed in 5–15% of patients who received first generation EGFR TKIs (25, 26). MET signaling activates MAPK and PI3K/AKT pathways, bypassing the requirement for EGFR signaling. To address the problem of the multiple mechanisms of resistance, second and third generation EGFR inhibitors have been developed. The defining characteristic of the third-generation EGFR TKIs is that they have significantly greater activity against EGFR mutant receptors than EGFR wildtype (WT), making them more sensitive for tumor cells (27). Osimertinib, a third-generation EGFR TKI, has shown objective response rates and progression-free survival compared with chemotherapy in the first-line setting and was recently approved as the first-line treatment for *EGFR*-mutated NSCLC (28). Despite its success, there are reports of an acquired mutation at C797S in exon 20 among the patients who received osimertinib which affects drug binding, rendering the TKI ineffective (29). Acquired resistance through activation of Aurora A kinase has also been reported (30).

Anaplastic Lymphoma Kinase (ALK) is a receptor tyrosine kinase that in normal settings signals to promote cell growth and inhibit apoptosis in a regulated manner. Rearrangement of the *ALK* gene results in the N-terminal fusion of the ALK tyrosine kinase domain with different fusion partners, mainly echinoderm microtubule-associated protein like 4 (*EML4*), producing EML4-ALK fusion proteins. In other cases, ALK is shown to also partner with kinesin family member 5B (*KIF5B*) (31) or TRK-fused gene (*TFG*) (32). The dimerization of ALK mediated by its fusion partner results in a constitutive activation of the

ALK tyrosine kinase activity and subsequently mediates an increase in pro-growth and anti-apoptotic signaling in NSCLC (33, 34). Similarly, there are described other fusion proteins resulting from the chromosomal rearrangement such as *RET* fusion with *KIF5B* (35), *ROS1* fusion with *CD74* (36), *NTRK1* fusion with myosin phosphatase Rho-interacting protein gene (*MPRIP*) or *CD74* (37). Approximately 5–7% of NSCLC patients harbor ALK fusions (38). In an initial Phase I trial, the patients with *ALK* rearrangements displayed a 60.8% objective response rate to the ALK/ROS1/MET TKI, crizotinib (39). The median progression-free survival (PFS) was 9.7 months with the probability of PFS at 6 months to be 87.9%. The second-generation ALK-inhibitor ceritinib also showed a 60% response rate among the 180 ALK-fusion positive NSCLC patients in a phase I trial (40). An *EGFR* L858R mutation, *ALK* gene amplification, *KRAS* mutation, and *KIT* gene amplification have been reported in ALK fusion positive patients with acquired resistance to crizotinib, suggesting that other genetic changes may confer crizotinib resistance. Novel therapeutic strategies to overcome the development of acquired resistance to ALK TKIs are currently being studied (18).

Kristen Rat Sarcoma (KRAS) is a small GTPase that is activated when a GTP is bound and deactivated when KRAS hydrolyzes GTP into GDP. Activation is mediated by the exchange of GDP to GTP and is facilitated by Guanine Nucleotide Exchange Factors (GEFs), whereas the deactivation mechanism of promoting GTP-GDP hydrolysis is mediated by GTPase Activating Protein (GAP). KRAS is a central protein that couples growth factor receptor signaling to downstream pathways including RAF-MEK-ERK and PI3K-AKT and is critical for cell proliferation and survival (41). Somatic mutations in KRAS are common in NSCLC occurring in ∼15–25% of NSCLC patients. Common mutations are in amino acid residues 12, 61, and rarely on 13. These mutations will block GAP leading to constitutive activation of RAS. In lung adenocarcinoma, the common G12C mutation is a distinct feature of exposure to tobacco smoke. In spite of intense research, there are currently no agents to directly target KRAS (42). Therefore, many have elected to target pathways downstream of RAS, especially the RAF-MEK-ERK and PI3K-AKT pathways (43).

The findings summarized above have changed the way that lung cancer patients are treated. The standard of care upon diagnosis today is to perform genetic analysis to identify driver mutations. Patients with targetable drivers are placed on specific agents, and in general show an initial response characterized by tumor shrinkage. However, since resistance eventually develops in these patients, and there is a large fraction of LUAD patients where the oncogenic driver cannot be targeted (e.g., K-Ras) or in which there is no identifiable driver, additional therapeutic approaches are required. The relationship between specific oncogenic drivers and engagement of the complement pathway has not been established. However, as discussed below, specific oncogenic drivers in LUAD are associated with different sensitivity to immunotherapy, and thus complement activation needs to be studied in the context of specific oncogenes.

RESPONSES TO IMMUNOTHERAPY

A second major advance in the treatment of lung cancer has been the advent of immunotherapy. While lung cancer was thought for many years to not be an immunological cancer, recent studies have clearly demonstrated the contrary. In fact, lung adenocarcinoma as a subtype is one of the most immunological tumor types, and immunotherapy has been actively investigated in both NSCLC and SCC (13, 44, 45). Cancer cells can be recognized by the immune system due to their ability to express altered levels of cellular proteins or the expression of mutated proteins. However, tumors are rarely eliminated by activated T cells. A model to account for this has been proposed and designated "immunoediting" (46). In this model there is initial recognition of cancer cells by the adaptive immune system; however, eventually cancer cells adapt by engaging immunosuppressive pathways to counter T cell-mediated tumor killing. In fact, immunoevasion has been designated as one of the "Hallmarks of Cancer" (47). Targeting immunosuppressive pathways will presumably lead to reactivation of cytotoxic T cells and tumor elimination (13, 48, 49).

A great deal of research has focused on pathways that regulate the function of T cells under non-cancerous conditions, designated immune checkpoints (50, 51). These pathways function through specific ligand-receptor interactions to inhibit T cell function (52). The PD-L1 pathway involves expression of PD-1 on activated T cells (both $CD8^+$ and $CD4^+$), and PD-L1 which is expressed on cancer cells as well as inflammatory cells of the tumor microenvironment including macrophages. Binding of PD-L1 to PD-1 results in inhibition of T cell receptor signaling and generates an "exhausted" phenotype, thereby allowing tumor progression. Monoclonal antibodies that block these interactions result in reactivation of T cells, and potentially tumor elimination. For lung cancer, monoclonal antibodies against both PD-1 and PD-L1 have shown clinical efficacy, leading to their approval by the FDA (53). To date immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway have been approved for lung adenocarcinoma. However, the overall response rate in unselected patients is approximately 20% (45), underscoring the need for additional therapeutic approaches. Even more discouraging are the data examining LUAD with driver mutations in tyrosine kinases (e.g., EGFR and ALK fusions). For this subgroup of patients the response rates are even worse, and for ALK fusions there are very few reports of a positive response to anti-PD-1/PD-L1 therapy (16). Current clinical trials are examining combinations of these agents, such as EGFR inhibitors and anti-PD-1 (54). There are a large number of factors that have been shown to correlate with responsiveness to checkpoint inhibitors. While correlations of mutational burden, the presence of neoantigens (55), cigarette smoking, and expression of PD-L1 have been associated with clinical response (45, 56), the cellular and molecular mechanisms mediating response to immune checkpoint inhibitors are not well understood. There is a concerted effort to combine checkpoint inhibitors with other agents, resulting in a large number of clinical trials, many with limited scientific rationale (57). To develop a more rational approach, a better understanding of the

immune response in lung cancer is required. This will entail a more comprehensive examination of the changes in the tumor microenvironment, focusing on both the innate and the adaptive immune response and the cross talk between these pathways. In particular, for LUAD, it will be critical to integrate how specific oncogenic drivers regulate this interaction.

COMPLEMENT PATHWAY

The complement pathway is part of the innate immune system that complements the ability of immunoglobulins and phagocytic cells to clear microbes and damaged cells, promotes inflammation through recruiting both the innate and adaptive immune cells, and attacks the pathogen's cell membrane itself. The complement pathway has been extensively reviewed (58–61), and therefore we will briefly discuss aspects of this pathway relevant to cancer progression. Many of the proteins that are involved in the complement pathway are synthesized by the liver and circulate as inactive precursors, or pro-proteins (see **Figure 2**). When stimulated, proteases in the system cleave the complement proteins in an amplifying cascade to release cytokines while complement fixation tags the triggering cells for opsonization. On the surface, the complement pathway may appear to be merely an antimicrobial mediator, but in the past few decades it has become apparent that such an intricate system has the potential to recognize surface antigens and may have much broader functions in immune surveillance and homeostasis (61). Furthermore, more recent studies have broadened the reach of complement activation from just the confines of intravascular systems, to local secretion of complement components by tissue and infiltrating cells, and potentially even intracellular activation of complement (62). Due to such broad targets and even greater functional versatility, the complement system is under tight regulation through multiple mechanisms (63, 64).

The activation of complement component 3 (C3) may occur through three distinct pathways: (i) classical pathway, (ii) alternative pathway, and (iii) lectin pathway (65) (see **Figure 2**). Although different proteins are recognized and involved, these different pathways of activation converge upon a single event, the conversion of C3 into C3a and C3b. The classical pathway begins when circulating immunoglobulins such as IgM or certain subclasses of IgG first bind to the antigen on the surface of the pathogen or target cells. This mediates the recruitment and activation of C1 complex comprising C1q, C1r, and C1s which is a serine protease that will subsequently cleave C2 and C4. The activated products of C2 and C4 form C4b2b, an assembly of multiprotein complexes with enzymatic activity termed, C3 "convertases." The alternative pathway involves two distinct and separate initiation steps: (ii-a) properidin-mediated or (ii-b) $C3(H_2O)$-mediated activation of C3. In properidin-mediated alternative pathway, properidin binds to C3b and activates Factor B and Factor D to form C3bBb, another C3-convertase. On the other hand, $C3(H_2O)$-mediated activation involves the direct activation of C3 by Factor B and $C3(H_2O)$. $C3(H_2O)$ is the hydrolytic and conformationally rearranged product of C3 that functionally mimics C3b. This pathway results in $C3(H_2O)Bb$, another C3-convertase. Lastly, the lectin pathway is triggered by carbohydrates recognized by mannose-binding lectin (MBL), ficolins, or collectin-11 which activates serine proteases such as MASP-1 and MASP-2. MASP-1 and MASP-2 are responsible for cleaving C2 and C4 to form a C3-convertase analogous to the C3-convertase made by the classical pathway. None of these pathways are exclusive in any disease and may occur simultaneously.

The activation of C3 produces C3a and C3b. C3a is a potent anaphylatoxin that promotes inflammation, cell migration, and activation. C3b, on the other hand, binds covalently to the surface of target cells through a newly exposed thioester bond and aids in opsonization (a process that increases the efficacy of the phagocytic process) or recruits other proteins with proteolytic properties to continue the complement activation cascade. C3b can bind to an existing C3 convertase such as C4b2b or C3bBb to form a C5 convertase, C4b2b*C3b or C3bBb*C3b, respectively. Similar to C3, cleaving C5 leads to production of the anaphylatoxin, C5a, and C5b. C5b recruits C6-9 to form the membrane attack complex (MAC) that causes pore formation and eventually cell lysis. The C3b-mediated opsonization or formation of MAC are thought to be the two main direct mechanisms of complement-mediated innate immune response (60, 61, 66).

It was originally thought that activation of complement would represent a strategy to inhibit tumor formation and progression, specifically through antibody mediated killing of tumor cells. Consistent with this model, in a genetic mouse model of breast cancer, autochthonous mammary carcinoma formation is accelerated in mice with global deletion of complement C3 (67). This is associated with alterations in the TME, promoting a more immunosuppressive environment. In particular, increases in regulatory T cells (Tregs) are observed in the setting of C3 loss. This is consistent with other studies demonstrating that anaphylatoxins regulate the development and recruitment of Tregs (68, 69).

However, several research groups have shown that complement deficiency or therapeutic complement inhibitors slow tumor growth in animal models (70–75). Published data also shows that the complement system is activated in many human patients with lung cancer (72, 76). Furthermore, examination of data in the Cancer Genome Atlas (TCGA) reveals gene amplification or increased expression of the complement regulatory proteins, CD46 and CD55, in approximately 25% of human lung adenocarcinomas. This suggests that tumors evolve the ability to block complement activation. These findings present a paradox: complement activation can promote tumor growth, yet cancer cells overexpress proteins that limit complement activation.

ROLE OF ANAPHYLATOXINS

Activation of C3 and C5 generates C3a and C5a, respectively, and these anaphylatoxins are potent pro-inflammatory molecules that induce a multitude of effects on cells such as attracting

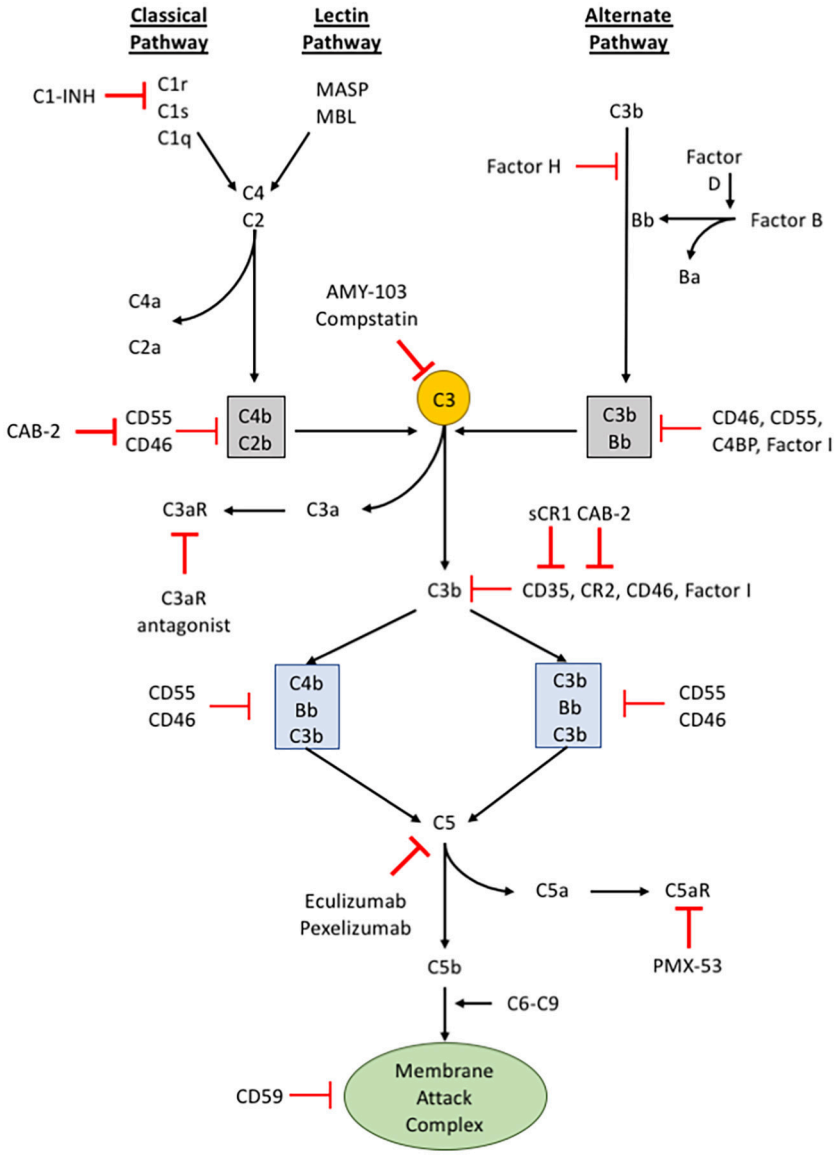

FIGURE 2 | The complement pathway. A schematic of the complement signaling pathway where all 3 pathways (classical, lectin, and alternative) converge on C3. The red, bolded inhibitory signs indicate points in the complement signaling cascade where pharmacologic inhibitors can be used to alter signaling within cells, while the red, unbolded inhibitory sign indicates regulatory proteins in the complement cascade.

neutrophils and monocytes to the site of complement activation. C3a and C5a sustains the inflammatory responses by activating granulocytes and macrophages, increasing vasodilation and vascular permeability and releasing pro-inflammatory mediators (61, 77). C3a and C5a exert their effects by signaling to their respective receptors, namely C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). Both C3aR and C5aR belong to a family of transmembrane G protein coupled receptors, but C5L2 is not coupled to G proteins (78, 79). C5L2 was first described by Ohno et al., though its exact biological effects of signaling remain unclear (80). Many different cells express receptors for C3a and C5a. These include cells of myeloid origin (81), non-myeloid origin (82), dendritic cells (83), monocyte/macrophages (84), and neutrophils (85).

The host immune response has major effects on cancer initiation, progression, and metastasis (86). Since C3aR and C5aR are expressed on multiple immune cells, it has been postulated that an important function of complement is to regulate immunomodulatory functions of the tumor microenvironment. However, the role of anaphylatoxins in cancer progression is likely to vary between types of cancer and be context dependent. C5a stimulation has been shown to increase the release of matrix metalloproteinases (MMP) *in vitro*, while C5a-C5aR signaling enhances invasion of a human

cholagiocellular carcinoma cell line (HuCCT1) *in vivo* (87). On a similar note, C5a-overexpressing lymphoma cells significantly accelerated tumor progression. The authors attributed this to increased recruitment of Gr-1$^+$CD11b$^+$ myeloid cells in the spleen and overall decreased CD4$^+$/CD8$^+$ T cells in the tumors overexpressing C5a (88). However, more recently, anaphylatoxins generated by complement activation in the tumor microenvironment frequently associated with inhibition of anti-tumor immune responses important in metastatic spread. Pentraxin-related protein 3 (PTX3) has been identified as a tumor suppressor negatively regulating complement-mediated inflammation. PTX3 is shown to interact with C1q and Factor H to impede complement activation, resulting in lower C5a production, macrophage infiltration, and angiogenesis (89) (see **Figure 2**). In addition, Markiewski et al. showed that C5a aided in the recruitment of myeloid-derived suppressor cells (MDSCs) into tumors and inhibited anti-tumor T cell responses through the generation of reactive nitrogen and oxygen species (73). Consequently, blockage of C5a to C5aR signaling impaired tumor growth and lowered the percentage of MDSCs in spleens of lung cancer-bearing mice (90). C3a was also shown to be implicated with tumor progression. Namely, in a syngeneic primary murine B16-F0 melanoma model, the absence of C3aR signaling slowed tumor progression. In the same study, the authors showed that the antitumor effects of C3aR inhibition are linked with a decrease in tumor-associated macrophages and an increase in tumor-infiltrating neutrophils and CD4$^+$ T lymphocytes (74). Moreover, anaphylatoxins are also shown to induce inflammation through the induction of bioactive molecules within the tumor microenvironment. For instance, C3a and C5a signaling enhances IL-6 production in astrocytoma cells (91), and blocking C5aR signaling down regulated the expression of IL-6 in a mouse model of lung carcinogenesis (90). Clinical studies have revealed that increased serum IL-6 in patients are associated with advanced tumor stages of various cancers, including multiple myeloma (92), non-small cell lung carcinoma (93), colorectal cancer (94), renal cell carcinoma (95, 96), breast cancer (97), and ovarian cancer (98). Taken all together, anaphylatoxins frequently hinder anti-tumorigenic immune responses and may be a potential target for therapeutics.

COMPLEMENT INHIBITORS AND REGULATORS

Complement activation is important for clearance of foreign agents, but pathogens have developed a number of strategies to evade the complement-mediated immune response. Most pathogens express soluble and surface-bound complement regulators that delay or even block complement effector functions in order to protect themselves from elimination. However, during a persistent infection, complement activation must be tightly regulated in order to protect host bystander cells. Therefore, it is not surprising that dysregulation of the complement cascade can result in autoimmune disease. Although the activation and deposition of complement products in tumor tissue has been demonstrated, the functional implication remains unclear.

Complement pathway participates in all facets of immune surveillance by collaborating with both the innate and adaptive immune systems. This "bridging" ability seems to continue as the activation products of C3 degrades into iC3b and C3dg. iC3b and C3dg are shown to bind to CR2 (CD21) on B cells (see **Figure 2**) to augment the immune response when limited amounts of antigen are available (99, 100). Furthermore, iC3b and C3dg aid in memory B cell induction and maintenance in the germinal centers and facilitates the shuttling of antigens between B cells and follicular dendritic cells by opsonizing cellular particles (66, 99, 101). In turn, the robust production of antibody against specific antigens improves the innate immune response by facilitating C1q-mediated activation of complement mentioned above.

Given the regulatory role of complement inhibitors on complement activation, it is tempting to hypothesize that cancer cells actively escape complement and immune surveillance by expressing complement inhibitors. The expression of membrane-bound inhibitors (CD46, CD55, and CD59) are upregulated among bladder cancer patients (102). It appears that different cancer types utilize different complement regulators to evade the complement mediated immune surveillance.

PATHWAYS OF COMPLEMENT ACTIVATION IN CANCER

Although the mechanisms of complement activation in NSCLC are incompletely understood, pre-clinical and clinical data suggests that activation occurs at least in part through the classical pathway (72). IgM is a potent activator of the classical pathway, and we have observed deposits of IgM in experimental and human NSCLC (72). "Natural" IgM refers to germline encoded IgM that is produced even without exposure to specific antigen (103, 104). It is frequently poly-reactive, and there is evidence that natural antibodies bind to epitopes expressed on cancer cells (105). Although anaphylatoxins can suppress anti-tumor immunity, the MAC is directly cytotoxic. To protect themselves from MAC-mediated lysis, cancer cells express high levels of complement regulatory proteins, including CD46, CD55, and CD59 (64) (see **Figure 2**). Regulatory proteins CD46 and CD55 inhibit complement activation by binding with either C3b or C4b and preventing the formation of C3 and C5 convertases, while CD59 inhibits the MAC complex (82). We propose that complement is activated in the setting of cancer or precancer due to binding of natural IgM to neoantigens on the cell surface. This may lead to lysis of some target cells, but the tumor cells evolve mechanisms to evade complement-mediated elimination (such as overexpression of the regulatory proteins), and in fact employ byproducts of complement activation (anaphylatoxins) to suppress anti-tumor immunity in the TME. Thus, tumors can co-opt the immunosuppressive effects of complement activation while escaping its cytotoxic effects. Although IgM is primarily a classical pathway activator, in some instances mannose binding lectins bind to glycosylated IgM and activate the lectin pathway (106). Furthermore, even when complement is activated through the classical or

lectin pathway, the alternative pathway amplifies the process and can account for the majority of overall activation (107). Thus, the complement system can be activated by many different protein-protein interactions, and activation generates multiple biologically active fragments. Drugs that selectively block activation through specific pathways are being developed and may be more effective and safer than the currently available drugs (108). Therefore, identification of the specific mechanisms of complement activation in NSCLC may lead to new treatment strategies for this disease. In addition, a more detailed examination of the role of individual regulatory proteins needs to be undertaken in preclinical models.

ROLE OF CANCER CELL COMPLEMENT

In addition to systemic complement activation, recent studies have demonstrated that cancer cells can also produce complement (see **Figure 3**). In ovarian cancer cells an autocrine loop in which expression of C3 by the cancer cells results in production of C3a which signals through the C3aR to promote growth (109). In this setting the role of cancer cell expression appears to be more critical for tumor progression than production by the TME, since these tumors grow equally well in $C3^{-/-}$ mice as in WT. Overexpression of C5aR has been detected in both human lung cancer cell lines and in samples of human tumors (110). Elevated levels of expression have been associated with increased metastasis, and negatively with levels of E-cadherin, suggesting a role for C5a/C5aR signaling in regulating the epithelial-mesenchymal transition (EMT) of cancer cells. Consistent with these findings, in ovarian cancer expression of C3 is regulated by TWIST, which controls EMT (111). Data from our laboratory has demonstrated endogenous expression of C3 and production of C3a by Lewis Lung Carcinoma cells, which represent a mesenchymal phenotype (72). Recently, studies have demonstrated that intracellular activation of complement in cancer cells can act as an immunosuppressive pathway to regulate expression of PD-L1 (112). The pathways whereby cancer cell-intrinsic complement acts, as distinct to activation in the TME are likely to be different. In particular, cancer-cell intrinsic complement may signal in an autocrine fashion to promote cancer cell growth. However, this will be dependent not only on the complement activation, but also the expression of receptors for C3a and C5a on the cancer cells themselves.

ROLE OF COMPLEMENT PRODUCTION BY CELLS OF THE TME

Recent studies have demonstrated that T cells produce complement proteins which can act in an autocrine fashion to promote T cell function (see **Figure 3**). Studies have demonstrated that the promotion of a Th1 phenotype is promoted through translocation of C3a to the surface of $CD4^{+}$ T cells, resulting in production of Th1 cytokines (113–115). Intracellular expression and function of C5 has also been shown (116). For both of these systems it appears that intracellular complement is critical for both the initiation and the contraction of T cell activation and IFNγ production. Thus, we anticipate that activation of intracellular complement would result in a greater proportion of anti-tumorigenic T cells (Th1), and thus blocking this pathway would be expected to promote rather than inhibit tumor progression. In other models, complement signaling has been shown to inhibit the function of Tregs, through pathways that involve both C3a and C5a (68). Since increased Treg infiltration of tumors is associated with immunosuppression, complement activation in this context would be predicted to inhibit tumor progression.

Complement activation has also been shown to occur in tumor endothelial cells (117). While the role of this pathway has not been extensively studied, data suggest that complement activation on endothelial cells allow for increased T cell homing and tumor infiltration. In this model activation of complement would appear to be critical for T cell infiltration associated with inhibition of tumor growth and increased sensitivity to immunotherapy, whereas complement inhibition would result in tumors with fewer T cells. Complement proteins are also expressed in other cells including macrophages and B cells (113, 118). Studies have shown production and activation in the setting of antigen presenting cells (APC) interacting with T cells, resulting in T cell activation. It would be expected that this activation would be associated with inhibition of tumor progression.

PRECLINICAL MODELS FOR LUNG CANCER

To develop a better understanding of the role of complement in LUAD preclinical models that reproduce the human disease are required. Murine models for the study of lung cancer have been the backbone of preclinical data to support human clinical trials. Before focusing on the complement pathway, we will briefly discuss these models, and describe their strengths and limitations in defining immunoregulatory pathways. For more detailed information there are a number of excellent reviews on this topic (119, 120).

Early models examined tumor initiation in mice using carcinogens, including compounds present in cigarette smoke (119, 121). These mice develop lung adenocarcinomas with molecular, morphologic, and histologic similarities to that of human lung tumors (122). Both human and mouse adenocarcinomas arise from the type II epithelial cells or Clara cells of the peripheral lung and follow the same stages of development beginning with an initiated cell with a genetic mutation that proliferates to become a hyperplasia to a carcinoma *in situ*. Studying molecular and cellular mechanisms of murine lung tumor progression throughout the multi-stage carcinogenesis offers a better understanding of the pathogenesis. However, carcinogen-induced lung tumors are largely characterized by KRAS mutations, and there are currently no chemical models which result in tumors with other oncogenic drivers (123). Furthermore, these tumors are generally benign adenomas, which may eventually become invasive but do

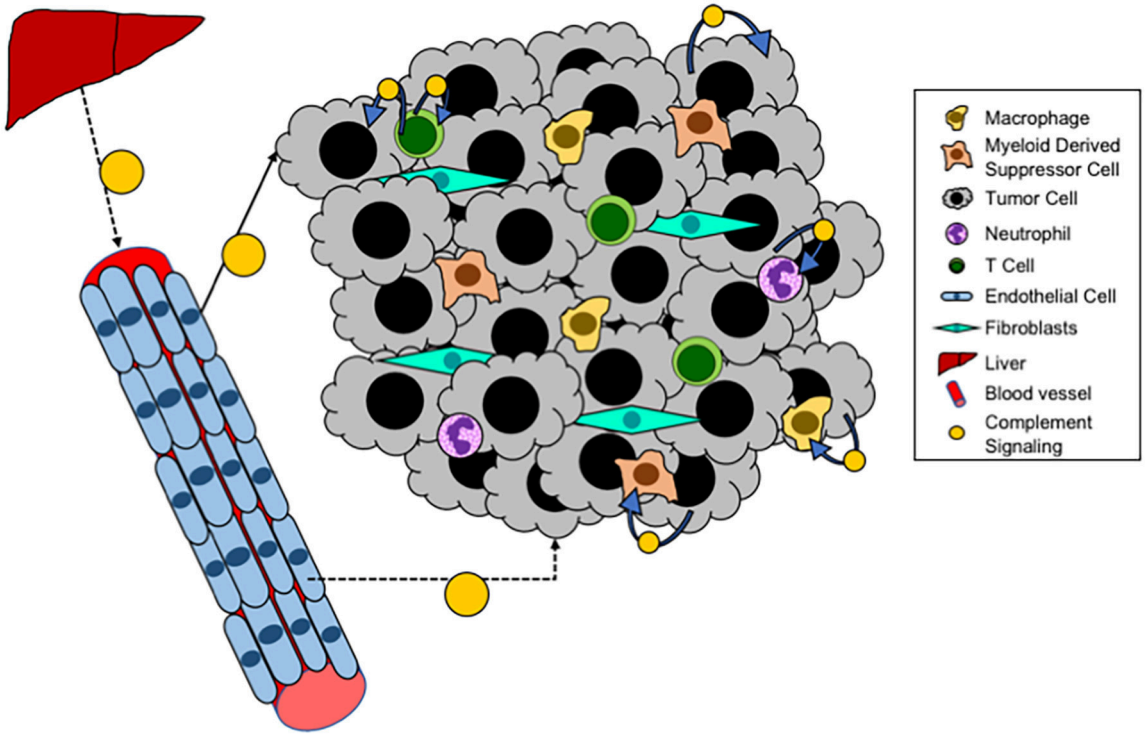

FIGURE 3 | Local complement signaling in tumors. A schematic of complement signaling that occurs within tumors. Systemic complement is produced by the liver and travels via the blood to distant sites. While locally, within a tumor, tumor cells, T cells, and endothelial cells can all produce complement that acts either in an autocrine or paracrine fashion.

not metastasize. There are many carcinogenic agents available. Namely, most potent of all are the cigarette smoke carcinogens, such as polycyclic aromatic hydrocarbons (PAH), tobacco-specific nitrosamine, and benzo[a]pyrene (121). However, a 5-month exposure period with cigarette smoke carcinogens must be followed by a 4-month recovery period (124).

Genetic mouse models (GEMMs) with specific drivers have been developed. Using Cre-Lox technology lung tumors have been generated with Kras mutations (125, 126), and mutations in Egfr (24, 127). Using CRISPR technology, lung tumors driven by the fusion kinase Eml4-Alk have also been generated (128). By selectively deleting tumor suppressor genes such as p53, these tumors can be made to be more aggressive (129). A strength of this model is that, like the earlier chemical models, the various stages of tumor development can be recapitulated, and changes in the microenvironment can be assessed in a dynamic fashion. However, one significant limitation in this model is the low degree on non-synonymous mutations in the tumors (130). The mutational burden in these tumors is at least an order of magnitude less than seen in human LUAD. A consequence of this is the poor response to immunotherapy, such as checkpoint inhibitors. This is likely due to lack of neoantigens and recognition by adaptive immune cells ($CD8^+$ and $CD4^+$). In examining how pathways such as complement interact with the adaptive immune system, this may be a problem with many of these GEMMs.

Patient-derived xenograft (PDX) models require the injection of patient-derived cancer tissue into immunodeficient mice, such as $Prkdc^{scid}$, Nude, or $Rag1^{null}$ mice. The detailed differences among these strains will not be discussed here, but these immune-deficient mouse models all lack functional T cells and B cells. PDX offers a powerful tool to assess human tumor biology, namely the identification of therapeutic targets for the donating patients (131). In addition, many immunodeficient models accept allogeneic and xenogeneic grafts making them ideal models for cell transfer experiments or to examine tumor response to therapy *in vivo* prior to translation into clinical trials (131). One of the obvious advantages of PDX over implantation of human cell lines is that PDX represents more accurate tumor heterogeneity compared to the established human cell lines. Despite the advantages, xenograft models do not give insights to the role of the immune system in controlling tumor progression. Recent advances in immunotherapies stressed the importance of the immune system in tumor biology, and many strides have been made to create the next-generation PDX models with humanized mice. To establish PDX models conditioned with human immune system, $CD34^+$ human hematopoietic stem cells (HSCs) are engrafted into host immunodeficient mice (132). HSCs give rise to various lineages of human blood cells in mice and to further improve the integrity of transplanted HSCs, immunocompromised mouse strains such as NOG-GM3, NSG-SGM3, and MISTRG are generated (133).

Implantable models involve the injection of cancer cells into mice. Earlier studies employed xenograft approaches where human NSCLC were injected subcutaneously into immunodeficient mice (134, 135). These studies allow a dynamic measurement of tumor growth, but suffer from the same limitations discussed above for PDX models. More recently, syngeneic models have been studied, in which immunologically compatible murine cancer cells are implanted into immunocompetent mice. The major disadvantage of this model is its limited number of cell lines in different mouse strains. For example, Lewis lung carcinoma (LLC) and CMT167 are the only Kras driven LUAD cell lines derived from the lung tumors of C57/BL6 mouse (136). Cell lines have been generated with Eml/Alk fusions derived from the CRISPR engineered mice (128), but there are currently to our knowledge no murine cell lines harboring Egfr mutations, and the current mouse models in which mutated Egfr is driven off a lung-specific promoter are unlikely to generate cell lines *ex vivo*, due to turning off of the promoter. Implantable models have the advantage of monitoring the progression of a full-fledged tumor, and in most cases these tumors will metastasize. Most murine cancer cells have high levels of non-synonymous mutations, and express neoantigens which are recognized by the adaptive immune system of the host. This system is also amenable to genetic manipulation of either the cancer cells, through silencing or overexpressing specific genes, as well as the host through the use of genetic knockout and targeted knockout mice. In using syngeneic implantable models, we would argue that it is critical to implant the tumors into the lung, rather than subcutaneously. This allows tumor development in the correct microenvironment. For example, tumors implanted subcutaneously will not be exposed to alveolar macrophages and other lung-specific cells. Despite its disadvantages, a syngeneic model—especially when combined with orthotopic injections—is the only currently available approach in which the tumor microenvironment is accurately depicted in the animal (72, 136–140).

Studies of the complement pathway in orthotopic immunocompetent models of LUAD have compared the effects of this pathway using a panel of murine lung cancer cell lines encompassing different oncogenic drivers (72). Both genetic and pharmacologic inhibition of complement blocked tumor progression, similar to what has previously been reported looking at metastasis to the lung (73, 75, 141).

CLINICAL TARGETING OF COMPLEMENT

Several studies have examined complement activation in human cancers, including lung cancer. Using a specific antibody against complement C4d, it has been shown that levels of this protein in plasma from lung cancer patients assessed by ELISA were able to discriminate between benign and malignant nodules (142). Published data also shows that the complement system is activated in many human patients with lung cancer (72, 76). Furthermore, examination of data in the Cancer Genome Atlas (TCGA) reveals gene amplification or increased expression of complement regulatory proteins, CD46 and CD55 in ∼25% of human lung adenocarcinomas.

While preclinical data indicate that complement inhibitors may represent a novel therapeutic strategy for treating cancer in general and lung cancer in particular, there are currently no open clinical trials in any malignancy according to Clinicaltrials.gov. However, there is at least one FDA-approved complement inhibitor, ecoluzimab, which is a monoclonal antibody against C5. This agent has been approved for paraoxysmal nocturnal hemoglobinuria (PNH) (143). PNH is a hematological disorder where certain surface proteins are missing on erythrocytes (144). As related to the complement pathway, CD55 and CD59 expression is deficient in PNH (while CD46 is not normally expressed on human erythrocytes), thus preventing regulation of the complement cascade and leading to unregulated activation (144, 145). A number of other agents are being developed for other diseases (see **Table 2**). There are several important issues that need to be addressed to accelerate the application of complement inhibitors into the clinic. One of these is the choice of agent. Preclincal studies indicate that inhibition of either C3a or C5a signaling inhibit cancer progression in lung cancer models and in other malignancies. It is not clear if these signal through redundant pathways and thus the choice of agent needs to be more clearly developed. A second major issue is related to patient selection. In lung cancer, trials with unselected patients have in general been less successful than targeted trials with clear criteria for patient selection. As discussed above, the majority of currently ongoing clinical trials have focused on subsets of LUAD based on oncogenic drivers. In preclinical models, there is insufficient data to determine if complement inhibitors will be more effective for lung tumors with specific drivers, e.g., KRAS mutations vs. EGFR mutations vs. fusion kinases. This is complicated by the lack of

TABLE 2 | Current drug candidates to target complement proteins.

Target	Product (company)	Suggested indications
C5aRA	PMX-53 (Peptech Ltd.)	RA, psoriasis
C5	Eculizumab/Soliris (Alexion Pharmaceuticals)	PNH
C5	Pexelizumab (Alexion Pharmaceuticals)	Clinical phase 3 for AMI, CABG
CD35 (CR1)	sCR1/TP10 (Avant Immunotherapeutics)	Clinical phase 2 for CABG
CD55 (DAF) and CD46 (MCP)	CAB-2/MLN-2222 (Millenium Pharmaceuticals)	Clinical Phase 1 for CABG
fH	fD inhibitor (Ra Pharma)	AMD, orphan renal diseases
C3	AMY-103 (Amyndas)	Transplant
C3	Compstatin/POT-4 (Potentia Pharmaceuticals)	Clinical phase 1 for AMD
C1-INH	Phucin/rhC1INH (Pharming Group N.V.)	Clinical phase 3 for HAE
C1r/C1s	C1-INH (Cetor, BerinertP, Leve Pharma)	Clinical phase 3 for HAE

C5aRA, C5a receptor antagonist; RA, rheumatoid arthritis; PNH, paraoxysmal nocturnal hemoglobinuria; AMI, acute myocardial infraction; CABG, coronary artery bypass grafting; AMD, age-related macular degeneration; HAE, hereditary angioedema (146).

appropriate models for many of these oncogenic drivers. Thus, additional preclinical studies will be needed to answer this. There is also a critical need to define biomarkers predictive of response to complement inhibitors. Since an important mechanism of complement inhibitors is modulating the immune system, it would be predicted that more immunogenic tumors with higher levels of infiltrating T cells would be more responsive to these agents. Thus, mutational burden might be predictive of response. However, in preclinical studies, EML4-ALK tumors, which have a relatively low mutational burden were shown to be sensitive to both C3aR or C5aR inhibition (72).

To date, there has been limited examination of complement activation using samples from human lung cancer. Early studies demonstrated that human NSCLC cell lines express high levels of complement inhibitory proteins and are resistant to complement-mediated lysis (147); these studies did not associate this with specific oncogenic drivers. Studies have used immunostaining of human lung tumor samples for expression of C3 and demonstrated association of expression with progression (148). These data suggest that complement activation in biopsies from cancer patients could represent a potential biomarker for local tumor activation of complement. Data from our laboratory has confirmed that complement activation as assessed by immunostaining for C3d represents a fairly frequent event in human lung cancer, with positive staining observed in approximately 40% of cases (72). However, additional studies are required to examine specific subgroups based on oncogenic drivers.

In KRAS driven lung cancer, response rates to anti-PD-1 therapy are approximately 20%. At least one study has demonstrated additivity of complement inhibitors targeting C5aR with anti-PD-1 in a mouse model where cancer cells are implanted subcutaneously (71). While these studies need to be extended to more clinically relevant models of lung cancer, they support a clinical trial using combinations of C5aR inhibitors and immune checkpoint inhibitors. Less is known regarding the effectiveness of complement inhibition for lung cancers with other drivers. Our laboratory has used a panel of murine lung cancer cells expressing the oncogenic fusion kinase Eml4-Alk. These cell lines were derived from a genetic mouse model employing a CRISPR construct to engineer the fusion kinase (128). Interestingly, there tumors appear to be resistant to anti-PD-1 therapy (72) similar to what is observed in clinical trials of patients with ALK fusion drivers. However, in an orthotopic mouse model these tumors were sensitive to inhibitors of either the C3aR or C5aR.

TARGETING COMPLEMENT IN LUNG CANCER PREVENTION

While there is much active research focusing on treating established lung cancer, there is less known regarding the role of complement in the early stages of tumor initiation. Since lung cancer usually presents as advanced disease, it is appealing to develop strategies to prevent the initiation of lung tumors and/or inhibit the early stages of transformation. In that regard, there has been extensive efforts to develop chemopreventive agents for lung cancer (6, 149, 150). Preclinical studies have tested numerous agents preventing the development of lung tumors in mice. For example, recent studies have demonstrated that elevated levels of the lipid mediator prostacyclin can inhibit induction of lung tumors in response to either carcinogens or exposure to cigarette smoke (151, 152). This resulted in a clinical trial in which orally active prostacyclin analogs were able to inhibit progression of dysplastic lesions in smokers (153).

The role of the complement pathway has not been studied in any of these models. However, recent data suggest that complement activation is required for the formation of sarcomas (89). In these studies, chronic inflammation driven by the loss of PTX3 resulted in activation of the complement cascade which was critical for tumor formation. However, other studies have suggested that increased levels of Factor H are associated with increased risk of developing lung cancer (154, 155). Thus, additional studies are needed to define the precise role of the complement pathway in tumor initiation. These will require the appropriate mouse models, and a detailed examination of how differences in the oncogenes driving tumor initiation interact with the complement system.

CONCLUDING REMARKS

In examining the findings regarding complement activation, it is clear that activation can result in pathways that can either promote or inhibit tumor progression. While anaphylatoxins can lead to engagement of immune-evading mechanisms, localized activation of intracellular complement in T cells can lead to production of $CD4^+$ subpopulations which are associated with inhibition of tumor progression (113). Thus, there are competing pathways, and the net effect of inhibiting (or activating) complement in a particular cancer subtype such as lung cancer must be considered with great care. In addition, elevated expression of complement regulatory proteins have the potential to block the inhibitory effects of complement on cancer cell progression, while enabling the pro-tumorigenic and inflammatory effects (63, 156).

The efficacy of immunotherapy in multiple cancers including lung cancer support examining other pathways that regulate the immune response to tumors. Complement has emerged as a critical link between the innate and adaptive immune system, and therefore targeting this pathway as a therapeutic and preventative strategy in lung cancer has great potential. The complexity of complement has increased as our understanding has encompassed not just systemic complement activation, but also localized and intracellular complement regulation. From these studies it has become evident that complement can regulate both pro- and anti- tumorigenic pathways, and that activation in different cell types potentially will have opposing effects. This does not appear to be unique to the complement pathway; for example studies have shown in hepatocellular carcinoma that activation of NF-κB in hepatocytes vis a vis macrophages has opposing effects on tumor progression (157). Therefore,

administration of therapeutic inhibitors (or activators) of the complement pathway may target competing, and potentially opposing pathways. Developing a strategy to selectively target the pro-tumorigenic effects of complement, and potentially simultaneously stimulate the anti-tumorigenic effects will require a deeper understanding of the role of these pathways in the tumor microenvironment. It is also likely that complement inhibitors will be used in combination with other therapies. For lung cancer these will likely be chemotherapy, targeted therapies, or immunotherapy. Currently, therapeutic strategies are dictated by the identification of specific oncogenic drivers. Therefore, examining complement in the context of these drivers in relevant preclinical models will be important in designing these trials.

Targeting the immune system as a therapeutic for cancer has revolutionized oncology. However, it is early days, and many potential targets regulating anti-tumor immunity have yet to be studied. Complement, as a bridge between innate and adaptive immunity is certain to be a potential target for treating lung cancer in the future.

AUTHOR CONTRIBUTIONS

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

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frontiers in Immunology

REVIEW
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The Role of Membrane Bound Complement Regulatory Proteins in Tumor Development and Cancer Immunotherapy

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It has long been understood that the control and surveillance of tumors within the body involves an intricate dance between the adaptive and innate immune systems. At the center of the interplay between the adaptive and innate immune response sits the complement system—an evolutionarily ancient response that aids in the destruction of microorganisms and damaged cells, including cancer cells. Membrane-bound complement regulatory proteins (mCRPs), such as CD46, CD55, and CD59, are expressed throughout the body in order to prevent over-activation of the complement system. These mCRPs act as a double-edged sword however, as they can also over-regulate the complement system to the extent that it is no longer effective at eliminating cancerous cells. Recent studies are now indicating that mCRPs may function as a biomarker of a malignant transformation in numerous cancer types, and further, are being shown to interfere with anti-tumor treatments. This highlights the critical roles that therapeutic blockade of mCRPs can play in cancer treatment. Furthermore, with the complement system having the ability to both directly and indirectly control adaptive T-cell responses, the use of a combinatorial approach of complement-related therapy along with other T-cell activating therapies becomes a logical approach to treatment. This review will highlight the biomarker-related role that mCRP expression may have in the classification of tumor phenotype and predicted response to different anti-cancer treatments in the context of an emerging understanding that complement activation within the Tumor Microenvironment (TME) is actually harmful for tumor control. We will discuss what is known about complement activation and mCRPs relating to cancer and immunotherapy, and will examine the potential for combinatorial approaches of anti-mCRP therapy with other anti-tumor therapies, especially checkpoint inhibitors such as anti PD-1 and PD-L1 monoclonal antibodies (mAbs). Overall, mCRPs play an essential role in the immune response to tumors, and understanding their role in the immune response, particularly in modulating currently used cancer therapeutics may lead to better clinical outcomes in patients with diverse cancer types.

Keywords: mCRP, complement cascade, oncology, immunotherapy, combination therapy

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INTRODUCTION

The complement system is an evolutionarily primordial component of the innate immune response that functions through a series of over 30 coordinated cascading proteins and zymogens to protect the body from invading pathogens (1). The proteins of the complement system can be found both in the plasma and as inactive precursors on the surface of cells within the body, and when activated by foreign pathogens lead to opsonization and eventual lysis of foreign cells. Though complement is an essential part of the immune response against microbes, the complement system also plays crucial roles in maintaining homeostasis through such mechanisms as the removal of apoptotic cells, the regulation of coagulation, angiogenesis, and lipid metabolism and, importantly, the surveillance of neoplastic cells (2–6). Furthermore, as in all cases of homeostasis, just as the complement pathway can be activated, it too must be kept under the tight control of negative regulators so as to prevent excessive damage to self-tissues. Atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy (C3g), and paroxysmal hemoglobinuria (PNH) are all examples of serious pathological clinical conditions resulting from inadequate control of the complement system, highlighting the importance of complement regulation (7). Membrane-bound Complement Regulatory Proteins (mCRPs) are one such factor that exerts tight regulatory functions on the complement system thus protecting the body from the deleterious effects of overactive complement. While the regulation of the complement system is becoming relatively well-studied, the relationship between the regulation of the complement system and the surveillance of neoplastic cells is not well-understood, mainly due to the fact that there exists a dichotomy in the understanding of the relationship between tumorigenesis and complement. On one hand it is thought that complement is a necessary check to neoplastic cells, and thus the expression of mCRPs allows tumor cells to proliferate unchecked, while on the other hand it has been observed that chronic inflammation can promote carcinogenesis indicating that, to a certain extent, mCRP expression may be protective against tumor growth. In this review we will discuss what is known about the role of mCRPs in regulating tumor growth, how their expression may be used as a biomarker to assess malignancy in certain cases, and how this evolving knowledge of mCRPs can be combined with the growing arsenal of immunotherapy to create improved outcomes for cancer patients.

THE COMPLEMENT SYSTEM

The complement system recognizes foreign pathogens and self-cells expressing aberrant surface molecules indicative of damage through three converging pathways: the classical, lectin, and alternative pathways. The classical pathway is activated by immune complexes of antigens and antibodies. The C1 complex, consisting of C1q and two serine proteases, C1r and C1s, circulates in the serum in an inactive state. When the inactive C1q component binds to the Fc region of IgM or IgG complexed with antigen, a conformational change occurs which results in the activation of C1r and C1s (8). Activated C1s will cleave C4 into C4a and C4b, and C2 into C2a and C2b. Subunits C4b and C2a will then bind non-covalently resulting in the creation of C4bC2a, a C3 convertase enzyme complex (9, 10). In the lectin pathway, pattern-recognizing mannose-binding lectins (MBLs) and ficolins bind to carbohydrate ligands, such as mannose, present on the surface of pathogens and together with MBL-associated serine protease 2 (MASP2) forms a C1-like complex that cleaves C4 and C2 resulting in a C3 convertase C2aC4b. Finally, in the alternative pathway, stimulation occurs through spontaneous hydrolysis of C3 or the sensing of a foreign surface structure. In this process, hydrolyzed plasma C3 [C3(H20)] and factor B bind, with the help of Factor D, create $C3(H_20)Bb$. The $C3(H_20)Bb$ complex will cleave plasma C3, resulting in C3b, which will bind to cell surfaces and to Bb, resulting in C3Bb. C3Bb is the functional C3 convertase of the alternative pathway.

C3 is the point of convergence between the three complement pathways, where despite different mechanisms of activation, the effector result becomes synonymous. The cleavage of C3 results in the production of C3a, a major anaphylatoxin, and C3b, an important molecule known as an opsonin which is able to coat the surface of antigens thereby marking them for phagocytosis by circulating macrophages. C3 convertase will also create a C5 convertase by binding to available C3b molecules. C5 convertase cleaves C5 to create C5b, which then binds with C6, C7, C8, and multiple C9 to form the C5b-9 complex. This complex is also known as the Membrane Attack Complex (MAC) and will be deposited into the lipid bilayer of cells eventually resulting in membrane destruction and cellular lysis.

While the MAC is an important effector arm of the complement system, there are several pathogens which are resistant to MAC lysis due to such structures as the cell wall found in gram-positive bacteria (11) or the generation of microbial complement inhibitors, such as the streptococcal inhibitor of complement (SIC) which is capable of preventing MAC formation through interference with the C5b-C7 and C5b-C8 complexes (11, 12). For these reasons the pro-inflammatory signaling and the phagocytic functions of complement are just as, if not more important than the direct effects of cell lysis. During amplification of the complement system, C3a and C5a are released in a constant stream, which functions through G-protein coupled receptors (GPCRs) C3aR and C5aR, respectively, to signal as powerful chemo-attractants for neutrophils, monocytes, eosinophils, mast cells and macrophages (13–17). Furthermore, opsonins C3b and C4b aid in phagocytosis by binding to proteins and polysaccharides on microbial and foreign surfaces and receptors, such as CR1 expressed on phagocytes. With regards to cancer, both the chemoattractant and opsonization properties of complement activation have serious implications for the immune composition of the tumor microenvironment.

The Complement System and It's Interaction With Tumor Cells

The expression of various surface markers on tumor cells has been found to activate all three pathways of the complement system. The classical pathway has been found to be activated

by specific molecules expressed on the surface of tumorigenic cells. The general mechanism involves the recognition of post-transcriptionally modified tumor-specific antigens by natural IgM, which unlike IgG, is capable of binding C1q with only a single molecule (18). Natural IgM is IgM produced without prior antigenic stimulation and without the intervention of adaptive immune responses to an antigen. It exists in low levels to help the body maintain homeostasis and to recognize cells that have been invaded by a foreign pathogen, and senescent, apoptotic, precancerous, and cancerous cells (19–22). In one such example, the expression of gangliosides GD3 and GD2 expressed on the surface of melanoma and neuroblastoma cells can be recognized by natural IgM antibodies in the sera of a limited number of healthy individuals, resulting in complement mediated cell lysis (23, 24). In another study, an antibody, SC-1, was isolated from a patient with signet ring cell carcinoma of the stomach and found to be reactive to all diffuse-type stomach cancer cells, and around 20% of intestinal-type adenocarcinomas. Upon reaction, the antibodies were found to induce apoptosis of the cancerous cells through a complement mediated pathway, and in clinical studies, SC-1 was able to induce regression of primary stomach cancers (25–28).

The lectin pathway has been shown to be activated in numerous glioma cell lines, where glioma cells expressing high levels of mannose-glycoproteins are easily bound by MBL, resulting in C3 and C4 activation (29). Finally, in cancers driven by virus-dependent transformation, such as EBV-infected B lymphoblastic cell lines and HIV infected T-cell lines, the alternative pathway is quickly able to recognize aberrantly expressed viral carbohydrate particles on the surface of infected cells, resulting in complement activation (30–33).

Overall, while complement is shown to be activated by tumor cells, whether this activation is actually beneficial to tumor eradication has come under intense scrutiny. A simple explanation for this is that while to a certain extent inflammation is beneficial for the control of neoplastic cells, prolonged inflammation, which could be caused by activated complement cascades, actually promotes oncogenesis (34). This theory is supported by the clinical example of the link between intraprostatic inflammatory lesions, prostatic intra-epithelial neoplasia, and cancer (35). The association of an inflammatory state and cancer is further supported by evidence that non-steroidal anti-inflammatory drug use is associated with reduced incidence of colorectal and gastric cancers (36, 37).

The first correlation between the complement cascade and increased tumor growth came from a study by Markiewski et al. where cervical tumors were transplanted into C3-deficient mice and wild-type (WT) mice. In this study tumors grew faster in WT mice as compared to C3-deficient mice, indicating that C3 may promote tumor growth. They then used the same experimental design in C5a receptor-deficient mice and found that C5a also aids in tumor growth by binding to C5a expressed on myeloid-derived suppressor cells (MDSCs). Binding to MDSCs prompted granulocytic/neutrophil-like MDSCs to migrate to the tumor, and also increased ROS and reactive nitrogen species production in monocytic MDSCs, both of which resulted in stronger suppressive MDSC effects on T-cells (38, 39). Bulla et al. performed a similar study where they found that as compared to WT mice, C1q deficient mice bearing syngeneic B16 melanoma had a slower tumor growth, fewer lung metastases, and prolonged survival. It has also been noted that the expression of complement and complement reactive proteins is present in measurable quantities in many malignant cancers (40). A final example of the deleterious effects of complement on the control of oncogenesis comes from a study by Wang et al. which showed that C3, acting through C5aR and C3aR on the surface of CD8+ tumor-infiltrating lymphocytes (TILs), is able to constitutively suppress IL-10 production. This data ultimately showed that complement activation in the tumor microenvironment suppresses the anti-tumor effects of CD8+ TILs (41, 42).

mCRPs

As is the case in any homeostatic process, there are several regulatory mechanisms in place to ensure that the complement system does not become over activated, thus causing harm to self-tissues. There are several soluble regulatory proteins such as C1 inhibitor, C4b binding protein, and factors H, B, D, and I. In addition, mCRPs are another control mechanism that includes CD35 (Complement receptor 1, CR1), CD46 (membrane cofactor protein, MCP), CD55 (decay acceleration factor, DAF,), and CD59 (protectin) (43, 44). In fact, complement regulatory proteins are expressed on every cell in the body (45), though the expression of these mCRPs varies across tissue type. It can be hypothesized that because different tissues face different immune interactions, the mCRP expression across tissue type is variable (46).

CD35

CD35 is primarily expressed on erythrocytes, lymphocytes, phagocytes and dendritic cells, with rare expression on tumor cells (47, 48). It functions as a cofactor for the cleavage of C3b into iC3b (49). Additionally, CD35 binds to C4b and promotes the degradation of C4b into C4c and C4d. Importantly, CD35 is also involved in accelerating the decay of C3/C5 convertases, resulting in an inhibition of complement activation at the level of the C3 cascade. Previously it had been shown that CD35 expression could be found in follicular dendritic cell tumors, malignant endometrial tissue, and leukemic blasts (44, 50, 51). More recently, studies have also linked the expression of CD35 on both tumor and on immune cells to a susceptibility for gallbladder cancer (52), advanced clinical stage and poor overall survival in patients diagnosed with nasopharyngeal cancer (53).

THE FUNCTION OF CD46

CD46, CD55, and CD59 are the mCRPs whose function most relates to tumors. Together, these surface proteins are also known to inhibit complement responses, and of late have also been a focus of research related to human malignancy. CD46 is a transmembrane glycoprotein that is expressed on all nucleated cells, and like CD35, functions to protect excessive complement activation by acting as a cofactor in the proteolytic cleavage of C3b and C4b, mediated by Factor I (54) (**Figure 1**).

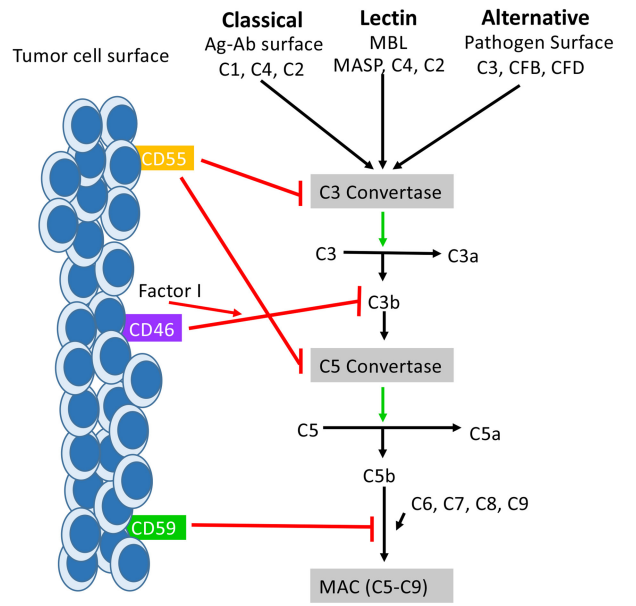

FIGURE 1 | How mCRPs regulate the complement cascade: mCRPs CD55, CD46, and CD59 exert a regulatory influence on the complement cascade to prevent complement from becoming overly activated. CD55, CD46, and CD59 are known to exert control on all three pathways of complement activation. CD55, also known as DAF, accelerates the decay of the C3 convertases (C4bC2a and C3bBb) and consequently the C5 convertases into constituent elements and prevents re-association (55). The outcome is destabilization of the C3 and C5 convertases which results in decreased anaphylatoxin (C3a,C4a, C5a) formation, decreased opsonin formation (C3c and iC3b), and prevention of MAC formation. CD46 functions as a cofactor for Factor I in the cleavage of C3b and C4b (not shown), leading to inactivation of both (56). CD59 prevents the polymerization of C9 and insertion of additional C9 molecules into the C5b-9 complex (57). It also directly interferes with pore formation of C5b-8, resulting in inhibition of MAC formation. While the distribution of CD55, Cd46, and CD59 is varied across tissues of the body, they are all found expressed on the surface of various tumor cells where they serve as biomarker for tumor formation.

Though CD46 may initially have been thought to primarily function as a mCRP, CD46 has also been found to have functionality in mediating immune responses. For example, CD46 has been found to act through distinct mechanisms to regulate different T-cell subsets during an immune response, where CD46 actually acts as a costimulatory molecule for T-cells. Specifically, the binding of CD46 on CD4+ T cells has been found to result in an initial proliferation and activation of T helper type 1 cells (T_H1 cells), with a characteristic production of Interferon γ (IFN γ) (58). However, a simultaneous expansion of effector cells leads to an accumulation of interleukin 2 (IL-2), which provides a switch signal for CD4+ T-cells to take on a T regulatory (Treg) phenotype. CD4+ cells then begin producing IL-10 in order to control the expanding immune response (**Figure 2**). When CD46 is dysregulated, this switch to a Treg phenotype does not occur, which clinically has been related to chronic inflammatory diseases such as relapsing and remitting Multiple Sclerosis (MS) (59), asthma (60), and Rheumatoid Arthritis (RA) (61). Additionally, as discussed above, such a chronic inflammatory state can allow pre-metastatic cells to thrive (62). CD46 activation on γδ T-cells has also been shown to directly suppress their IFNγ and TNFα production, which can further lead to a pro-tumor environment (61, 63, 64). Together this data suggests a temporally and spatially regulated role of CD46 in adaptive immune responses, which also serves as an important indication that the complement cascade is capable of exerting a driving influence on adaptive T cell responses during anti-tumor responses.

In terms of the regulation of CD46, it has been shown that CD46 is highly glycosylated, and that CD3 stimulation alters the O-glycosylation of CD46 in activated T-cells, resulting in decreased CD46 processing and T-cell singling, which ultimately leads to a T-reg phenotype characterized by the dominance of IL-10. Nuclear factor κB (NF-κB) has also been shown to regulate CD46 expression, where activation of NF-κB is critical for CD46 expression (65).

CD28, which is a receptor on T-cells that provides a secondary activation signal for T-cells in conjunction with the primary TCR signal (66), has also been identified to have an important role in regulating CD46 signaling. Not only has CD28 been shown to control CD46 expression on activated T-cells, but Charron et al. also showed that the engagement of CD28 and CD46 mediates T-cell responses. In regards to the IFNγ:IL-10 production ratio, as compared to CD28 stimulation alone, CD28/CD46 co-stimulation was shown to promote regulatory function, while compared to CD46 activation alone, CD28/CD46 co-stimulation was shown to decrease regulatory function (67). Together this data indicates the intricate role of CD28 in regulating CD46, and the important cytokine-related role that these two may play in tumor specific adaptive responses.

CD46 AS A BIOMARKER FOR CANCER

Combining this data of adaptive T cell responses, which seem to be anti-tumor in certain circumstances, and pro-tumor in others, with the fact that it is still not unanimously agreed upon whether complement expression is beneficial to tumor defense, it seems the role of mCRP CD46 is not as clear cut as originally hypothesized. For this reason, investigators have sought to characterize CD46 expression on various tumors, with the potential goal of using CD46 as a biomarker to predict immune response and patient outcome. In ovarian cancer for example, CD46 expression was linked to shorter revival-free time, defined as the time from the primary surgical treatment until the time of diagnosis of a recurrent tumor or death, and an overall less favorable outcome (68). Similar findings have also been found in breast cancer cases, where CD46 expression and involvement of lymph nodes represent independent risk factors for disease-free survival, and CD46 expression was found to be linked to less favorable diagnoses (69). Other cancers found to express higher levels of CD46 than adjacent normal tissues, which also relates to a worse clinical prognosis, include hepatocellular carcinoma (HCC), colon cancer, and Multiple Myeloma (70–72).

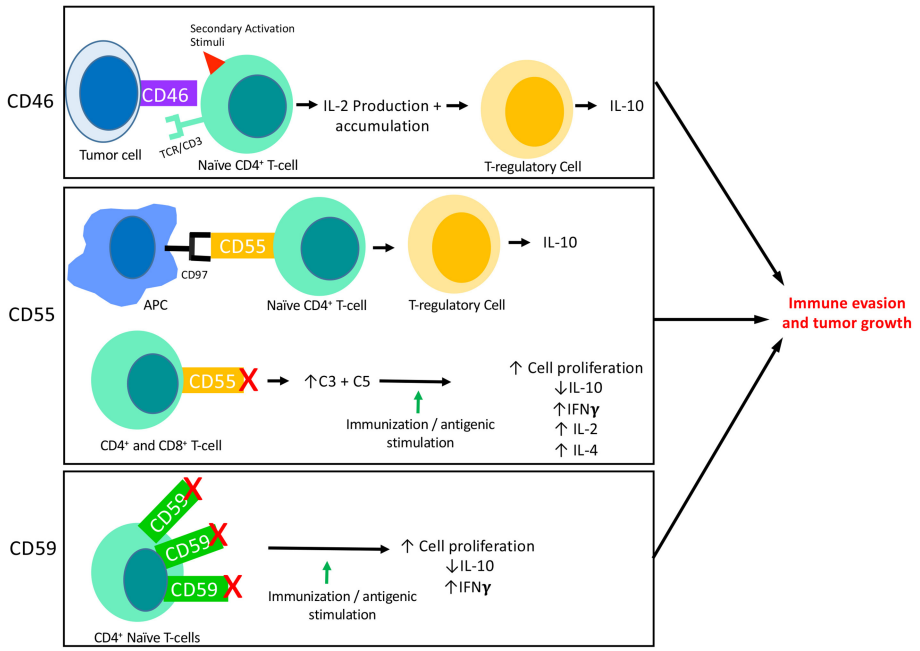

FIGURE 2 | The interaction of mCRPs with the adaptive immune response: CD46, CD55, and CD59 all have known interactions with the adaptive immune response. This figure summarizes what is currently known about each of their interactions with adaptive responses, specifically T-cell responses. CD46 is known to be expressed on the surface of tumor cells and its binding to a naïve CD4+ T-cell in the presence of a secondary activation stimuli results in IFNγ and IL-2 production . Though initially immuno-stimulatory, as IL-2 accumulates it causes activated CD4+ T-cells to undergo a transformation into a Th1 Regulatory cell that produces high levels of IL-10. Two important aspects of CD55 activity are shown here. First, CD55 on the surface of T-cells are known to interact with CD97 displayed on the surface of Antigen Presenting Cells (APCs). This interaction leads to a shift in T-cell functionality, resulting in T-cells that function like TRegs and produce IL-10. The blockade of CD55 on the surface of T-cells has also revealed the immunosuppressive function of CD55. When CD55 is blocked on both CD4+ and CD8+ naïve T-cells followed by immune stimulation (*in vitro*) or immunization (*in vivo*), T-cells are shown to proliferate and to produce increased IFNγ, IL-2, and IL-4 and decreased IL-10 as compared to cells or animals that were untreated. This effect appears to be dependent on the increased levels of C3 and C5 present due to blocked functionality of CD55. In certain circumstances, CD59 is found to be overexpressed on CD4+ T-cells which results in downregulation of CD4+ activity. Accordingly, blockade of CD59 results in enhanced T cell responses consisting of increased cell proliferation, decreased IL-10 production and increased IFNγ production.

CD55 AND CD97

CD55 also functions as an inhibitor of both the classical and the alternative pathway of complement activation where, unlike CD35 and CD46 which act in a proteolytic fashion, it accelerates the decay of C3 and C5 convertases. CD55 does this by inducing a rapid dissociation of C2a or Bb catalytic subunit present in convertases on the cell surface (73) (**Figure 1**). Like CD46, CD55 has also been shown to have important effects on the adaptive immune response, where CD55 has been linked to the suppression of adaptive immune responses *in vivo*. For example, in mice lacking the Daf1 gene, which encodes the murine homolog of human DAF, CD4+ T-cells were found to produce more IFNγ and IL-2 and less IL-10 in response to active immunization (74). Other investigators have found that during primary T-cell activation, the absence of CD55 on APCs and T-cells enhances the proliferation and leads to enhanced effector cell frequency (75). In a model of CD8+ T-cell immune responses to lymphocytic choriomeningitis virus infection, mice lacking Daf had increased CD8+ T-cell expansion in spleen and lymph nodes, and an increased number of antigen-specific CD8+ T-cell, which resulted in faster infection clearance (76). These effects were ultimately linked to the presence of increased complement proteins due to a lack of CD55 expression and not CD55 itself, as knocking out C3 expression in CD55$^{-/-}$ mice restored normal responses (77). Overall however, the expression of CD55 decreases complement mediated cell lysis in tumors, and a lack of CD55 increases the overall inflammatory response (78–81) (**Figure 2**). In an effort to explain the observation of enhanced T-cell responses in DAF$^{-/-}$ mice, another group investigated whether CD55 expression influences the stimulatory power of antigen presenting cells (APCs). In this study APCs from DAF$^{-/-}$ mice treated with an inflammatory stimuli elicited more potent T-cell responses in a complement dependent manner, and also had decreased PD-L1 and increased CD40 on the cell surface (82). Natural killer (NK) cell responses have also been shown to be inhibited by CD55 (83).

The regulation of CD55 is also versatile as the synthesis and expression of CD55 on tumor cells has been shown to be influenced by IL-1α, IL-1β, IL-4, EGF, TNF-α, IFNγ, and Prostaglandin E2 (84–88).

Another important factor regarding CD55 relates to CD97, an EGF-TM7 receptor expressed primarily on monocytes and granulocytes that acts as a natural ligand for CD55 (89). Together,

this complex acts as a T cell receptor co-stimulatory protein complex (90). A study by Capasso et al. showed that direct CD55 engagement with CD97 and co-stimulation with CD3 results in T-cell activation involving increased T-cell proliferation, IL-10, and GM-CSF production, and expression of activation markers CD69 and CD25 (77). Importantly, the naïve T cells that are stimulated in response to CD55 and CD97 binding are shown to produce cells that behave like Tregs, which would promote tumor progression if expressed in the TME (91).

CD55 as a Biomarker

It is not entirely clear whether CD55 is expressed by tumors to help defend against the deleterious effects of complement activation, or whether CD55 expression on tumors is more functionally related to its role as a ligand for CD97 in T-cell activation. Either way, it is clear that CD55 is not only present in cancer tissues, but also that it plays an important permissive role in the progression of tumorigenesis. In many cases, for example in colon cancer, CD55 serves as a marker of tumor aggression and decreased 7-year survival (92). In the setting of breast cancer, it was found that cells expressing high CD55 levels were more resistant to apoptotic stimuli, have a higher growth rate, and in human cancer, are an independent prognostic factor for recurrence (93). Other cancers that show high expression of CD55 and worse clinical prognoses as a result include prostate cancer, ovarian cancer, AML, CML, ALL, gastric carcinoma, and cervical cancer (94–99).

Overexpression of CD55 in Barrett's esophagus has also been associated with esophageal adenocarcinoma risk (100). Interestingly, in this instance it appears that rather than CD55 being a marker of tumor cells, CD55 expression instead lends to a microenvironment that is favorable for a malignant transformation. In some sense, it leads to a question of the chicken or the egg—is CD55 expression upregulated which then leads to an ability for tumor cells to proliferate unchecked by complement and a microenvironment permissive to tumor growth, or do tumor cells form, and then as a secondary defense mechanism express CD55 to protect against complement destruction. Such a clarification has not been made, though it is important as the distinction could indicate clinical treatment using mAbs to be more appropriate for premalignant vs. malignant states. This distinction may also be helpful in understanding the seemingly dual role that complement plays in tumor cells. On one hand, the expression of CD55, which results in a downregulation of complement activity may be a protective mechanism to the inflammatory milieu of a premalignant state, aiming to protection against further inflammatory stimuli and a malignant transformation. Alternatively, the expression of CD55 could prevent complement mediated killing of premalignant cells, resulting in decreased control of tumor growth.

CD97 as a Biomarker

With CD55 showing such impressive potential as a biomarker for malignant states and prognosis, it is logical that CD97, which binds to CD55 and controls adaptive T cell responses, would also have utility as a biomarker. In intrahepatic cholangiocarcinoma, for example, CD97 and CD55 together were associated with histological grade, and increased biliary soluble levels of CD97 specifically was an independent risk factor for patient survival (101). CD97 and CD55 are also upregulated in pancreatic cancers, and are associated with lymph node involvement, metastasis, and vascular invasion. Wu et al. identified CD97 and CD55 to be upregulated in human gallbladder carcinoma (102), and Mustafa et al. showed that CD97 is a specific biomarker for dedifferentiated oral squamous cell carcinoma and that it accurately predicts grading and staging of disease (103, 104). Rectal adenocarcinoma, cervical squamous cell carcinoma, medullary thyroid carcinoma, and gastric carcinoma were also shown to exhibit similar trends (99, 105–107).

In a study by Steinert et al. histopathological staining showed that in human colorectal cancer, normal colorectal epithelium did not stain for CD 97, while 75% of carcinomas did express CD97. Further, the most significant staining of CD97 occurred at the invasion front. A dispersed pattern of CD97 was correlated with a poorer clinical stage as compared to those tumors that expressed CD97 in a uniformed pattern (108). This information indicates that CD97 is involved in tumor migration, invasion and differentiation (109). Others hypothesize that CD97 and CD55 may facilitate the adhesion of cells to surrounding surfaces, facilitating metastasis (103). Thus, CD97 may not only serve as a biomarker of tumor aggressiveness and early metastasis, but it may also serve as an effective therapeutic target.

CD59

CD59 inhibits the polymerization of C9 and it's binding to C5b-8 through competitive inhibition of an epitope on C8, resulting in inhibition of MAC assembly and cell lysis (110–112) (**Figure 1**). CD59 plays a critical role in the protection of self-tissues and is widely expressed on most tissues in the human body including erythrocytes, monocytes, heart, spleen, liver, and kidney (113). The protective effects of CD59 are so important that pathogenically low levels of CD59 are associated with autoimmune diseases such as diabetes, multiple sclerosis, and chronic hemolysis (114–116). Like CD46 and CD55, CD59 is also involved in T-cell responses, where CD59 is upregulated on CD4+ T cells and leads to down regulation of CD4+ activity. Accordingly, blockade of CD59 results in enhanced T cell responses (**Figure 2**) (117).

CD59 as a Biomarker

Predictably, CD59 also has been shown to have a biomarker related function for various tumors. Increased expression of CD59 is associated with reduced survival in colorectal cancer patients (118), and with decreased overall survival and progression-free survival in patients with diffuse large B cell lymphoma and adenocarcinomas of the prostate (119, 120). The opposite is true in breast tumors however, where loss of CD59 expression in breast tumors correlates with poor patient survival. The authors of this finding hypothesize that the loss of CD59 may provide a "selective advantage" for breast cancers, which results in more invasive tumors (121). This may also relate to the findings regarding the potentially deleterious role that complement activation can play in tumors.

mCRPs and Tumor Therapy

Because of the great deal of data showing that CD46, CD55, and CD59 expression are linked to worse clinical outcomes, and are in some cases highly specific for tumor cells, many approaches to block mCRP expression on tumor cells have been studied. The first and perhaps most studied of these approaches is neutralizing mAbs. Overall these have shown effective enhancement of tumor cell susceptibility to complement mediating killing in a wide range of tumor types (122). For example, neutralization of CD55 has led to increased complement activation and complement-mediated killing in Burkitt lymphpoma (81), leukemia (123), melanoma (124), and breast cancer (125). The same can be said for the blockade of CD59 with neutralizing mAb and neuroblastoma (126), leukemia, breast (127), ovarian (128), and renal cancers (129). Small interfering RNAs (siRNAs) (130) and anti-sense phosphorothioate oligonucelotides (S-ODNs) (131) have also been successfully used to downregulate mCRP expression in tumors, which in many cases leads to mitigation of tumor burden.

Recently, neutralizing mAbs have also been employed concomitantly with chemotherapeutic drugs to achieve improved outcomes, especially in patients who are non-responsive to initial chemotherapeutic treatment, often due to an initial overexpression of mCRP. CD20-postitive Burkitt lymphoma Raji cells and primary CLL cells are generally resistant to the complement-dependent cytotoxicity induced by rituximab treatment. Mamidi et al. and Weiguo et al. independently showed that inhibition of mCRP expression, specifically CD59 (132), sensitizes cancerous leukemia cells to complement attack, resulting in enhanced effectiveness of rituximab (122). Similarly, the use of mAbs blocking CD55 and CD59 in addition to Rituximab treatment leads to increased tumor toxicity in non-Hodgkin's lymphoma (133). Results have shown that in Herceptin treatment for non-small cell lung cancer (NSCLC), neutralization of CD55 and CD59 results in markedly increased Herceptin-mediated complement cytotoxicity. Even more interesting, this study showed that overexpression of mRPs on tumor cells is likely largely responsible for Herceptin resistance in NSCLC (134). CD55 and CD59 expression were also correlated with the protection of HER2-overexpressing breast cancer and uterine serous carcinoma cells from trastuzumab-induced complement dependent cytotoxicity (135, 136). CD55 has been identified as a signaling protein responsible for self-renewal and therapeutic resistance to cisplatin in endometroid tumors, and blockade of CD55 using saracatinib sensitizes chemo-resistant cells to cisplatin (137). A human CD59 inhibitor has been shown to enhance complement dependent cytotoxicity of ofatumumab against rituximab-resistant B-cell Lymphoma cells and CLL (138). In a slightly different approach, Su et al. used a model of prostatic cancer, where CD46 was found to be overexpressed in primary tumor tissue in metastatic castration-resistant prostate cancer (mCRPC) but not on normal tissues, and was able to show excellent selective killing of cancer cells by using an antibody-drug conjugate (ADC) consisting of a tubulin inhibitor and a macropinocytosing anti-CD46 ADC. Their CD46 ADC caused regression and elimination of a mCRPC cell line xenograft, showing the efficacy of targeting CD46 in combination with a tubulin inhibitor as a means to treat cancer (139).

Though the inhibition of mCRPs has shown marked efficacy in harnessing the power of the complement cascade to control tumor growth, such therapies pose a threat of causing over activation of the complement cascade in normal tissues, as mCRPs are expressed on normal tissues ubiquitously throughout the body. As a result, a fear of non-specific mCRP blockade is the development of autoimmune-like disease, as could be expected considering the auto-immune diseases associated with genetic mutations of specific mCRPs (140). Despite these fears, there are several examples of anti mCRPs therapies being used both successfully and safely. For example in a study using both transgenic mice and macaques, the transient depletion of CD46 on the cell surface using a recombinant protein was not only able to sensitize tumors to complement mediated cytotoxicity, but was also shown to be safe and well-tolerated as defined by body weight and blood and chemistry analyses (141). In addition, to prevent possible off-target effects, efforts have been made to specifically deliver mCRP targeting therapeutics to the tumor site. One way to do this is to create antibodies with one F(ab) region specific to an mCRP and another F(ab) region with high affinity to a tumor-restricted antigen (43). In doing so, the potential side-effects of generalized anti-mCRP therapy can be extenuated. An example of the successful use of this strategy can be seen in a study by Gelderman et al. where the group designed a bispecific anti-CD55 and anti-Ep-CAM antibody that was able to precisely target and cause C3 deposition in cervical and colorectal carcinomas, which overexpress Ep-CAM (142, 143). These targeted therapies certainly provide an excellent approach to developing safer and more effective anti-cancer therapeutics, though more in-depth clinical studies are needed in order to further categorize potential toxicities of the various mCRP targeting drugs.

A New Paradigm to Understand mCRP Expression

The successful use of mAbs directed against mCRPs suggests that targeting mCRP, especially when in combination with other chemotherapeutic drugs, does have valuable therapeutic value. While this may be true, it also remains the case that the role of complement in the TME is likely more deleterious to controlling tumor growth than it is helpful. The implication of this is that the expression of mCRPs in tumors should indicate less complement activation and therefore a better prognosis. The actuality is that mCRP expression by and large is indicative of increased TNM staging and worse overall patient survival. If put into the current paradigm of complement activation, where increased complement activation in a tumor results in enhanced tumor killing and thus increased patient survival, these ideas seem irreconcilable. In order to reconcile the role of mCRPs in tumor expression, we argue that mCRPs should be viewed as more of a biomarker of an aggressive tumor phenotype involving intense generalized inflammation rather than a functional measure of the amount of complement activation present in a given TME.

mCRPs have been found to be upregulated by inflammatory cytokines and in inflammatory conditions (82, 84, 85, 144), likely as a reactionary attempt to prevent pathological activation of complement. In a TME however, there are constant sources of inflammation and especially once tumor cells have escaped initial immune control, there is an intense infiltration of immune cells and activation of the complement cascade. As a result, mCRPs levels could continually rise in response to snowballing inflammation, despite being unable to fully control activation within the TME. As a result, mCRPs would be expressed most intensely in the most inflammatory environments, which as discussed above is an advantageous environment for tumor growth. In this paradigm, mCRPs would serve as an excellent biomarker for invasive and progressive disease though less of a therapeutic target. This understanding would also concurrently explain why both mCRP expression and complement activation in the TME are positively correlated with a worse overall patient survival.

Complement and Checkpoint Inhibitor Therapy

Components of the complement cascade interact with adaptive immune responses in a myriad of ways. We have already discussed how almost all mCRPs are capable of downregulating T-cell activation and effector function through either complement-dependent or independent mechanisms. Further, with the recent success of PD-1 immune checkpoint blockade therapy, understanding the role that complement plays specifically in responses to therapy, and generally in responses of the adaptive immune system is of extreme importance.

We have already discussed that mice lacking CD55 mount more potent T cell responses upon stimulation than mice expressing CD55, which is requisite on C3 and C5aR signaling. Further, APCs in these $CD55^{-/-}$ mice expressed decreased PD-L1 and increased CD40 after stimulation as compared to WT (82). Several other complement constituents have been found to regulate adaptive immune responses in similar ways. It has been established that T cells express C3a and C5a receptors, which when bound by ligand result in IL-10 production and suppression of tumor-specific CD8+ T cell mediated cytotoxicity in melanoma (145). C5a, which causes tissue damage by inducing pro-inflammatory cytokine and chemokine production, neutrophil migration and blood vessel permeability, has been shown to stimulate IL-10 and TGF-β production from myeloid cells which promotes Treg generation (146, 147). In another study, C5a was shown to induce PD-L1 expression on monocytes through the activation of ERK1/2 and JNK signaling pathways, showing yet another interaction of complement with T cell responses (148). Interestingly, PD-L1 blockade has also been shown to result in the production of massive amounts of C5a suggesting a synergistic relationship between the two (148, 149). Exploiting this relationship, one group examined the therapeutic efficacy of PD-1/PD-L1 blockade in $C5aR^{-/-}$ mice, and found that C5a negatively regulates the efficacy of PD-1/PD-L1 blockade. Increased T-cell ratios and functions in the tumor tissue were observed when PD-1/PD-L1 agonists were used in combination with a C5aR antagonist (149). Clinically, dual blockade of PD-1 and C5a/C5aR has been shown to work synergistically to protect against NSCLC (150). It is hypothesized that these effects are due to C5a recruitment of MDSCs to the TME. PD-1/PD-L1 blockade cannot overcome the suppressive T cell activity of the MDSCs, so blockade of C5a thus reduces MDSCs in the TME and creates a niche more susceptible to PD-1 blockade (151). Finally, in a study where mass spectrometry was used to correlate baseline serum protein signatures with response to nivolumab in metastatic melanoma, patient survival could be partially predicted by the signature of proteins associated with acute phase reactant and elements of the complement cascade. In this study, the presence of complement pathway proteins was associated with poor outcomes in patients treated with checkpoint inhibition (152). Overall this data surprisingly points to the idea that the presence of complement proteins negatively regulates response to checkpoint inhibitor therapy.

Though clearly there exists ample data on the interaction of complement with checkpoint inhibitor therapy, there do not yet exist any studies linking the expression of mCRPs specifically to the efficacy of PD-1/PD-L1 blockade. Considering the widespread use of immunocheckpoint inhibitor therapy and the considerable interaction of mCRPs with T-cell activation, further understanding of how mCRPs impact PD-L1 expression, and impact PD-1/PD-L1 blockade therapy is of vital importance. It may be hypothesized that because complement products, for example C5a, negatively regulate PD-L1 responses, the use of neutralizing mAbs against mCRPs that increase C3a and C5a production in the TME such CD35, CD45, and CD55, would not be a useful combinatorial therapy. It could be argued however that because CD59 is acting on inhibition of the MAC formation which is more directly and immediately responsible for tumor killing, the blockade of CD59 in conjunction with immunocheckpoint inhibitor therapy may be useful. Additionally, because mCRPs have been shown to be a specific biomarker for many cancer types, therapies that take advantage of the capability of mCRPs to identify malignantly transformed cells in order to deliver immunocheckpoint inhibitors directly to a tumor tissue, while at the same time sparing normal tissue, could be extremely useful and lead to even better clinical outcomes in cancer patients treated with these regimens. Realistically, the same is true of almost any chemotherapeutic drug; mCRPs could be used to identify cancerous cells, and therapies could be designed to traffic to areas strongly exhibiting mCRPs or specific isoforms indicative of tumorigenesis depending on specific tumor type. Ultimately, more research is needed on the interaction of mCRPs and the growing arsenal of immunocheckpoint inhibitor therapies.

CONCLUSION

mCRPs have complex effects on the TME, and in order to further exploit mCRPs as cancer targets, a deeper understanding of how mCRPs impact both the innate and adaptive immune responses is needed. First and foremost, mCRPs act locally in

the TME to tightly regulate the activation of the complement cascade at various steps. But more than that, recent data is showing that mCRPs interact with aspects of the adaptive immune response, where by and large, mCRPs are being shown to downregulate T-cell responses to cancer. Generally, this points to an anti-inflammatory role of mCRPs. With mounting evidence that inflammation in the TME is actually beneficial for tumor growth and immune evasion however, it becomes necessary to revisit the role of mCRPs in tumorigenesis and the regulatory mechanisms that may lead to mCRP expression in the first place. What can be established is that mCRP expression in a tumor is overwhelmingly associated with more aggressive TNM staging and, worse overall, patient prognoses. In addition, mCRP expression seems to be specific for tumorigenic tissue and serves as a way to differentiate tumor tissue from adjacent normal tissues. In this review we suggest a new paradigm for understanding mCRP expression in relation to cancer therapy, which is that in the midst of widespread and mounting inflammation within a TME, mCRP expression continually increases as a way to limit pathological complement activation. In doing so, mCRPs become an excellent biomarker for TMEs that are extremely inflammatory, and thus most permissive for aggressive tumor growth and metastasis. In addition to their role as a biomarker, evidence is emerging that neutralizing mAbs against mCRPs can be used to sensitize patients to other chemotherapeutic drugs. Combination therapy of neutralizing mAbs against mCRPs and conventionally using chemotherapy shows great clinical promise. That being said, the role of mCRP expression in cancer is extremely complex and the staging, distribution and intensity of mCRP within the tumor, along with the type of tumor and interactions with combination drugs, need to be taken critically into account when deciding what treatments to use. Finally, it is relatively unknown how mCRPs interact with immunocheckpoint inhibitor therapy, and with the success and widespread use of these therapies, more work needs to be done to elucidate this relationship.

AUTHOR CONTRIBUTIONS

AG and JY conceptualized, strategized, and planned the manuscript together. AG wrote and researched the body of the manuscript, and JY advised and edited throughout.

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The Complement Cascade as a Mediator of Human Malignant Hematopoietic Cell Trafficking

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The complement cascade (ComC) cleavage fragments C3a and C5a regulate the trafficking of normal, differentiated hematopoietic cells, although they do not chemoattract more primitive hematopoietic stem/progenitor cells (HSPCs). By contrast, human myeloid and lymphoid leukemia cell lines and clonogenic blasts from chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML) patients respond to C3 and C5 cleavage fragments by chemotaxis and increased adhesion. Consistent with this finding, C3a and C5a receptors are expressed by leukemic cells at the mRNA (RT-PCR) and protein (FACS) levels, and these cells respond to C3a and C5a stimulation by phosphorylation of p44/42 MAPK and AKT. However, neither of these ComC cleavage fragments have an effect on cell proliferation or survival. In parallel, we found that inducible heme oxygenase 1 (HO-1)–an anti-inflammatory enzyme, is a negative regulator of ComC-mediated trafficking of malignant cells and that stimulation of these cells by C3 or C5 cleavage fragments downregulates HO-1 expression in a p38 MAPK-dependent manner, rendering cells exposed to C3a or C5a more mobile. We propose that, while the ComC is not directly involved in the proliferation of malignant hematopoietic cells, its activation in leukemia/lymphoma patients (e.g., as a result of accompanying infections or sterile inflammation after radio-chemotherapy) enhances the motility of malignant cells and contributes to their dissemination in a p38 MAPK–HO-1 axis-dependent manner. Based on this idea, we propose that inhibition of p38 MAPK or upregulation of HO-1 by available small-molecule modulators would have a beneficial effect on ameliorating expansion and dissemination of leukemia/lymphoma cells in clinical situations in which the ComC becomes activated. Finally, since we detected expression of C3 and C5 mRNA in human leukemic cell lines, further study of the potential role of the complosome in regulating the behavior of these cells is needed.

Keywords: complement cascade, leukemia, chemotaxis, cell migration, inflammasome, Hmgb1, S100a9, HO-1

INTRODUCTION

Leukemia that is resistant to standard chemotherapy or that recurs after myeloablative treatment and transplantation of hematopoietic stem/progenitor cells (HSPCs) remains an important clinical problem. This malignancy is first initiated locally in hematopoietic tissue and, depending on the type of leukemic cells, has the tendency to spread to other areas of bone marrow (BM), spleen, or lymph nodes and may finally infiltrate solid organs, such as liver, brain, or skin. The origin of cells that initiate leukemia is still disputed and may include mutated HPSCs or even more differentiated cells from hematopoietic or lymphoid lineages (1–3).

Since leukemic cells express several receptors that are also functional on the surface of normal hematopoietic cells and that regulate migration and adhesion, they are highly responsive to similar growth factors, cytokines, chemokines, bioactive phospholipids, and extracellular nucleotides (EXNs) that direct migration of normal hematopoietic cells (3, 4). These pro-migratory factors are already expressed in the hematopoietic microenvironment under steady-state conditions and, what is important here, can be upregulated in response to tissue-damaging chemotherapy or radiotherapy. Upregulation of these pro-migratory factors leads to unwanted side effects—namely, metastasis to hematopoietic and non-hematopoietic organs (5, 6).

We will present here the concept that chemo/radiotherapy-induced tissue/organ damage is a form of sterile inflammation that involves activation of innate immunity. According to the definition, sterile inflammation is an inflammatory process triggered in the absence of microbial pathogens; however, most of the innate immune pathways that sense microbial infections are also involved in this process (7, 8). For example, chemotherapy- or radiotherapy-induced cell damage leads to release of several danger-associated molecular pattern molecules (DAMPs) that may induce the innate immune response by activating the complement cascade (ComC) (7, 9). DAMPs released from damaged cells activate the ComC in a mannan-binding lectin (MBL)–mannan associated protease (MASP)-dependent manner. As a consequence, MASP-1/2 activates downstream elements of the ComC but in parallel also triggers activation of the coagulation cascade (CoaC) and the fibrinolytic cascade (FibC) (10–12). DAMPs, depending on their molecular structure, may also bind to the family of Toll-like receptors (TLRs). One of the most important DAMPs released from damaged cells is extracellular ATP, which binds to P2 purinergic receptors and, after binding to the P2X7 receptor, activates the inflammasome (13).

Based on this mechanism, a chemotherapy or radiotherapy-induced pro-inflammatory microenvironment in hematopoietic organs and other tissues leads to release of several peptide- and non-peptide-based mediators, including bioactive lipids and ExNs, such as ATP, and activate the three ancient cross-interacting proteolytic cascades, the ComC, CoaC, and FibC (5, 6, 9, 13, 14).

In this review we will focus on the key role of the ComC and address the most important pathways that lead to its activation after chemo/radiotherapy. First, we will focus on the important role of the MBL pathway of ComC activation, which is triggered by release of DAMPs and reactive oxygen species (ROS) from damaged cells (11–13). Next, we will highlight the role of EXNs, and in particular ATP, in activating the inflammasome and the release from cells of IL-1β, IL-18, and several DAMPs, including high mobility group box 1 (Hmgb1) and calgranulin B (S100a9) (15). In particular, Hmgb1, as an important DAMP, is recognized by the circulating pattern-recognition receptor MBL, and the Hmgb1–MBL interaction potentiates activation of the ComC in an MBL–MASP-dependent manner (6).

Moreover, since the ComC and sterile inflammation can be inhibited by anti-inflammatory treatment, including upregulation of heme oxygenase 1 (HO-1) in the cells or downregulation of the inflammasome, we will also address the potential application of HO-1-activating molecules or inflammasome inhibitors in ameliorating a chemotherapy-induced pro-metastatic microenvironment (16, 17). Chemotherapy/radiotherapy-induced sterile inflammation in collateral tissues could also be easily ameliorated after administration of non-steroid inflammatory drugs or anti-inflammatory steroids (5, 6).

Response of Normal HSPCs to ComC Activation and Stimulation by C3 and C5 Cleavage Fragments

Receptors for soluble C3 and C5 cleavage fragments (C3aR and C5aR, also known as C5aR1 or CD88) are expressed by normal HSPCs as well as several differentiated cells from hematopoietic and lymphatic lineages, including leucocytes, monocytes, lymphocytes, and dendritic cells (7, 9, 12). Both C3a and C5a are reported to be potent activators and chemo attractants for mast cells, granulocytes, and monocytes and, as anaphylatoxins, play an important role in activation and degranulation of granulocytes. Both C3a and C5a are degraded to $_{desArg}$C3a and $_{desArg}$C5a, and the second of these retains significant biological activity. However, neither C3a nor C5a chemoattract normal HSPCs, C3a may enhance the responsiveness of these cells to stromal-derived factor 1 (SDF-1), an important α-chemokine playing a role in retention of HSPCs in BM niches as well as directing the homing of HSPCs after transplantation to the BM. This effect relies on a C3a-mediated increase in incorporation of the SDF-1 receptor, CXCR4, into membrane lipid rafts (18). Evidence has accumulated that CXCR4, if incorporated into membrane lipid rafts, responds much more strongly to an SDF-1 gradient, which is explained by the fact that lipid rafts are membrane domains associated with downstream signaling molecules involved in signal transduction from activated CXCR4. This has been demonstrated by direct confocal colocalization studies at the single-HSPC level and by western blot analysis demonstrating the presence of CXCR4 in lipid raft-enriched cell membrane fractions. As a result, HSPCs briefly exposed (primed) with C3a before transplantation into lethally irradiated animals show enhanced seeding efficiency to BM niches (19). This proposed strategy could potentially be employed in the clinic to facilitate engraftment of transplanted HSPCs and to accelerate hematopoietic recovery after the procedure. Thus, while ComC cleavage fragments do not directly chemoattract normal HSPCs, they play an important role in

enhancing the responsiveness of these cells to a retention/homing SDF-1 gradient.

Moreover, ComC cleavage fragments play a pivotal role in mobilization of HSPCs from BM into peripheral blood (PB), as seen for example in inflammation, tissue/organ injury, or during pharmacological mobilization—a clinical procedure to harvest for transplantation purposes normal HSPCs that are mobilized from BM into PB (18, 19). In this procedure, pro-mobilizing agents, such as cytokine granulocyte colony stimulating factor (G-CSF), activate granulocytes to release several proteolytic and lipolytic enzymes, ROS, and DAMPs, which facilitate release of HSPCs from their BM niches. This effect is subsequently enhanced in a positive feedback loop by terminal products of ComC activation, C5a, and to some extent $_{desArg}$C5a. Granulocytes activated by C5a are subsequently chemo attracted to a C5a gradient present in PB and are the first cells that egress from BM into PB during mobilization and thus pave the way for HSPCs to follow in their footsteps across the PB–BM endothelial barrier (18–21).

In light of the most recent observations, the role of the ComC in regulating cell biology, including that of hematopoietic stem cells, may seem even more surprising. Namely, in addition to the long-prevailing classical view of the ComC as a serum-operative danger sensor and first line of defense system in the organism, a novel concept has been recently proposed in which the ComC regulates the biology of normal stem cells in an autocrine-dependent manner. In support of this notion, experimental evidence has accumulated that C3 is present inside cells as a component of the "complosome," and its activation may impact cell biology (22–24). Thus, further studies are needed to better understand the role of the complosome in normal and malignant hematopoiesis. Intracellularly expressed autocrine C3a may mask the responsiveness of exogenously added C3a as a stimulating molecule in several biological assays. Confirming that this is a potential complication, we detected the presence of C3 and C5 by PCR in normal human and murine hematopoietic stem cells and in several leukemia cell lines (**Figure 1**). Therefore, further work is needed to understand the implications of the endogenous expression of C3 both in normal and in malignant hematopoietic cells (25).

In summary, despite the fact that normal human HSPCs express C3a and C5a receptors and, after being primed by C3a, respond significantly more strongly to an SDF-1 gradient, surprisingly, they do not show spontaneous chemotaxis in response to C3 and C5 complement cleavage fragments. This is in contrast to immortalized leukemia and lymphoma cell lines and clonogenic leukemic progenitors, which will be discussed below in this review.

Induction of a Pro-metastatic Microenvironment in BM and Other Organs in Response to Chemotherapy in a Sterile Inflammation-Dependent Manner

Accumulating evidence shows that leukemia contains some rare, primitive (cancer stem cell-like) cells that are highly mobile and, if they survive applied chemotherapy or a myeloablative procedure prior to hematopoietic transplantation, are responsible for leukemia regrowth (1–3). These recurring leukemic cells, like normal hematopoietic cells, respond by chemotaxis to several chemo attractants present in hematopoietic organs, PB, and lymph as well as to those upregulated in pre-metastatic niches in solid organs (3, 4, 13, 14). Therefore, this chemotactic microenvironment, creating a fertile soil for metastasizing leukemic cells, is induced as an unintentional side effect of chemotherapy. A well-known example of such a phenomenon is that SDF-1, an important chemottractant for normal and malignant hematopoietic cells, as mentioned above, is upregulated in BM after conditioning for transplantation by myeloablative administration of cytostatics or irradiation (26). It is known that upregulation of SDF-1 in myeloablated BM along with other factors is required for homing and engraftment of HSPCs infused into PB during hematopoietic transplantation. Simiarly, SDF-1 induced in BM after radio/chemotherapy applied for other non-transplantation-related reasons, such as systemic chemotherapy, has been postulated to promote metastasis of therapy-resistant cancer cells to the bones (27).

In addition to SDF-1, chemotherapy induces expression of several other chemo attractants in the BM microenvironment, including bioactive phospholipids, such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), and releases EXNs, including ATP, from damaged cells, which may all chemoattract solid tumor and leukemia cells (3–6). This unwanted effect seen in BM is a result of therapy-induced sterile inflammation in tissues exposed to cytostatics or irradiation and could be ameliorated by anti-inflammatory treatment. Our group has already demonstrated this phenomenon in more detail in the metastasis of solid tumors, with evidence that simple anti-inflammatory treatment of experimental animals with relatively simple compounds, such as non-steroid anti-inflammatory drugs (e.g., ibuprofen) or steroids (e.g., dexamethasone), given shortly after chemotherapy ameliorates induction of pro-metastatic niches in various organs and reduces the metastatic spread of intravenously infused cancer cells (5, 6).

In summary, by inducing a prometastatic microenvironment, chemotherapy or irradiation could be a double-edged sword that limits the therapeutic benefits of anti-cancer treatment. More importantly, this concept applies also to leukemia, and we will address this issue below.

Activation of the ComC in Response to Chemotherapy Due to Induction of Sterile Inflammation Mediated by Activation of the Inflammasome

It is known that the ComC plays a role in the pathogenesis of several solid tumors by modifying their growth, adhesion, affecting metastatic potential, and affecting their response to therapeutics (28). By contrast, much less information has been reported on the role of the ComC in the pathogenesis and progression of leukemia, and lymphoma and cancer cells may become exposed to ComC cleavage fragments, as seen, for example, during sterile or microbe-mediated inflammation (17).

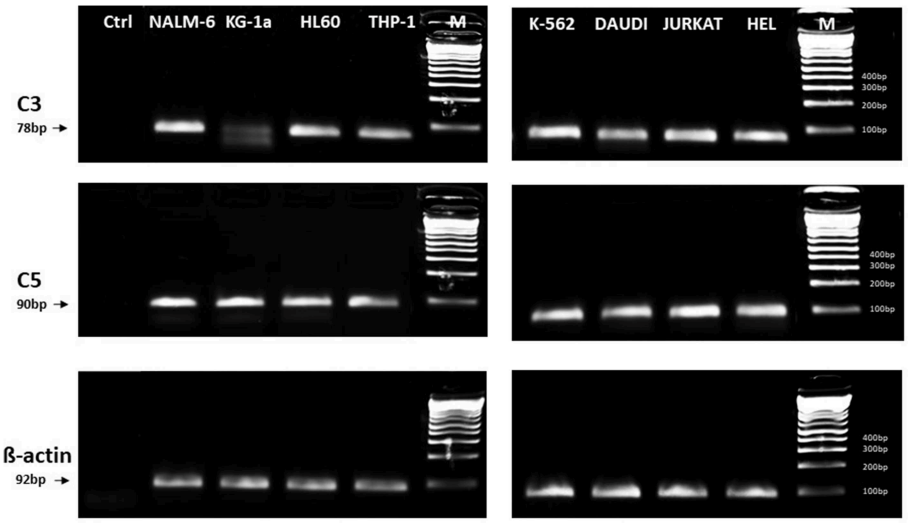

FIGURE 1 | Expression of C3 and C5 mRNA in various myeloid and lymphoid leukemia cell lines. Expression was detected in purified mRNA samples by reverse transcription polymerase chain reaction (RT-PCR). Samples containing only water instead of cDNA were used in each run as negative controls. Representative agarose gels of the RT-PCR amplicons are shown. Sequence of primers employed is shown in **Supplementary Materials**.

We propose that activation of the ComC after chemotherapy or irradiation occurs in patients via DAMP-induced sterile inflammation (3, 8, 29). We also propose that the inflammasome, and in particular one of its family members, NLRP3, is actively involved in this process. Overall, inflammasomes as mentioned above are intercellular multimeric complexes, and the NRLP3 inflammasome seems to play the most important role in the response of this family to a variety of physiological and pathogenic stimuli. Activation of the NRLP3 inflammasome complex leads to cellular release of IL-1β and IL-18, which are activated by proteolytic processing to active forms by caspase 1 before secretion. In a parallel result of inflammasome activation, cells release any of several DAMPs, including the abovementioned Hmgb1, which are recognized by MBL and directly activate the ComC in an MBL-dependent manner (15). However, one has to remember that the functional output of the inflammasome is much broader and, after sufficiently strong activation, may induce an inflammatory form of cell death called pyroptosis. Thus, as recently proposed, the inflammasome operates inside cells at the intersection of the inflammatory response with fundamental cellular processes, including cell death (15).

It is widely accepted that a crucial mediator activating the NLRP3 inflammasome in hematopoietic cells is extracellular ATP, which is one of the crucial components released, as mentioned above, in response to stress related to tissue or organ injury, as seen for example after chemotherapy or irradiation (3, 11, 13). As an extracellular signaling molecule, ATP activates several purinergic G protein-coupled receptors on the surface of cells, and P2X7 receptor activation is particularly crucial in triggering activation of the NRLP3 inflammasome response. In this review, we present the concept that the NRLP3 inflammasome acts as a "cogwheel" that couples a purinergic signaling mediator, ATP, which is released from damaged cells, with activation of the MBL pathway of the ComC (**Figure 2**).

As it is shown in **Figure 3** we confirmed activation of ComC in that AML patients exposed to chemotherapy. Next, to demonstrate involvement of the inflammasome in the ComC response to chemotherapy-induced sterile inflammation, we exposed mice to vincristine. We then evaluated (i) ELISA activation of the ComC by detecting the C5a cleavage fragment in PB and (ii) activation of inflammasome components in PB-derived mononuclear cells by looking for upregulation of the mRNA level for genes encoding interleukin-1β (*Il-1β*), interleukin-18 (*Il-18*), Asc (*Pycard*), NLRP3 (*Nlrp3*), caspase 1 (*Casp1*), high mobility group box 1 (*Hmgb1*), and calgranulin B (*S100a9*) (**Figure 4**). We confirmed that, like the response to radiotherapy (30), the inflammasome is activated by exposing experimental animals to cytostatic treatment. Our results also suggest that DAMPs released from the cells (e.g., Hmgb1), as known ligands for MBL, activate the ComC in an MBL-dependent manner (7, 11, 12). Activation of the ComC and release of C3a and C5a initiates several processes involving activation of innate immunity cells and release of proteolytic and lipolytic enzymes. Moreover, the C3a and C5a ComC cleavage products maintain a positive amplification loop to sustain the sterile inflammation response.

Thus, as proposed in **Figure 2**, and supported by our results, activation of the inflammasome in an ATP-dependent manner and the release of DAMPs seems to be an important mechanism of ComC activation in response to chemotherapy. The same mechanism seems to operate after irradiation (30). Nevertheless, the inflammasome, in addition to ATP, may also be activated by other factors released in response to chemotherapy or irradiation, such as S1P (3, 5, 6). On the other hand, the ComC could also be activated by other mechanisms in leukemic patients who

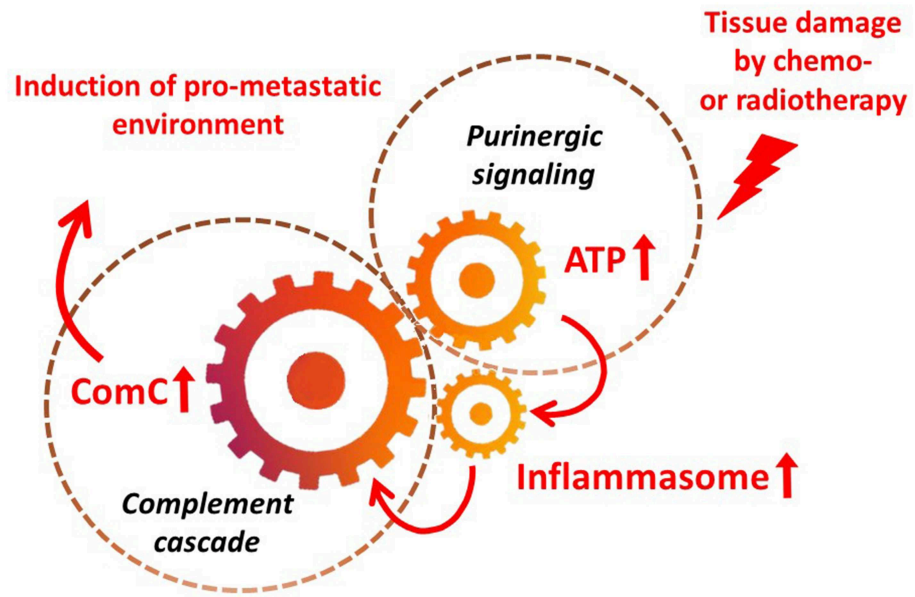

FIGURE 2 | The inflammasome as a "cogwheel" coupling purinergic signaling with the complement cascade. As proposed, tissue damage after exposure to chemotherapy or irradiation activates release of several mediators from damaged tissues, including ATP. As part of purinergic signaling, ATP activates P1 receptors, including the P2X7 receptor, on the surface of bone marrow mononuclear cells and stromal cells, which activates the inflammasome. Danger-associated molecular pattern molecules (DAMPs; e.g., Hmgb1) released from cells in which the inflammasome has become activated and from other cells directly damaged by chemo- or radiotherapy activate the complement cascade (ComC) in an MBL–MASP-dependent manner. Activation of the ComC maintains sterile inflammation in damaged tissues.

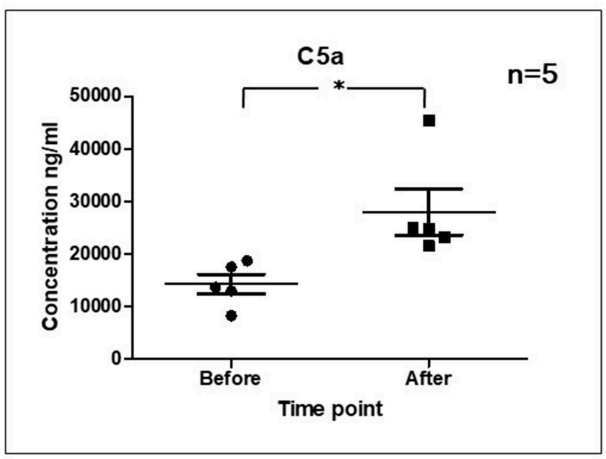

FIGURE 3 | Activation of ComC in patients undergoing chemotherapy. Patient serum samples were isolated from 5 primary AML patients peripheral blood (4°C/4,000 g, 15 min) before or 24 h after chemotherapy, respectively. All patients received "3+7" IA (IDA+Ara-C) regimens induction treatment. Serum complement 5a(C5a) level was measured by ELISA assay (Human complement C5a ELISA Kit; SAB Cat No. EK5444). Data represent the mean value ± SEM for two independent experiments. $^{*}p < 0.05$; (independent-sample *t*-test).

suffer from accompanying infections as a response to pathogen-associated molecular pattern molecules (PAMPs), which also trigger the classical and alternative pathways of ComC activation.

In addition, as with normal hematopoietic cells, further studies are needed to shed more light on the potential role of inflammasome activation in directly regulating biological processes in human leukemic blasts (31). It is also important to investigate the interplay of inflammasome activation with the intracellular C3 and C5 complesome (22–24). In fact, intracellular C5 activation has been shown to be required for NLRP3 inflammasome assembly in human $CD4^{+}$ T lymphocytes, and this is modulated by the differential activation of C5aR vs. the surface-expressed alternate receptor C2L2 (C5aR2) (32). In further support of such a mechanism, we found, as mentioned above, that human leukemia cells lines express endogenous mRNA for C3 and C5 (**Figure 1**) and express several elements of the inflammasome complex (not shown). It is worth mentioning that there have been initial attempts to modulate activity of the inflammasome in leukemic cells by employing small-molecule inhibitors of this pathway (33). Such treatments may have a positive effect on inhibiting leukemia cell progression and spread, and it has been reported that NLRP3 overexpression or activation inhibits cell proliferation and stimulates apoptosis in chronic lymphocytic leukemia cells (34).

The Response of Leukemic Cells to C3 and C5 Cleavage Fragments

The role of the ComC in solid tumor malignancies has already been the subject of several extensive studies. It is also well known that the C3 cleavage fragments (C3a and C5a anaphylatoxins) directly promote migration of normal

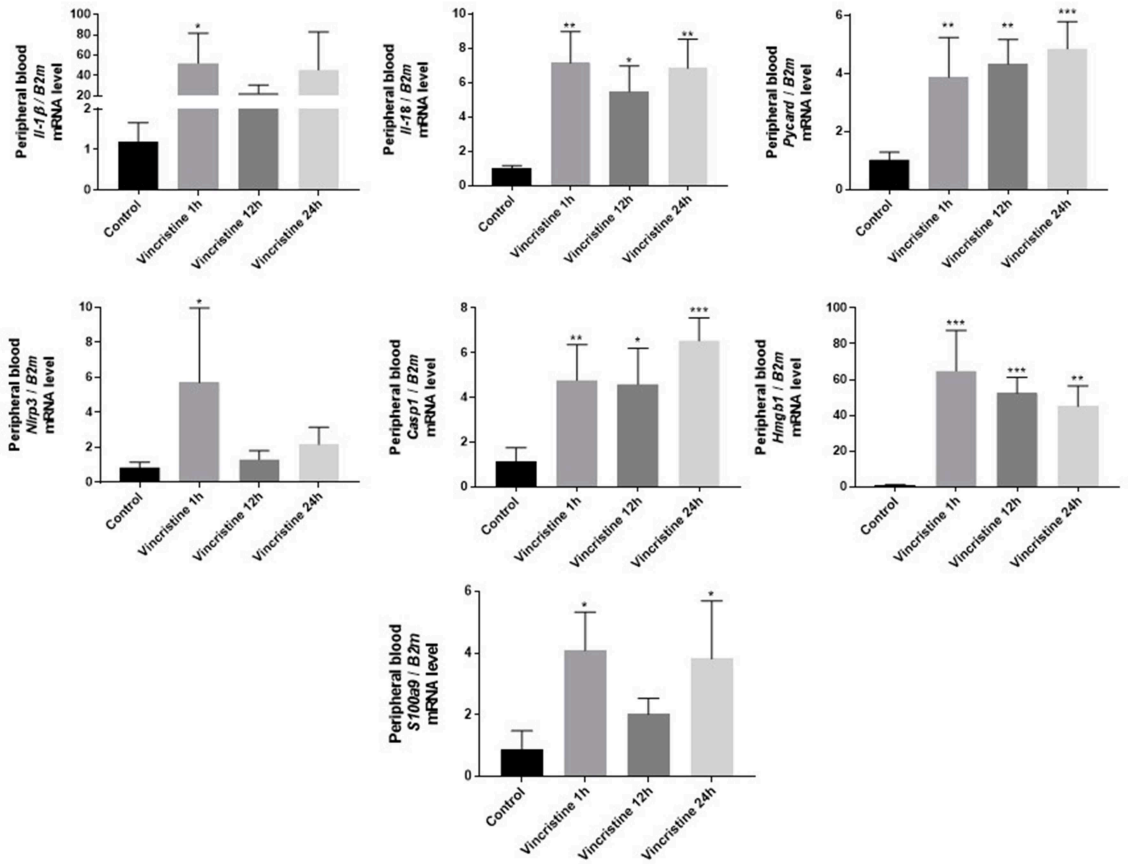

FIGURE 4 | Expression of genes involved in inflammasome activation and propagation in mouse peripheral blood after 1-, 12-, or 24-h vincristine treatment. Expression of genes encoding interleukin-1β (*Il-1β*), interleukin-18 (*Il-18*), Asc (*Pycard*), NLRP3 (*Nlrp3*), caspase 1 (*Casp1*), high mobility group box 1 (*Hmgb1*), and calgranulin B (*S100a9*) in mouse peripheral blood after 1-, 12-, or 24-h treatment with 0.5 mg/kg vincristine, as measured by qRT-PCR. Results were normalized to the β2 microglobulin (*B2m*) level. Data represent the mean value ± SEM for four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.001$ compared with control (one-way ANOVA followed by Bonferroni *post hoc* test). Sequence of primers employed is shown in **Supplementary Materials**.

differentiated hematopoietic cells, including leucocytes, monocytes, lymphocytes, and NK cells. The additive role of ComC cleavage fragments in co-regulating migration of normal HSPCs was presented earlier in this review. However, as mentioned above, in contrast to normal human hematopoietic cells, there is relatively little evidence concerning ComC involvement in leukemia, and there are limited reports on the expression of C3aR and C5aR by leukemic cells. It has been demonstrated, for example, that the HL-60, THP-1, and U-937 cell lines express both functional receptors and that their expression is regulated by interferon gamma (IFN-γ) and phorbol myristate acetate (35–40).

To fill in this knowledge gap, we analyzed seven myeloid (HEL, K-562, THP-1, U937, KG-1a, HL-60, DAMI) and five lymphoid (DAUDI, RAJI, NALM-6, JURKAT, MOLT4) cell lines as well as primary $CD33^{+}$ AML and CML patient leukemic blasts to see whether they express C3aR and C5aR, according to RT-PCR (17). The expression of these receptors was subsequently evaluated at the protein level by FACS. We also asked whether release of C3a and C5a anaphylatoxins due to ComC activation affects the biology of these cells and whether C3aR and C5aR are functional on the surface of leukemic blasts (17).

We found that all cell lines evaluated in our studies expressed mRNA for both receptors, except for K-562, which does not express C3aR, and DAMI and JURKAT cells, which do not express C5aR. The expression of C3aR and C5aR mRNA was corroborated by expression at the protein level by FACS. We also found expression of C3aR and C5aR receptors on the surface of $CD33^{+}$ blasts purified from CML and AML patients. Both receptors were expressed at the mRNA and protein levels (17).

To assess the functionality of C3aR and C5R, we stimulated cells with C3a or C5a and evaluated the effect on proliferation, survival, migration, and adhesion of the tested leukemic cell lines. First, we evaluated the potential effect of these anaphylatoxins on proliferation of leukemic cells by adding C3a or C5a to liquid cultures of leukemic cells or by adding both ComC cleavage fragments to clonogenic colony-forming units of granulocyte-monocyte (CFU-GM) assays of $CD33^{+}$ blasts isolated from patient AML or CML cells. While C3 and C5 cleavage fragments were reported to stimulate proliferation of some solid cancer

cell lines, in our hands we did not observe any effect of C3a and C5a on proliferation of human leukemic cell lines. Similarly, C3a or C5a also did not affect survival of leukemic cells in serum-free cultures supplemented with 0.5% bovine serum albumin (17).

As mentioned above, C3 and C5 cleavage fragments directly stimulate migration of normal differentiated hematopoietic cells. Therefore, we next became interested in the role of C3a and C5a anaphylatoxins in regulation of the migration of human leukemic cell lines. Again we performed Transwell migration assays with established human cell lines and $CD33^+$ blasts isolated from AML and CML patients. We found that C3a and C5a induced migration of human leukemic cell lines and clonogenic AML and CML blasts. Thus, our data indicates that the responsiveness of clonogenic leukemic cells to C3a and C5a is most likely a result of their malignant transformation (17).

Since the motility of cells in response to migration stimulatory factors may be the result of either gradient-orchestrated unidirectional cell movement (chemotaxis) or random migration (chemokinesis) (41), we tested whether the effect of C3 and C5 cleavage fragments (C3a and C5a) on the migration of human leukemic cell lines in a Transwell assay depends on one process or the other. We found that chemokinesis is the main phenomenon responsible for the enhanced migration of leukemic cells. Moreover, we also observed C3aR and C5aR expression-mediated adhesion of our leukemic cell lines to fibronectin-coated plates. Our receptor expression results and the observed cell responsiveness in migration and adhesion assays corresponded with activation of the p44/42 MAPK and AKT signaling pathways (17).

The Molecular Basis of ComC-Mediated Migration of Human Leukemic Cells

Evidence has accumulated that activation of the ComC is negatively regulated by the anti-inflammatory effect of HO-1, and, *vice versa,* ComC activation leads to downregulation of HO-1 in the cells (16, 42, 43). HO-1 is an inducible anti-inflammatory enzyme that is upregulated in response to several oxidative stress stimuli, and the anti-inflammatory functions of HO-1 have been very well-demonstrated in HO-1 knockout (KO) mice as well as in rare cases of human HO-1 deficiency. These *in vivo* HO-1 deficiencies provide evidence that HO-1 somehow balances the effects of ComC activation. Moreover, it has been reported that HO-1 is also a negative regulator of cell motility (16, 42). Downregulation of HO-1 inside cells leads to enhanced migration, whereas upregulation of HO-1 has the opposite effect. This process involves p38 MAPK, which negatively regulates intracellular HO-1 expression (44, 45).

To better understand this phenomenon, we stimulated two human leukemic cells lines, U937 and KG-1a, with C3a or C5a and confirmed that these pro-migratory factors downregulate the expression of HO-1 at the mRNA and protein levels in leukemic cells by upregulating p38 MAPK. This finding suggests the possibility of inhibition of the *in vivo* spreading of leukemia cells by intracellular inhibition of p38 MAPK and/or upregulation of HO-1 expression (17, 42).

To address this issue, we downregulated expression of p38 MAPK in U937 and KG-1a cells by employing the specific small-molecule inhibitor SB203580, and, as expected, SB203580 was a potent inhibitor of leukemic cell migration in response to C3a, C5a, or SDF-1 gradients in Transwell chemotactic assays. A similar effect on the migration of leukemic cells was obtained by upregulation of HO-1 activity by exposing cells to the HO-1 small-molecule activator CoPP (17). Thus, activation of the ComC in leukemia/lymphoma patients (e.g., as the result of accompanying microbial infections or chemotherapy-induced sterile inflammation) and release of C3 and C5 cleavage fragments could be ameliorated by inhibition of p38 MAPK or upregulation of HO-1. Such a treatment strategy would have a beneficial effect on decreasing the risk of *in vivo* spread of leukemia/lymphoma cells. The efficacy of this potential therapeutic approach has been confirmed in immunodeficient mice injected with human leukemia cells. Another therapeutic possibility for inhibiting ComC activation would be application of ComC inhibitors (e.g., compstatin) or small-molecule inhibitors of the inflammasome (e.g., MCC950) (46).

Modulation of the ComC in Leukemia Patients

Both extracellular activation of the ComC and, very likely, also its intracellular activation (a complosome effect) plays a pleiotropic role in leukemia development as a link between the tumor and the host immune system (22–24). Specifically, this mutual relationship regulates tumor growth in different ways. On the one hand, it is well-known that activation of the ComC is an important element in antibody-dependent cellular toxicity, complement-dependent cytotoxicity, and the clearance of apoptotic cells (47, 48). Elements of the ComC may protect tumor cells from NK attack by membrane-bound or soluble regulators (e.g., CD55, CD59, and factor H) and by suppression of anti-tumor T cell immunity. However, more studies are needed to assess the role of complosome activation in leukemia blasts and in cells involved in a potential immune response.

These unwanted effects of ComC activation could be ameliorated by C3aR and C5aR inhibitors as well as inhibitors of membrane-bound and soluble regulators. One may also consider the clinical application of more general ComC inhibitors, such as eculizumab or compstatin, to inhibit the C3a- and C5a-mediated spread/dissemination of leukemia cells (49). In addition, some small molecules that upregulate expression of HO-1 and inhibit p38 MAPK could also potentially find practical application, and our *in vivo* results lend support to this possibility. Of note, inhibition of p38 MAPK has already been employed in the clinic to inhibit progression of myelodysplastic syndrome (MDS) and to improve hematopoiesis in MDS patients (50).

Based on the scheme shown in **Figure 2,** activation of the ComC in leukemia patients could potentially be inhibited by employing inhibitors of inflammasome activation or by inhibiting ATP-mediated activation of the P2X7 receptor (51, 52). These possibilities, however, require further study, as targeting these mechanisms may lead to unwanted side effects.

CONCLUSIONS

In conclusion, leukemia cell lines employed in our studies, both myeloid and lymphoid, as well as clonogenic blasts isolated from AML and CML patients express functional C3aR and C5aR receptors, and as we reported in the past leukemic cells respond to stimulation by these anaphylatoxins by an enhanced random migration known as chemokinesis (17). This is a relevant phenomenon to all the situations in which leukemic cells become exposed to active ComC fragments. Both sterile inflammation induced by chemotherapy or radiotherapy and accompanying microbial-induced infections in leukemia patients enhance the migratory potential of malignant blasts, and this enhanced motility may contribute to the systemic spread of leukemic cells. This process is mediated by downregulation of HO-1 in leukemic cells in a p38 MAPK-dependent manner. Therefore, inhibition of this axis by employing activators of HO-1 or inhibitors of p38 MAPK may have a beneficial effect in ameliorating this unwanted phenomenon. This has to be balanced by a better understanding of the role of the ComC in enhancing the migration of leukemia cells and, on the other hand, in its potential involvement in the immune response to leukemia. More studies are also needed to understand the role of the ComC in regulating other aspects of leukemogenesis, such as the potential involvement of intracellular autocrine C3 activation (involving the complosome). Finally, at a mechanistic level, we propose that both chemotherapy and radiotherapy activate a purinergic signaling–inflammasome–ComC axis and lead to the occurrence of sterile inflammation in collateral tissues.

AUTHOR CONTRIBUTIONS

MR conceived idea and wrote a paper. AL generated data for **Figure 4**. KB generated data for **Figure 1**. BX generated data for **Figure 3**. KB-J and other authors contributed to writing manuscript and approved it.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01292/full#supplementary-material

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REVIEW
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The Complement Receptors C3aR and C5aR Are a New Class of Immune Checkpoint Receptor in Cancer Immunotherapy

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Cancer immunotherapy has made remarkable clinical advances in recent years. Antibodies targeting the immune checkpoint receptors PD-1 and CTLA-4 and adoptive cell therapy (ACT) based on *ex vivo* expanded peripheral CTLs, tumor infiltrating lymphocytes (TILs), gene-engineered TCR- and chimeric antigen receptor (CAR)-T cells have all shown durable clinical efficacies in multiple types of cancers. However, these immunotherapeutic approaches only benefit a small fraction of cancer patients as various immune resistance mechanisms and limitations make their effective use a challenge in the majority of cancer patients. For example, adaptive resistance to therapeutic PD-1 blockade is associated with an upregulation of some additional immune checkpoint receptors. The efficacy of transferred tumor-specific T cells under the current clinical ACT protocol is often limited by their inefficient engraftment, poor persistence, and weak capability to attack tumor cells. Recent studies demonstrate that the complement receptor C3aR and C5aR function as a new class of immune checkpoint receptors. Complement signaling through C3aR and C5aR expressed on effector T lymphocytes prevent the production of the cytokine interleukin-10 (IL-10). Removing C3aR/C5aR-mediated transcriptional suppression of IL-10 expression results in endogenous IL-10 production by antitumor effector T cells, which drives T cell expansion and enhances T cell-mediated antitumor immunity. Importantly, preclinical, and clinical data suggest that a signaling axis consisting of complement/C3aR/C5aR/IL-10 critically regulates T cell mediated antitumor immunity and manipulation of the pathway *ex vivo* and *in vivo* is an effective strategy for cancer immunotherapy. Furthermore, a combination of treatment strategies targeting the complement/C3aR/C5aR/IL-10 pathway with other treatment modalities may improve cancer therapeutic efficacy.

Keywords: complement, cancer immuno therapy, complement receptor C3aR, complement receptor C5aR, IL-10 (interleukin-10), PD-1 - PDL-1 axis, immune check point

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INTRODUCTION

As a major component of the innate immunity, the complement system also directly regulates lymphocyte function (1, 2). Recent studies have shed important insights to the role of complement and its receptors in antitumor immunity. Clinical observations and animal studies suggest that complement signaling inhibits antitumor immunity. It was reported that tumor or circulating

complement levels are positively correlated with tumor size and poor outcome in different types of cancers, such as neuroblastoma, colorectal, lung, ovarian cancer, chronic lymphocytic leukemia, and carcinomas of the digestive tract (3). Extensive animal studies have also demonstrated that the complement system functions to inhibit antitumor immunity (4–15). Mechanistically, complement may inhibit antitumor immunity by promoting recruitment of myeloid-derived suppressor cells (MDSCs) into the tumor microenvironment (TME) (4–6, 9, 12, 13) or by suppressing dendritic cells (DCs)/NK cell activation (7, 8). Recent studies suggest that a new mechanism plays an important role in complement signaling-mediated suppression of antitumor immunity: direct inhibition of IL-10 production in $CD8^+$ tumor infiltrating lymphocytes (TILs) in TME (11, 14). Here, we summarize relevant findings and propose that C3aR and C5aR function as a new class of immune checkpoint receptors that should be targeted for cancer immunotherapy.

COMPLEMENT SUPPRESSES ANTITUMOR IMMUNITY THROUGH C3aR AND C5aR

In addition to the many clinical reports positively correlating complement levels with tumor size and poor outcome in various human cancers [reviewed by Pio et al. (3)], animal studies testing different tumor types in different mouse models also show that the complement signaling pathway exerts potent inhibition on antitumor immunity (4–15) (**Table 1**). The tested mouse tumor models include TC-1 cervical cancer, Lewis lung cancer, RMA lymphoma, 4T1, and E0771 breast cancer, B16 melanoma, HPV16 skin cancer, and MC38 colon cancer with either transplanted tumor cells or spontaneously developed cancers. Complement signaling was disrupted in these animal studies by using genetic models including mice deficient for C3, C4, C3aR, or C5aR1 (4, 9–15) or inhibitors to complement C3, C3aR, and C5aR1 (4–6, 8, 10–12, 14). The reported results are highly consistent in that tumor growth is suppressed when complement-mediated signaling is inhibited or removed. In studying the underlying cellular mechanisms, Markiewski and colleagues first showed that C5a/C5aR1 interaction promotes the migration of MDSCs into tumors and enhances the suppressive capacity of tumor-associated MDSCs (4). The regulation of myeloid suppressor cells in tumors by complement signaling is also observed by several other studies (5, 6, 9, 12, 13). Thus, a major immune suppressive role by complement signaling may be mediated through recruitment of MDSCs into tumors. In addition to MDSCs, other innate cell populations such as neutrophils, DCs and NK cells are also involved in complement-mediated immune suppression of antitumor immunity (7, 8, 10). When complement C3 is exhausted using cobra venom factor, NK cells are greatly increased in tumors and depletion of NK cells nullifies the enhanced antitumor activity induced by cobra venom factor treatment (8). Although complement signaling modulates innate immune cell activities, the enhanced antitumor immunity exhibited in mice following disruption of complement signaling is T lymphocyte dependent. Not only effector $CD4^+$ and $CD8^+$ TILs are enhanced in these mice but also depletion of T cells through TCRα genetic deletion or antibodies against $CD4^+$ or $CD8^+$ T cells diminishes the enhanced antitumor immunity in the complement signaling deficient models (4, 8–15). These studies suggest that multiple immune suppressive mechanisms are induced by C3aR and C5aR1 signaling (**Table 1**).

C3aR AND C5aR-MEDIATED IMMUNE SUPPRESSION ON T LYMPHOCYTES

Three small cationic peptides, C3a, C4a, and C5a, generated by complement activation are termed as anaphylatoxins. These peptides induce chemotaxis, cell activation, and inflammatory signaling by binding to their respective G-protein-coupled receptors (GPCR), referred to as C3aR and C5aR1. The models for anaphylatoxins binding to their cognitive receptors have been proposed after the molecular cloning of C3aR and C5aR1 (19). In the immune system, C3aR is predominantly distributed on leukocytes of myeloid lineages, such as neutrophils, basophils, eosinophils, mast cells, monocytes/macrophages (20–23). Ligand-receptor engagement induced receptor phosphorylation leads to receptor desensitization, internalization, and activation of diverse downstream signaling pathways in different cell types. C3aR is highly expressed on neutrophils, and C3a induces calcium influx in response to C3a (24); however, C3aR inhibits neutrophil mobilization *in vivo* in an intestinal ischemia-reperfusion model (25). In mast cells, C3a activates PI3K signaling pathways and subsequent Akt-phosphorylation, as well as MAP kinases Erk1/Erk2 to promote cytokine expression (26). In human monocyte/macrophage, engagement of C3a to Ca3R, together with TLR signaling induces secretion of proinflammatory cytokines such as IL-1β, IL-6, and TNFα (27, 28). C3aR signaling modulates IL-1β secretion through NLRP3 inflammasome activation by regulating ATP efflux (29). Similar to C3aR, C5aR is abundantly expressed in neutrophils, eosinophils and basophils, monocytes/macrophages, and mast cells (30–33). C5a binding to C5aR causes calcium flux as well as activation of several components of different signaling pathways, including PI3K-γ kinase, phospholipase C, phospholipase D and Raf-1/B-Raf mediated activation of MEK-1 (34–37). In addition to a similar proinflammatory function of C3aR, C5aR1 is also a chemotactic receptor. Upon engagement with C5aR1, C5a serves as a chemoattractant for monocytes, neutrophils, eosinophils, and basophils (38).

It is well established that complement components and their receptors C3aR and C5aR1 are expressed in not only myeloid and tumor cells but also $CD4^+$ T lymphocytes (39–44). Furthermore, endogenously or locally produced C3a and C5a bind to C3aR and C5aR on $CD4^+$ T cells and regulate T cell function, such as differentiation, survival and cytokine production (40, 41, 45, 46). Interestingly, in contrast to the lack of C3aR and C5aR1 expression on peripheral $CD8^+$ T cells in naive mice, both receptors are strongly upregulated on $CD8^+$ TILs from mouse and human tumors (11). Overall, ∼20% of $CD8^+$ TILs are C3aR and C5aR double positive. To determine the source of complement that mediates immune suppression on $CD8^+$ TILs,

TABLE 1 | Mouse models on the complement/C3aR/C5aR1/IL-10 pathway in antitumor immunity.

Reference	Animal strain/Treatment	Tumor type	Phenotype
Ajona et al. (12)	PD-1/C5a double blockade	Lung cancer	Growth and metastasis inhibition
Cho et al. (16)	$C3^{-/-}$ mice, C5a silencing in tumor	Ovarian cancer	C5a recruits MDSCs to tumor microenvironment
Corrales et al. (5)	C5aR antagonist	Lung cancer	C5a recruits MDSCs to tumor microenvironment
Emmerich et al. (17)	$IL10Rb^{-/-}$ mice, IL-10 treatment	Squamous carcinoma	IL-10 promotes anti-tumor $CD8^+$ T cell response
Gunn et al. (6)	SCID mice, C5a overexpression	Lymphoma/ovarian cancer	C5a recruits MDSCs
Janelle et al. (8)	cobra venom factor treatment	Melanoma	Complement inhibits NK function
Kwak et al. (14)	$C3^{-/-}$ mice,C3aR, C5aR antagonists	lung Cancer	Complement inhibits $CD4^+$ T cell function
Markiewski et al. (4)	$C3^{-/-}$, $C4^{-/-}$, factor $B^{-/-}$, $C5aR^{-/-}$ mice	Cervical cancer	Complement recruits MDSCs to tumor
Medler et al. (15)	K14-HPV16 Tg, $C3^{-/-}$mice	Squamous cell carcinoma	C5a/C5aR regulate macrophage/mast cell
Mumm et al. (18)	$IL\text{-}10^{-/-}$, $IFNg^{-/-}$, MMTV-rtHer2 Tg mice	Squamous tumor/thymoma	IL-10 promotes $CD8^+$ T cell function
Nabizadeh et al. (10)	$C3aR^{-/-}$mice, C3aR/C5aR antagonists	melanoma, colon, breast cancer	Complement inhibits $CD4^+$ T cell and neutrophil
Qing et al. (7)	$C3^{-/-}$ and $C5aR^{-/-}$ mice	Melanoma	Complement inhibits DC-NK function through MDSCs
Vadrevu et al. (9)	$C5aR^{-/-}$ mice, C5aR antagonist	Breast cancer	Complement inhibits T cell through Treg and MDSCs
Wang et al. (11)	$C3^{-/-}$, $IL\text{-}10^{-/-}$, $TCR^{-/-}$mice, C3aR and C5aR antagonists	Melanoma/colon/breast cancer	Complement inhibits antitumor $CD8^+$ T cell by
Zha et al. (13)	$C5aR^{-/-}$ mice, PD-1 blockade and C5aR antagonist	Melanoma/colon cancer	C5a/PD-1 blockade enhances antitumor efficacy

chimeric mice with either lymphocytes or host cells lacking C3 were used as tumor-bearing hosts. C3-deletion in $CD8^+$ T cells was sufficient to remove complement-mediated suppression on antitumor immunity (11), suggesting that autocrine C3 production and the interaction of activation products with Ca3R/C5aR plays a critical role in suppressing effector $CD8^+$ TIL function.

C3aR AND C5aR SIGNALING INHIBITS IL-10 PRODUCTION IN TUMOR INFILTRATING T LYMPHOCYTES

How does autocrine complement signaling inhibit effector $CD8^+$ T cell function? Several clues suggest a possible mechanism underlying C3aR/C5aR signaling-mediated immune checkpoint function: complement signaling may inhibit IL-10 production in effector T lymphocytes given the role of IL-10 in $CD8^+$ TIL expansion and immune activating function in antitumor immunity (see Discussion in next section). First, it was shown that a fraction of $CD8^+$ effectors expresses IL-10 at the peak of coronavirus infection and the $IL\text{-}10^+CD8^+$ T cells show superior CTL activity and *in vivo* protection against chronic infection (47). Second, we found that complement pathway related genes are enriched in the $IL\text{-}10^+CD8^+$ T cells (11), suggesting a possibility of mutual or reciprocal regulation. Indeed, in $C3^{-/-}$ *Il10* reporter (Tiger) mice, $CD8^+$ TILs within B16 tumors but not peripheral blood readily express IL-10 (11). Kwak and colleagues also observed enhanced IL-10 expression in $CD4^+$ and $CD8^+$ T lymphocytes in lungs of tumor-bearing C3-deficient mice (14). Furthermore, antagonists to C3aR and C5aR1 also promote IL-10 production in $CD8^+$ TILs as well as *in vitro* activated $CD8^+$ T cells (11). Importantly, the enhanced antitumor immunity in complement-deficient mice or wildtype mice treated with antagonists to C3aR and C5aR1 depend on IL-10. Depletion of the IL-10 gene in these mice completely abolishes the enhanced antitumor immunity in both melanoma and breast cancer tumor-bearing C3-deficient mice (11). The suppression of IL-10 production in $CD8^+$ TILs is mediated through endogenously produced complement and its autocrine interaction with C3aR and C5aR on $CD8^+$ T cells. The inhibition on IL-10 production by signaling through C3aR and C5aR is redundant as antagonism to one of these receptors alone does not promote IL-10 production. Accordingly, antagonism to C3aR and C5aR1, but not to a single receptor, suppresses tumor growth and the antitumor effect depends on IL-10 *in vivo* (11). Therefore, inhibition of antitumor immunity through suppression of IL-10 production in $CD8^+$ TILs in response to complement/C3aR/C5aR signaling represents a new mechanism of complement-mediated immune supression (48).

IL-10 FUNCTIONS AS AN IMMUNE ACTIVATING CYTOKINE IN CANCER IMMUNOTHERAPY

IL-10 is a pleiotropic cytokine produced by many cell populations, including but not limited to activated T cells, B cells, macrophages as well as mast cells (49, 50). Although it was initially identified as a cofactor for thymocytes growth and T cell activation, IL-10 was further recognized as a regulatory cytokine due to its anti-inflammatory functions. IL-10 impairs the maturation of dendritic cells and macrophages by interfering with upregulation of costimulatory molecules such as CD80, CD86, MHCII, and CD83 on activated dendritic cells and macrophages (51, 52). In addition, IL-10 skews the Th1/Th2 balance to Th2 by selectively blocking IL-12 synthesis in activated dendritic cells (53). Macrophages can be polarized

to M1 (inflammatory) or M2 (anti-inflammatory) phenotypes depending on the microenvironmental stimuli. IL-10 inhibits the activation and proliferation through Stat3-dependent and -independent pathways and polarizes macrophage to a M2 like phenotype (54, 55). IL-10 directly acts on $CD4^+$ T cells to differentiate T helper cells into inducible regulatory T cells and maintain the expression of key transcription factor Foxp3 (56, 57). Regulatory T cells also express IL-10 and mice deficient for IL-10 in regulatory T cells did not display systemic autoimmunity; however, these mice developed spontaneous colitis, skin and lung hyperreactivity, suggesting an organ specific role of IL-10 on regulatory T cells (58).

Although IL-10 is often associated with an immune suppressive function, recent clinical studies have unequivocally shown that IL-10 is an immune activating cytokine promoting antitumor immunity (59–61). In a phase I clinical trial, pegylated recombinant human IL-10 (rhIL-10) has shown encouraging clinical efficacy in several types of solid tumors (59). Among the 24 patients treated with rhIL-10 monotherapy at 20–40 μg/kg active dose, the overall objective response rate is 21%. Furthermore, IL-10 treatment increases serum levels of pro-inflammatory cytokines IL-18 and IFNγ as well as FasL in cancer patients and the induced cytokine levels are strongly correlated with clinical responses (59, 61). Pegylated rhIL-10 treatment dramatically expands $PD\text{-}1^+LAG\text{-}3^+$ activated $CD8^+$ T cells in the blood of cancer patients. Importantly, both the number and effector function of $CD8^+$ TILs from these patients are increased (61). These results support that the major function of rhIL-10 is to expand the number as well as enhance the effector function of antitumor $CD8^+$ T cells

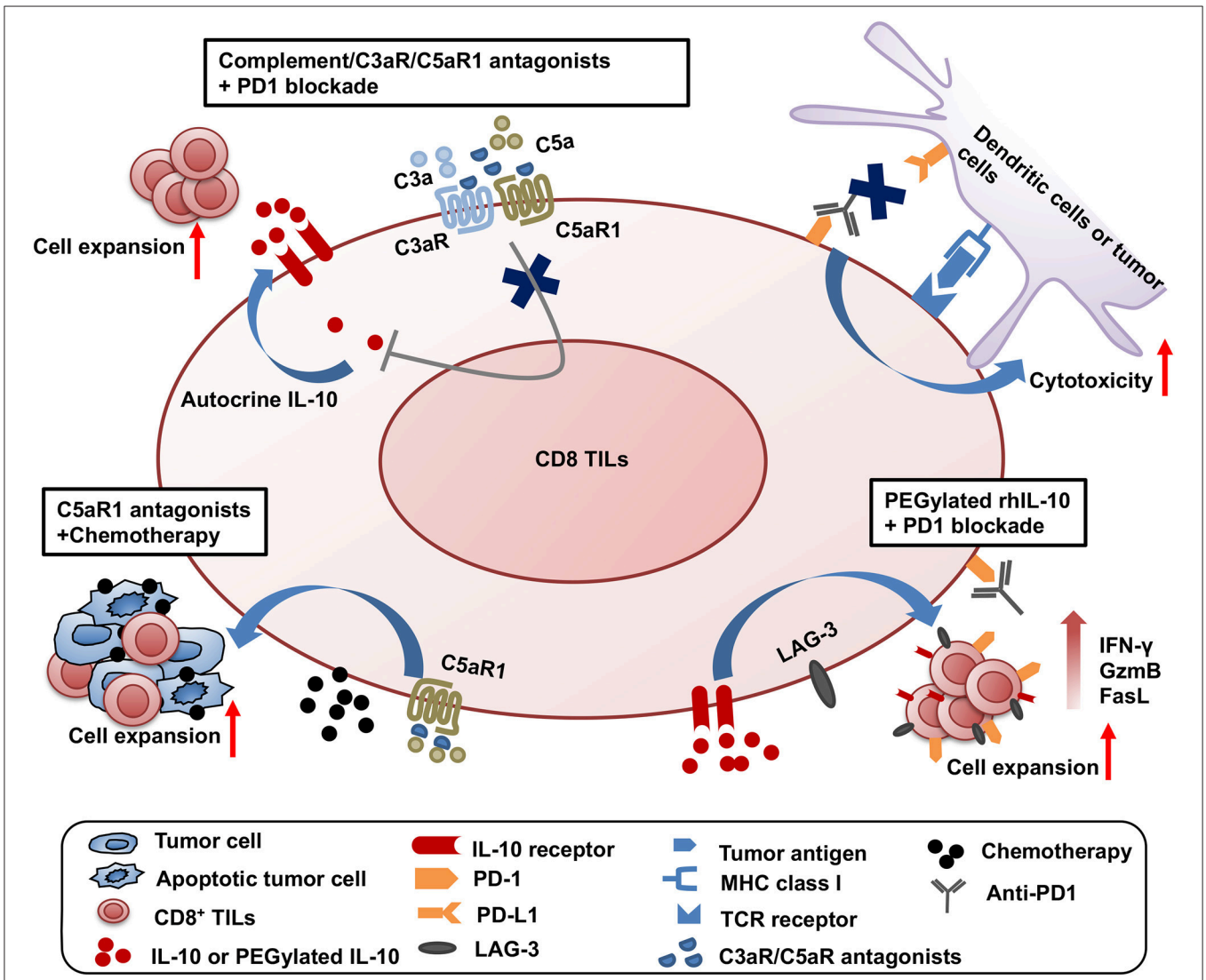

FIGURE 1 | Synergistic effect of three combined strategies by targeting complement/C3aR /C5aR1/IL-10 pathway and other treatment modalities. The three combined strategies were shown as follows: (1) dual blockade of complement signaling and immune checkpoint receptor PD-1; (2) complement signaling blockade and chemotherapy; (3) the clinical use of pegylated rhIL-10 with anti-PD-1 antibody.

in cancer patients. A Phase 3 clinical trial (NCT02923921) in patients with metastatic pancreatic cancer is being conducted based on promising efficacy data from early clinical studies. Mechanistically, checkpoint inhibition, and IL-10 treatment together enhances the number and quality of pre-existing TILs. The efficacy of PD-1/PD-L1 inhibitors is highly associated with tumor microenvironment such as TIL density, PD-1/PD-L1 expression; tumor intrinsic feature, such as tumor mutational burden, microsatellite instability; as well as gut microbiota (62). The clinical trials of pegylated recombinant human IL-10 are focused on several solid tumor types. Its efficacy on solid and blood tumor types needs to be tested clinically in the future.

Another potential application for IL-10 and C3aR/C5aR antagonists is to incorporate them into *in vitro* expansion protocols of T cells for ACT. IL-2 is the primary cytokine used in the *in vitro* expansion of TILs and gene-engineered T cells for clinical use, however, the *in vivo* efficacy of expanded T cells under the current clinical ACT protocol is often limited by their inefficient engraftment, poor persistence, and weak capability to attack tumor cells (63–68). It was shown long ago that IL-10 augments IL-2-induced proliferation and promotes CTL activity of activated $CD8^+$ T cells (69–72). Consistent with animal studies and human clinical trial data showing that IL-10 promotes $CD8^+$ TIL proliferation (11, 17, 18, 61), addition of IL-10 to *in vitro* culture of TILs from human lung cancer with IL-2 drastically enhances the quantity and quality of the expanded human TILs and upregulates genes related to several signaling pathways, such TCR signaling, Notch signaling, cell cycle and CTL killing (11). Furthermore, pegylated rhIL-10 also prevents continuous TCR-stimulation induced apoptosis of activated human T cells (61). Interestingly, remissions in lymphoma patients treated with anti-CD19 chimeric antigen receptor (CAR-T) cells are associated with high serum levels of IL-10 and IL-15 (73). These results strongly suggest that the addition of IL-10 to the IL-2-supported *in vitro* T cell expansion protocol may improve the clinical efficacy of adoptive T cell therapy. In addition to IL-10, antagonists to C3aR/C5aR1 may also be used in such protocol as the *in vitro* culture of activated $CD8^+$ T cells in the presence of C3aR/C5aR1 antagonists induces IL-10 production (11).

SYNERGISTIC EFFECT BY TARGETING COMPLEMENT/C3aR/C5aR/IL-10 PATHWAY AND OTHER TREATMENT MODALITIES

Significant progress has been made on testing the synergistic effect of combined treatment targeting the complement/C3aR/C5aR/IL-10 pathway and other cancer treatment modalities. The first combined strategy is dual blockade of complement signaling and immune checkpoint receptor PD-1 (**Figure 1**). The complement signaling/IL-10 pathway is independent of the PD-1/PD-L1 pathway as modulation of this pathway does not affect the expression of PD-1 on T cells and PD-L1 on tumor cells (11). Two different experimental systems in which (1) PD-L1-silenced B16F10 tumors were inoculated in C3-deficient mice or (2) B16F10 tumor-bearing wildtype mice were treated with anti-PD1 and antagonists to C3aR/C5aR clearly show that blockade of complement signaling and PD-1/PD-L1 interaction has dramatic synergistic antitumor effect (11). This synergistic antitumor effect is subsequently confirmed by two other studies (12, 13). These data provide important clues to rational design of future clinical trials.

The second combined treatment strategy uses complement signaling blockade and chemotherapy (**Figure 1**). In a squamous cell carcinoma (SCC) model, antagonist to C5aR1 enhances the treatment efficacy of paclitaxel chemotherapy and the synergistic effect depends on $CD8^+$ T lymphocytes (15). Increased $CD8^+$ TILs and the expansion of specific T cell clones were associated with enhanced efficacy (15).

The third combination is the clinical use of pegylated rhIL-10 with the anti-PD-1 antibody pembrolizumab in a cohort of heavily pretreated patients with melanoma, no-squamous cell lung cancer or renal cell carcinoma (61) **(Figure 1)**. This combination achieved a 42% objective response rate, in contrast to the 21% objective response rate by pegylated rhIL-10 monotherapy (59). A combination of pegylated rhIL-10 with anti-PD-1 promotes persistent proliferation and expansion of $LAG\text{-}3^+PD\text{-}1^+$ $CD8^+$ T cells in the cancer patients. These exciting clinical trial results have opened new avenues for effective cancer immunotherapy.

In summary, we have identified that tumor infiltrating $CD8^+$ T cells express complement receptors C3aR and C5aR and complement signaling inhibits anti-tumor functions through repression of endogenous IL-10 production in $CD8^+$ TILs. We and other groups have also confirmed that endogenous and exogenous IL-10 enhances anti-tumor functions of $CD8^+$ T cells in human and mouse *in vitro* and *in vivo*. The independence of complement/C3aR/C5aR/IL-10 from the PD-1/PD-L1 signaling pathway makes it possible to block complement receptors and PD-1/PD-L1 as a combined therapy to treat cancer patients clinically. Results from other groups also suggest that the combined blockade of complement and PD-1/PD-L1 signaling with antibodies improves the efficacy of treatment through other mechanisms. Together, we and other groups provide clear evidences that complement receptors C3aR and C5aR are a new class of immune checkpoint receptors.

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