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Context-dependent roles of complement in cancers

Lubka T. Roumenina^{*,#,1}, Marie V. Daugan^{*,1}, Florent Petitprez^{1,2}, Catherine Sautès-Fridman¹
and Wolf Herman Fridman^{#,1}

¹ INSERM, UMR_S 1138, Centre de Recherche des Cordeliers, Sorbonne Universités, Université de Paris, F-75006, Paris, France

² Programme Cartes d'Identité des Tumeurs, Ligue Nationale Contre le Cancer, F-75013, Paris, France

* these authors contributed equally to the study.

Correspondence to:

Lubka T. Roumenina, Ph.D.

Cordeliers Research Center, INSERM UMRS 1138;

15 rue de l'Ecole de Medecine; 75006 Paris, France

Phone: 33-1-44-27-90-96/ Fax: 33-1-40-51-04-20,

e-mail: lubka.roumenina@crc.jussieu.fr

and

Wolf H. Fridman, PhD, MD.

Cordeliers Research Center, INSERM UMRS 1138;

15 rue de l'Ecole de Medecine; 75006 Paris, France

Phone: 33-1-44-27-91-02/ Fax: 33-1-40-51-04-20,

e-mail: herve.fridman@crc.jussieu.fr

Abstract

The tumor microenvironment (TME) highly influences the growth, spreading of tumors and therefore patient's clinical outcome. In this context, complement system plays a major and complex role. It may either kill antibody-coated tumor cells or support local inflammation, hamper anti-tumor T cell responses favoring cancer spreading. Recent studies demonstrate that these opposite effects depend of the sites of its activation, the composition of the TME and the tumor cell sensitivity to complement attack. In this review, we present the implication of complement activation and its effects on cancer control and clinical outcome in different TME contexts. We also provide an overview of the publicly available transcriptomic data on the prognostic value of complement genes expression in 30 cancer types. We argue that the interplay of complement within each cancer is unique, governed by the properties of the tumor cells and the TME. This concept is of critical importance for the design of efficient therapeutic strategies.

Introduction

The interactions of malignant cells with supporting and reactive non-transformed host cells are orchestrated by the density, location and functional activity of the latter and by soluble mediators, released in the tumor microenvironment (TME)¹. Frequently forgotten elements of the TME are the components of the complement system, produced by the tumor and infiltrating cells or coming from the circulation². Complement is a key player in the innate immune defense against pathogens and in the maintenance of host homeostasis. It is composed of more than 50 plasma components produced mainly by the liver and released in the circulation or expressed on the cell membrane. They interact with each other in a cascade manner in the extracellular space³. Recent discoveries made clear that complement effectors can be generated also intracellularly and that complement proteins have non-canonical functions, independent of the cascade^{4, 5}. Cumulating evidences over the years have proven that complement proteins are present in the TME and that malignant and infiltrating cells gain capacity to produce in situ a large spectrum of these components⁶. Their functionality and level of expression by malignant cells or in the TME modulate the fate of the tumor. In cancer, the impact of complement is versatile, ranging from anti-tumor defense to potent tumor promotion. The data in the literature, mostly focused on animal models and in vitro studies, yield heterogeneous and sometimes contradictory conclusions. Analyses of human cancers are scarce and it is still unclear whether complement is overactivated or, on the contrary, inhibited in cancer patients. This review presents the high diversity of actions of complement components in cancer and the heterogeneity of their production and activation pathways. Using data in human cancers and in mouse models, mechanisms of tumor control and tumor promotion are discussed. We compare the expression of complement genes and their clinical impact in different cancers, using publicly available databases and show the context-dependent impacts of complement in different cancers. Finally, we argue that the most appropriate therapeutic approaches to activate or neutralize complement are dependent on the tumor context and are difficultly transposable from one cancer to another.

The complement system

Complement is a central part of the immunity that serves as a first line of defense against pathogens and stressed host cells³. The complement system is composed of plasma proteins that react with one another to opsonize pathogens, inducing a series of inflammatory responses that concomitantly help the immune cells to fight against infections and to maintain homeostasis². The complement cascade can be initiated, depending on the context and the location (Figure 1A).

Conventional complement activation pathways: Historically, complement was considered to be initiated by three distinct pathways – classical, lectin and alternative. Immune complexes and apoptotic cells activate the classical pathway, after recognition of the target molecules by C1q. The lectin pathway is triggered after recognition of sugar motives, foreign for a

healthy tissue. The alternative pathway is permanently active at low grade, serving as a sentinel to attack any surface, which is not specifically protected.

Each of these pathways leads, through a sequence of conformational changes and enzymatic reactions, to the cleavage of the central component C3 into bioactive fragments C3a and C3b, followed by generation of C5a and the C5b-9 membrane attack complex (MAC)^{2, 7}. The anaphylatoxins C3a and C5a, the opsonizing C3 activation fragments and the MAC are the canonical effectors of the complement system. C3a and C5a bind to their receptors C3aR and C5aR and play a critical role in inducing inflammation and activation of immune cells as well as endothelial, epithelial cells, fibroblasts and certain malignant cells, which express the anaphylatoxin receptors³. In support of inflammation, anaphylatoxins induce oxidative burst on macrophages, eosinophils and neutrophils. Moreover, C3a and C5a induce histamine release by basophils and mast cells to provoke vasodilatation. In a physiological context these events contribute to the acute inflammation and eradication of the pathogen. In the context of cancer, complement anaphylatoxins are constantly generated and in the majority of the studied models lead to tumor-promoting chronic inflammation⁶.

MAC assembly creates a transmembrane pore that causes prompt osmotic lysis of certain bacteria and metabolically inert targets (erythrocytes, lysosomes). Nucleated host cells resist lytic killing by MAC due to high expression of membrane regulators. Nevertheless, when formed, C5b-9 complex can have profound effects on cell functions, leading to activation and adaptation or cell death depending on the context⁸. To avoid accidental healthy host tissue damage, complement is a tightly regulated cascade, constantly kept in check. However, cancer cells adapt escape mechanisms for MAC. Hsp90 protected tumor cells from complement-dependent cytotoxicity by inhibiting, together with mortalin, C5b-9 assembly and/or stability at the plasma membrane⁹.

Considering these effects of complement on cell activation and survival as well as on the modulation of the entire immune system, it is not surprising that the tumors are evolving to adapt to its presence and to subvert it for their benefit.

Non-canonical and intracellular complement initiation: Complement could also be activated by an unconventional, convertases-independent pathway, by enzymes cleaving C3 and C5¹⁰⁻¹⁴. Although generated by non-canonical mechanisms, these C3a and C5a as well as C3b and C5b are often identical in sequence with the convertase-generated anaphylatoxins. They are, therefore, canonical effectors, but generated in a non-canonical manner. This cleavage could occur in the circulation, within the tissues but also intracellularly. Interestingly, thrombin can cleave C5 at a different site, generating even more potent equivalent of C5b, with higher lytic activity¹².

Complement activation inside the cells has been described in T-cells and exerts homeostatic and immunological functions^{15, 16}. This intracellular complement system is referred to as a complosome¹⁷. In T-cells C3 is cleaved by cathepsin L, generating C3a and C3b¹⁸. Again in T cells (Th1), intracellular C5 activation occurs but the mechanism is still not fully defined¹⁹.

Intratumoral initiation of the complement cascade

Despite the strong evidences of complement activation in human tumors and mouse models, only few studies addressed the pathway, by which these anaphylatoxins are generated.

Canonical mechanisms of initiation: Lung cancer is probably the best characterized model for classical pathway activation in mouse tumors. Indeed, in the seminal paper describing the pro-tumoral role of complement, a lung epithelial cell line was used (TC-1) and the complement activation was nailed down to a C4-dependent classical or lectin pathways²⁰. Recently, we found that it was the classical pathway that was activated in this model²¹. In other lung cancer mouse models, the classical pathway was also activated, likely by intratumoral immunoglobulins²². Although not necessarily implicated in the complement initiation, the alternative pathway can amplify the C3 activation fragments deposits, perpetuating thus intratumoral complement activation. Little evidence is available for the implication of the lectin pathway^{23, 24}.

Non-canonical initiation: C3 and C5 can be cleaved also by complement cascade-independent proteases, bypassing thus the initiating recognition events in cancer models¹²⁻¹⁴. C5 is cleaved by macrophages-derived urokinase in mouse models of squamous carcinogenesis, leading to C3-independent release of C5a¹³. Using C5-producing tumor cell lines, it was shown that C5a can be generated by a still not identified cell membrane-bound serine protease¹⁴. Thrombin is produced in tumors and has potent pro-tumoral activity²⁵. Therefore, it is tempting to speculate that the cleavage of C5 will be yet another mechanism of its pro-tumoral activity in situ.

Certain tumor cells contain intracellular pools of C3 and C5. Although currently poorly studied, we postulate that the discoveries for the intracellular cleavage of C3 and C5 made for the T cells^{18, 19} are not restricted to this population and that the intracellular generation of C3a and C5a may have a major role in for the biology of the tumor cells, and the non-immune constituents of the TME, such as endothelial cell and fibroblasts.

Complement effectors and immune contexture of the tumor

Canonical functions of the complement effectors on the immune TME. The immune contexture of the tumor, which is determined by the density, composition, functional state and organization of the leukocyte infiltrate, is a key determinant of the tumor progression¹. Complement receptors are expressed on the immune cells. C3a and C5a promote leucocyte attraction and impact their phenotype (Figure 2). After the discovery that C5a recruits MDSC to the tumor microenvironment and elicit them to suppress the effector T cells²⁰, it was found that C3a and/or C5a exert a profoundly influence on the TME by inducing a series of context-dependent functions, including: 1) recruitment of tumor-promoting macrophages and CCL2 production²⁶, 2) decrease of CD4+ T-cells and neutrophils recruitment²⁷, 3) decrease of NK cells recruitment²⁸, 4) stimulation of a pro-tumoral phenotype for CD4+ T-

cells²², 5) inhibition of IL10 expression in intratumoral CD8+ T-cells²⁹, 6) stimulation of the protumorigenic properties of mast cells and macrophages, including suppression of CD8+ T cell cytotoxicity¹³, 7) promotion of pro-tumoral NETosis by neutrophils³⁰, etc.

The level of intratumoral C5a may be a key determinant for the composition of the immune TME. Mouse lymphoma producing low level of C5a grow slower in mice and have increased IFN- γ -producing T-cells in spleen and tumor-draining lymph nodes³¹. Conversely, tumor-bearing mice with high C5a-producing cells had accelerated tumor progression with more Gr-1+CD11b+ myeloid cells in spleen and overall decreased T-cells in tumor, tumor-draining lymph nodes, and spleen.

The C3 activation fragments are potent effectors, modulating the immune response³. Chemo- as well as immuno-therapeutical approaches induce apoptosis in tumor cells. Opsonization of apoptotic tumor cells with iC3b prevents the maturation of the dendritic cells via interaction with CR3 and contribute to the induction of antigen-specific silencing and tolerance³². Moreover, iC3b-CR3 interaction results in dysregulation of NK-dependent tumor surveillance³³.

Even though the majority of the experimental models agree on the pro-tumoral role of C3a and especially C5a, the mechanisms described above are context-specific. Rarely the same mode of action of the anaphylatoxins is found in different cancer models. This could reflect the differences in the composition of the immune microenvironment of every cancer. Indeed, in mice, as in humans, the immune infiltration is largely controlled by the properties of the tumor cells themselves^{1, 34}. Whereas a large body of data concerning the impact of complement in different murine models and in vitro are available, those in human cancers remain scarce.

Non-canonical impact of complement components on immune cells. By their non-canonical functions, complement components modulate the fundamental processes of immune cells, including immune cells proliferation, migration, metabolism, and even transcriptional activity³⁵⁻³⁷. Studies on human T cells show unconventional intracellular C3 cleavage by cathepsin L¹⁸. This “tonic” intracellular C3a is required for homeostatic T cell survival. At least in part this C3 was internalized as C3(H2O) from the extracellular milieu³⁸. The cleavage of C3 by cathepsin L is species-specific and does not operate in mice¹⁸. This should be taken into account if this mechanism is studied in animal models of cancer. Again in human T cells (Th1), intracellular C5 activation and stimulation of intracellular C5aR1 results in the assembly of the NLRP3 inflammasome, needed for the optimal production of IFN- γ ¹⁹. Moreover, novel functions of intracellular C3 emerge, such as its implication in immune cells gene transcription³⁷ or regulation of autophagy³⁹. C1q was shown to modulate CD8+ T cell metabolism in the context of autoimmunity and viral infections³⁶. All these processes have not been studied in the context of cancer yet. Nevertheless, it is tempting to speculate that the non-canonical functions of complement will shape the immune TME and will play a key role in anti-tumor immunity.

Neovascularization and complement

Neovascularization is critical for the oxygen and nutrients supply to the tumor. Complement contributes to this process via its canonical effectors C3a and C5a as well as by non-canonical, cascade-independent effects of the individual components (Figure 3A)

Mode of action of the canonical effectors: It has been known for long that C5a promotes migration and tube formation of endothelial cells *in vitro*^{40, 41}. In addition, C3^{-/-} and C5aR^{-/-} endothelial cell have impaired angiogenesis capacity⁴². Nevertheless, the impact of C3a and C5a in mouse tumors seems to be model-dependent. C3^{-/-}, C3aR^{-/-} or C5aR^{-/-} showed either impaired tumor angiogenesis^{13, 42}, increased blood vessel permeability without effect on microvascular density⁴³ or no impact⁴¹. The context-dependent impact of the anaphylatoxins on neovascularization requires further investigation.

Mode of action of the non-canonical effectors: Recent evidences point towards a major role of C1q in cancer neovascularization via non-canonical, cascade-independent mechanism. A fraction of tumor vessels endothelial cells produce C1q in mouse models and in human tumors^{21, 44}. The microvascular density was either decreased or the vascular network was disorganized in tumors, growing in C1q^{-/-} mice^{21, 44}. This could be explained by alteration of the C1q-mediated expression of VEGFs and VEGFRs, as shown in tumor models²¹ and in studies of pregnancy complications of these mice⁴⁵. The pro-angiogenic effect of C1q is again context-dependent, since neovascularization was enhanced in neuT-C1q^{-/-} breast cancer model⁴⁶.

Although mouse models provide insights for the role of complement cascade and its individual components in tumor neovascularization, most of the data are generated with subcutaneously implanted tumor cell lines. Endothelial cells from different organs have unique properties, including different spectrum of complement proteins expression^{47, 48}, which may differentially impact the neovascularization in the cancer types. Therefore, further studies are needed to determine the relative impact of complement in neovascularization of human tumors.

Direct impact of complement effectors on tumor cell biology

Impact of the canonical effectors: In addition to promoting inflammation, C3a and C5a could affect the fundamental processes of the tumor cells, such as survival, proliferation, migration, stemness, etc (Figure 3B,C). Anaphylatoxin receptors are expressed on certain cancer cells⁶. Multiple reports show that these cells express also C3 and/or C5 and generate C3a and C5a, acting in an autocrine manner. The impact of the signaling is related to stimulation of proliferation^{49, 50}, multipotent state of glioblastoma glioma stem-like cells⁵¹, the epithelial to mesenchymal transition^{52, 53}, invasiveness and morphology alteration⁵⁴, stemness etc. For example C3a enhanced cell proliferation, migration and stemness in cutaneous squamous cell carcinoma and this activity was correlated with activation of the Wnt and β -catenin pathway⁵⁵. If the cascade proceeds to the terminal MAC formation, it was

shown that the sublytic levels of C5b-9 mediate signaling, promoting cancer cell cycle progression⁵⁶. Cancer cells-derived C3a also adapts the cerebrospinal fluid for leptomeningeal metastasis by activation of C3aR on the choroid plexus epithelium, disrupting thus the blood-cerebrospinal fluid barrier⁵⁷. The role of complement for cancer metastases is reviewed elsewhere⁵⁸.

Complement cascade may lead to tumor cell killing if sufficiently strongly activated by host anti-tumoral IgM or IgG or by therapeutic antibodies and if abundant MACs are inserted into the cell membrane. In the context of cancer, little evidence suggests that complement can proceed to cell-killing MAC without treatment with targeted therapeutic (like tumor-cell targeting monoclonal antibodies⁵⁹). This escape from complement killing is in part linked to a high expression of complement regulators at tumor cell surface.

Non-canonical functions of the complement proteins: Many complement components, such as C1q, C1s, C3, properdin, FH, FI, etc have non-canonical, extracellular and intracellular functions, modulating the fundamental processes of the tumor cell in selected models, promoting proliferation and tumor progression when tested in animal models^{44, 60-62}. In embryonic development and in cancer models, intracellular C3 impacts epithelial to mesenchymal transition⁵². Properdin, which is the only positive regulator of the cascade, has here also opposite non-canonical functions, compared to the majority of the other tested complement proteins. It suppresses breast cancer cell growth by control of transcription⁶³. C1q could exert anti-tumoral effects by activation of tumor suppressor WWOX to induce apoptosis in prostate and breast cancer cells^{46, 64}. WWOX is only weakly expressed in the majority of the tumor types, suggesting that this effect of C1q will be context-dependent, relevant particularly of the hormone-regulated tissues (breast, ovary, prostate, etc).

Complement components and receptors in human tumors

The potential roles of complement as a part of the interplay between malignant cells and the TME in human cancer begins to unveiled. Although quite dispersed, literature accumulates to show that tumors develop in a complement rich milieu. Many of the cells, present in the TME, produce complement components and/or bear complement receptors and complement regulators⁶⁵, suggesting a potential *in situ* activation of the complement pathways (Figure 1B). In a physiological context the complement components produced by the immune cells regulate the fundamental processes of the cells and help to fight infection. Nevertheless, in the context of the tumor, the malignant cells may produce various complement components, resulting in a disturbed complement milieu, impacting local complement activation. From the analysis of the literature, two striking observations can be made. The first one is the presence of complement receptors, particularly C3aR and C5aR on most of the cell types in the TME, suggesting that the activity and functions T and B lymphocytes, neutrophils, macrophages, MDSCs, DCs, endothelial cells and fibroblasts can be modulated by the activation fragments of C3 and C5. The second is the presence at high levels of complement regulators, capable to inhibit complement activation, particularly the

terminal pathway, on malignant cells. This is an underscored mechanism of escape of the tumor cells from the attack of complement, which can add up to the immune escape mechanisms that could guide cancer Immunotherapy^{66, 67}. This may explain also why intratumoral C5b-9 staining has not been frequently reported⁶⁸. Unfortunately, only very limited number of studies address the expression and activation of complement in large cohorts of patients with different cancers. This problem can be at least in part resolved now, thanks to the data mining and bioinformatics analyses of The Cancer Genome Atlas (TCGA) database and the pathology atlas of the human cancer transcriptome (protein atlas)^{69, 70}.

Expression of the complement genes in human cancers

To draw an overall picture of the impact of complement in different cancers, we compared the gene expression levels and the prognostic impact of the main complement components in 29 tumor types, using publicly available datasets^{69, 70}. Figure 4 shows a non-supervised hierarchical clustering of the expression of 50 complement-related genes in solid tumors, encompassing 30 cancers. Several conclusions can be drawn from this analysis. Overall, there is a strong heterogeneity in expression among genes but, surprisingly, not that much between cancer types. The gene encoding C3, the pivotal complement component, is expressed in all cancer types together with genes of the components of the classical pathway (*C1QA*, *C1QB*, *C1QC*, *C1R*, *C1S*, *C4A* and *C2*). In contrast, genes for the components of the lectin pathway are poorly expressed in all tumor types (*MBL2*, *MASP2*, *FCN2*) or heterogeneous with poor expression in the majority of the cancers (*MASP1*, *FCN1*, *FCN3*), arguing against a major implication of this pathway in *in situ* activation of the complement cascade. As for the alternative pathway, *CFB* and *CFD* are heterogeneously expressed with a particular low expression in kidney chromophobe (KICH), uveal melanoma (UVM), prostate adenocarcinoma (PRAD). The remaining tumors exhibit a higher expression of *CFB* and *CFD*. This, together with the high local expression of *C3*, suggests that complement could be activated via the classical or the alternative pathway.

A striking feature is the very low expression of *C6*, *C8A*, *C8B* and *C9* genes (with the exception of cholangiocarcinoma (CHOL, bile duct cancer), which suggests that terminal pathway is unlikely to be activated via *in situ* produced components. Moreover, the genes encoding complement regulators acting at the level of C1 (*SERPING1*, C1 inhibitor) and at the level of the C3 convertases, (*CFH*, *CFI*, *CD46* and *CD55*) are highly expressed in most cancers. Moreover, the terminal pathway regulator *CD59* is among the highest expressed complement genes in all studied tumors, suggesting efficient protection of the malignant cells from complement-mediated killing. This pattern of gene expression is perfectly in line with the examples from the literature, demonstrating high expression of these regulators in different types of cancer^{68, 71-76}. Complement-mediated cytotoxicity may act as a selective pressure for tumor overexpression of complement regulators. Indeed, hypoxic tumor cells are resistant to complement-mediated cytotoxicity due, in part, to hypoxia-induced expression of complement regulator CD55 in colorectal cancer⁷⁷. This is again context-dependent, since in an NSCLC model, on the contrary, hypoxia decreased the regulators expression and increased susceptibility of the tumor cells to complement attack *in vitro*⁷⁸.

The low levels of terminal pathway genes expression together with the high expression of complement regulators reinforce the hypothesis that malignant cells evolve and adapt to avoid potentially killing MAC formation. Instead, intratumoral complement activation can be sustained via locally expressed classical and alternative pathway components, generating thus the largely pro-tumoral anaphylatoxins C3a and C5a.

The prognostic impact of the complement expression in cancer patients

Further, we evaluated the impact of the expression of genes encoding components of the classical and alternative pathways on overall survival of patients with different malignancies, utilizing data available in the TCGA (Figure 5). Four groups of cancers could be defined. The first contains tumor types, for which the overexpression of the genes of the components of the classical and alternative pathway are associated with good prognosis. This group of cancers with “protective complement” involves prostate adenocarcinoma (PRAD), mesothelioma (MESO), sarcoma (SARC) and skin cutaneous melanoma (SKCM). A second group in which C3 expression correlates with longer overall survival (OS) comprises kidney chromophobe (KICH), adrenocortical carcinoma (ACC) and thyroid cancer (THCA), although significance is not reached in the latter. The third group contains cancers in which high expression of classical and alternative pathway genes correlates with poor prognosis. This group of “aggressive complement” tumors includes uveal melanoma (UVM), low grade glioma (LGG), glioblastoma (GBM), kidney clear cell renal cell cancer (KIRC), lung squamous cell carcinoma (LUSC) as most significantly impacted types. It includes also digestive tract cancers such as: rectum adenocarcinoma (READ), colon adenocarcinoma (COAD) and stomach adenocarcinoma (STAD), as well as uterine cancers, like: uterine corpus endometrial carcinoma (UCEC) and uterine carcinosarcoma (UCS), for which significance was not reached for most of the genes. The fourth group encompasses a large number of tumor types in which the gene expression analysis did not reveal any robust clinical impact, named therefore of “uncertain significance”. It comprises cholangiocarcinoma (CHOL), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), breast invasive carcinoma (BRCA), pancreatic adenocarcinoma (PAAD), lung adenocarcinoma (LUAD), urothelial bladder carcinoma (BLCA), head-neck squamous cell carcinoma (HNSC), ovarian cancer (OV), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC) and thymoma (THYM).

Cancers with “protective complement”: It is striking that within this group, the density of B cells and B cell transcriptomic signatures were associated with longer survival and response to immunotherapy with immune check point blockers in melanoma (Jennifer Wargo, unpublished results) and in soft tissue sarcoma (WHF, unpublished results). Therefore, it is tempting to speculate that in these cases complement is involved in the beneficial effect of B lymphocytes. Indeed, early studies from the 60s have already noticed a potential link between the presence of anti-sarcoma antibodies and anti-tumoral effect of complement in mice⁷⁹ and complement-fixing antibodies were detected in sera from sarcoma patients⁸⁰. High expression of C5, produced mainly by the tumoral cells, correlated with better event-

free and overall survival Ewing's sarcoma⁸¹ (not included in the TCGA dataset). In these cases, C5aR was mainly detected on tumor cells in situ.

Despite the abundant set of data about complement in mice grafted with melanoma cell lines (yielding often contradictory conclusions^{27-29, 44}), little is known about the complement activation in human melanoma and its association with clinical parameters in patients' cohorts.

Very limited data is available for complement and prostate cancer. Sublytic complement C5b-9 protects prostate cancer cells from tumour necrosis factor- α -induced cell death⁸². Interestingly, proteolysis of iC3b and C5 by the serine protease prostate-specific antigen in prostatic fluid was detected, inhibiting the terminal pathway⁸³.

The mesothelioma (MESO) presents an interesting case. The analysis presented above includes only 82 patients with heart, mediastinum and pleural MESO and shows overall tendency of good prognosis associated with classical and alternative complement genes. Experimental evidences are available only for pleural MESO. Evaluation of C4d and C1q staining in a malignant pleural MESO cohort revealed absence of C1q staining in the majority of the tumors (only few positive infiltrating immune cells) and absence of C4d deposits on malignant cells⁸⁴. Membranous C4d deposits were found only in tertiary lymphoid structures and this staining was associated with bad prognosis. Patients with low C4d plasmatic levels at diagnosis had a significantly better overall survival. In another cohort, C1q staining of tumor and infiltrating myeloid cells was strong⁸⁵. In this context C1q was shown to bind to hyaluronic acid and promote cell adhesion and proliferation in complement cascade-independent manner.

Further studies are needed to clarify the potential link between complement and cancer progression in the group of "protective complement" in order to provide experimental validation whether the concerted expression of these proteins occurs indeed in situ and whether complement is indeed activated. A staining of large cohorts of patients for C1q, C4d, C3d and C5b-9 is necessary to determine the real impact of complement for cancer progression and prognosis in these groups of patients.

Cancers with "protective C3": In thyroid cancer (THCA) cellular deposits of IgG and complement factors C3d, C4d, and C5 were shown in up to 80% of the cases, but data for prognostic impact was not reported⁸⁶. Since these cancers are of particularly good prognosis, this may go in line with the lack of prognostic impact of complement in this cancer. No data from the literature was available to verify *in situ* the impact of complement in KICH and ACC, for which the high expression of C3 gene is associated with particularly good prognosis. In these cancers the protective role of C3 could potentially be driven by non-canonical functions.

Cancers with "aggressive complement": Our analysis reveals a particularly strong negative prognostic impact of the complement genes in uveal melanoma (UVM). A high expression

level of complement regulators has been reported for this cancer⁸⁷ but no data are available for the status of complement activation *in situ*. Further studies are warren to test whether indeed in UVM complement expression/deposits may impact prognosis and to evaluate whether patients with this cancer may benefit from complement blocking therapy.

We suggest to classify gliomas as cancers with “aggressive complement”. The complement components C1QA, C1S, C2 and C7 were found upregulated in high risk relative to low risk glioma (GBM and LGG) patients⁸⁸. The activation of B cells around high-risk gliomas is also likely, as indicated by the enrichment of a B cells related gene set, over-expressed in high risk compared to low risk glioma. Other studies also suggest that complement is activated in GBM, but the deleterious impact remains to be proven by *in situ* analyses^{51, 89}.

To date the best examples of “aggressive complement” tumors, for which genes expression and bioinformatics analyses corroborate with assessment at protein level *in situ* are KIRC and non-small cell lung cancer (NSCLC). In both cases all components for classical pathway activation could be produced intratumorally and classical pathway activation, detected by presence of C1q and C4d-positive deposits was associated with poor prognosis^{21, 90}.

The classical pathway requires a trigger. C1q can bind over 100 different targets, but the major ones are the IgG and IgM-containing immune complexes². Indeed, IgM and IgG antibodies have been detected in several tumor types such as in NSCLC⁹¹, ovarian^{92, 93}, KIRC²¹ and breast⁹⁴ cancers. These antibodies may come from the circulation or be produced at the tumor site^{91, 93} potentially initiating the complement cascade. Indeed, in KIRC²¹ and in NSCLC⁹⁰, C1q-mediated classical pathway activation was detected, leading to C4d deposits. In both cases high level of intratumoral C4d staining was associated with poor prognosis^{21, 90}. Also, C4d in plasma was increased in NSCLC patients, associated with poor prognosis^{90, 95}. Data in the literature do not present separately the two subtypes of NSCLC: lung squamous cell (LUSC) and lung adenocarcinoma (LUAD). Although LUSC falls in the “aggressive complement” group, for LUAD the significance of complement is uncertain.

The assessment of complement genes expression in different tumors strongly suggest that when complement is activated *in situ*, this happens thanks to locally produced complement proteins. Data are lacking for the majority of the cancer types, but within the group of tumors with “aggressive complement”, we evaluated the complement status at protein level in KIRC patients²¹. We found that there is an *in situ* orchestrated production of C1q by tumor-associated macrophages (TAMs) and C1r, C1s, C4 and C3 by tumor cells, concomitant with IgG deposits. This allowed C1 complex assembly and complement activation. Interestingly, what conferred poor prognosis in KIRC patients was the presence of intratumoral C1q producing TAM, as well as the concomitant local production and deposition of C4 activation fragments on the tumor cells. A surprising finding was that the local production of C3 by tumor cells conferred poor prognosis, while C3d-positive deposits were not associated with prognostic impact. This, together with the clear negative impact of C3 in different mouse models, it is important to evaluate the spectrum of deposits of C3 activation fragments in human tumors. Indeed, the used antibodies did not allow to determine the exact nature of the fragments, the anti-C3d recognizing C3, C3b, iC3b as well as the C3dg and C3d fragments, each having different function. In another cohort, though,

the C5a-positive staining (marking indistinguishably the presence of the anaphylatoxin C5a and the intact C5) and the presence of C5aR+ staining were correlated with poor prognosis^{96, 97}. The malignant cells in KIRC, therefore, hijack macrophage-produced C1q to complement the remaining components made by the cancer cell, to promote tumor growth. The proposed mechanism of action in tumors with “aggressive complement” is depicted in Figure 6, based mainly on the data from KIRC and NSCLC, as well as on mouse and *in vitro* data. Further studies are needed to evaluate to what extent this mode of action of complement is valid for other cancers.

Cancers with “complement of uncertain significance”: This group encompasses a large number of cancers, for which either sporadic significance for the prognostic impact was detected or no significance at all, using the median cutoff. Interestingly, in pancreatic adenocarcinoma (PAAD) tumor cells release exosomes, which harbor B cell targets and bind anti-tumoral IgG to exert decoy function against potential complement-mediated cytotoxicity⁹⁸. This could explain why, despite the high expression of classical and alternative pathway genes and proteins⁹⁹ in PAAD, they do not seem to be associated with prognosis. Complement may not be efficiently activated *in situ*, when IgGs are subverted from the tumor cells and targeted to exosomes.

Cascade-independent impact of complement in human cancers

As evidenced in *in vitro* and mouse models, most of the complement proteins have functions outside the cascade. They can operate alone and/or in parallel with the cascade in any of the tumor groups defined here. An indication for such phenomena could be if one gene or expressed/deposited protein is associated with good or poor prognosis, while the remaining members of the same pathway are not. Another indication could be if the presence of the protein within the cell is associated with a prognostic impact, while its deposits are not.

This could be illustrated with the case of C1q-producing TAM in KIRC. We found that they were a robust marker for poor prognosis in three independent cohorts. Since C1q was produced by M2-like subtype of TAM, the question arose whether this negative impact illustrates the well-known pro-tumoral impact of the immunosuppressive and pro-angiogenic M2¹⁰⁰⁻¹⁰², C1q being an additional biomarker for this population or whether C1q plays a role in the balance between TAM phenotypes, influencing their mode of action. Interestingly, a recent in-depth immune profiling revealed that TAMs in KIRC (but likely in other cancers as well) represent a heterogeneous cell population with a subset called M-5 being associated with T-cell exhaustion¹⁰³. TAMs of the M-5 subset express higher levels of C1q genes as well as C1q receptors and C3aR, making them responsive to C1q and C3a²¹. In addition, they overexpress PD-L2. Both M-5 and C1q⁺ TAM were associated with T-cell exhaustion. Since immunosuppressive action of C1q was already described for T-cells^{36, 104, 105}, it is tempting to speculate that M-5 macrophages exert their immunosuppressive activity at least in part via C1q²¹.

Warnings on the use of transcriptomic data for complement genes to predict patients' outcome

Gene expression analyses may inform on the potential production level of their corresponding protein, but in the complement cascade the generation of the effectors is a matter of activation and cleavage. Although transcriptomic analyses are useful to pinpoint associations between complement components and clinical outcome, only *in situ* analyses of the complement proteins, their activation fragments deposits (C4d, C3b, iC3b, C3d), the anaphylatoxins (C3a, C5a), their receptors (C3aR, C5aR1, C5aR2) and their regulators will allow to understand how complement modulates tumor cells and the TME, resulting in control or development of cancers.

A clear example for such discrepancy is the modest prognostic impact of C3AR and C5AR1 genes, which reach statistical significance at median cutoff only for 3 and 5 cancers respectively. Nevertheless, C5aR seems to be the key effector of the deleterious impact of complement, even when C5a it is generated by cascade-independent proteases^{13, 14, 20, 26, 29, 106}. In addition, a clear negative prognostic impact was detected for C1s in bladder urothelial carcinoma (BLCA) at protein level¹⁰⁷. Detailed gene expression analyses of the TCGA cohort revealed that *C1S* as the most significantly upregulated gene related to advanced disease both in urothelial carcinoma of upper tract and in urinary bladder cancer. It correlated with a panel of disease markers, but the gene expression failed to reach significance in terms of prognostic impact. This case illustrates the importance to perform staining at protein level to determine the prognostic impact of a given protein.

Another hurdle is that different activation fragments of the complement proteins have different biological functions. Therefore, the exact deposited activation fragment has to be distinguished *in situ*. Well characterized and validated antibodies with known fragment specificity should be used. Detection of complement and its activation fragments is performed in routine pathology laboratories and the difficulties to work with paraffin embedded tissues are well documented¹⁰⁸. Nevertheless, nowadays reliable protocols exist for staining of complement components and activation fragments in paraffin-embedded tumor tissue²¹, with antibodies, validated by competition tests with intact proteins or fragments. With the current state of the image analyses, distinction between intracellular production and deposits in automated algorithms is tricky. This requires experienced observers to stratify the patients. Moreover, proteomic analyses allow spatially resolved profiling of the proteins within different tumor regions. The complement exploration in cancer will benefit from the advent of this technology, which will allow to better evaluate the complement production and deposits *in situ*.

A further challenge, when a complement protein is produced both by malignant and by infiltrating cells, is to ascribe the pro- or anti-tumoral effect to this complement protein and its canonical and/or non-canonical functions, or to the presence of the cell itself, as it was the case for C1q+ TAM²¹.

Indeed, our prognostic data need to be interpreted within the limits of using median gene expression as a cutoff to stratify patients. Even if the median cut off did not reveal significant prognostic impact for complement genes in the “uncertain significance” group of patients, use of other cut-offs may have unveiled significant correlations.

Therapeutic perspectives.

An analysis on a large panel of cancers (Figure 4) revealed simultaneous intratumoral expression of genes coding for proteins involved in complement activation (C1q, C1s, C1r, C3) or sensing activation products (C3aR and C5aR) as well as in complement regulation (C1Inh, FH, FI, CD59). Taken together, the local production and increased activation of complement in the tumor microenvironment is associated with dampening of the anti-tumor immune responses and promotion of cancer development in a large subset of cancers. Despite the heterogeneity of the data in the literature, it can be concluded that in the majority of the tumors the anaphylatoxin receptors C3aR and C5aR are novel class of immune checkpoints that could be targeted for tumor immunotherapy. Indeed, C5aR blockade showed decreased tumor growth in variety of mouse models alone⁶ or in combination with a checkpoint inhibitors (anti-PD1, anti-PDL1)^{29, 109, 110}. Moreover, a phase 1 clinical trial for administration of anti-C5aR monoclonal antibody (IPH5401) in combination with anti-PDL1 Durvalumab in patients with advanced solid tumors is now recruiting (STELLAR-001, NCT03665129). C5aR blockade could improve also the efficacy of chemotherapy. Indeed, C5aR targeting with PMX-53 improved efficacy of paclitaxel chemotherapy, by promoting antitumoral T cell response¹³. Many more complement-targeting molecules are in the pipeline for variety of disease indications¹¹¹ and can be adapted for cancer therapy, acting at different steps of the cascade¹¹². Nevertheless, the therapeutic combinations have to take into account the large variety of functions of complement proteins, such as in the context of radiotherapy, where C3a and C5a are crucial to the anti-tumor immune response¹¹³.

Cancer vaccines are a promising approach to stimulate the immune system to efficiently recognize and kill tumor cells. Endothelial quiescence prevents tumor-specific T-cells homing. This endothelial quiescence was reversed by cytokine-mediated activation of the tumor vasculature followed by upregulation of C3 and local generation of C5a in the TC-1 mouse model of tumor vaccination¹¹⁴. The C5a-dependent upregulation of endothelial adhesion molecules, resulted in efficient T-cell extravasation, infiltration into the tumor and malignant cells killing. These results highlight once again the context-dependent action of complement. In the same model complement activation is pro-tumoral^{20, 21}, but turns anti-tumoral in case a robust antitumor immune response¹¹⁴. Indeed, these data suggest that when effector T cells are present, complement facilitates tumor rejection, whereas it may promote inflammation and tumorigenesis when other immune cell types predominate over antitumor T cells¹¹⁴.

Big efforts are now focused to design anti-cancer monoclonal antibodies with enhanced complement mediated cytotoxicity in order to kill the tumor cells to which they are directed.

The recent discovery of the potentiation of the C1q binding and complement activation by IgG hexamerization brought a new trend for generation of a new generation of therapeutic antibodies¹¹⁵⁻¹¹⁷. If given in the right context, they could indeed confer benefit in the eradication of the tumor. Although limited, the data from the literature suggest that the impact of malignant cells-binding IgG on tumor growth is context dependent. In an immunostimulatory milieu, such antibodies can induce powerful anti-tumor immunity that can potentially be harnessed for the treatment of patients with cancer. Potential benefit to harm ratio has to be evaluated for each subset of patients even within one tumor type to avoid potential enhancement of the pro-tumoral impact of complement. Intervention strategies, recently summarized by Fishelson and Kirschfink¹¹⁸ have to be established to overcome the resistance of the tumor cells to complement mediated killing, which will improve the efficacy of these therapeutic antibodies.

In order to design efficient complement targeted therapeutics for cancer we have to better understand the mechanism by which these complement proteins contribute to tumor development. This will allow to tip the fine-tuned balance of the complement reaction and to decide whether to block the activation, prevent the regulation and/or act on the functions of these proteins outside complement system.

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Figure legends

Figure 1. The complement system in the TME. A) The mechanisms of activation and regulation of the complement system. The complement system can be activated by three pathways; the classical, lectin and alternative pathway leading to the generation of the C3 convertase. The classical pathway is activated after the recognition by C1 complex (composed of C1q, C1r and C1s) of immune complexes/apoptotic cells; the lectin pathway is initiated after the fixation of the complex MBL (Mannose Binding Lectin)/MASP (MBL-associated serine proteases) to terminal mannose residues. The alternative pathway is constitutively activated at low grade by the spontaneous hydrolysis of C3 into C3 H₂O. These initiation events lead to the formation of enzymatic complexes - C3 convertases, which cleave the central component C3 into C3a (anaphylatoxin) and C3b which can opsonize the cells and allows the formation of the C5 convertase. The C5 convertase cleaves C5 into C5a (anaphylatoxin) and C5b which initiate the terminal pathway of the complement system, leading to the formation of the membrane attack complex. Some proteases, extrinsic to the cascade, can also cleave C3 or C5 independently to the convertases formation. To avoid host tissue damage, this system is tightly regulated by soluble or membranous proteins at different levels of the cascade.

B) The composition of the TME and the complement proteins, produced by different non-malignant cell types. The tumor has a rich complement environment. All stromal and tumor cells participate to the local production of complement proteins. The immune cells, especially the myeloid ones, can produce components especially of the classical and alternative pathways, express high level of complement receptors and multiple regulators. The endothelial cells and fibroblasts are also key actors the TME and produce complement proteins, express regulators and a lower level of complement receptors. Finally, the participation of tumor cells depends of cancer type but a key feature is the high expression of complement regulators to protect against complement-dependent cytotoxicity.

Figure 2. Modulation by complement of the pro- and anti-tumoral impacts of the immune contexture in cancers. A) pro-tumoral action of complement effectors on the immune cells; B) anti-tumoral action of complement effectors on the immune cells

Figure 3. The pro- and anti-tumoral impact of complement on neoangiogenesis and on the fundamental processes of tumor cell. The complement system can A) promote angiogenesis through the actions of anaphylatoxines of C1q or can act on the key characteristics of tumor cells and B) induce a pro-tumoral phenotype by increasing EMT, autophagy, proliferation, migration and stemness or C) have a anti-tumoral impact by decreasing proliferation and apoptosis. These effects are cancer type dependent and concentration dependent.

Figure 4. Expression of complement genes in human cancers. TCGA PanCanAtlas¹¹⁹ data used are downloaded through cBioPortal¹²⁰ and come from TCGA Data Coordination Center (DCC). RNASeq expression data, RNASeqV2, from TCGA is processed and normalized using RSEM to generate TPM (transcripts per million). Specifically, the data_RNA_Seq_v2_expression_median file in cBioPortal corresponds to the rsem.genes.normalized_results file from TCGA. 30 solid tumor types are used in this analysis. In order to avoid bias, liver hepatocellular carcinoma (LIHC) was excluded from the study because of the capacity of the liver to express very high amounts of complement genes. The mean of TPM of each complement gene for the patients of the different TCGA cohorts is calculated and then converted in $\log_2(1+TPM)$ data. Using R package “pheatmap” and the clustering method “complete”, the heatmap is generated and allows the visualization of the TPM mean of each complement gene for the different TCGA cohorts. ACC, adenocortical carcinoma; BLCA, bladder carcinoma; BRCA, breast carcinoma; CESC, cervical squamous carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, lower grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

Figure 5. Impact of the expression of the complement genes on the survival of cancer patients. The survival analysis is performed by using Gene Expression Profiling Interactive Analysis (GEPIA) tool¹²¹. Overall survival (OS) analysis based on gene expression with a median cut-off is used to calculate hazards ratio based on Cox PH Model and Log-rank p-value. Considering the very low number of events, TGCT cohort is excluded from the survival

analysis. The heatmap representing the log₂ HR is generated by using the R package “pheatmap” and the clustering method “ward.D2”. The heatmap allows the visualization of the log₂ HR with a scale centered in 0 of each complement gene for the different TCGA cohorts. The surrounded boxes correspond to a log-rank p-value <0.05.

Figure 6. Proposed mechanisms for the classical complement pathway activation and its consequences on tumor progression in tumors with “aggressive complement”. C1q is produced by TAM (1) and contributes to a tumor-promoting phenotype of these cells (2) and T cell exhaustion. Secreted C1q promotes adherence of tumor cells (3) and neoangiogenesis (4). A particular feature of ccRCC is that the tumor cells produce C1r and C1s (5) and allow formation of a functionally active C1 complex (6), capable to activate the classical pathway. Moreover, IgG deposits on tumor cells serve as C1 ligand (7) to initiate the cascade. The tumor cells produce also the subsequent components and allow C4 and C3 activation fragments deposition (8). Anaphylatoxins C3a and C5a are released, exerting their action on the tumor cells and on their microenvironment. The ensemble of these processes contributes to tumor progression and poor prognosis of patients. This model is based on the data for KIRC and is potentially applicable in other tumors from this group.

Figure 1A

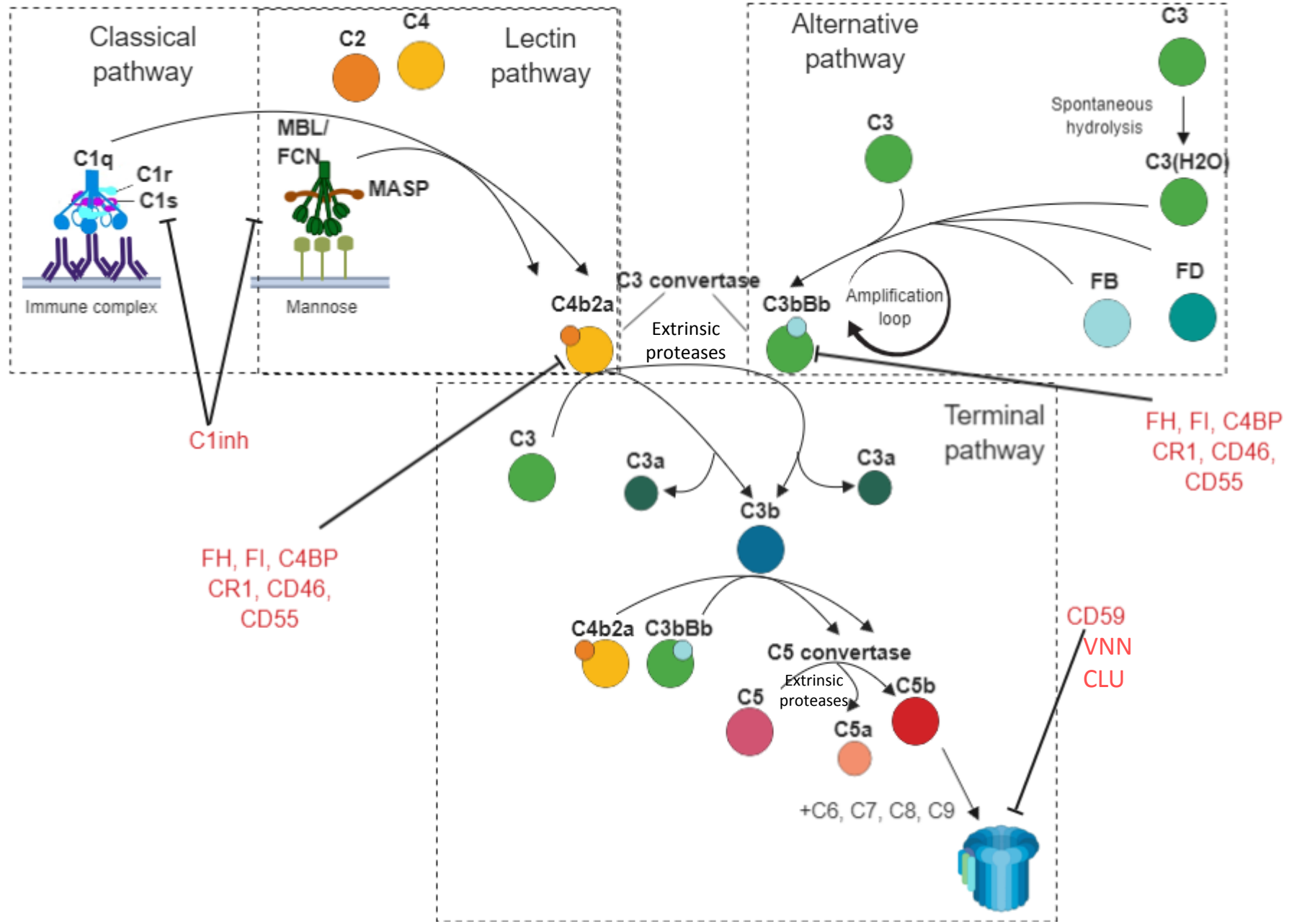


Figure 1B

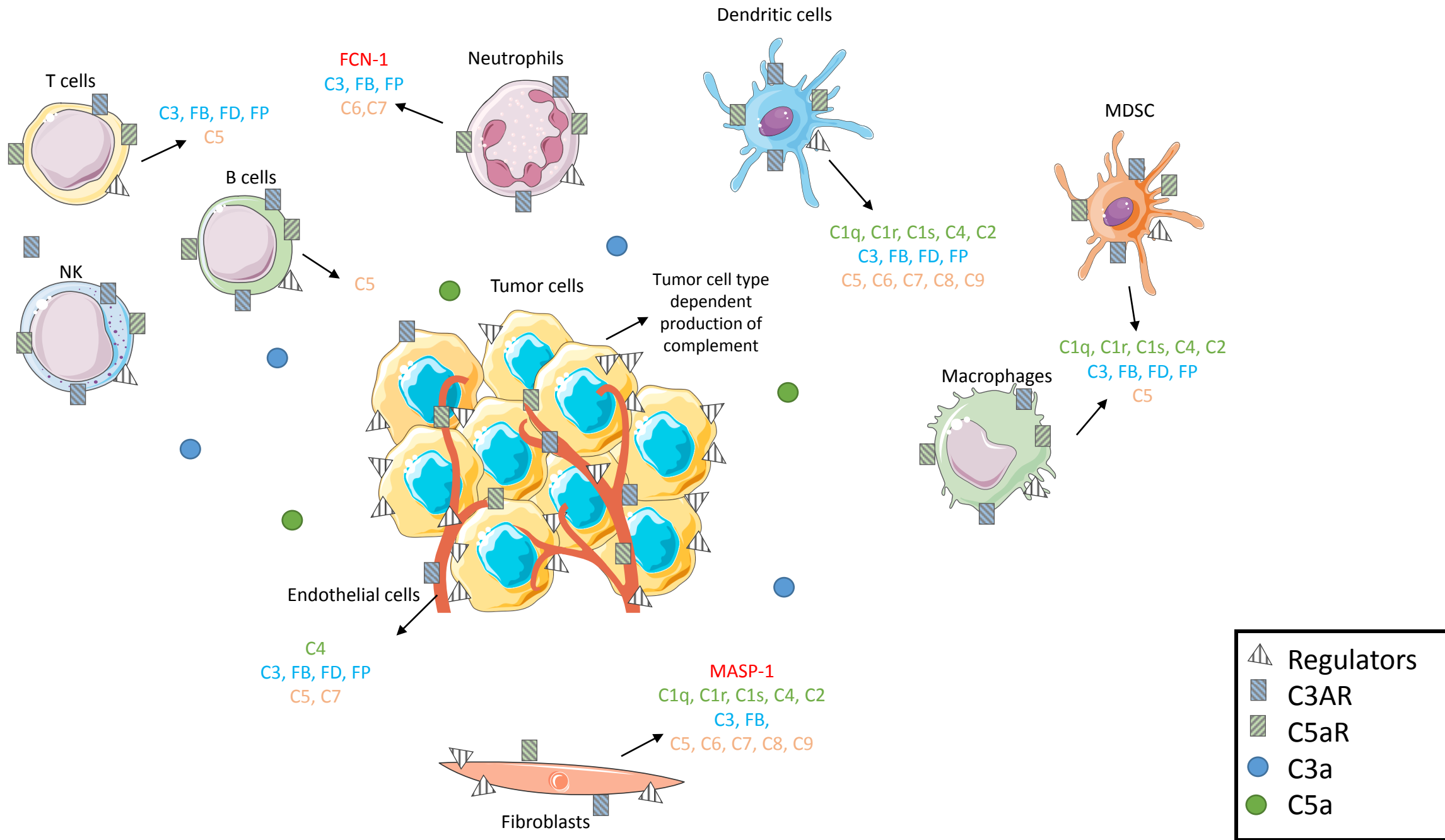
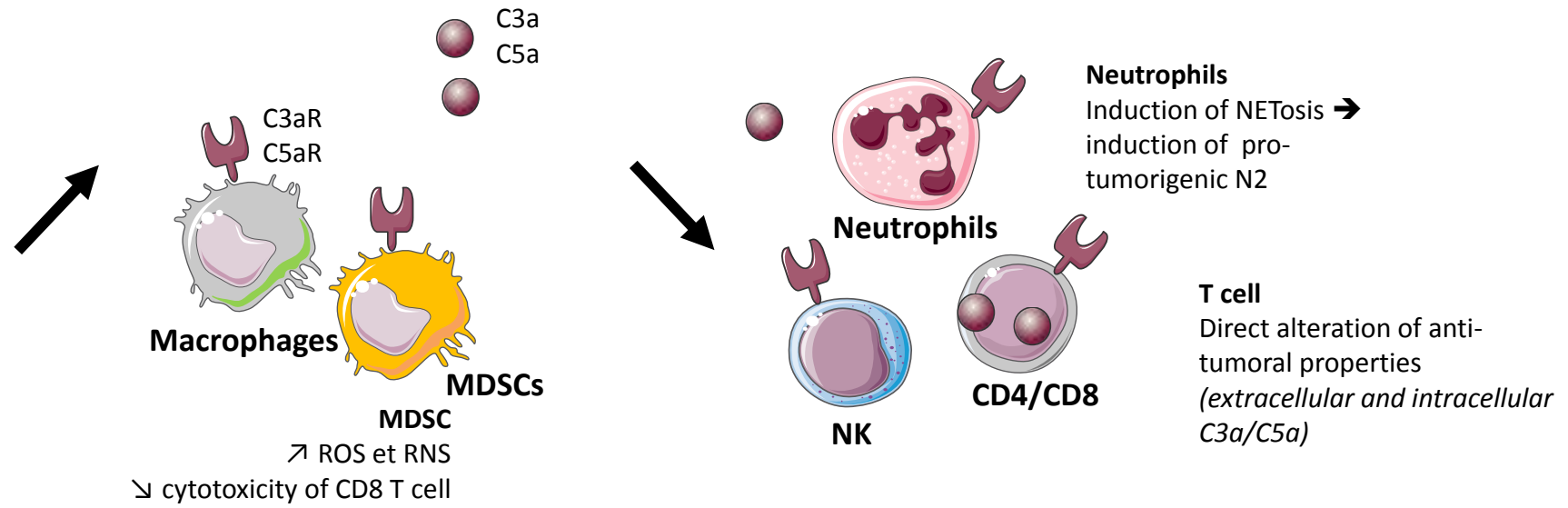


Figure 2

Immune microenvironment

A) Pro-tumoral impact



B) Anti-tumoral impact

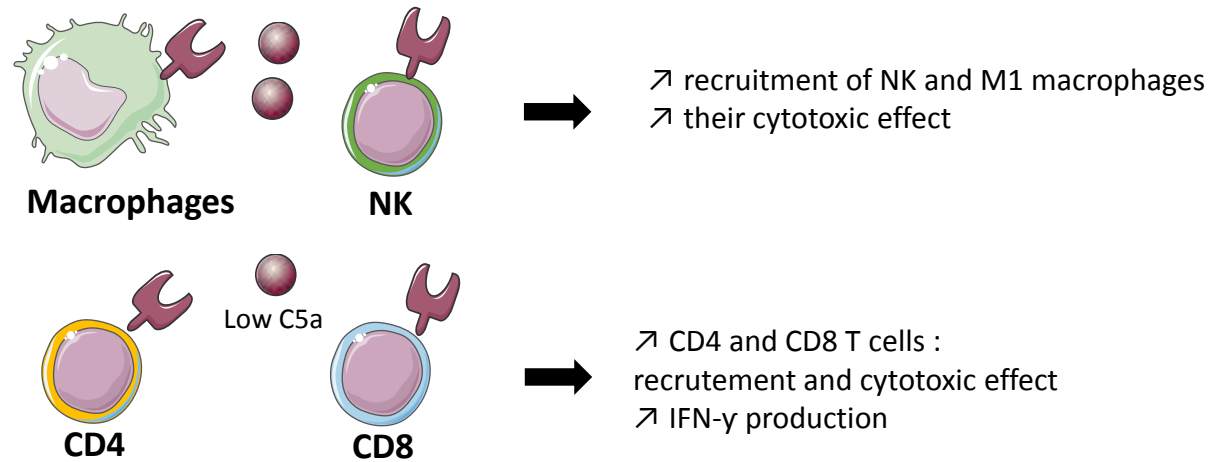
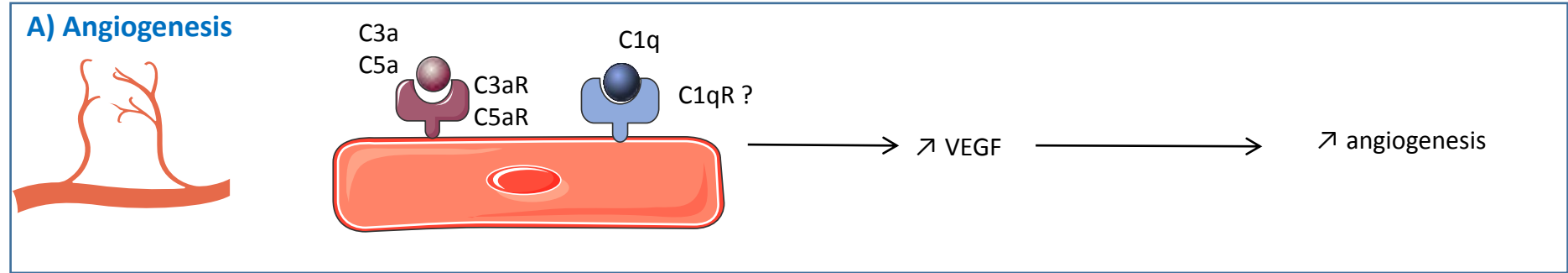


Figure 3

Angiogenesis



Tumor cells

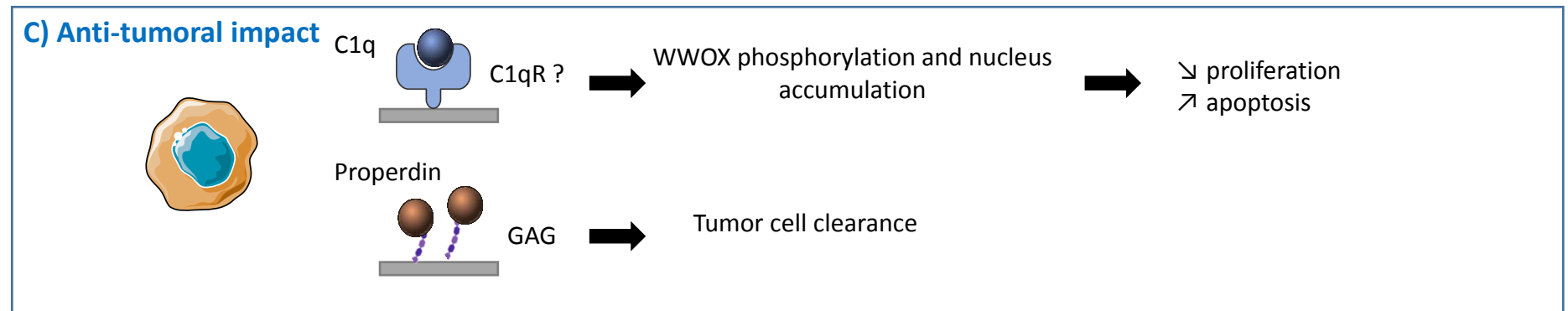
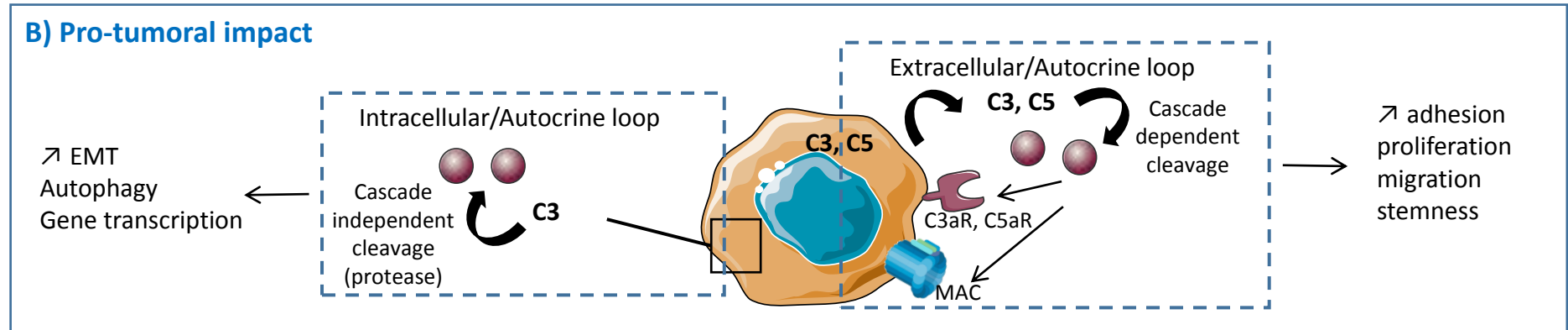


Figure 4

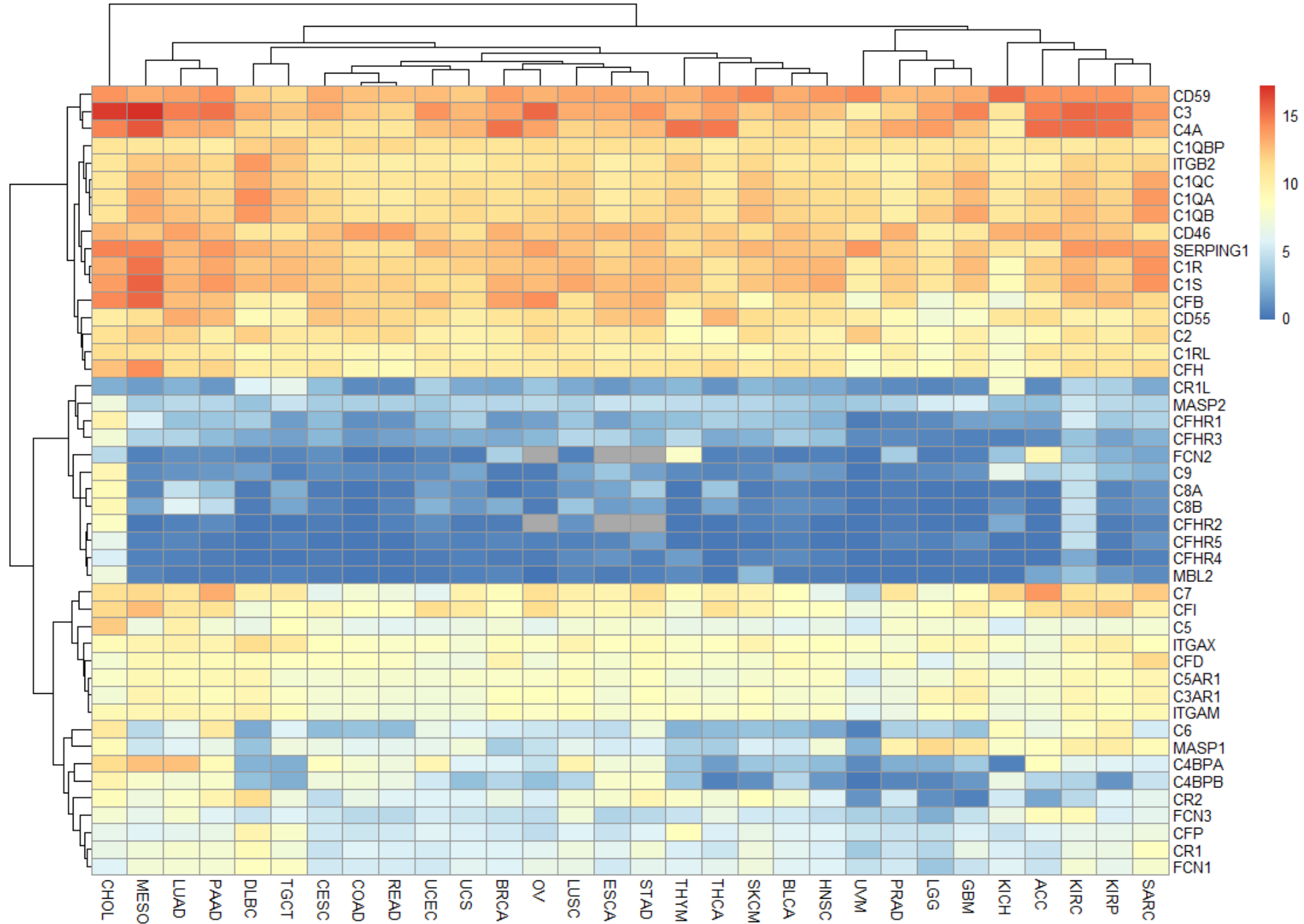


Figure 5

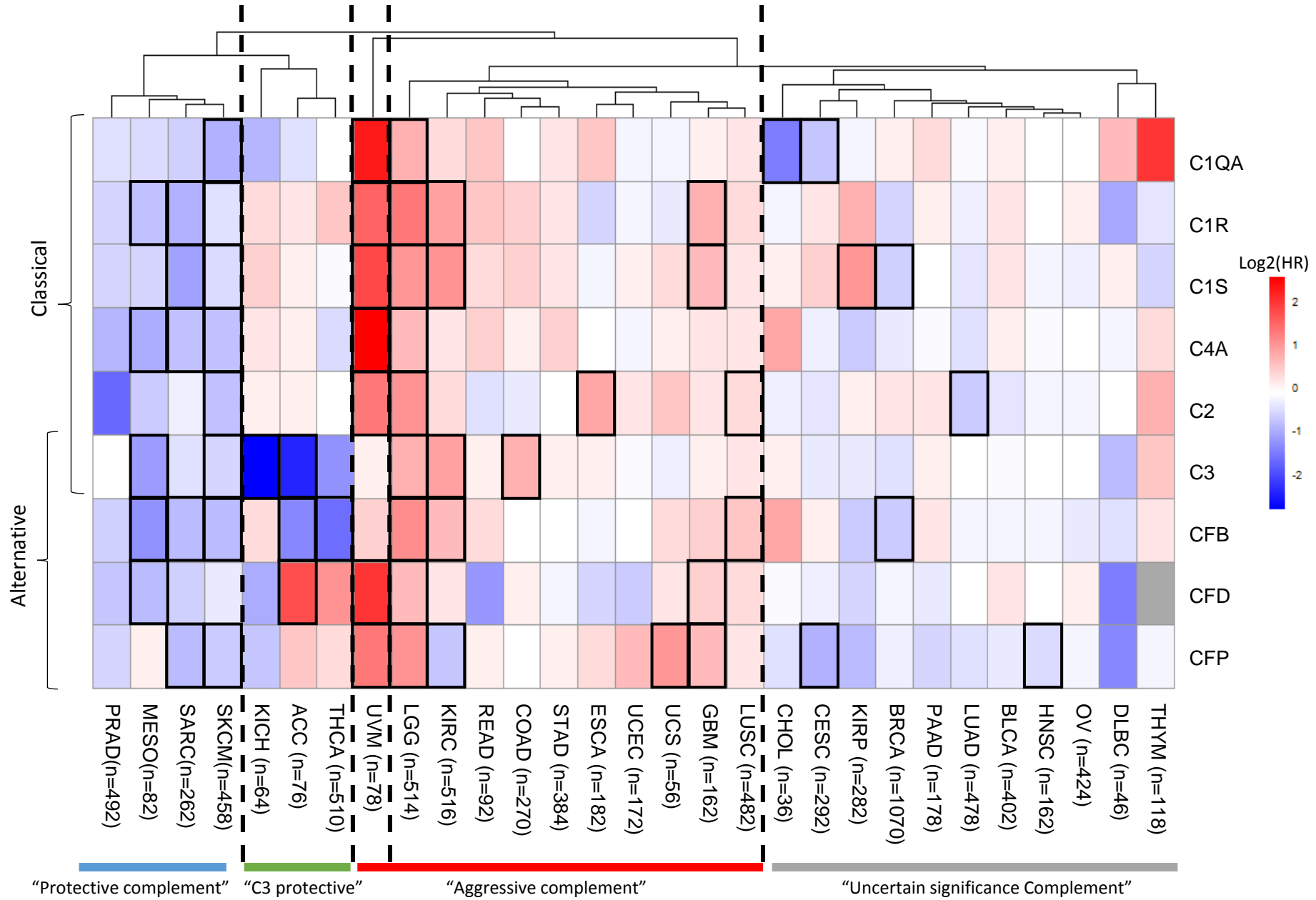


Figure 6

