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Role of the complement system in tumor progression

Marie Daugan

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Marie Daugan. Role of the complement system in tumor progression. Immunology. Université Paris Cité, 2019. English. NNT : 2019UNIP5038 . tel-04803795

HAL Id: tel-04803795

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Role of the complement system in tumor progression

Par Marie Daugan

Thèse de doctorat d'immunologie

Dirigée par Dr. Lubka Roumenina

Présentée et soutenue publiquement le 25 novembre 2019

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ACKNOWLEDGEMENT

I would like to thank all the people who participated in the success of this thesis. Thanks for your personal and professional support.

To my supervisor, Dr. Lubka Roumenina, thank you for giving the strength and envy to do my best during these three years of my thesis. You have been an amazing mentor that always encourages me to go further and allows me to fully develop myself. Thanks for giving the chance to present my work in different congress, to write excellent review and to supervise many students. You have initiated me to the world of complement and now I love it and I want to continue working on it.

To Pr. Catherine Sautès-Fridman and Pr. Wolf H. Fridman, the knowledge keepers in the lab, you are my others mentors in this thesis. Thanks for sharing with me your strong expertise in cancer immunity. Working with you was an excellent opportunity for me to improve my knowledge in this field but also to challenge my scientific thinking to always move forward.

To my team director, Dr. Véronique Frémeaux-Bacchi, thanks for helping me to improve my work every monday at the “complement meeting”. Your incredible knowledge of the complement system and all your relevant comments and advices have taught me what really is scientific rigor.

To Dr. Marie-Agnès Dragon-Durey, thanks for supporting me in this thesis and for your precious help about our common friend “FH”.

To Dr. Isabelle Cremer and Dr. Diane Damotte, thanks for your help and participation in this project.

To all members of team 13, thanks for helping me to improve my work through your advices in our inter-team meeting and by giving me access to cohorts of patients.

To the members of the jury, I thank Dr. Christine Gaboriaud and Pr. Ruben Pio for being reviewers and their kind advices to ameliorate my thesis. I thank Pr. Eric Tartour for accepting being the president of the jury and Dr. Marta Biedzka-Sarek for being examiner of my work.

A Laila, ma co-thésarde préférée du labo. Malgré ta timidité du départ ces quelques années de galère nous ont bien rapprochées. Nous avons partagé beaucoup de moments ensemble plus ou moins joyeux mais notre amitié et notre soutien mutuel a permis de les surmonter. J'ai partagé de merveilleux instants en ta compagnie, merci d'avoir été là pour moi, sache que tu pourras toujours compter sur moi. J'admire ton courage et ta détermination, elles te mèneront loin dans ta vie, ne baisse jamais les bras !

A Carine, la plus crazy d'entre toutes. Dès que je t'ai vu la première fois j'ai tout de suite été convaincue que j'allais bien m'entendre avec toi. Ton originalité te rend unique mais ta gentillesse et ton soutien m'ont permis d'évoluer sereinement durant ces quelques années. Au-delà d'être mon amie, un énorme merci pour ton travail sur les souris, sans toi, rien n'aurait été possible. Girl power !

A Rémi, mon co-équipier sur le projet complément cancer. Notre complémentarité a permis de former une équipe au top durant les premières années de ma thèse. Maintenant tu es parti dans une autre équipe (pas si loin ;-)) et je te souhaite de d'épanouir le plus possible car tu le mérites.

A Nicolas, premier padawan de Lubka, tu as mis la barre très haute pour passer derrière toi. Tes précieux conseils mais aussi ton énergie et ta bonne humeur m'ont grandement aidée à réussir cette thèse même maintenant que tu es loin ;-). Complément power !

A Margot, mon M&Ms, on s'est croisée durant ton stage de licence et le complément t'a attrapé dans ces filets, car deux ans après te voilà de retour pour ton stage de M2 dans l'équipe. On a partagé beaucoup de moments ensemble durant ces derniers mois que je ne suis pas prête d'oublier. Parfois à l'opposé de moi avec ton côté rentre dedans, tu es la meilleure personne sur qui j'ai pu compter durant ma thèse et en qui j'accorde toute ma confiance. Je crois que maintenant je ne dois plus t'appeler stagiaire... mais co-thésarde ! Une chose est sûre, avec toi, la relève est assurée.

A Tania, le pilier de l'équipe, mon petit chat. Vous avez dit manipulation du génome ; Crispr-cas9, siRNA, plasmid, lentivirus ? Pas de crainte la reine Tania est là. Merci de m'avoir transmis ton savoir mais surtout merci de m'avoir supportée durant ces trois années de thèse. Maintenant futur pro de l'IHC ?

A Vico, petit poulpi. Carine m'avais longtemps parlé de toi avant ton arrivée comme étant une personne formidable et il faut dire que ce n'est pas assez pour te décrire. Tu t'es épanouie dans cette personne que tu es devenue avec ce cœur en or, cette empathie, cette gentillesse et cette douceur mais sache aussi que tu fais ressortir le meilleur de chacun d'entre nous.

A Valérie Jupit', a non Nathalie, je me suis trompée de prénom ;-). Tu as été comme une maman du labo pour moi. Tu prends soin de nous comme personne avec toujours le bon mot

pour nous réconforter mais je pense qu'il est temps que ce soit nous qui veillons sur toi. Tu es formidable, ne change pas !

A Béné, Mahmud et Erwan, mes co-thésard. Avec le temps, on a appris à mieux se connaître et maintenant je vais partir en ayant découvert trois personnes au grand cœur. Merci d'avoir partagé le bureau avec moi, je vous souhaite le meilleur pour la fin de votre thèse !

Aux différentes personnes que j'ai pu encadrer, Julie, Sonia, Romane, je vous remercie d'avoir participé à ce travail et d'avoir fait de cette thèse une expérience si enrichissante.

A Florent, monsieur bioinformatique, merci de m'avoir fait découvrir et apprécier le dark world of bioinformatics et R. Florent <- Meilleurprof\$R (, bigdata) Error « Meilleurprof » not found, try Meilleurprofdumonde ;-).

A Sophie et Noémie, les néphros de la team. Merci pour votre expertise et vos conseils tout au long de la thèse, vous avez contribué à son succès.

A toute la bande des M2, Lucas, Manon, Hajar, Idris, Alice et Melchior, merci d'avoir apporté avec vous la bonne humeur au labo. Lucas tu es le seul survivant avec Margot, si tu en es arrivé là ce n'est pas pour rien, tu as fait preuve d'une persévérance incroyable et je suis certaine que tu vas être au top ces prochaines années.

A la dream team de Catherine, Laetitia, Irelka, Guillaume, Antoine, Marco, Ilenia, ça a été vraiment super de travailler en collaboration avec vous ! J'espère que les marquages complément ne vous ont pas trop marqués ;-)

Aux post-docs, nouveaux, anciens ou revenants, Aditi, Irati, Pauline et Jules. Merci pour ces bons moments passés ensemble et pour votre aide.

A Nathalie, Tessa, Lucie, Solène, Myriam, vous avez participé à un moment ou un autre dans l'évolution de cette thèse. Vamoooooss !!!

Au personnel de la plateforme, Christophe, Estelle, Hélène, merci pour votre précieuse aide durant ces trois années de thèse.

A toutes les personnes extérieures à l'équipe qui m'ont apporté leur aide et notamment à Jordan Dimitrov et à l'équipe de Guido Kroemer ; Valentina, Oliver et Allan, merci de m'avoir accordé de votre temps pour m'aider à faire avancer mon projet.

A mes amies d'enfance Emma et Anna, merci pour avoir toujours cru en moi et m'avoir soutenue durant ces nombreuses années.

A mes amies de la fac, Popo, Lane et Lorph, vous m'avez supporté et fait grandir durant ces 6 années de pharmacie ensemble. Même si la distance nous sépare maintenant, vous avez toujours la même place dans mon cœur.

A Rose, Joao, Stacie et Nico, même si ne comprenez pas tout à mes études vous m'avez toujours soutenue. Merci de m'avoir accueillie dans votre famille.

A mes grands parents, votre intérêt pour ma recherche et vos yeux remplis de fierté m'ont toujours poussée à donner le meilleur de moi-même. Merci d'être ces personnes aimantes et affectueuses.

A mes parents et mon frère, vous avez été mon moteur durant toutes ces années. Vous pouvez être fiers car sans vous, je n'en saurais jamais arrivée là. Merci pour votre amour et votre soutien quotidien.

A mon mari, Kevin, on a tout partagé ensemble et tu as toujours été mon supporter n°1. Durant toutes ces années tu m'as soutenue dans mon projet de devenir chercheur et aujourd'hui c'est l'aboutissement de ce rêve. Cette réussite, tu en es grandement responsable et je te dédis cette thèse. Aujourd'hui, nous avons pleins de projets en tête et je suis pleinement confiante sur le fait que l'on va les mener avec succès car à deux rien ne nous arrête.

RESUME

Le système du complément fait partie intégrante du système immunitaire et permet via ses capacités d'opsonisation, de chimiotactisme et de lyse, de protéger l'hôte contre les pathogènes. Compte tenu de ces propriétés, le système du complément a longtemps été considéré comme un élément anti-tumoral. Ainsi, des anticorps monoclonaux ont été développés afin de permettre la mort de la cellule cancéreuse par des mécanismes dépendent de la cytotoxicité du complément. Cependant de récentes études ont permis de repositionner ce système en montrant des effets pro-tumoraux associés au complément dans certains types de cancer et notamment à travers une modulation de la prolifération tumorale, l'angiogenèse ou le microenvironnement immunitaire. Ces effets résultent d'un mode d'action extracellulaire des protéines du complément mais également d'une action non conventionnelle en intracellulaire.

Ainsi, le but de mon projet de thèse est de déterminer l'impact pronostique du complément dans la progression tumorale et de comprendre les mécanismes sous-jacents avec pour objectif l'identification de nouveaux marqueurs pronostiques et de nouvelles cibles thérapeutiques. Pour répondre à cette question, j'ai utilisé des outils bioinformatiques, des modèles *in vitro*, *in vivo* ainsi que différentes cohortes de patients.

Une analyse transcriptomique pan-cancer a permis de mettre en évidence une hétérogénéité de l'expression des gènes du complément dans plus de 30 types de cancers différents. De façon intéressante, on observe une forte expression des gènes de la voie classique et alterne ainsi qu'une forte expression des régulateurs de la voie terminale. Ces données suggèrent une adaptation de la tumeur lui permettant de profiter des effets inflammatoires liés à la génération des anaphylatoxines tout en s'affranchissant des effets cytotoxiques du complément. Selon le type de cancer, les protéines du système du complément peuvent exercer des effets pro ou anti-tumoraux se traduisant par un effet agressif ou protecteur sur le pronostic des patients. De plus, j'ai identifié que le niveau de C4d plasmatique représente un nouveau biomarqueur non invasif permettant de prédire la survie des patients atteints de carcinome rénéale à cellules claires (ccRCC).

Le ccRCC fait partie des tumeurs présentant une surexpression des gènes du complément associée à un mauvais pronostic. Dans différentes cohortes de patients atteints de ccRCC, j'ai pu mettre en évidence surexpression locale de C1q, C4 et C3 ainsi qu'une activation locale de la voie classique associée à une diminution de la survie des patients. Cette activation résulte d'une interaction dynamique entre les cellules tumorales et le microenvironnement immunitaire en présence de complexes immuns. De plus, certaines protéines du complément peuvent également avoir une action indépendante de la cascade. Mes études ont montré que le Facteur H et le C1s pouvaient exercer des fonctions intrinsèques non canoniques en modifiant les caractéristiques cellulaires clés de la cellule tumorale telles que ; la prolifération, la survie ou encore la migration. Dans le ccRCC, l'expression de FH et de C1s confère un pronostic péjoratif. Cet effet sur la survie des patients résulte d'une action intracellulaire du FH spécifique des cellules tumorales. L'ensemble de ces données révèle le potentiel du système du complément en tant que marqueur pronostique mais également en tant que cible thérapeutique.

ABSTRACT

The complement cascade is a component of the innate immune system and is involved in cell homeostasis and defense against pathogens through its opsonization, chemotaxis and lysis actions. Due to these properties, it has been for long considered as an anti-tumoral effector. Thus, anti-cancer monoclonal antibodies were developed to allow tumor cell death through complement-dependent toxicity. However, recent studies have highlighted that complement has pro-tumoral effects by promoting tumor cell proliferation, angiogenesis and immunosuppressive microenvironment. These may be linked to a classical, extracellular activation of the complement cascade. Nevertheless, I have hypothesized that complement components may exert pro-tumoral effects via unconventional, intracellular mode of action.

The objective of my PhD project was to characterize the prognostic impact of complement in tumor progression and to understand the mechanisms that governs it. The ultimate goal was to uncover novel mechanisms, explaining the tumor growth and to identify reliable prognostic biomarkers or therapeutic targets. To answer these questions, bioinformatic, in vitro, in vivo tools and cohorts of patients were used.

A pan-cancer transcriptomic analysis revealed an important heterogeneity of the expression of complement genes among 30 different tumor types. Interestingly, a high expression of genes belonging to the classical and the alternative complement pathways, together with a high expression of regulators, and low levels of terminal pathway components was observed. This finding suggests that a fraction of the tumors developed an adaptation in order to benefit from complement activation through the generation of the anaphylatoxins, while escaping from complement attack. On the contrary, in another group of cancers, overexpression of complement proteins confers favorable prognosis. This context-dependent impact of complement effects, ranging from pro- to anti-tumoral depending on cancer type, allowed to classify the tumors either with protective or aggressive complement.

The clear cell renal cell carcinoma (ccRCC) belongs to the tumors with high expression of complement genes that are associated with poor prognosis, falling into the “aggressive complement” group. In different cohorts of ccRCC patients, I showed that the local overexpression of C1q, C1s, C4 and C3 as well as the C4 activation fragments deposits by the classical pathway occurs and it is associated with a decreased survival. This activation resulted from a dynamic interaction between tumor cells and immune microenvironment in presence of immune complexes. Moreover, I identified the plasmatic C4d as a novel non-invasive prognostic biomarker for the survival of ccRCC patients.

Complement proteins may play a role outside of the cascade. I found that Factor H and C1s exert non canonical, intracellular functions, modulating key characteristics of the tumor cell such as its transcriptional program, its morphology, proliferation, survival or migration. This could explain the poor prognosis of FH and C1s overexpression in ccRCC. The intracellular action of FH was cancer cells-specific, detected in ccRCC and lung adenocarcinoma cells, but not in normal tubular and endothelial cells.

Taken together, these data suggest that the complement system and its individual components can be useful prognostic biomarkers and therapeutic targets with a great potential.

GLOSSARY

ABOi: ABO incompatible
aHUS: Atypical hemolytic uremic syndrome
Akt: protein kinase B
AM: Adrenomedullin
AMD: Age-related macular degeneration
AMI: Acute myocardial infarction
ANCA: Antineutrophil cytoplasmic-antibody-associated vasculitis
APC: Antigen-presenting cell
APS: Antiphospholipid syndrome
ATG16L1: Autophagy related 16 like 1
ATP: Adenosine triphosphate
BCR: B cell receptor
BSP: Sialoprotein
BTLA: B and T lymphocyte associated
C1inh: C1 inhibitor
C1qR: C1q receptor
C3aR: C3a receptor
C3G: C3 glomerulopathy
C4BP: C4 binding protein
C5aR: C5a receptor
CAD: Cold Agglutinin disease
CAFs: Cancer associated fibroblasts
CAGB: Coronary artery bypass grafting
CCP: Complement control protein
ccRCC: Clear cell renal cell carcinoma
CDC: Complement-dependent cytotoxicity
CDCC: Complement dependent cell-mediated cytotoxicity
CL: Collectin
CNV: Choroidal neovascularization
COPD: Chronic obstructive pulmonary disease
CR: Complement receptor
CRP : C-reactive protein
CRP: Complement regulatory protein
crRCC: Chromophobe renal cell carcinoma
CSC: Cancer stem cells
CSF: Cerebrospinal fluid barrier
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4
CTSL: cathepsin-L
DAF: Decay-accelerating factor
DCs: Dendritic cells
DDD: Dense deposit disease
DDIT3: DNA Damage Inducible Transcript 3
DDR1: Discoidin domain receptor 1
DFS: Disease free survival

DMP1 : Dentin matrix 1
DNA : Deoxyribonucleic acid
ECM: Extracellular matrix
EMT: Epithelial to mesenchymal transition
Erk: Extracellular signal-regulated kinases
Ext-C: Extracellular complement
Ext-FH: Extracellular FH
FACS: Fluorescence-activated cell sorting
FB: Factor B
Fc: Fragment crystallizable
FCN: Ficolin
FD: Factor D
FGF: Fibroblast growth factor
FH: Factor H
FHL-1: FH like protein 1
FHR: FH related protein
FI: Factor I
FOXO1: Forkhead box protein O1
FP: Properdin
GA: Geographic atrophy
GAG: Glycoaminoglycans
GAPDH: Glyceraldehydes-3-phosphate dehydrogenase
gC1qR: Receptor for the globular heads of C1q
GPCR: G protein-coupled receptors
GVHD: Graft versus host disease
HA: Hyaluronic acid
HAE: Hereditary angiodema
HCC: Hepatocellular carcinoma
HGF: Hepatocyte growth factor
HIV: Human immunodeficiency viruses
HMGB1: High-mobility group box 1
HUS: Typical hemolytic uremic syndrome
HVEM: Herpesvirus entry mediator
IA/IE: Mouse MHC class II
IDO: Indoleamine 2,3-dioxygenase
IFN γ : Interferon-gamma
Ig: Immunoglobulin
IGF1: Insulin-like growth factor 1
IGFBP-5: Insulin-like growth factor binding protein-5
Il: Interleukin
Int-C: Intracellular complement
Int-FH: Intracellular FH
IPCV: Idiopathic polypoidal choroidal vasculopathy
JNK: Jun amino-terminal kinase
LAG-3: lymphocyte-activation gene 3
LAIR1: Leukocyte Associated Immunoglobulin Like Receptor 1
LN: Lupus nephritis
LPS : Lipopolysaccharide
LRP6: Lipoprotein receptor related protein

MAC: Membrane Attack Complex
MAPK: Mitogen-activated protein kinase
MASP: Mannan-Associated Serine
MBL: Mannan-Binding Lectin
MCP: Membrane cofactor protein
MCP-1: Monocytes chemoattractant protein-1
MDSCs: Myeloid derived suppressor cells
MHC: Major histocompatibility complex
miRNA: Micro RNA
MMP: Matrix metalloproteinases
MN: Membranous nephropathy
NCL: Nucleolin
NET: Neutrophil extracellular trap
NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
NK: Natural killer
NLRP3: NOD-like receptor family, pyrin domain containing 3
NMP1: Nucleophosmin
NSCLC: non-small cell lung cancer
OPN: Osteopontin
OS: Overall survival
OXPHOS: Oxidative phosphorylation
PAMP: Pathogen-associated molecular pattern
PD-1: Programmed cell death 1
PD-L1: Programmed death ligand 1
PD-L2: Programmed death ligand 2
PFS: Progression free survival
PI3K: Phosphoinositide 3-kinase
PMN: Polymorphonuclear
PNH: Paroxysmal nocturnal haemoglobinuria
pRCC: Papillary renal cell carcinoma
PSA: Prostate-specific antigen
PTX3: Pentraxin 3
RA: Rheumatoid arthritis
RCC: Renal cell carcinoma
RhoA: Ras homolog gene family, member
RNA : Ribonucleic acid
RPTEC : Renal proximal tubular epithelial cells
RSPS19: Ribosomal protein S19
SAPK: Stress-activated protein kinases
SIRS: Systemic inflammatory response syndrome
SLE: Systemic lupus erythematosus
TAMs: Tumor associated macrophages
TANs: Tumor associated neutrophils
TCGA: The cancer genome atlas
TCR: T cell receptor
TGF- β : Transforming growth factor beta
Th: Helper T lymphocytes
THBS1: Thrombospondin-1
TIM-3: T-cell immunoglobulin and mucin-domain containing-3

TLRs: Toll-like receptors
TLS: Tertiary lymphoid structure
TM: Thrombotic microangiopathy
TME: Tumor microenvironment
TNF: Tumor necrosis factor
Tregs: Regulatory T lymphocytes
TWIST1: Twist Family BHLH Transcription Factor 1
UV: Ultraviolet
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor
VHL: Von Hippel-Lindau
wAIHA: Warm autoimmune haemolytic
WWOX: WW Domain Containing Oxidoreductase
 β 2M: Beta 2-microglobulin

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Introduction

I. Cancer

I.1 Epidemiology

Cancer is a worldwide big scourge responsible for 18 078 957 new cases of cancers and 9 555 027 deaths worldwide in 2018 and that continues to grow despite important efforts in prevention and treatment. Indeed, taking into account population growth and ageing, epidemiologists project a rise in the annual number of new cancer cases from 18.1 million in 2018 to 29.4 million in 2040¹.

Lung, breast, colorectum, prostate and stomach constituted the top 5 of the most frequent cancers. In terms of mortality, the lung cancer reaches the first place with 1 761 007 deaths worldwide, followed by stomach, liver, breast and colon cancers² (Figure 1).

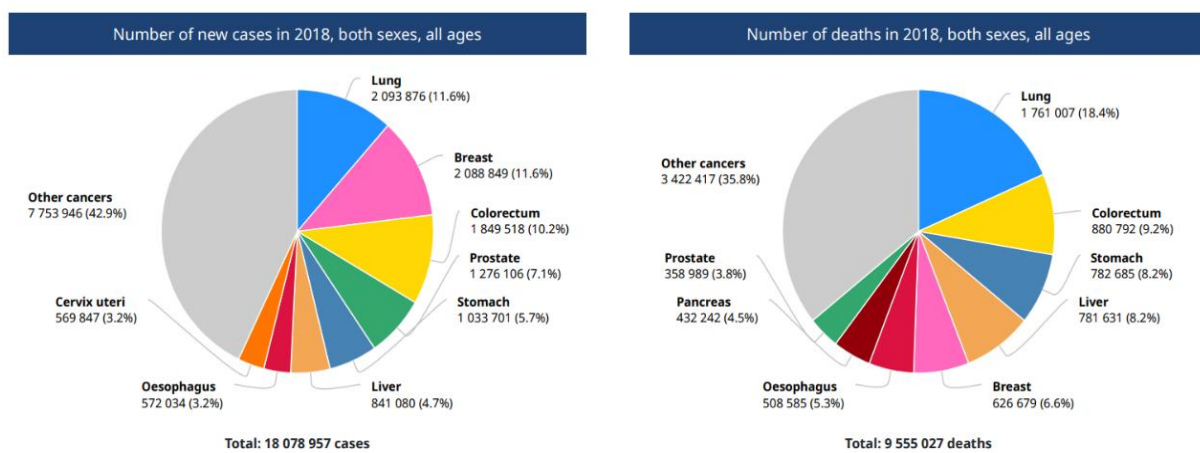


Figure 1: Prevalence and mortality of cancer (available on <http://gco.iarc.fr/today/home>.)

I.2 Carcinogenesis

Cancer is not just one disease but a group of more than 100 diseases. Each cancer type is named according to its organ of origin. Importantly, cancer types share some principal features but they can be very different in a biological point of view and also in terms of aggressiveness and response to treatment.

Cancer is a complex disease that affects the genome, in which an accumulation of successive mutations leads to a formation of a mass of abnormal cells called tumor³. Every cells

experience up to 10^5 lesions per day. Fortunately, the occurrence of sporadic mutations is a process that is tightly regulated by a DNA repair system. However, in some cases, this system is surpassed and the mutation is not eliminated. The maintenance of this mutation in the genome follows Darwinian selection. If the mutation confers a selective advantage for the cell, like inactivation of tumor suppressors or abnormal activation of oncogenes, this mutation is considered as “driver” and will be fixed and transmitted to its progeny⁴. The accumulation of driver mutations allows the transformation of a normal cell to a cancerous cell (Figure 2).

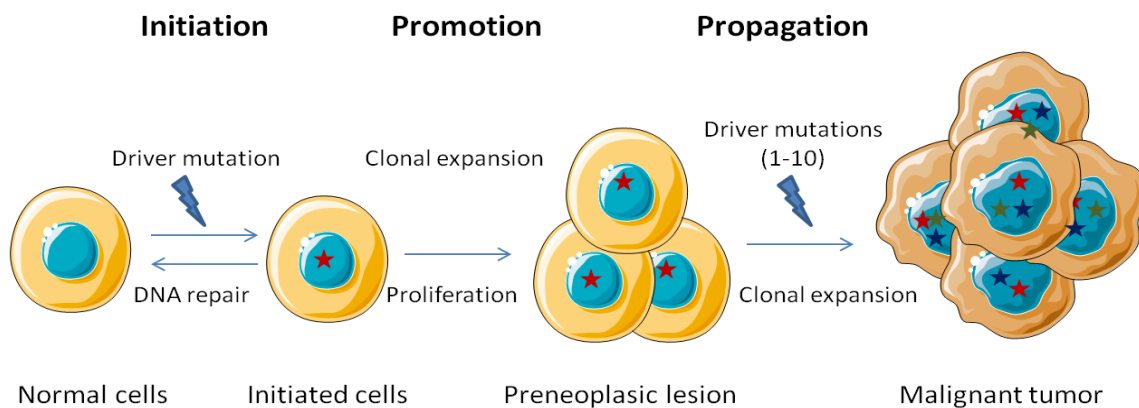


Figure 2: Carcinogenesis steps. Carcinogenesis is a multistep process where mutations accumulate in order to transform a normal cell to a malignant tumor.

The multistep evolution is not a linear process. When a cell acquires a driver mutation, a process of clonal selection occurs and the clone becomes dominant within the tumor. However, this step takes time and if the mutation rate is high, successive driver mutations can occur before the end of the selection, leading to an intratumoral heretogeneity⁵ (Figure 3).

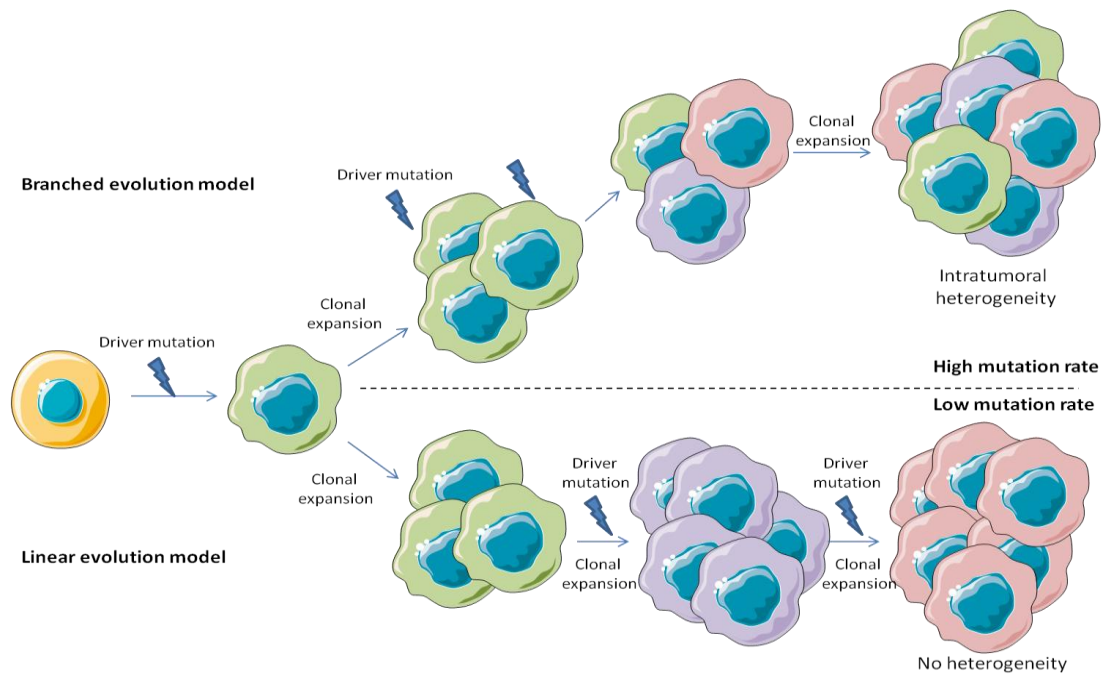


Figure 3: Clonal evolution of cancer. Depending on the mutation rate, the tumor can evolve through a linear or a branched evolution model that leads to different level of tumor heterogeneity.

I.3 Tumor microenvironment (TME)

Beyond the mass of proliferating cells, the tumor is a complex tissue growing into a microenvironment composed by many different other cells types, forming tumor-associated stroma, which are active participants in tumorigenesis (Figure 4)^{6,7}. These stromal cells can comprise more than 50% of the mass of primary tumors and their metastases⁸. The TME is a very dynamic site in which tumor cells, extracellular matrix, stromal cells and soluble factors interact with each other to positively or negatively regulate tumor growth⁷. Except tumor cells, the TME is composed by cancer stem cells, lymphatic/vascular endothelial cells, pericytes, fibroblasts, adipocytes and immune cells⁹. Nevertheless, the proportion and activation state of each cell can be very different according to cancer types and among patients.

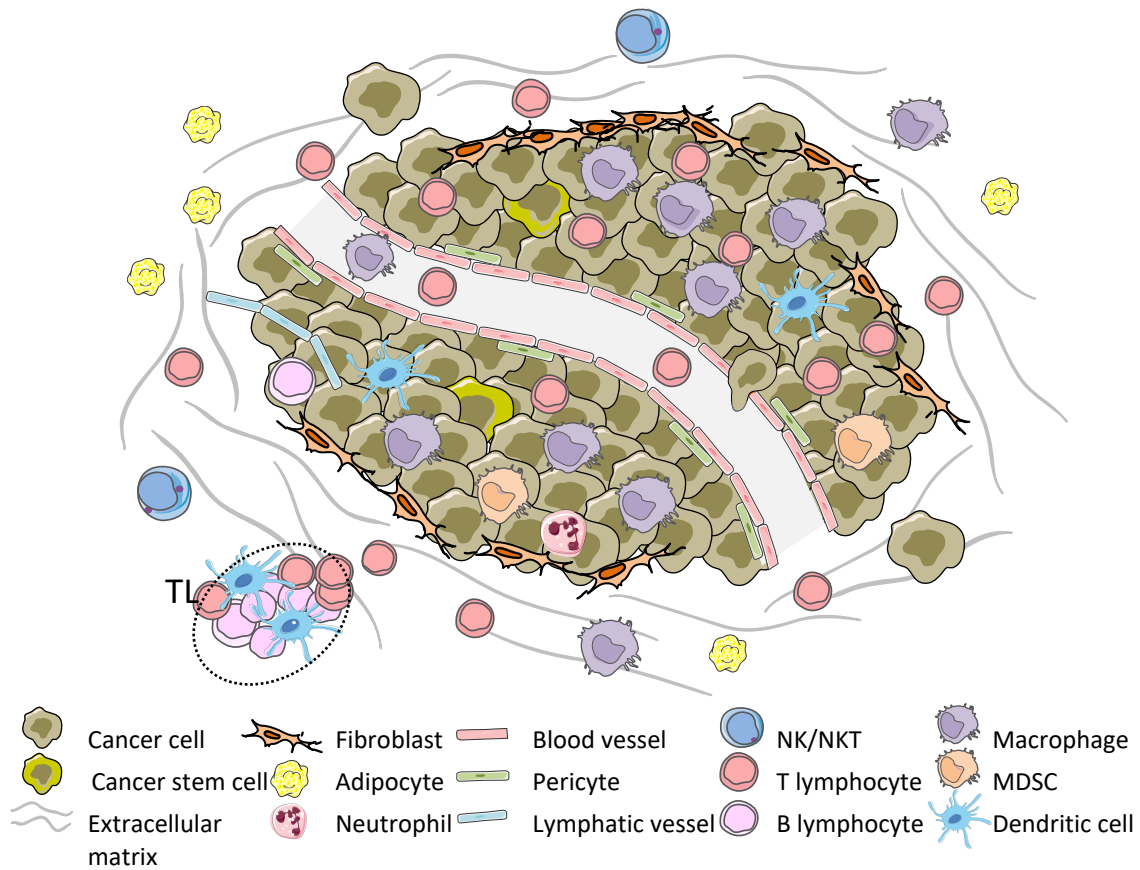


Figure 4: Tumor microenvironment. The TME is composed by many cell types which act together to suppress or support tumor growth.

Cancer stem cells

Cancer stem cells (CSC) are a rare population within the TME. CSCs display some particular features such as self-renewal capacity, long-term quiescence and resistance to conventional therapy (e.g chemotherapy) that targets proliferating cells. Thus, CSC can regenerate tumor after treatment and may be responsible for relapse¹⁰.

Endothelial cells

Vascular endothelial cells. Tumor-associated endothelial cells form vessels and are the key effector of angiogenesis to provide nutrient and oxygen for tumor growth¹¹. Tumor cells secrete growth factors such as vascular endothelial growth factor A (VEGFA) to induce the formation of new vessels that are often leaky and have chaotic branching. The interaction between tumor cells and endothelial cells is bidirectional, endothelial cells can also interact with tumor cells through the release of angiocrine factors to stimulate tumor progression but also with immune cells to control their local recruitment¹².

Lymphatic vessels are also an important player in tumor growth and dissemination of malignant cells in the draining lymph nodes.

Pericytes

Pericytes provide structural support for blood vessels^{11,13}. A low pericyte coverage of the vasculature can induce cancer cell intravasation into the circulation and dissemination and correlates with poor prognosis and metastases.

Fibroblasts

Cancer associated fibroblasts (CAFs) exert a key role in supporting and fueling tumor growth and dissemination by providing growth factors (HGF, FGF and IGF1), chemokines (CXCL12), cytokines (TGF- β) and extracellular matrix components (ECM). Fibroblasts are also implicated in the promotion of immunosuppressive microenvironment¹⁴.

Adipocytes

In some cancers, adipocytes help tumor growth by providing fatty acids as fuel for cancer cells and participate in the recruitment of malignant cells through adipokines secretion¹⁵.

Immune cells

While acute inflammation helps to control tumor growth on a first place, the induction of a chronic inflammation can sustain tumor development. This duality of immune cells effects operates within the TME with the presence of both tumor-antagonizing and tumor-promoting leukocytes.

T lymphocytes. T cells represent a major population within the TME that infiltrate tumor core and the invasive margin. T cells can have pro- or anti-tumor phenotypes depending on disease type and stage. CD8⁺ cytotoxic T cells, CD4⁺ Th1 helper and $\gamma\delta$ T cells are usually associated with a good prognosis whereas T regulatory cells (Tregs), CD4⁺ Th2 helper and Th17 cells are usually associated with poor prognosis^{9,16}.

B lymphocytes. The majority of B cells are found in tertiary lymphoid structure (TLS) adjacent to the tumor. B cell infiltration is associated with good prognosis in some cancers¹⁷. An immunosuppressive population of B cells that produced IL-10 and TGF β called Bregs is also detected within tumors

NK and NKT cells. NK and NKT cells are usually detected outside the tumor area and exert anti-tumoral effects through their cytotoxic properties. However, the presence of TGF β in the TME is responsible for anergic phenotype of NK cells.

Tumor associated macrophages (TAMs). TAM constitute the most abundant immune population and are usually associated with a pro-tumoral¹⁸ effect by promoting tumor growth, immunosuppression and angiogenesis. The macrophages are classically separated into M1 and M2 macrophages^{19,20}. While M1 are pro-inflammatory cells which promote an anti tumor Th1 response, M2 macrophages display anti-inflammatory properties. These anti-inflammatory properties are linked to secretion of cytokines such as Il-10, TGF- β or arginase-1 which enhance Th2 response. M2 also promote angiogenesis through secretion of VEGF and metastasis via production of MMPs.

Myeloid derived suppressor cells (MDSCs). MDSCs are a heterogeneous population of immature myeloid cells that are not present at steady state and appear in pathological conditions with chronic inflammation like cancer. MDSCs can be divided into two groups granulocytic (PMN-MDSC) or monocytic (M-MDSC) according to phenotypically and morphologically resemblance to neutrophils or monocytes respectively²¹. Both populations display immunosuppressive properties mainly by modulating T cell response. While PMN-MDSCs mainly use ROS production to induce T cell tolerance, M-MDSC modulate T cell response by utilizing iNOS, arginase and Il-10²²⁻²⁴.

Tumor associated neutrophils (TANs). Two types of TAN can infiltrate the tumor; N1 and N2. N1 display anti-tumoral effects and in the opposite, N2 exert pro-tumoral effects through arginase-1 secretion and growth factor production (VEGF and HGF).

Dendritic cells (DCs). DCs in the TME are often immature and unable to stimulate immune response to tumor-associated antigens through downregulation of MHC II, co-stimulatory signals and decrease production of IL-12.

Mast cells. In cancer, mast cells are found to exert pro-tumoral effect t by supporting tumor growth and dissemination as well as angiogenesis and immunosuppressive immune environment.

I.4 Hallmarks of cancer

Carcinogenesis is a multistep process in which a normal cell accumulates some genetic or epigenetic “driver” alterations (single nucleotide errors, deletions and insertions) that confer a selective advantage for its growth, survival and dissemination. This selective process referred to Darwinian principle and 5 to 15 mutations are needed to progressively convert a normal cell into cancer cell. The main processes affected during this multistep development of human tumor are called hallmarks of cancer and include proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming of cellular energy, evading immune destruction and activating invasion and metastasis^{11,25} (Figure 5).



Figure 5: Hallmarks of cancer (Hanahan et al., Cell, 2011)

I.4.1 Sustaining proliferative signaling

Tumor cells acquire the ability to deregulate growth-promoting signals in their favor in order to sustain chronic proliferation.

This capacity results from different mechanisms:

- Tumor cells produce growth factors that act in an autocrine manner to stimulate proliferation.

- Tumor-associated stroma produces growth factors that stimulate tumor cells proliferation in a paracrine way.
- Tumor cells can upregulate level of receptors at cell surface or facilitate ligand-receptor interactions
- Tumor cells can become totally independent from growth factor by constitutively activate downstream component of signaling pathway.

I.4.2 Evading growth suppressors

Tumor cells circumvent processes that negatively regulate cell proliferation. The regulation of cell proliferation is controlled by different genes, named tumor suppressor genes such as TP53. TP53, also called “guardian of the genome”, responses to genetic alteration by inducing cell cycle arrest and apoptosis in order to preserve genome integrity²⁶. Thus, it is not a surprise that TP53 gene is one of the most frequently mutated gene in human cancer with 36% of cancer patients that display a mutation²⁷.

I.4.3 Resisting cell death

Tumor develops different strategies to limit cell death. On one hand, tumor cells block multiple apoptosis-inducing signals such as TP53 or the pro-apoptotic (Bax, Bim, Puma). On the other hand, tumors also circumvent apoptosis through increase expression of anti-apoptotic regulators (Bcl-2, Bcl-xl).

I.4.4 Enabling replicative immortality

The tumor cells acquire the ability to growth and divide in an unlimited way through breakdown of senescence and crisis. This immortality behavior comes from a protection of telomeres from erosion that governs the number of growth-division cycles.

I.4.5 Inducing angiogenesis

According to its high proliferation rate, tumor needs a lot of energy in order to fuel their nutrient and oxygen consumption and evacuate metabolic wastes. This particular need is achieved with the development of new vessels from existing ones, called angiogenesis. This process occurring early in the tumor progression is governed pro-angiogenic signals such as vascular endothelial growth factor A (VEGF-A) or fibroblast growth factor (FGF). Moreover,

angiogenesis can be supported by innate immune cells like macrophages, neutrophils or mast cells.

I.4.6 Activating invasion and metastasis

The majority of cancer-associated deaths (about 90%) are caused by metastatic disease rather than primary tumors. Invasion is a multistep process in which tumor cells can invade first local tissue then passed through blood or lymphatic vessel (intravasation) and transit to a distant site where tumor cells traverse through the vascular wall (extravasation) and invade and colonize a distant tissue to form metastasis^{11,28}.

In order to propagate, tumor cells can acquire an invasive phenotype and disseminate from tissue of origin to local invasion and distant metastasis. The invasion phenotype is characterized by a modification in their shape and their attachment to other cells and to the extracellular matrix (ECM). This change called epithelial to mesenchymal transition (EMT) is controlling by different transcriptional factors such as Snail, Slug, Twist and Zeb1/2 and is associated with a loss protein implicated in cell-to-cell adhesion or cell-to ECM adhesion like E cadherin and acquired expression of cell migration molecules like N-cadherin.

EMT is a reversible process. In absence of EMT inducing signals, tumor cells revert their phenotype to a non-invasive one. This plasticity allows the invasion of tumor cells from their initial site to a distant one and then the reverse process (mesenchymal-to epithelial transition) result in the formation of new tumor colonies.

EMT program is linked to the “mesenchymal” invasion. However, there are other modes of invasion such as “collective invasion” which referred to the invasion of masses of cancer cells and “amoeboid” in which tumor cells slither through existing interstice in the ECM rather than clearing a path for themselves like in “mesenchymal” or “collective” invasion.

I.4.7 Reprogramming cellular energy

Tumor cells adapt their metabolism in order to support cell growth and proliferation. This adaptation goes through a switch from mitochondrial oxidative phosphorylation to glycolysis to produce energy. This metabolic switch called “Warburg effect” enable less ATP production (18 fold lower) but allows more biosynthesis of nucleosides and amino-acids that are essential for active cells proliferation^{11,29}.

I.4.8 Avoiding immune destruction

The interaction between tumor cells and the immune system is complex and can be viewed as a balance between a protective immune system that provides an anti-tumor response and an immunosuppressive microenvironment that fuels tumor growth. This balance is subject to many variations over time and follows the 3E rule, elimination, equilibrium and escape³⁰⁻³² (Figure 6).

I.4.8.1 Elimination

Tumor cells express at their surface signals of danger that can be recognized locally by dendritic cells, macrophages and NK cells and lead to chemokines secretion and recruitment of new innate immune cells. Tumor cells killing by innate immune cells generate a source of tumor antigens that drive the development of tumor-specific adaptive immune response by T and B lymphocytes. This immunosurveillance allows a complete elimination of tumor cells.

I.4.8.2 Equilibrium

Sometimes, tumor cells survive from the elimination phase and enter into an equilibrium in which the immune system exerts a sufficient pressure to contain tumor development but not fully extinguish tumor cells leading to a quiescent phase. Equilibrium is the longest of the three phases and can last for many years. Gradually, the constant immune pressure leads to selection of tumor clones with reduced immunogenicity.

I.4.8.3 Escape

Tumor cell clones selected in the equilibrium phase develop multiple mechanisms to escape from the innate and adaptive immune system that allow them to grow and become clinically detectable. These immunoevasive strategies occur at different levels: the tumor cells and the immune microenvironment. In tumor cells, different mechanisms are developed to become immune silent such as loss of tumor antigen expression, loss of MHC I, expression of immune checkpoint), resistance to apoptosis, immunosuppressive cytokines secretion (VEGF, TGF- β , IDO). Within the tumor microenvironment, a switch toward an anti-inflammatory immune response occurs with recruitment of tumor associated macrophages, MDSC, Tregs or fibroblasts.

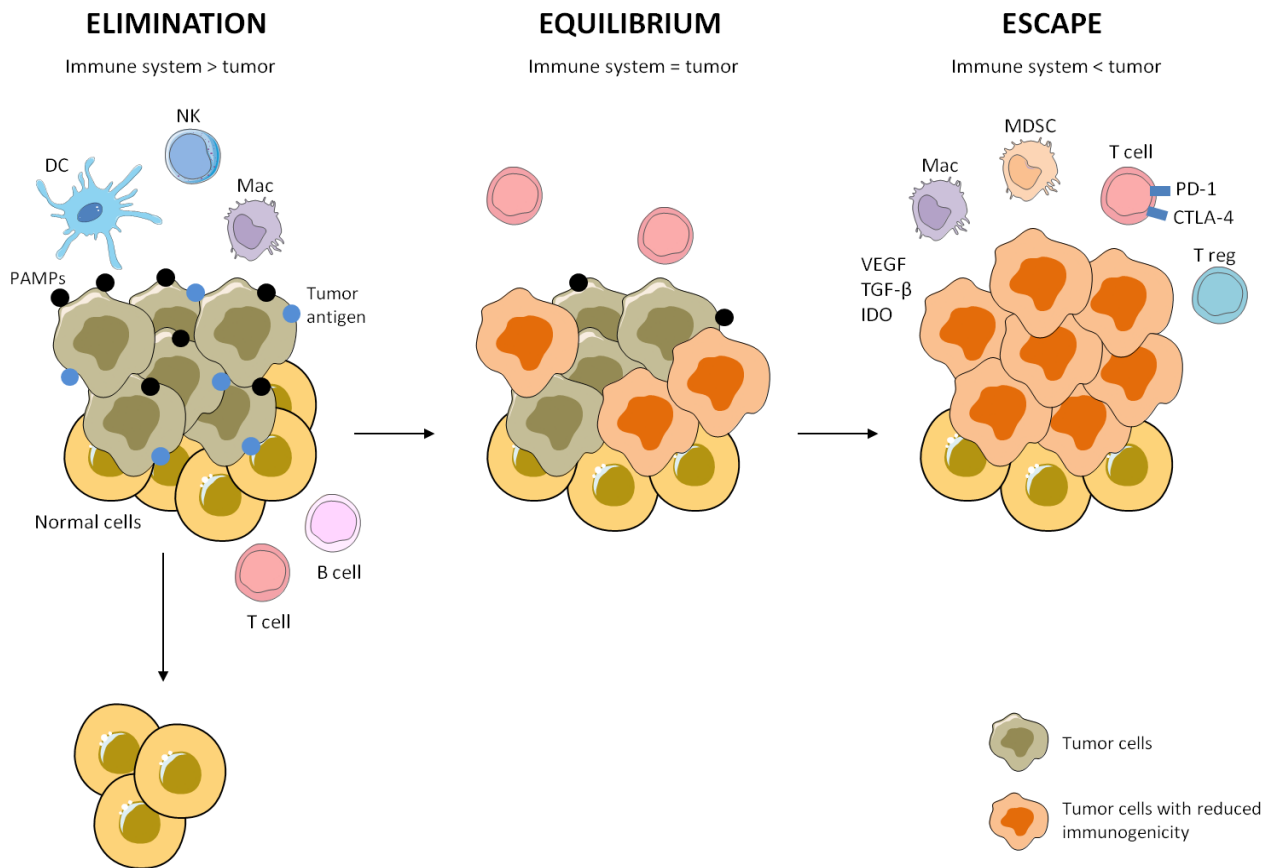


Figure 6 : Cancer immunoediting (adapted from Schreiber et al., Science 2011). After transformation from normal to tumor cell, the immune system is required and act by three sequential phases : elimination, equilibrium and escape

I.4.8.4 Immune checkpoint

In response to antigen presentation, the engagement of TCR results in T cell activation. However, to avoid an overactivation of T cell response, this system is tightly regulated by inhibitory signals provided immune checkpoints^{33,38}. Within the TME, tumor cells can escape from immune response by stimulation of an overexpression of immune-checkpoint proteins such as PD-1, TIM-3, CTLA-4, LAG-3 or BTLA that regulate CD8+ T cells expansion. These proteins interact with their receptors presents at the surface of myeloid or cancer cells (Figure 7).

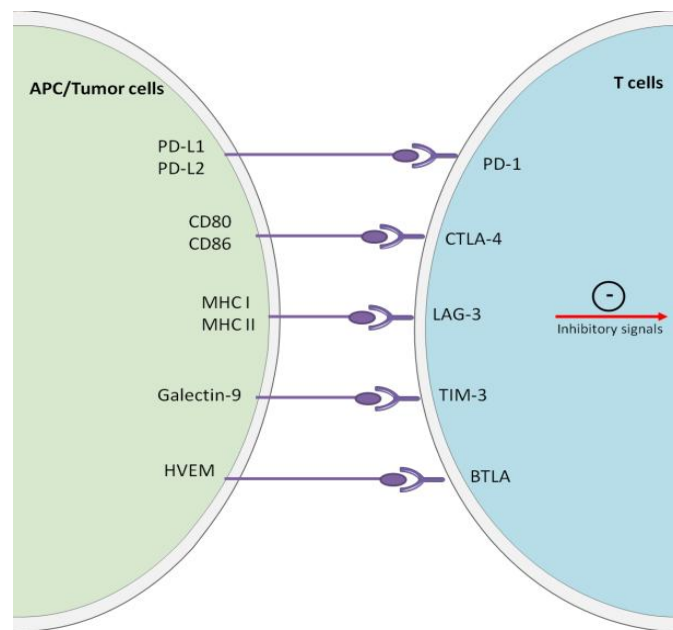


Figure 7: Immune checkpoints that negatively regulate T cell response (adapted from Pardoll et al., Nat Rev Cancer, 2012). APC: antigen-presenting cell, BTLA: B and T lymphocyte associated, CTLA-4: cytotoxic T-lymphocyte-associated protein 4, HVEM: Herpesvirus entry mediator, LAG-3: lymphocyte-activation gene 3, MHC : Major histocompatibility complex, PD-1: Programmed cell death 1, PD-L1: Programmed death ligand 1, TIM3: T-cell immunoglobulin and mucin-domain containing-3

I.5 Risk factors for cancer

Every factor that increases the mutation rate or modulation of key cancer modulating genes increases the susceptibility to develop cancer. Thus, an impaired DNA repair system, exposure to genotoxic agents (e.g cigarettes, alcohol, drugs, virus, UV ⁴) or endogenous factors (e.g age, hormones, inflammation) are considered as risk factor for cancer³⁵ (Table 1).

The prevalence of risk factor is not uniform worldwide and changes over time. For example, chronic infections are associated with one in three cancers in sub-saharan Africa whereas it represent only one in thirty in North America¹.

Risk factor	Impact
Age	Most important risk factor for cancer overall. The median age of cancer diagnosis is 66 years
Alcohol	Alcohol is associated with increased risk of cancer of mouth, throat, esophagus, larynx, liver and breast
Chronic inflammation	Chronic inflammation can cause DNA damage and cancer. Chronic inflammatory bowel diseases (ulcerative colitis, Crohn disease) increase the risk of colon cancer
Hormones	Estrogen and progesterone increase the risk of breast and endometrial cancers
Immuno-suppression	Drugs or HIV can induce immunosuppression and increase the risk of certain cancers
Infection agents	<p><u>Viruses</u></p> <p>Epstein-Barr virus: lymphoma, nose and throat cancer</p> <p>Hepatitis B and C virus: liver cancer</p> <p>HIV: kaposi sarcoma, lymphoma, cervix, anus, lung, liver and throat cancer</p> <p>Human papillomaviruses: cervical, anal, oropharyngeal, vaginal, vulvar and penile cancers</p> <p>Human T-Cell Leukemia/Lymphoma Virus Type 1: non-hodgkin lymphoma</p> <p>Kaposi sarcoma associated herpesvirus: Kaposi sarcoma</p> <p>Merkel cell polyomavirus: merkel cell carcinoma</p> <p><u>Bacteria</u></p> <p>Helicobacter pylori: stomach cancer, lymphoma</p> <p><u>Parasites</u></p> <p>Opisthorchis viverrini: cholangiocarcinoma</p> <p>Shistosoma hematobium: bladder cancer</p>
Obesity	Obesity is a risk factor of several types of cancer including breast, colon, rectum, endometrium, esophagus, kidney, pancreas and gallbladder
Radiation	Radon increases the risk of lung cancer
Sunlight	UV exposure increases the risk of skin cancer
Tobacco	Leading cause of cancer and of death from cancer. Tobacco increase the risk of lung, larynx, mouth, esophagus, throat, bladder, kidney, liver, stomach, pancreas, colon, rectum, cervix cancers and acute myeloid leukemia

Table 1: Risk factors of cancer. Intrinsic or extrinsic risk factors for cancer development.

I.6 Cancer treatment

The landscape of cancer therapy has evolved dramatically over the last four decades with the development of new drugs and biomarkers. In advanced or metastatic stages, where surgery is not sufficient, others therapeutic strategies are needed. A large arsenal of drugs targeting all cancer hallmarks are currently available or under development. However, primary or acquired resistance limited the effectiveness to cancer therapy and lead to clinical relapse^{36,37}. To overcome this resistance and improve efficacy, the use of biomarkers that predict response to treatment and combination of drugs represent an effective strategy.

I.6.1 Chemotherapy

Chemotherapy is classically used to treat cancer and targets proliferative cells in a non-specific manner. This leads to numerous severe adverse effects. Depending on cancer types, the proliferation level is not equal and the efficacy of chemotherapy can vary considerably. Moreover, cancer stem cells have a very low level of proliferation making them resistant to chemotherapy and responsible for relapse. Taking into account the complexity of the tumor and its microenvironment and the close and dynamic relationship between both, it is not surprising that the targeting of one population can impact the others populations within the TME. For example, chemotherapy can induce immunogenic cell death that stimulate an anti-tumor response³⁸.

I.6.2 Radiotherapy

Radiotherapy is an important component of cancer treatment with around 50% of all cancer patients receiving radiation therapy³⁹. Radiotherapy is a loco-regional strategy inducing damages on genetic material and leading to cell death.

I.6.3 Hormonotherapy

Some cancers such as prostate cancer and breast cancer use hormonal receptors to sustain tumor growth. In these cases, removing of hormones or blocking their effect by hormonotherapy can be useful to treat cancer⁴⁰.

I.6.4 Anti-angiogenic

Anti-angiogenic inhibitors that block VEGF (bevacizumab) or its receptor (sunitinib, sorafenib) have been developed and approved in different cancers such as colorectal cancer,

non-small cell lung cancer (NSCLC), glioblastoma or renal cell carcinoma. However, anti-angiogenic agents can induce hypoxia and a switch of malignant cells toward a more invasive phenotype leading to resistance to treatment.

I.6.5 Targeted therapies

Contrary to chemotherapy, targeted therapy attacks a specific alteration of cancer cells⁴¹. Considering that every tumor is unique in its genetic characteristics, the choice of the treatment must be adapted to the patients. This concept referred to personalized medicine⁴². Taking into account the large spectrum of molecular alterations in cancer, many targeting therapies have been developed in the past decades. These targeted therapies belong to 3 categories; tyrosine kinase inhibitors (e.g imatinib), serine-threonine kinase inhibitors (e.g. vemurafenib) and monoclonal antibodies (e.g trastuzumab). Tumor cells can adapt to targeted therapy by using redundant signaling pathway leading to clinical relapse. The development of this adaptive resistance occurs through mutation, epigenetic reprogramming or remodeling of the TME. Moreover, the intra-tumoral heterogeneity leads to a non-homogeneous response to therapy and a selection of resisting clones that can also induce relapse after treatment⁴³.

I.6.6 Immunotherapy

Another key actor of the TME that can be target is the immune microenvironment. A successful immunotherapy results in a long term modification of the immune infiltrate toward a pro-inflammatory phenotype that promotes an anti-tumor response allowing tumor cells destruction. Unfortunately, a minority of the patients respond to immunotherapy. Thus, a characterization of the immune environment before treatment is necessary to predict the response and adapt the treatment. Tumors can be classified into 3 categories according to the immune infiltrate; tumors without pre-existing antitumor immunity (immune desert), tumors with T cells accumulated at the periphery of the tumor (immune-excluded) and tumors with high infiltration of T cells that display an exhausted phenotype (inflamed)⁴⁴⁻⁴⁷. According to the immune class, the treatment needs to be adapted. In immune desert tumor, a T cells response must be generated through vaccine or immunogenic cell death. In immune-excluded tumor, an enhancement of T cell infiltration is required. Finally, in inflamed tumors, a reversion of the exhausted phenotype toward an active one is needed and can be achieve by immune-checkpoint blockade. Among the multiple immune checkpoint that can be targeted, monoclonal antibodies acting on PD-1/PD-L1 or CTLA4 are approved for the treatment of some cancer⁴⁸ (Table 2).

Target	Drug name	Indications
PD-1	Nivolumab	Metastatic melanoma, metastatic squamous NSCLC, non-squamous NSCLC, advanced RCC, Hodgkin lymphoma, urothelial carcinoma, colorectal cancer with microsatellite instability, HCC
	Pembrolizumab	Hodgkin lymphoma, metastatic non squamous NSCLC, urothelial carcinoma, colorectal cancer with microsatellite instability, gastric adenocarcinoma
PD-L1	Avelumab,	Metastatic Merkel cell carcinoma, urothelial carcinoma
	Atezolizumab	Urothelial carcinoma, metastatic NSCLC
	Durvalumab	Urothelial carcinoma, NSCLC
CTLA-4	Ipilimumab	Metastatic melanoma

Table 2: List of immune-checkpoint inhibitors approved in cancer therapy. HCC: Hepatocellular carcinoma; NSCLC: Non-small cell lung cancer, RCC: renal cell carcinoma

I.7 Focus on clear cell renal cell carcinoma

In 2018, kidney cancer (RCC) was responsible for 403262 new cases and 175098 deaths worldwide. It affects preferentially men with 2 fold increase incidence in comparison to women². Recognized risk factor for RCC cancer include cigarette smoking, obesity, hypertension and environmental exposures to carcinogenic agents⁴⁹.

Kidney cancers are divided into 3 major histologic subtypes depending on the cell of origin; clear cell renal cell carcinoma (ccRCC, KIRC) which represents more than 75% of all kidney cancer cases, chromophobe renal cell carcinoma (5%) (crRCC, KICH) and papillary renal cell carcinoma (10%) (pRCC, KIRP)⁵⁰. The origin of ccRCC is proximal tubular epithelial cells⁵¹, pRCC is distal tubular epithelial cells and crRCC is intercalated cells of the distal tubules. At the cellular level, ccRCC tumor cells present a big clear cytoplasm due to a high content in lipid and glycogen.

Most ccRCC are sporadic (95%) and the remaining 5% have hereditary genetic background such as von Hippel-Lindau disease characterized by a mutation in VHL gene, a tumor suppressor implicated in VEGF regulation through HIF transcription factor⁵². Moreover, VHL is mutated not only in inherited ccRCC but also in most sporadic ccRCC with 86% of patients with both copies lost⁵³. This ubiquitous genetic alteration, that affects the regulation of angiogenic process, leads to highly vascularized tumor. Besides VHL, other driver mutations such as the tumor suppressors; polybromo-1 (*PBRM1*), SET domain-containing 2 (*SETD2*), and BRCA1-associated protein-1 (*BAP1*) as well as gene alteration of the PI3K/Akt/mTOR pathway participate in the ccRCC carcinogenesis⁵⁴.

TNM stages and Fuhrman grade are commonly used to classify the tumor and predict their aggressiveness. TNM classification consists of analysis of the spreading of the tumor by determining tumor size and location (T), lymph node spreading (N) and presence of metastases (M). Fuhrman grade is another staging method based on nuclei and nucleoli size and morphology. In a general manner, ccRCC tumors grow relatively slowly. However, survival can vary a lot between patients according to TNM stage and Fuhrman grade. For instance, the five-year survival rate for localized ccRCC is 73.2% whereas it reaches 10.5% for non-localized ccRCC patients⁵⁵.

In addition to the anatomopathological parameters, molecular classification can also represent a useful prognostic biomarker. Molecular analyses indicate a correlation between worsened prognosis and metabolic shift with reprogramming of glucose and fatty acid metabolism as well as modifications in tricarboxylic acid cycle^{56,57}

Moreover, immune parameters may also provide key information on ccRCC aggressiveness. Surprisingly in ccRCC, a high level of CD8+ T cells in the invasive margin is associated with a shorter PFS and OS⁵⁸. This negative impact can be linked to an ineffective CD8+ T response due to a low density of TLS and immature DCs present in the TME that is responsible for a defective education of T cells^{58,59}.

Partial or complete nephrectomy is the standard of care for local ccRCC. In metastatic ccRCC, the choice of the therapy depends on the risk score that takes into account TNM stage, Fuhrman grade and necrosis. In low risk patients, the first line treatment is targeted therapy (sunitinib, pazopanib, tivozanib) and anti-VEGF monoclonal antibody (bevacizumab). On the contrary, for patients with intermediate or high risk, the use of immunotherapy is recommended (nivolumab, ipilimumab)^{60,61}.

II. Complement system

The complement system is an evolutionarily ancient component of the immunity that is well conserved among species⁶². Complement system refers to a set of more than 50 membrane or plasmatic proteins. It was first described in the late 19th century by Jules Bordet as a heat-labile component of plasma that causes the killing of bacteria and was called “alexin”. Paul Ehrlich renamed it “complement” because of its ability to support/complement the functions of antibodies to kill pathogens. In early 20th century, Ferrata recognized complement to be a multi-component system, present in the sera of many species⁶³.

The vision for the complement system has evolved during the past decades and it is no more confined to the notion of a supportive part for antibody-mediated bacterial killing, but as a major effector of the immune system. Indeed, it is now appreciated that complement is involved in many processes to protect host against foreign or damaged elements such as pathogens but also apoptotic cells. To exert these functions, the complement system works as a sentinel system operating in the circulation and in tissues. In presence of pathogens or stressed cells, the complement system is activated and a rapid sequential cleavage of complement proteins occurs to allow the generation of bioactive fragments that play a key role in pathogen elimination and host homeostasis by inducing inflammation, opsonization/phagocytosis and lysis⁶⁴ (canonical functions). Nevertheless, there is now growing evidence that complement activity goes well beyond “classical” functions and that this system is also required for multiple other processes including adaptive immune response, development, cell metabolism or coagulation (non-canonical functions)⁶⁵.

In order to avoid host cells damage, this system is tightly controlled by an army of soluble or plasmatic regulators⁶⁶. Indeed, an imbalance in complement system towards either an excessive or a defective activation can cause adverse effects for host cells and may be involved in the development of various severe pathologies such as; autoimmune diseases, renal diseases, infections, neurodegenerative diseases or cancer⁶⁴. This implication in the physiopathology of many diseases, whose number continues to grow, leads to the development of new drugs targeting the complement system⁶⁷.

II.1 Source of complement proteins

In the whole body, the main producer of complement proteins is the liver and especially the hepatocytes. Hepatocytes produce most of the proteins of the complement system except C1q,

Factor D (FD) and Factor P (FP)⁶⁸ and contribute up to 90% of the plasmatic concentration of complement proteins⁶⁹. The remaining 10% come from immune cells, epithelial cells and endothelial cells or specialized cells within different organs. The transcription of complement genes can be modulated by various stimuli including cytokines (e.g Il-6, Il-1, IFN γ)⁷⁰ or hormones⁷¹.

In contrast to the systemic compartment, in tissues, the contribution of local complement production by a large panel of immune or non-immune cells (Table 3)^{68,72} is not negligible and plays a major role in local processes⁷³. Indeed, the complement proteins produced locally can be secreted and act in an autocrine manner but surprisingly the complement proteins can also remain within the cells to act intracellularly. This pool of intracellular complement proteins is called “complosome”^{72,74} (Figure 1).

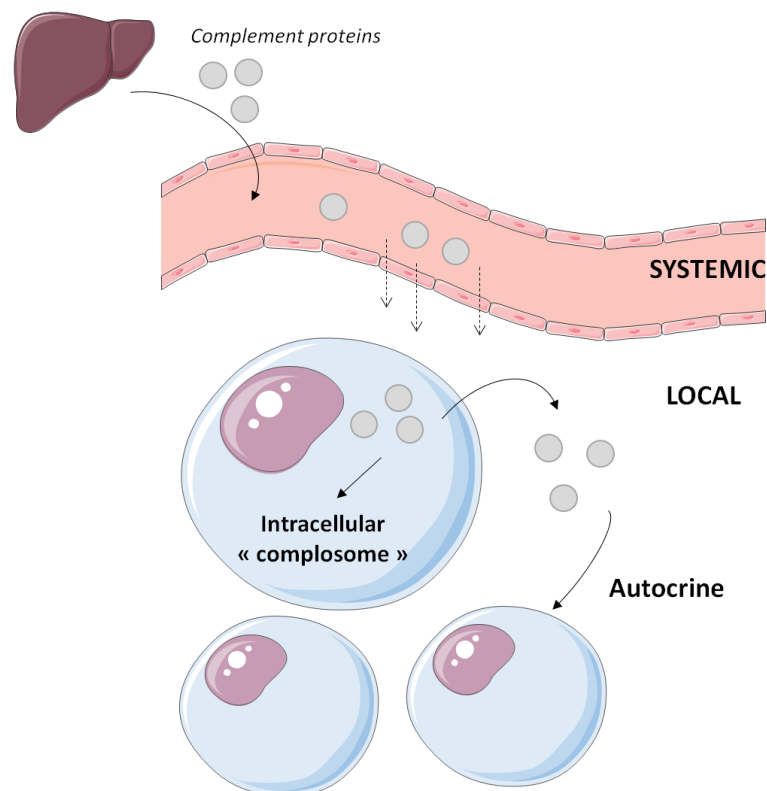


Figure 8: Source of complement proteins. In the systemic compartment, the complement proteins mostly come from the liver. In the local compartment, the complement proteins are produced by various cell types and can act through autocrine or intracellular ways.

Cell types	Complement components	Complement regulators	Complement receptors
Hepatocytes	C1r, C1s, C2-C9, FB, MBL, Ficolin- 2, Ficolin-3, CL-10, CL-11, MASP-1, MASP-2, MASP-3	C1INH, C4BP, FH, FI	C1qR, C3aR, C5aR1/2
T cells	C3, C5, FB, FD, FP	CD46, CD55, CD59	CR1, CR2, CR4, C3aR, C5aR1/2
B cells	C3, C5	FH, FI, CD46, CD55, CD59	C1qR, CR1, CR2, CR4, C3aR, C5aR1
Monocytes	C1q, C1r, C1s, C2-C9, FB, FD, FP, MBL, Ficolin-1, CL-11, MASP-1, MASP-2, MASP-3	C1INH, C4BP, FH, FI, CD46, CD55, CD59	C1qR, CR1, CR3, CR4, C3aR, C5aR1/2
Macrophages	C1q, C1r, C1s, C2-C9, FB, FD, FP, CL-11	C1INH, FH, FI	C1qR, CR1, CR3, CR4, CR1g, C3aR, C5aR1/2
PMN	Ficolin-1, C3, FB, FP, C6, C7	CD46, CD55, CD59	CR1, CR3, CR4, C3aR, C5aR1
Mast cell	C1q, C3, C5,	CD46, CD55, CD59	CR1, CR4, C3aR, C5aR1
Dendritic cell	C1q, C1r, C1s, C2-C9, FB, FD, FP	C4BP, FH, FI, CD46, CD55, CD59	CR1, CR3, CR4, CR1g, C3aR, C5aR1
NK		CD46, CD55, CD59	CR3, CR4, C3aR, C5aR1/2
Platelet	C5-C9	C1INH, FH	C1qR, CR1, CR4, C3aR, C5aR1/2
Fibroblasts	C1q, C1r, C1s, C2-C9, FB, MASP1	FH, CD46	C1qR
Endothelial cells	C1q, C1s, C2, C3, C4, FB, CL-11, CL-12	C1INH, FH, FI, CD46	C1qR, CR1, C5aR1/2
Epithelial cells	C1q, C1r, C1s, C2-C5, FB, Ficolin-1, Ficolin-3	C1INH, FH, CD46	C1qR, CR3, C5aR1/2
Keratinocytes	C3, FB	C1INH, FH	CR1, CR2, C5aR1/2
Adipocytes	C1q, C1r, C1s, C3, FB, FD, FP	FH, FI	CR1, CR2, C5aR1/2
Astrocytes	C1q, C1r, C1s, C2-C9, FB, FD	C1INH, FH, FI	C1qR, CR1, CR2, C3aR, C5aR1/2
Pancreatic islets	C1q, C1s, C3-C7, FB	C4BP, FH, FI	
Retinal cells	C1q, C1r, C1s, C2-C5, FB, MBL, MASP1, MASP2	FH, FI, CD46	CR1
Chondrocytes	C1q, C1r, C1s, C2-C4	C1INH	
Myocytes	C3-C5	FH, FI, CD46	

Table 3: Cell types involved in local complement production (adapted from Reichhardt et al., 2018 and Lubbers et al., 2017). C1inh: C1 inhibitor, C1qR: C1q receptor, C3aR: C3a

receptor, C4BP: C4 binding protein, C5aR: C5a receptor, CL: collectin, CR: complement receptor, FB: Factor B, FCN: Ficolin, FD: Factor D, FH: Factor H, FI: Factor I, FP: Properdin, MAC: Membrane Attack Complex, MASP: Mannan-Associated Serine, MBL: Mannan-Binding Lectin, NK: natural killer, PMN: polymorphonuclear

II.2 Pathways of activation

The activation of the complement system consists of a cascade processes, driven by conformational modifications and inducing a rapid and local reaction. The complement system can be activated by two different ways: an intrinsic or extrinsic activation. The intrinsic activation consists of proteolytic cascade through three different pathways; the classical pathway, the lectin pathway and the alternative pathway. In contrast, the extrinsic activation refers to an unconventional direct cleavage of C3 or C5 generating bioactive fragments that bypass the three established pathways. Surprisingly, the extrinsic activation occurs in both extracellular and intracellular levels.

II.2.1 Intrinsic complement activation

The complement system can be activated by 3 different pathways; the classical pathway, the lectin pathway and the alternative pathway (Figure 9). All of these pathways converge to the cleavage of the central component, C3, and the activation of a common terminal pathway.

II.2.1.1 Classical pathway activation

The activation of the classical pathway occurs after the fixation of the C1 complex to an activator. C1 is a large complex of 790kDa composed by a recognition molecule C1q that associate to a heterotetramer of four serine proteases; two C1r and two C1s molecules (C1s-C1r-C1r-C1s), in a Ca²⁺ dependent manner^{75, 76}. In physiological conditions, C1q is associated with proenzymes C1r and C1s to form an inactive C1 complex in the plasma. However, in the presence of an activator, the binding of C1 induce a major conformational change and an autoactivation of C1r occurs. C1r is able to cleave and activate C1s and the active C1s cleaves is canonical substrates C4 and C2⁷⁶. The cleavage of C4 generates a bioactive fragment C4b and a small fragment C4a. C4b binds covalently to the surface of adjacent cells and interacts with C2 which is cleaved by active C1s and allow the generation of the C2a and C2b⁷⁷. C2b is released in the circulation and C2a stays bound to C4b to form the central enzyme; the classical C3 convertase (C4b2a). Of note, to standardize complement

nomenclature the usual denomination of C2a and C2b has to be changed in order that the smaller fragment retains the earlier letter than larger fragments. In this case, the classical C3 convertase is renamed C4b2b⁷⁸.

Besides immune complexes, C1q can recognize a variety of others ligands including pathogen structures or apoptotic cells (Table 4).

Immunoglobulins	Fc fragment of IgG (IgG3> IgG1>IgG2), IgM
Apoptotic cells	Phosphatidyl serine, double stranded DNA, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), annexins A2 and A5 and calreticulin
Virus	HIV-1
Bacteria	Lipopolysaccharide (LPS), bacterial porins
Pentraxins	C-reactive protein (CRP), pentraxin 3

Table 4: Ligands of C1q

The efficiency of classical pathway activation is very dependent of the nature and the density of the C1 ligands. For example, IgM are more efficient to activate complement than IgG. Moreover, a cluster of IgG leads to a stronger activation of complement than an isolated IgG⁷⁹. Indeed, the affinity of C1q for single-IgG-molecule is very low and IgG clustering or aggregation are required to form a recognition pattern and activate complement like in case of IgM that are already multivalent⁸⁰.

II.2.1.2 Lectin pathway activation

The ficolins (H, L and M), collectins and MBL are the recognition molecules that trigger the activation of the lectin pathway. C1q and MBL display similar structure and analogy in the activation pathway. MBL associate to MBL-associated serine proteases (MASP-1 and MASP-2) in a calcium dependent manner. This complex allows the cleavage of C4 and C2 after the fixation to mannose residues and enables the formation of the C3 convertase C4b2b (previously C4b2a) as it occurs for classical pathway.

II.2.1.3 Alternative pathway activation

Under normal physiological conditions, the alternative pathway is constitutively activated at a low level in order to maintain a surveillance level for pathogen invasion. This constitutive activation occurs by the spontaneous hydrolysis of C3 into bioactive C3(H₂O) in the fluid phase, called tick-over. C3(H₂O) can bind to Factor B (FB) which is cleaved by the serine protease Factor D (FD) into Ba (released) and Bb fragments and form to a fluid phase C3

convertase complex C3(H₂O)Bb. This convertase is able to cleave small amount of C3 into C3a and C3b. The opsonin C3b can bind to cell surface through thioester bond or surface molecule that serve as platforms but it is rapidly degraded by complement regulators. In case of infections, C3b binds to pathogen surfaces that lack complement regulators to inactivate it. FB interact with C3b and FD cleaves C3b-bound FB to form a surface bound C3 convertase (C3bBb) which cleaves large amount of C3 into C3a and C3b which can form new convertases (amplification loop). The C3bBb is a short-lived complex but can be stabilized by properdin (FP)⁸¹.

II.2.1.4 Common terminal pathway

Each pathway leads to the formation of the central C3 convertase which cleaves C3 into C3a and C3b. The C3 convertase is a very short-lived complex (around 90s) but the half-life can be increased 5- to 10- fold by association with properdin⁸². Increasing concentration of generated C3b provokes binding of C3b to a C3 convertase and modifies the specificity of the enzyme that starts to cleave C5⁸³⁻⁸⁵. This new enzymatic complex is called C5 convertase. C5 convertase cleaves C5 into C5a, a powerful anaphylatoxin and C5b which can initiate the terminal part of the cascade. However, the structure of C5 convertase is still not well understood and the exact mechanism of cleavage is still under debate. C5b interact with C6, C7, C8, insert into the membrane and C9 polymerize to form the membrane-attack-complex (MAC). The alternative pathway is mainly responsible of the terminal complement activity thanks to the presence of the amplification loop⁶⁶.

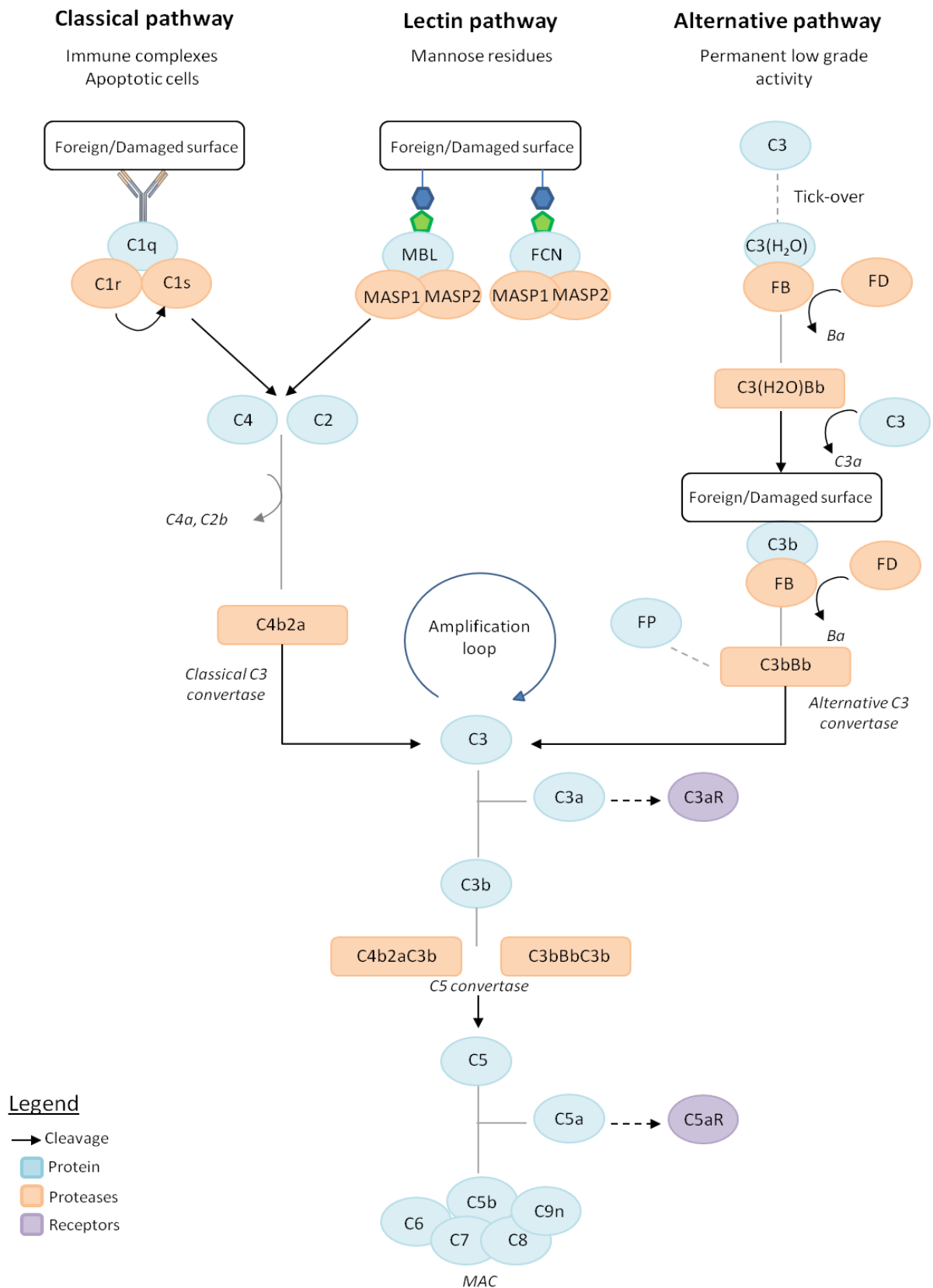


Figure 9: Complement cascade. MBL: Mannan-Binding Lectin, FCN: Ficolin; MASP: Mannan-Associated Serine Protease; FB: Factor B, FD: Factor D, FP: Properdin, MAC: Membrane Attack Complex

II.2.2 Extrinsic complement activation

Extrinsic activation consists of an unconventional activation of the complement system independently of the three established pathways. Indeed, the cleavage of C3 and C5 can occur outside the cascade through actions of different proteins such as thrombin⁸⁶, plasmin⁸⁷, coagulation factors IXa, Xa, XIa⁸⁷, kallikrein⁸⁸, or cathepsin-L⁸⁹ (Table 5). The generated fragments are for the majority biologically active and sometimes even more than those generated through the convertases. For instance, thrombin can induce the cleavage of C5 and allows the generation of an alternative C5b9 with more powerful lytic capacity than its analog, C5b-9. However, the functionality of these fragments is controversial. In another study, C5aT fragment generated by thrombin does not exhibit C5a-like chemotactic/migration properties *in vitro*^{90,91}. In the case of plasmin, besides C3, it can also cleave C3b and generate iC3b that differ in size from those generated by FI and thus lose their capacity to induce phagocytosis through CR3/4^{92,93,88}

Another limitation is that the majority of the studies are performed *in vitro* with purified protein or *ex vivo* with plasma samples and don't reflect the physiological conditions⁸⁷. Indeed, in normal conditions, proteases are tightly regulated and their residual activity is not sufficient to activate complement. In this context, *ex vivo* incubation of baboon serum with thrombin, plasmin, or FXa did not show noticeable complement cleavage unless supraphysiologic amounts of enzymes were used⁹⁴. Nevertheless, in some pathological context where the regulation processes are circumvented, like in sepsis or disseminated intravascular coagulopathy, extrinsic activation could represent an important player for complement activation⁹¹.

In vivo, the exact contribution of intrinsic activation in the generation of C3 bioactive fragments remains unclear. For example, while thrombin is able to cleave C5 *in vitro* into bioactive C5a fragment, it has limited function *in vivo*. In C3 deficient mice, thrombin actively participates in the generation of C5a in injured lungs whereas in wild type mice it exerts only a minor effect suggesting that thrombin acts as a compensatory mechanism to produce C5a in case of C3 deficiency⁹⁵. Another problem with these studies is that they are often reported in isolated papers and no validation studies by independent groups are available.

	Substrate	Fragments
Thrombin	C5	Different cleavage site than C5 convertase. Biologically active intermediate, C5bT-9 MAC is more lytic than its analog, C5b-9
Plasmin	C3, C5	Different cleavage site than C3/C5 convertase. The cleavage of C3 by plasmin is 100 times less efficient than C3-convertase cleavage. Urokinase can activate plasmin and its cleavage of C5 into C5a ⁹⁶
caFIXa, FXa, FXIa	C3, C5	Identical bioactive fragments than those generated with convertases FXa has the most potent proteolytic effectiveness
Kallikrein	C3, FB	Same cleavage site than C3/C5 convertase Bioactive C3a and C3b
Cathepsin-L	C3	Intracellular cleave of C3

Table 5: List of proteins responsible for C3/C5 cleavage independently of complement activation

II.3 Effector functions of complement

The activation of the complement system leads to the generation of bioactive fragments that play a key role in pathogen elimination and host homeostasis. These functions are linked to complement activation and are called canonical functions. They refer to inflammation, opsonization/phagocytosis and lysis. Moreover, complement proteins can also have other, unconventional functions that are linked to adaptive immune response, development, cell metabolism or coagulation (Figure 11).

II.3.1 Canonical functions of complement

The host cells express high levels of regulators in order to escape from complement attack. However, any pathogen, cell or debris that lack these “don’t attack me” molecules are susceptible to complement attack. This complement attack is characterized by 3 effector pathways working in concert: the releasing of pro-inflammatory signals, the opsonization of the target and the induction of lysis (Figure 10).

II.3.1.1 Inflammation

C3a and C5a, also called anaphylatoxins, are small peptides (10-14kDa) that are released during complement activation. In plasma, these two fragments are rapidly converted into

C3adesArg and C5a desArg by C-terminal cleavage mediated by carboxypeptidase N⁹⁷. C3adesArg cannot bind to C3aR and C5adesArg presents a 90% decrease of its pro-inflammatory activity⁹⁸ unlike in mice, where C5adesArg retains some functional activity⁹⁹.

The anaphylatoxins support inflammation by acting as chemokines and allow the recruitment of C3aR- and C5aR-positive immune cells (monocytes/macrophages, eosinophils, neutrophils, basophils, mast cells, dendritic cells, T cells, B cells) and the modulation of the activity of these cells. They are also involved in the increase of vascular permeability¹⁰⁰.

C3aR, C5aR1 and C5aR2 are G protein-coupled receptors (GPCR) which induce intracellular signaling pathway after ligand binding. Depending on the context, the anaphylatoxins have either a pro or anti-inflammatory roles¹⁰¹.

On one hand, C3a acts in an anti-inflammatory way by decreasing cytokine release from B cells^{102,103}, regulates polyclonal immune response and inhibits recruitment and degranulation of neutrophils¹⁰⁴. On the other hand, C3a displays pro-inflammatory properties by increasing cytokine release by macrophages^{105,106}, inducing degranulation in eosinophils¹⁰⁷ and mast cells¹⁰⁸ and induces Th1-polarized response¹⁰⁹.

C5a acts as a chemoattractant molecule for C5aR1 expressing cells and stimulates the release of histamine from mast cells¹¹⁰, facilitates leucocytes extravasation, stimulates angiogenesis¹¹¹ or degranulation of neutrophils, induces Th1 phenotype expansion^{112,113} and promotes B cells trafficking in secondary lymphoid organs¹¹⁴. But in some cases like tumors, C5a can also exhibit anti-inflammatory effects by a decrease in T cell recruitment and response¹¹⁵⁻¹¹⁷. At the opposite, C5aR2 exert more anti-inflammatory actions and may serve as a decoy receptor sequestering C5a/C5a des Arg from C5aR1 or block C5aR1 signaling¹¹⁸.

II.3.1.2 Opsonization

The second major effector arm of the complement system is to protect the host from infection or damaged cells by phagocytosis of cells coated with opsonins, generated as a result of complement activation.

Fragments of C3 (C3b, iC3b, C3d) can opsonize target structures such as immune complexes, pathogens or dying cells which will be recognized by phagocytes through different receptors such as CR1, CR2, CR3, CR4 and CR1g. Depending on the type of C3 fragments, the phagocyte can generate either a pro or anti-inflammatory response.

The binding of C3b to CR1, expressed on monocytes, macrophages, neutrophils, erythrocytes and renal podocytes serves in the phagocytosis, clearance of immune complexes and promotion of proinflammatory molecules secretion^{119,120}.

The binding of iC3b/C3d/C3dg to CR2, expressed on B cells lowers the activation threshold of B cells and influences the generation of memory B cells¹²¹. Thus, complement may be considered as a natural adjuvant of the humoral immune response. Despite many *in vivo* evidences of B cell response modulation after interaction between CR2 and C3 fragments¹²², the relevance of this effect in human remains is not clear. Nonetheless, CR2 deficiency in patients is associated with immunodeficiency, reduced B cell memory and hypogammaglobulinemia suggesting that the effects on B cells reported in mice may potentially be translate in humans^{123,124}.

CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are receptors for iC3b and are expressed by macrophages, monocytes, dendritic cells, neutrophils and NK cells. Upon ligand binding, they play a role in phagocytosis, leucocytes trafficking, degranulation, adhesion, migration and costimulation^{125,126}. The binding of iC3b to CR3 results in TGF- β 2 and Il-10 production and induction of tolerogenic response and silent clearance of apoptotic cells¹²⁷.

C3b and iC3b can bind to CR1g, expressed on restricted subset of tissue-resident macrophages, including Kupffer cells, and induce phagocytosis.

II.3.1.3 Lytic/sublytic MAC

MAC consists of a pore of 10nm that induce calcium flux and pathogen lysis by colloid osmosis¹²⁸. MAC can induce the lysis of gram negative bacteria such as Neisseria meningitis but not gram positive bacteria because of their extremely thick wall that MAC cannot penetrate. Nevertheless, pathogens can escape from complement attack by recruiting host soluble regulators to their membrane or by producing proteases that degrade complement proteins and inhibitors of C3 convertase^{66,129}.

Moreover, nucleated cells are also resistant to complement attack due to a rapid MAC recovery from cell surface. Thus, multiple MACs must be inserted in the cell membrane to have a lytic activity¹²⁸. Nevertheless, pore formation is not the only action of MAC. Indeed, a sublytic MAC at the surface of nucleated cell can induce different effects depending on cell types such as increase/decrease proliferation, induction/inhibition of apoptosis, change in cell motility, inflammation.¹³⁰

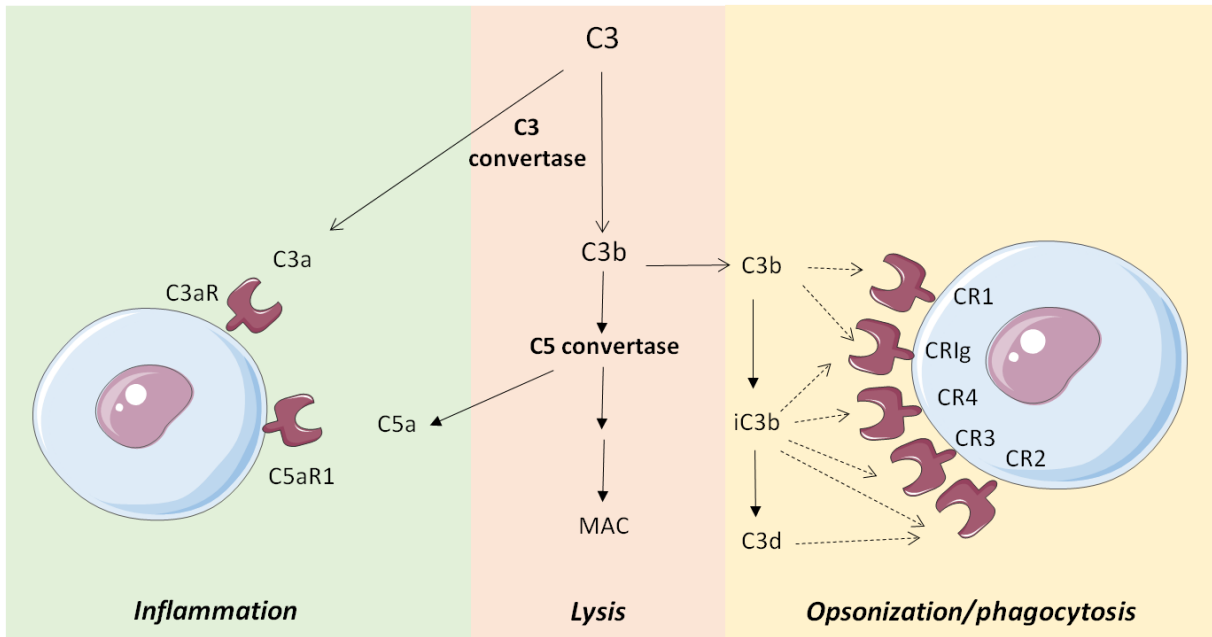


Figure 10: Canonical functions of the complement system. To protect host from pathogens and stressed cells, the complement system is activated and act by three major ways: induction of inflammation, lysis and opsonization.

II.3.2 Non canonical functions

Accumulated evidences show that complement proteins can have other functions that the classic inflammation, opsonization and lysis that protect against pathogens or stressed cells. Indeed, the complement turns out to be a key effector of adaptive immune response, inflammation, metabolism, coagulation and development. Surprisingly, these functions are mediated by complement proteins extracellularly or intracellularly but outside of the complement cascade. These novel exciting functions lead to repositioning of the role of complement in diseases.

II.3.2.1 Immune related functions

Recent work has revealed a crosstalk between complement and the inflammasome¹³¹. Extracellular engagement of C3aR¹³², C5aR1¹³³ and sublytic MAC¹³⁴ activate NLRP3 inflammasome. On the contrary, C1q suppresses NLRP3 activation during phagocytosis of apoptotic cells by macrophages¹³⁵

Moreover, complement proteins can interfere with T cell immunity in both extracellular and intracellular manner.

In the extracellular space, CD46 regulates the expression of Il-7R that enhances Th1 and Th17 cell response⁷⁴ and regulate amino-acid influx and fatty-acid metabolism¹³⁶. A dysregulation of CD46-mediated signals on T cells have been shown to be associated with multiple sclerosis progression¹³⁷. Moreover, CD46 regulates Notch receptors and ligands expression and modulate T cell activation through interaction with Jagged1¹³⁸. In patients, CD55 deficiency has been associated with gastrointestinal diseases through an induction of intestinal inflammation linked to an increase production of pro-inflammatory cytokines by CD55 deficient CD4+ T cells¹³⁹. Furthermore, CD59 downregulates T cell activity independently of complement activation¹⁴⁰.

In the intracellular space, cathepsin-L (CTSL) can cleave intracellular C3 into C3b and C3a that can bind to intracellular C3aR at the surface of lysosomes and induced survival mechanism through mTOR¹¹². However, the results of this study is based on only one C3 deficient patient thus other patients must be included to have an idea of the exact reliability of C3 deficiency involvement in T cell modulation. Furthermore, intracellular C5 activation and C5aR1 stimulation in human T cells allow ROS generation and NLRP3-inflammasome assembly, a key effector of T cell inflammatory response¹⁴¹.

Emerging evidences indicates that complement may interplay with Toll-like receptors (TLRs) in order to coordinate host defense. For example, CR3 suppress TLR2, TLR4, TLR7 and TLR8 response¹⁴². C1q, MBL¹⁴³, CD46, C3aR and C5aR1 are also implicated in this crosstalk¹⁴⁴.

Finally, some proteins of the complement system can act outside the cascade to allow tolerogenic clearance of apoptotic cells. C1q plays a major in the silent clearance of apoptotic cells. Indeed, when a cell dies, it exposes many “eat-me” signals such as phosphatidylserine, double stranded DNA, GAPDH or calreticulin that can be recognized by C1q. The opsonization by C1q modulates the immune response towards a tolerogenic one with silent phagocytosis and anti-inflammatory response^{135,145,146}. Moreover, a recent work shows that FH can bind to histone and induces nucleosome release from apoptotic cells that facilitate their phagocytosis¹⁴⁷.

II.3.2.2 Non-immune related functions

Some complement proteins can also have a role in tissue development and homeostasis. For example, CD46 play a role in human epidermal progenitor cell proliferation and self-renewal¹⁴⁸. C1q is required for neuron maturation. C3a and C5a are implicated in multiple development processes such as maintenance of the pluripotent state of human embryonic stem cells¹⁴⁹, mobilization of hematopoietic stem cells from the bone marrow¹⁵⁰, migration and

proliferation of cardiac pluripotent progenitor cells¹⁵¹, osteoclast differentiation and bone formation¹⁵².

There are also some interconnections between individual complement proteins and coagulation. In one hand, MASP2 can convert prothrombin to thrombin¹⁵³ and C5a/C5aR1 pathway is involved in the initiation of coagulation cascade through an upregulation of tissue factor expression in endothelial cells. On the other hand, some coagulation proteins can activate the complement cascade through the cleavage of C3 and C5 (kallikrein, thrombin, plasmin)¹⁵⁴.

Complement is also involved in the regulation of cell metabolism in an extracellular and intracellular manner. C3adesArg and C5adesArg interact with C3aR and C5aR2 at cell surface to stimulate triglyceride accumulation and glucose transport and increase insulin resistance in adipocytes¹⁵⁵ and pancreatic β -cells and may play a role in obesity and type II diabetes⁶⁵. Complement can also can on T cell metabolism and thus interfere with T cell activity. Engagement of extracellular CD46 by C3b increases expression of glucose transporters and amino acid channel and induces glycolysis and OXPHOS⁶⁵. In an intracellular way, C5a activate NLPR3 inflammasome and C3a promote T cell survival through mTOR signaling¹¹². Moreover, C3 in the cytosol of pancreatic beta cells can interact with ATG16L1 and induces autophagy¹⁵⁶.

Some complement proteins can also interfere with kinin system that play a role in inflammation and vasodilatation. In particular, gC1qR can induces bradykinin release and C1inh can control the activation of this pathway¹⁵⁷. Interestingly, C1-inh deficiency is associated with hereditary angioedema¹⁵⁸.

These recent studies opened a new avenue for research and led to the conclusion that the complement proteins are multitasking agents, whose functions are not limited to the complement cascade. Nevertheless, the evidences come from few laboratories and validation of the data by independent studies are necessary in order to confirm the conclusions.

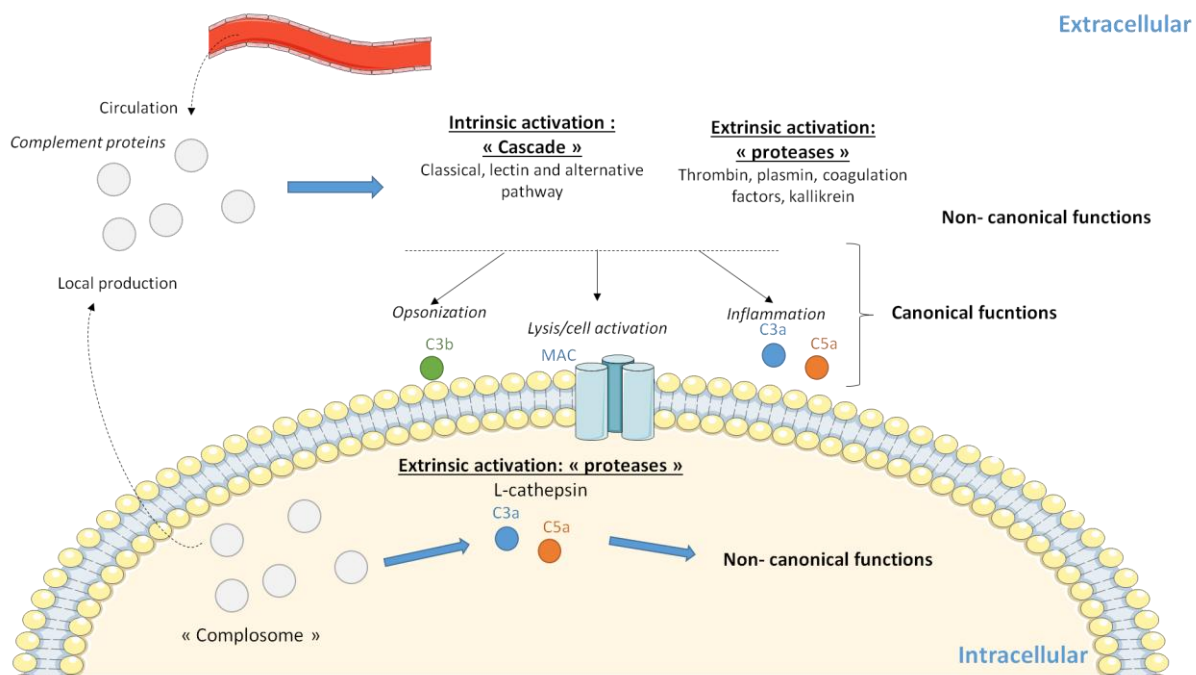


Figure 11: Complement activation in extracellular and intracellular spaces. The presence of complement proteins in a tissue can come from a local production or recruitment from the systemic compartment. In the extracellular space, complement can be activated by three pathways (classical, lectin and alternative) but also in an extrinsic manner by protease cleavage. Complement activation fragments exert classical functions referring to pathogen elimination and cell homeostasis called canonical functions. However, complement protein can also have unconventional functions called non canonical functions. In the intracellular space, the presence of complement proteins is called composome and can be associated with various function impacted cello physiology. The generation of complement bioactive fragments locally can occur through protease actions.

II.4 Regulation of complement system

To avoid any damage on host cells, the complement system is tightly regulated by a large panel of soluble or membranous regulators that act at different levels of the cascade (Figure 12). The initiation of the classical and the lectin pathway can be inhibited by C1-inh. Factor I, Factor H, MCP, CR1, C4BP and DAF regulated the complement activation at the level of C3 convertase. Finally, CD59, clusterin and vitronectin blocked the formation of the membrane attack complex (Table 6).

Initiation of classical and lectin pathway	
C1-inh (SERPING1)	C1 inhibitor binds to the active sites of C1r and C1s, MASP-1 and MASP-2 and blocks their enzymatic activity. Moreover, C1-inh also dissociates the heterotetramer (C1s-C1r-C1r-C1s) from C1 and release free C1q.
C3 convertase	
Factor I (FI)	Soluble serine protease that cleaves C4b and C3b into inactive fragments. The cleavage of C4b by FI into inactive fragments C4c and C4d occurs in presence of cofactors (CR1, MCP (=CD46), C4BP). The inactive fragments C4c is released and C4d stays covalently bound to the membrane. The cleavage of C3b by FI into inactive C3 (iC3b) occurs in presence of cofactor (FH, MCP, CR1). The generated iC3b is unable to find to FB and to form a C3 convertase. In presence of the cofactor CR1, FI can induce second cleavage of iC3b into C3c which is released and C3dg which remains bound to the surface. C3dg is then rapidly converted to C3d by tissue proteases.
Factor H (FH)	Soluble regulator that compete with FB for the binding to C3b, cofactor of FI and induces alternative C3/C5 convertase dissociation.
DAF (CD55)	Membrane regulator that prevents the assembly and accelerates the dissociation of the C3/C5 convertase (AP, CP)
Terminal pathway	
CD59	Membrane regulator that prevents the insertion of MAC to the membrane by blocking the uptake and incorporation of multiple C9 into the complex.
Clusterin and vitronectin	Soluble regulators that prevent the insertion in the membrane by rendering it water-soluble

Table 6: List of complement regulators and their functions

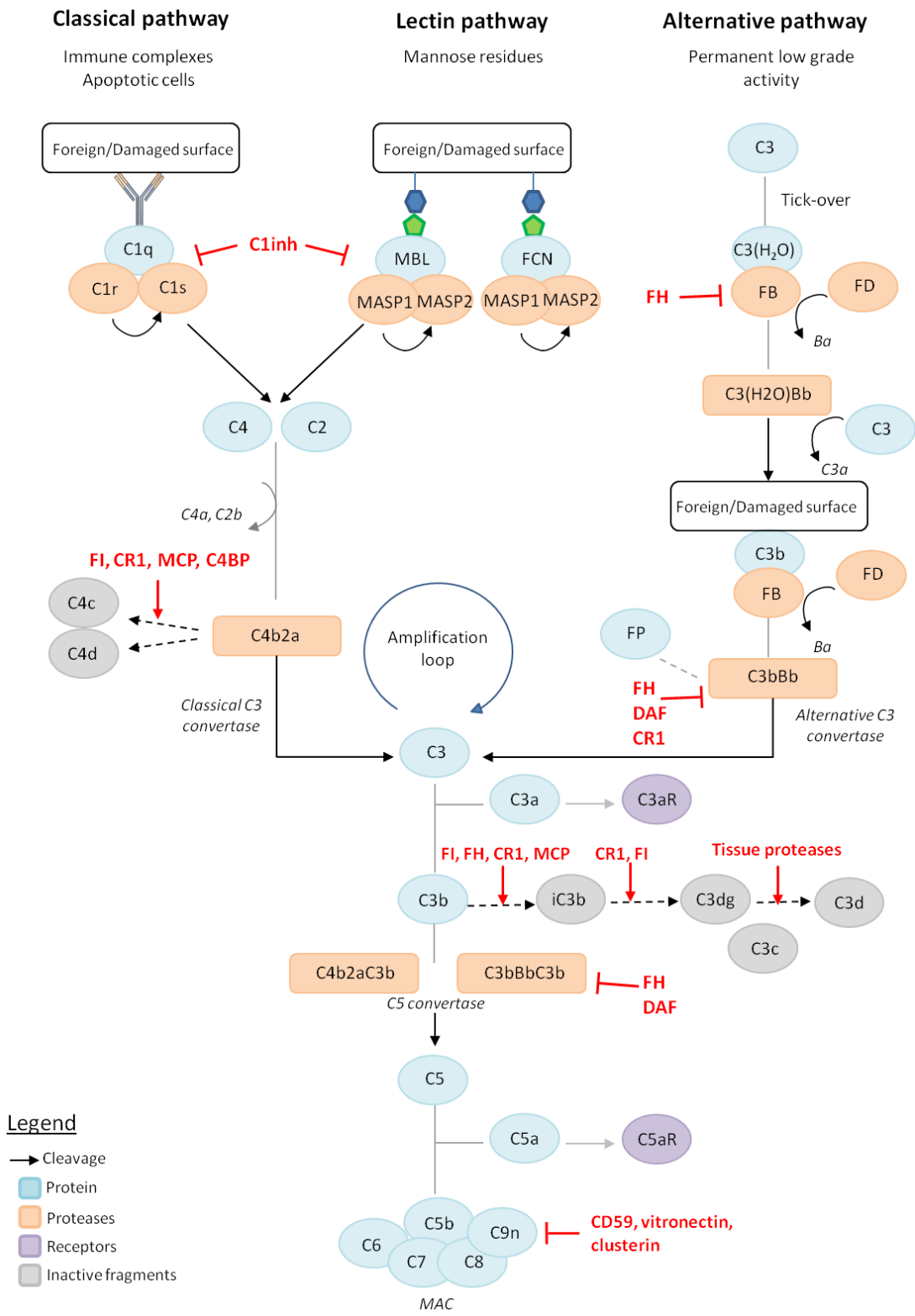


Figure 12: Regulation of the complement system. Soluble regulators: C1inh: C1 inhibitor, FH: Factor H, FI: Factor I, C4BP: C4 Binding Protein, Membrane regulators: CR1: Complement Receptor 1, MCP: Membrane Cofactor Protein, DAF: Decay Accelerating Factor

II.5 Focus on selected complement proteins

II.5.1 C1q

C1q is the first subcomponent of the classical pathway and acts as a recognition molecule for the classical pathway. C1q is a 460kDa constituted by 6 subunits composed by three types of chains (A-chain, B-chain and C-chain). Each subunit has a triple helical collagen like region in N-terminal (cC1q) linked to a globular head in C-terminal (gC1q) to form a bouquet-like architecture^{66,76,77,159}. The gC1q part allows the recognition of the Fc part immune complexes.

Contrary to most of complement proteins, C1q has an extra-hepatic origin and it is mainly produced by innate immune cells including dendritic cells, monocytes, macrophages and mast cells and in a less extend by endothelial cells, epithelial cells, mesenchymal cells, trophoblast, microglial cells, fibroblasts^{160,161}. The production of C1q is regulated by factors of microbial and host origin. Toll-like receptor (TLR) ligands, lipopolysaccharide (LPS), some cytokines (Il-1, Il-6, IFN- γ) and drugs can regulate C1q expression¹⁶².

C1q recognizes a large panel of molecules, which continues to grow over the years. Among them, immune complexes, pentraxins, molecules at the surface of bacteria or apoptotic cells are the best described ligands of C1q^{163,164} (Table 4). The binding of C1q to a ligand on pathogens or apoptotic cells surface triggers its canonical role as an initiator of the classical complement pathway. Moreover, C1q also have non-canonical role by acting independently of the cascade to modulate immune response (DC, T cells and B cells regulation), wound repair¹⁶⁵, chemotaxis¹⁶⁶, angiogenesis^{165,167}, autoimmunity¹⁶², preeclampsia¹⁶⁸, carcinogenesis^{160,167,169,170} and neurodegenerative diseases.

In autoimmunity, C1q is a tolerogenic mediator that allows silent clearance of apoptotic cells¹⁶². Indeed, C1q can bind to apoptotic molecules at the surface of apoptotic cells such as phosphatidyl serine, double stranded DNA, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), annexins A2 and A5 and calreticulin and enable a rapid silent phagocytosis without the release of inflammatory molecules¹⁷¹. A deficit in C1q has been associated with more than 90% risk of developing systemic lupus erythematosus (SLE). Indeed, in absence of C1q, the clearance of apoptotic bodies is impaired and apoptotic neo-antigens accumulate that induce autoimmune response. Recently, an alternative mechanism explaining the role of C1q in autoimmunity has emerged. Indeed, in a mouse model of SLE, C1q restrains the response to self-antigens by modulating the mitochondrial metabolism of CD8⁺ T cells¹⁷²,

In neurodegenerative diseases, C1q has a neuroprotective role by inducing phagocytosis by microglial cells of apoptotic neurons and amyloid peptides and by suppressing pro-inflammatory cytokines production¹⁷³.

On immune response, C1q interacts with LAIR1 and stops the differentiation of monocyte to DC and blocks the activation of plasmacytoid DC¹⁷⁴. On T cell, C1q can exert a direct antiproliferative effects through gC1qR¹⁷⁵. On B cells, C1q negatively influences BCR signaling and promotes a tolerogenic phenotype through its binding to gC1qR. Moreover, C1q promotes IgG production via its collagen domain¹⁷⁶.

In wound healing, C1q promotes permeability, proliferation and migration of endothelial cells¹⁶⁵.

In human prostate cancer cells, C1q can activate the tumor suppressor WWOX which is required for the blockade of cancer cells proliferation and induction of apoptosis¹⁷⁷. In a melanoma mice model, C1q promote cancer cell adhesion, migration, proliferation and angiogenesis¹⁶⁷. In human ccRCC, C1q have pro-tumoral effects by promoting angiogenesis¹⁷⁰.

In aging, C1 complex can bind to Frizzled receptors and C1s induce the cleavage of low density lipoprotein receptor related protein (LRP6) resulting in stabilization of β -catenin and activation of Wnt pathway¹⁷⁸.

II.5.2 C1s

C1s is a plasmatic glycoprotein of 79.9kDa that is encoded by 12 exon gene on chromosome 12. C1s are mainly produced by hepatocytes but in a less extent by other cell types including endothelial cells, fibroblasts (skin and synovial membrane), chondrocytes¹⁷⁹, monocytes/macrophages¹⁸⁰, dendritic cells¹⁸¹. The C1s synthesis is upregulated by different cytokines stimulation like IFN- γ , IFN- β 1 and TNF¹⁸². In mouse, C1s gene is duplicated and codes for two variants; C1sA, the homolog of human C1s that is expressed by hepatocytes and C1sB, expressed in male reproductive tissues¹⁸³.

In circulation, C1s is a proenzyme that exist as a tetrameric structure, C1s-C1r-C1r-C1s, which assembles in the presence of Ca^{2+} with C1q to form the multi-molecular C1 complex.

In a structural point of view, C1s is composed by two distinct functional regions with 6 domains; CUB1-EGF-CUB2-CCP1-CCP2-SP. At the N-terminal part, the three first domains CUB1-EGF-CUB2 mediate the interaction between C1r and C1s and also the interaction of the heterotetramer with C1q collagen arms¹⁸⁴⁻¹⁸⁶. The C-terminal part composed by CCP1-

CCP2-SP domains correspond to the catalytic regions in which the SP domain is the active site consists of the catalytic regions⁷⁷. C1s is a serine protease that is known to mediate the C1 complex proteolytic activity towards its substrates C4 and C2. It cleaves a single Arg–Ala bond in C4 to yield C4a and C4b and a single Arg–Lys bond in C2 to yield C2a and C2b¹⁸³. Interestingly, C1s can cleave other proteins outside the complement cascade and the number of C1s substrates is currently expanding (Table 7).

Substrate	Effect	Ref
High-mobility group box 1 (HMGB1)	C1s cleaves HMGB1 and diminishes its ability to enhance pro-inflammatory cytokines production from monocytes, macrophages and dendritic cells thus resulting in a reduction of the immunogenicity of apoptotic tissue debris and decreases the likelihood of autoimmunity ¹⁸⁷ .	187
Lipoprotein receptor-related protein 6 (LRP6)	C1q can bind to Frizzled receptors and activate C1s which cleaves LRP6 to trigger aging-associated Wnt receptor signaling	178
Basic fibroblast growth factor (bFGF)	C1s cleaves bFGF and inhibits its activity	188
Insulin-like growth factor binding protein-5 (IGFBP-5)	C1s cleaves IGFBP-5 and allows the release of IGF-I to cell surface receptors.	189
Metalloproteinase 9 (MMP9)	C1s cleaves MMP9 and activates its protease activity	190
GP120	C1s cleaves recombinant gp120, the HIV envelope proteins	191
Heavy chain of MHC class I molecule (MHCI)	C1s cleaves the heavy chain of MHCI and generates soluble form of MHCI able to bind to antigen fragments and interact with T cell receptor and regulate immune response	192
Beta 2-microglobulin (β2M)	C1s cleaves β2M in the C-terminal domain and this cleavage can be block by C1inh	193
Decorin, type I gelatin and type II collagen	C1s can directly degrade component of the extracellular matrix such as decorin, type 1 gelatin and type 2 collagens	194
Nucleophosmin (NMP1), nucleolin (NCL)	On apoptotic cells, C1s within C1 complex cleaves nucleolar NMP1 and NCL and thus reduces autoimmunity	195

Table 7: C1s substratome

C1s has been reported to be associated with several pathologies. Rare cases of C1s deficiency have been associated with increased susceptibility to infections (bacterial, viral, fungal), renal diseases (glomerulonephritis), systemic lupus erythematosus and severe cutaneous lesions¹⁹⁶. Heterozygote mutation in C1s can cause Periodontal Ehlers-Danlos syndrome¹⁹⁷. Moreover, C1s overexpression have been found in some cancers¹⁹⁸ and neurodegenerative diseases such as Alzheimer’s disease¹⁸³.

II.5.3 FH

FH is a 155kDa plasmatic glycoprotein produced mostly by the liver, reaching a plasmatic concentration that varies from 110-615 μ g/mL^{199,200}. Moreover, FH can be locally expressed by other cell types including dendritic cells²⁰¹, monocytes²⁰², fibroblasts²⁰³, retinal pigment epithelial cells, endothelial cells, epithelial cells, platelets, and mesenchymal stem cells²⁰⁴. Its expression is upregulated by various cytokines and growth factor including IL-27²⁰⁵, IFN- γ ²⁰⁶, IL-1, TNF²⁰⁷ and VEGF²⁰⁸ and also the transcription factor STAT4²⁰⁹. In contrast, miRNA-125b, miRNA-146a²¹⁰ and miR-155²¹¹ downregulate CFH expression.

FH is the master regulator of the alternative pathway that control complement activation in blood and on cell surface in order to protect tissues from complement mediated-injury⁶⁶. It is a soluble regulator that exerts its regulatory function by preventing alternative C3 convertase formation and accelerating its decay and acts as a cofactor for FI inactivation of C3b. The ability of FH to discriminate self vs non-self or altered-self comes from its capacity to bind glycoaminoglycans (GAG)²¹² and sialic acids²¹³ that are highly expressed proteins on cell surface that are not expressed on pathogens. FH can also bind to a variety of other ligands present at the surface of host cells under specific conditions like oxidative modification, apoptotic cells and extracellular matrix protein that are summarized in Table 8.

FH is composed by 20 CCP (complement control protein) domains (Figure 13). The regulatory domain of FH is provided by CPP1-4 and allows the interaction with C3b or C3(H₂O) to control alternative pathway activation. The other CCPs and especially CCP7 and CCP19-20 play a major role in the recognition of host cell by interacting with two targets types; C3b or C3d and cell surface molecules like GAG or sialic acid^{214,215}.

Ligands	Effect	Ref
C3(H ₂ O), C3b, C3d	FH interact C3b through CCP1-4 and CCP19-20 and with C3d through CCP19-20	⁶⁶
GAG, Heparin	FH interact GAG or heparin through CCP7 and CCP20	66,214,216
Sialic acid	FH interacts with sialic acid through CCP20	212
Pentraxin 3 (PTX3)	FH interact with PTX3 though CCP 7 and CCP 19-20 and enhances iC3b deposition.	217
Adrenomedullin (AM)	FH enhances AM-mediated induction of cAMP in fibroblasts, augments the AM-mediated growth of a cancer cell line, and suppresses the bactericidal capability of AM on Escherichia coli. Reciprocally, AM influences the complement regulatory function of factor H by enhancing the cleavage of C3b via factor I.	218

Thrombospondin-1 (THBS1)	FH can bind to platelets through interaction with THBS1	219
Complement receptor 3 (CR3)/CD11b	The interaction between FH and CR3 enhanced neutrophil activation with an increase generation of hydrogen peroxide. FH interacts with CR3 on macrophages and prevent their elimination through thrombospondin-1/CD47 axis resulting in a pathogenic accumulation and inflammation in a model of AMD	220,221
C-reactive protein (CRP)	FH binds to CRP through CCP7, CCP8-11 and CCP19-20	222
SIBLINGs : Sialoprotein (BSP), osteopontin (OPN) and dentin matrix 1 (DMP1)	SIBLINGs bind to cell surface can interact with FH which regulates complement lytic activity	223,224
Siglec-9	FH can directly bind to Siglec-9 on human monocytes and promotes an anti-inflammatory response after phagocytosis	147
Annexin A2 (A2)	A2 binds to FH CCP6-8 resulting in a decrease binding of FH to cell surface leading to increase alternative pathway activation on cells.	225,226
DNA	FH binds to DNA through CCP 6-8 and 19-20 on the surface of apoptotic cells	226
Histones	FH binds to histone through CCP 1-4, 6-8 and 19-20 on the surface of apoptotic cells	226

Table 8: FH ligands

Among the FH protein family, FHL-1 (FH like protein 1) and FHRs (FH related proteins) share a high homology with FH. FHL-1 is a splicing product of FH composed by the first seven N-terminal domains plus four specific amino-acids in C-terminal and displays the same functions than FH²²⁷. CFHRs belong to the FH family and comprise five members (CFHR1-5) that present a high sequence homology with FH. Their roles in the complement system is controversial but it appears that CFHRs support complement activation by competing with FH for the binding to C3b, C3d and GAG and thus prevent FH action and enhance complement activation²²⁷. Indeed, CFHRs proteins share homology with FH with homologous CCP6-9 and CCP18-20, the key domains implicated in recognition and regulation, and thus could interfere with FH functions through competition²²⁸.

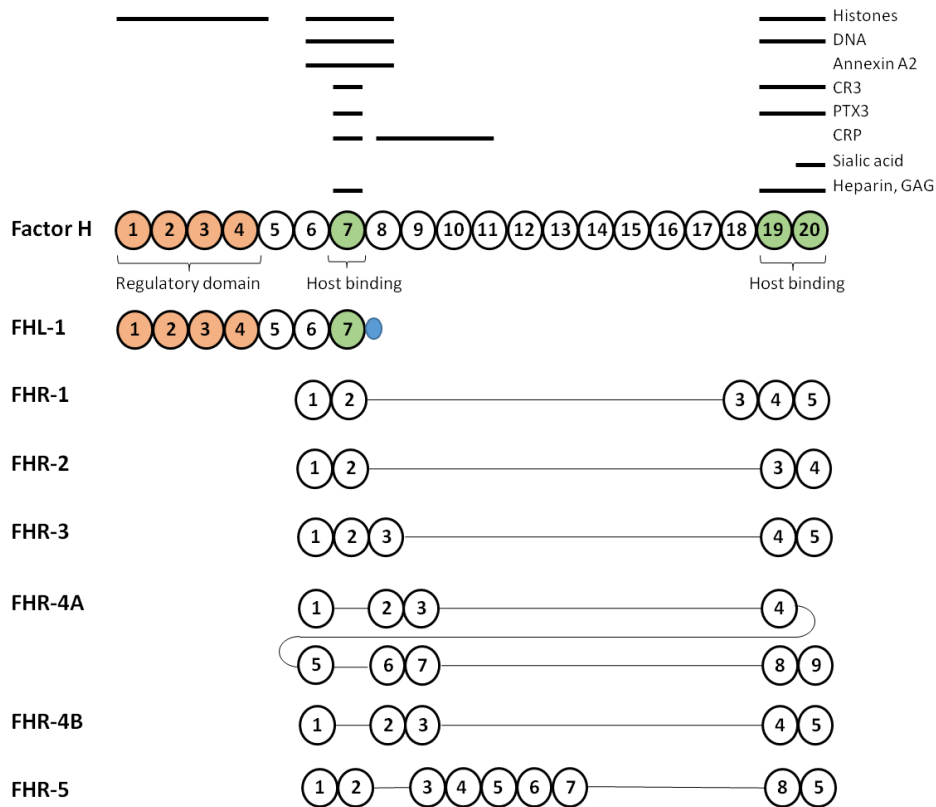


Figure 13: FH family proteins. The schematic representation of CCP domain structure of FH, FHL-1, and the FHR proteins. CCPs aligned vertically are homologous to FH domains. CCPs 1–4 of FH and FHL-1 mediate the complement regulatory functions (shown in orange). CCPs 7 and 19–20 (shown in green) constitute the main host surface-recognition sites; selected ligand binding sites are indicated by horizontal lines (adapted from Sanchez-Corral, 2018)²²⁹

In addition to its complement regulatory functions, FH binds to immune cells and modulates their activity and inflammatory state²³⁰. In the early stages of monocyte to dendritic cell (DC) differentiation, FH promotes a distinctive tolerogenic and anti-inflammatory profile²³¹. Moreover, FH production by DCs is upregulated by IFN- γ and results in a decrease of T cell proliferation²⁰¹. On neutrophils, FH supports migration and spreading and enhances IL-8 release and inhibits NET formation and ROS production²³². On B cell, FH blocks immunoglobulin secretion²³³ and supports their proliferation²³⁴.

Given its central role in the regulation of complement activation, an impairment in FH functions due to polymorphism, mutations or auto-antibodies has been described in association with several diseases such as typical and atypical hemolytic uremic syndrome (HUS²³⁵ and aHUS²³⁶, respectively), age-related macular degeneration (AMD)²³⁷ and dense deposit disease (DDD)²²⁸. Conversely, pathogens and altered self-cells such as cancer cells can use FH to induce an unwanted protection and thus an immune evasion strategy²⁰⁴.

II.6 Complement and therapeutics

Both inefficient and enhanced stimulation of complement can have detrimental effects on host cells and can be responsible for severe infectious or non-infectious diseases²³⁸. A defective complement activation can increase susceptibility to infections like meningitis or to autoimmune disease such as systemic lupus erythematosus (SLE). In the other hand, excessive complement activation is found in several diseases such as renal diseases (aHUS, C3 glomerulopathies), age-related macular degeneration (AMD) or cancer.

According to its implication in multiple pathologies, complement system represents a very promising therapeutics targets. However, in order to avoid potential adverse effect such as severe infections (*Neisseria meningitidis*), the modulation of complement system must be performed very carefully^{67, 239-241}. Eculizumab is, for the moment, the only approved drug that targets specifically complement. However, according to the increasing number of studies underlying the role of complement system in physiopathology, the number of clinical trials regarding complement targeting drugs is largely expanding⁶⁷ (Table 9). These drugs target different level of the cascade (initiation, amplification, effector function) depending on the clinical used and thus present various safety profile (Figure 14).

	Target	Drug	Mechanism of action	Indications
1	C1q	ANX005	Humanized anti-C1q monoclonal antibody	Guillain Barré Syndrome (Phase I)
2		ANX007	Fragmented humanized anti-C1q monoclonal antibody	Primary Open-angle Glaucoma (Phase I)
3	C1s	C1-inh (Cinryse, Berinert, Cetor, Ruconest)	Purified or recombinant protein that targets C1r, C1s, MASP1, MASP2	AMI, CABG, kidney transplantation (Phase I), trauma (Phase III), HAE (Approved)
4		BIVV009	Humanized antibody anti C1s	CAD, bullous pemphigoid, wAIHA, end-stage renal disease (Phase I)
5	MASP2	Narsoplimab	Human antibody anti MASP2	LN, MN, C3G (Phase II), IgA nephropathy, TM, aHUS (Phase III)
6	FP	CLG561	Human antibody anti properdin	AMD (Phase I), geographic atrophy (GA) (Phase II)
7	C3	Compstatin	Peptide C3 inhibitor, no generation of C3a/C3b	AMD (Phase I)
8		APL-1	Second generation of compstatin analog	Asthma, COPD (Phase I)

9		APL-2	Pegylated form of APL-1	AMD, GA, IgA nephropathy, LN, MN, wAIHA, CAD (Phase II), PNH (Phase III)
10		APL-9	Analog of compstatin with tridecapeptide linked to PEG	PNH (Phase I)
11		AMY-101/Cp40	Second generation of compstatin analog with increased half-life	C3G, PNH, kidney transplantation, periodontitis (Phase II)
12	C3 Converter-tase	Mirococept	Recombinant soluble CR1 that inhibits classical, lectin and alternative pathway	Shock (Phase I), inflammation (Phase II), Transplantation (Phase III)
13		TT30	Fusion protein between CR2 and FH	PNH (Phase I)
14	FB	IONIS-FB-LRx	Long lasting FB silencing	AMD, GA, IgA nephropathy (Phase II)
15		LNP-023	Small molecule inhibitor of FB	C3G, PNH, IgA nephropathy (Phase II)
16	FD	Lampalizumab	Humanized antibody anti FD	AMD, GA (Phase III)
17		ACH-4471	Small molecule inhibitor of FD	PNH, C3G (Phase II)
18	C5	Eculizumab	Humanized long-acting antibody against C5 that binds to C5 and prevents C5 convertase proteolytic cleavage.	Kidney transplantation (Phase I), CAD, Guillian Barre Syndrome, ANCA, Thrombocytopenia (Phase II), PNH, aHUS (Approved)
19		Pexelizumab	Humanized short-acting antibody against C5	AMI, CABG (Phase II)
20		Ravulizumab	Second generation anti-C5 antibody with long half-life	aHUS, PNH, generalized myasthenia gravis (Phase III)
21		Tesidolumab	Fully human anti-C5 antibody (different epitope than eculizumab)	Transplantation (Phase I), AMD, GA, PNH, uveitis (Phase II)
22		SKY59	Anti c5 antibody with increased half-ilfe	PNH (Phase II)
23		Pozelimab	Fully human anti-C5 antibody	PNH (Phase II)
24		ABP959	Biosimilar of eculizumab	PNH, aSHU (Phase I)
25		Coversin	Protein with dual activity against C5 and leukotriene B4	aHUS, PNH (Phase II)
26		RA101495	Peptide that binds C5 and allosterically inhibits C5 convertase cleavage	PNH (Phase II)
27		Zimura	Aptamer based C5 inhibitor	Stargardt disease, IPCV (Phase II), AMD (Phase III)
28		Cemdisiran	RNAi that impaired C5	PNH, aHUS (Phase II)

			synthesis	
29		SOBI002		AMD (Phase I)
30	C5a	ALXN1007	Antibody	GVHD, APS (Phase II)
31		IFX-I	Antibody	Sepsis, SIRS, complex cardiac surgery, hidradenitis suppurativa (Phase II)
32	C5aR1	Avacopan	Small molecule	aHUS, C3G, Hidradenitis suppurativa, Acne inversa, IgA nephropathy (Phase II), ANCA (Phase III)
33		PMX-53	Peptide	RA, osteoarthritis, psoriasis (Phase II)
34		IPH-5401	Antibody	Advanced solid tumors (Phase I)

Table 9: Complement targeting drugs approved or in development. ANCA, antineutrophil cytoplasmic-antibody-associated vasculitis; aHUS, atypical haemolytic uraemic syndrome; ABOi, ABO incompatible; AMD, age-related macular degeneration; AMI, acute myocardial infarction; APS, antiphospholipid syndrome; C3G, C3 glomerulopathy; CAD, Cold Agglutinin disease; CAGB, coronary artery bypass grafting; CNV, choroidal neovascularization; COPD, chronic obstructive pulmonary disease; GA, geographic atrophy; GVHD, graft versus host disease; HAE, hereditary angioedema; IPCV, idiopathic polypoidal choroidal vasculopathy; LN, lupus nephritis; MN, membranous nephropathy; RA, rheumatoid arthritis; PNH, paroxysmal nocturnal haemoglobinuria; SIRS, systemic inflammatory response syndrome; TM: Thrombotic microangiopathy; wAIHA, warm autoimmune haemolytic anaemia. (adapted from Ricklin, Nat. Rev. Nephrol. 2018).

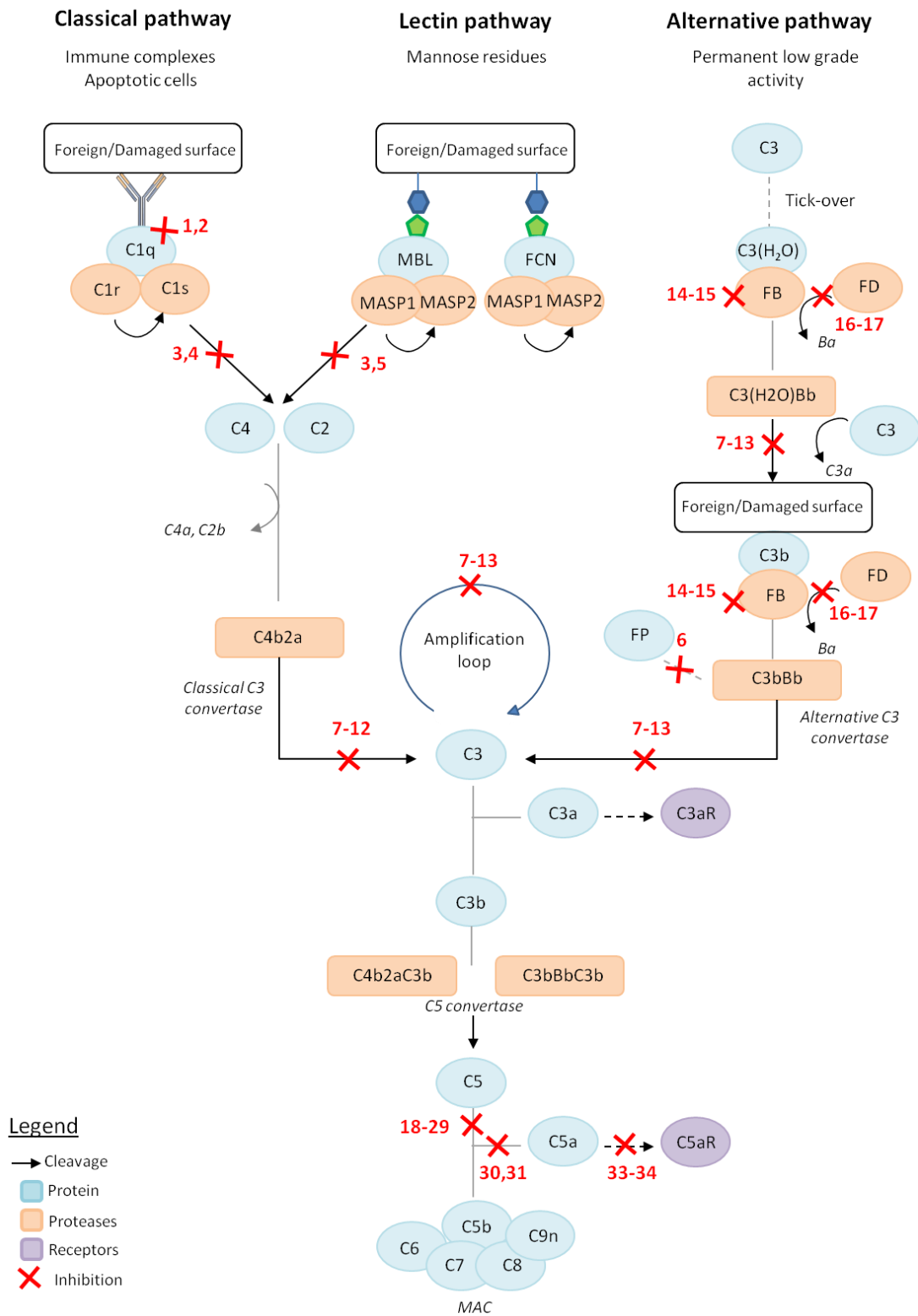


Figure 14: Complement and therapeutics. Numbers referred to Table 1. 1,2: drugs targeting C1q, 3,4: drugs targeting C1s, 5: drug targeting MASPs, 6: drug targeting properdin, 7-11: C3 inhibitors, 12-13: drugs targeting C3 convertase, 14-15: drugs targeting FB, 16-17: drugs targeting FD, 18-29: drugs targeting C5, 30-31: drugs targeting C5a, 32-34: drugs targeting C5aR1. (adapted from Ricklin, *J Immunol.*, 2013).

III. Complement in cancer

III.1 Complement status in tumors

The role of the immune system in tumor progression is an expanding field and many study focus on its characterization in different tumor types and its effect as therapeutic target. However, the role of the complement system, an important player of the innate immune system, is frequently forgotten.

Tumors are complement rich environment in which many cell types participate to the local production of complement component²⁴² (Figure 15). More specifically, protein of the classical and alternative pathway as well as complement receptor and regulators are commonly found in the TME suggesting a potential *in situ* activation. The high expression of C3aR and C5aR1 in most of the cell types within the TME (e.g immune cells, endothelial cells, fibroblasts and tumor cells) suggests that C3a and C5a can modulate the functionality of these cells. Tumor cells also expressed a high level of complement regulators that block the terminal pathway^{243,244}. These findings suggest that tumor cells can benefit from complement activation through the generation of anaphylatoxins but escape from complement cytotoxic effect by overexpression of regulators.

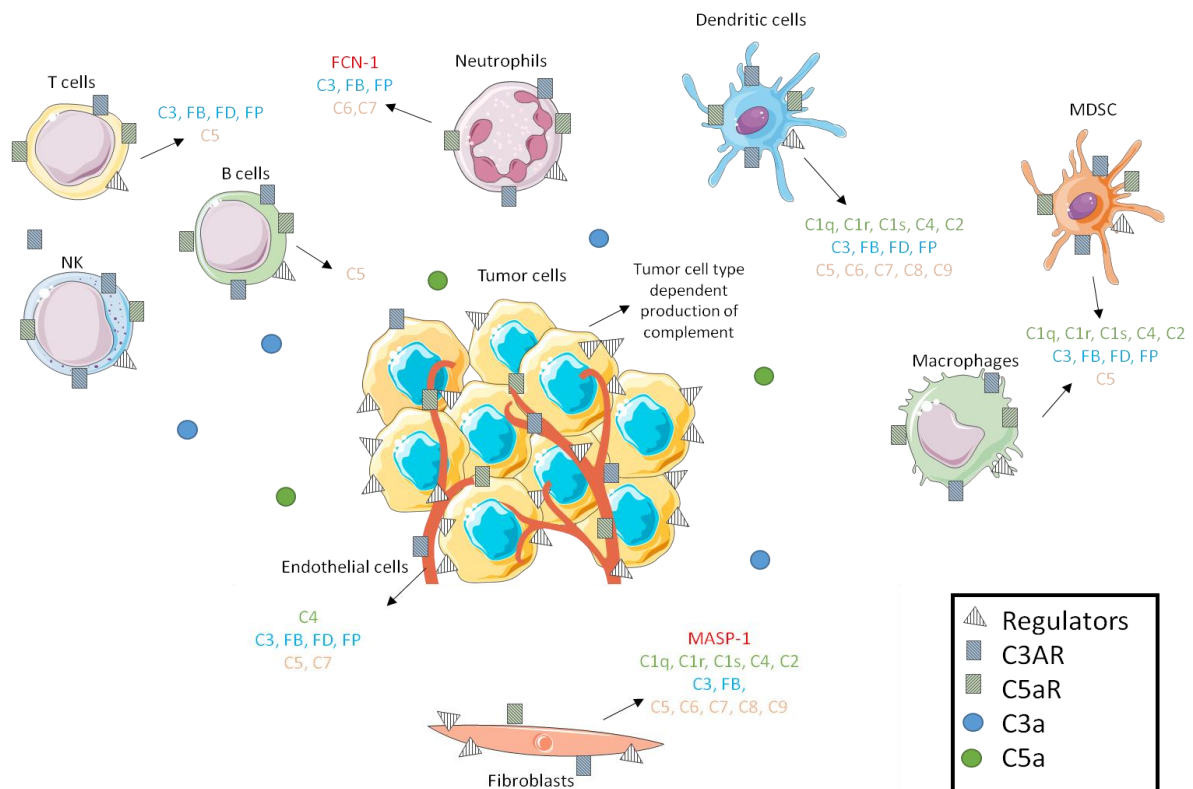


Figure 15: The complement tumor microenvironment. Tumor is a complement rich environment where different cell types participate in the local production of complement proteins. Blue: alternative pathway protein, green: classical pathway proteins, red: lectin pathway protein, orange: terminal pathway protein

III.2 Complement as a biomarker

III.2.1 Diagnostic biomarker

Tumor can induce an upregulation of complement component within the tissue but also in the systemic compartment. Intriguingly, an opposite effect can be observed depending of the nature of the complement proteins and the type of cancer (Table 10).

Overexpression			
	Type of cancer	Mechanism of action	Ref
C1q	Glioblastoma	Plasma: increased C1q in the sera of patients in comparison with healthy controls.	245
C1s	Lung cancer	Plasma: increased level of C1s in plasma of lung cancer patient in comparison with controls	246
	Prostate cancer	Tumor: Upregulation of C1s expression in prostate tumors compared to matched normal prostate tissues	247

C4	Lung cancer	Plasma: elevated C4 level in cancer patients in comparison to control group	248
C4a	Papillary thyroid cancer	Plasma: increase C4a in the sera of patients in comparison with healthy controls.	249
C4d	Lung cancer	Bronchial fluid: Higher levels of C4d in cancer patients than patients with control group.	250
	Lung cancer	Plasma: Higher levels of C4d in cancer patients than patients with benign nodules.	251
C3	Lung cancer	Plasma: elevated C3 level in cancer patients in comparison to control group	248
	Neuroblastoma	Plasma: elevated C3 level in cancer patients in comparison to healthy donors	252
	Pancreatic ductal adenocarcinoma	Tumor: Higher level of C3 protein in cancerous tissues than in adjacent normal pancreatic tissues	253
	Pancreatic cancer	Tumor: Higher level of C3 protein in cancerous tissues than in normal pancreatic tissues	254
C3a	Esophageal cancer	Plasma: Higher C3a level in patients than healthy donors	255
C3a desArg	Breast cancer	Plasma: Higher C3a desArg level in patients than healthy donors	256
C5a	Non-small cell lung cancer	Plasma: Higher C3a level in patients than healthy donors	257
C5aR1	Gastric cancer	Tumor: higher expression of C5aR1 in gastric tumoral tissues than in adjacent non-tumoral tissues	258
FH	Squamous lung cancer	Plasma: Upregulation of FH in uranium exposed miners in comparison with exposed miners without lung disease	259
	Lung cancer	Bronchoalveolar lavage: Higher concentration of factor H in lung cancer patients than controls	260
	Cutaneous squamous cell carcinoma	Tumor: FH is more expressed in invasive cSCC than normal skin or in situ C5CC.	261
	Bladder cancer	Urines: FH and FH related protein are marker for bladder cancer	262 263
C9	Squamous cell lung cancer	Plasma: C9 and its fucosylated form are significantly higher in SQLC patients, as compared to healthy control	264
CD46	Colon cancer	Tumor: CD46 is higher in colon cancer tissues compared with normal adjacent colon tissues	244
CD55	Colon cancer	Tumor: CD55 is higher in colon cancer tissues compared with normal adjacent colon tissues	244
CD59	Colon cancer	Tumor: CD59 is higher in colon cancer tissues compared with normal adjacent colon tissues	244
MASP 2	Ovarian tumor	Tumor: MASP2 gene expression is higher with ovarian cancer compared with controls	265
MBL	Colon tumor	Plasma: MBL2 level increases in patients compared to healthy blood donors.	266
	Ovarian tumor	Tumor: MBL2 gene expression is higher with ovarian cancer compared with controls	265

Underexpression			
C1s	Ovarian cancer	Tumor: Downregulation of C1s mRNA in ovarian tumor vs healthy control	267
	Ovarian cancer	Tumor: Downregulation of C1s expression in stage III serous ovarian carcinoma compared to normal tissue	268
	Lung cancer	Tumor: decrease expression in lung tumor tissues in comparison with paratumor tissues	246
C4BP	Ovarian cancer	Tumor: Downregulation of C4BPA mRNA in ovarian tumor vs healthy control	267
C7	Ovarian cancer	Tumor: Downregulation of C7 mRNA in ovarian tumor vs healthy control	267
FB	Glioblastoma	Plasma : decreased level of FB in GBM	245
FI	Gastric cancer	Plasma: FI is significantly under-expressed in gastric cancer sera compared to normal sera. Declining expression with the advanced pTNM stage from stage I to IV of gastric cancer patients	269
	Cutaneous squamous cell carcinoma	Tumor: FI is more expressed in invasive cSCC than normal skin or in situ CSCC.	270
FH	Colon cancer	Plasma: Decrease FH protein level in the serum of colorectal cancer patients vs. normal control	271
	Ovarian cancer	Tumor: Downregulation of FH mRNA in ovarian tumor vs healthy control	267
CD55	Ovarian cancer	Tumor: Higher expression of CD55 in ovarian cancer than in control	272

Table 10: Modulation of complement component in tumor vs. normal tissue/plasma.

Besides the protein level, the existence of complement auto-antibodies can also be used as a diagnostic biomarker. In lung cancer patients, the presence of auto-antibodies against a specific reduced form of FH has been reported. Interestingly, such antibodies are present in the sera of early-stage lung cancer patient but less in late-stage patient or undetectable in control patient without cancer²⁷³.

III.2.2 Prognostic biomarker

The potential of complement protein as prognostic biomarker has been shown in various cancer types. Indeed, the presence of complement protein in the tumor or the plasma of the patients correlates with an impact on survival in numerous cancers (Table 11).

Pro-tumoral			
	Type of cancer	Mechanism of action	Ref
C1q	Clear cell renal cell carcinoma	Tumor: C1q within the stroma is associated with decrease OS and PFS	170
	Non-small cell lung cancer	Tumor: High level of C1q predicts poor DFS and OS in NSCLC patients	274
C1s	Urothelial carcinoma	Tumor : C1s expression predicted poor disease-specific and metastasis-free survival rates	275
MASP 1	Cervical cancer	Plasma : High level of MASP-1 is associated with poor prognosis	276
MASP 2	Cervical cancer	Plasma : High level of MASP-2 is associated with poor prognosis	276
C4	Metastatic renal cell carcinoma	Solitary copy of either C4 isoform patients have a longer survival in comparison with the control group	277
C4a	Pancreatic adenocarcinoma	Plasma : C4a plasmatic level correlate with OS	278
C4d	Mesothelioma	Plasma : High level of C4d at diagnosis is associated with decrease OS	279
	Clear cell renal cell carcinoma	Tumor : C4 produced by tumor cells or deposits at the surface of tumor cells is associated with decrease OS and PFS	170
	Astrocytoma	Tumor : C4d staining is associated with worsened patient outcome	280
	Lung cancer	Tumor: Higher C4d deposition is associated with decrease survival	281
	Non-small cell lung cancer	Tumor: High level of C4d predicts poor DFS and OS in NSCLC patients. C4d level is associated with low level of PDL1 in tumor cells	274
C3	Non-small cell lung cancer	Tumor : C3 within the tumor is related to poor prognosis	282
	Ovarian cancer	Tumor : high level of C3 mRNA is associated with decrease OS	283
	Lung cancer	Tumor : high level of C3 mRNA is associated with decrease OS	283
C3a	Pancreatic adenocarcinoma	Plasma : C3a plasmatic level correlate with OS	278
C5a	Pancreatic adenocarcinoma	Plasma : C5a plasmatic level correlate with OS	278
	Clear cell renal cell carcinoma	Tumor : high tumoral C5a level was associated with poor overall survival in ccRCC patients after nephrectomy	284
C5aR1	Ovarian cancer	Tumor : high level of C5aR1 mRNA is associated with decrease OS	283
	Lung cancer	Tumor : high level of C5aR1 mRNA is associated with decrease OS	283
	Urothelial cell carcinoma	Tumor: C5aR1-positive patients exhibited significantly lower overall survival and DFS compared with C5aR1-negative UCC patients	285

	Lung cancer	Tumor : High levels of C5aR1 in human lung tumors were significantly associated with shorter recurrence-free survival, overall survival, and bone metastasis	286
	Non-small cell lung cancer	Tumor: High level of C5aR1 predicts poor DFS and OS in NSCLC patients	274
	Gastric cancer	Tumor: High level of C5aR1 predicts poor OS	287
	Clear cell renal cell carcinoma	Tumor: High level of C5a/C5aR1 predicts poor OS and RFS	288
	Breast cancer	Tumor: High level of C5aR1 predicts poor survival	289
FB	Pancreatic cancer	Plasma: High CFB plasmatic correlates with a decreased OS and DFS	290
FI	Breast cancer	Tumor: High expression of FI protein in tumor cells was correlated with significantly shorter cancer-specific survival and recurrence-free survival.	291
FH	Lung cancer	FH variants Y402H and H402H are associated with increased risk of lung cancer among smokers.	292,293
CD46	Ovarian cancer	Tumor : high expression of CD46 is associated with reduced shorter revival-free time at primary laparotomy	294
CD59	Breast cancer	Tumor : patients with CD59 overexpression have a worse prognosis	295

Table 11: Impact of complement protein on patient survival

Taken together, these data highlight the complexity of the complement system and its effect in the context of cancer, ranging from protective to aggressive, depending on tumor type. In this respect, further studies are required to clearly establish the role and mechanism of action of complement proteins in order to consider it as a potential therapeutic target.

III.3 Local activation of the complement system

The existence of complement fragment deposition on tumor cells such as C3, C4^{280,296-303} are consistent with local complement activation. However, the way complement is initiated within the tumor is not clearly elucidated and can result from an intrinsic activation by the three conventional pathways but also through an extrinsic activation via proteases.

III.3.1 Intrinsic activation

Emerging evidences are in favor of an activation of the complement system mediated by the classical pathway.

In cervical mouse model, C3 and C5 deficient mice have a slower tumor growth. This retardation is also observed in C4 deficient mice but not in FB deficient mice suggesting an involvement of the classical or/and the lectin pathway. Moreover, the presence of C1q in the tumor together with the absence of MBL point the implication of the classical pathway³⁰⁴. Interestingly, in the same mouse model, the activation of the classical pathway inside the tumor is linked to the presence of immunoglobulins¹⁷⁰.

Furthermore, similar observations have been made in human cancer patients. In NSCLC, an activation of the classical pathway mediated by C1q binding is observed and leads to C4d deposits on tumor cells²⁸¹. In ccRCC patients, we showed that C4d deposits as well as C1q but not MBL are present in tumor. The complement deposition are associated with the presence immunoglobulins within the tumor and with poor prognosis¹⁷⁰. In papillary thyroid carcinoma, complement depositions of C3d, C4d and C5 as well as cellular deposits of IgG1 and IgG4 were shown in 80% of the cases^{296,305}. The activation of the classical complement pathway through immunoglobulins may result from a local generation of specific anti-tumor antibodies that can fix complement³⁰⁶.

Even if the alternative pathway is not necessarily implicated, it can amplify and perpetuate intratumoral complement activation. However, very few evidences of an implication of the lectin pathway are available^{266,307}

III.3.2 Extrinsic activation

The presence of C3a and C5a in the supernatant of tumor cells cultured under serum free conditions^{257,308} and its release inhibition after treatment with protease inhibitors²⁵⁷ suggest that C3/C5 cleavage can occur through proteases that do not contribute to the three conventional activation pathways³⁰⁹. In prostatic fluid from cancer patients, an uncharacterized C3 fragment has been detected and potentially come from a cleavage by prostate-specific antigen (PSA)³¹⁰. Moreover, in human colon adenocarcinoma and melanoma cell lines, cathepsin-L is responsible for intracellular C3 cleavage conferring high tumorigenic and metastatic properties^{89,311,312}. Regarding C5, a macrophage-derived urokinase can induce its cleavage by plasmin in mouse models of squamous carcinogenesis⁹⁶. In addition, tumor cell promote the generation of thrombin³¹³, a protease involved in C5 cleavage.

III.4 Complement effects on tumor growth

The complement system has been for long considered as an anti-tumoral element according to its classic properties of opsonization, inflammation and lysis. However recent discoveries,

especially from in vitro and mouse data, provide new perspectives for the role of complement in cancer (Table 12, 13). Indeed, new findings highlight the effects of complement proteins and especially C3a and C5a which provide the tumor a growth advantage by modulating different key characteristics of the tumor such as tumor cell behavior, angiogenesis and immune microenvironment³¹⁴. Nevertheless, the complement system exerts ambivalent effect depending on cancer types ranging from anti-tumor defense to potent tumor promotion. This difference in mode of action could reflect the heterogeneity of TME composition between cancer types.

III.4.1 Effect on immune response

The anaphylatoxins C3a and C5a can modulate the functionality of the cells that expressed their receptors; C3aR and C5aR1 and notably immune cells. The pro-tumoral effects of C3a and C5a are linked to an alteration of T cell response through a direct modulation of their cytotoxic functions or by an indirect manner through the recruitment of immunosuppressive cells. This T-cell-dependent action of C5a is underlined in a mouse model of cervical cancer by the abrogation of the impact of C5aR1 deficiency when T cell are depleted³⁰⁴ (Table12). In the same line, the depletion of CD4+ but not CD8+ reverses the inhibitory effects of C3 deficiency in a lung model¹¹⁵(Table12).

The best characterized effect of the anaphylatoxins is the recruitment of immunosuppressive cells in the TME and especially macrophages³¹⁵ and MDSCs³⁰⁴. C3 deficient mice were protected from sarcoma carcinogenesis through a mechanism involving PTX3 (Table12). Indeed, PTX3 promotes the regulatory action of FH resulting in a decrease production of C3a and less recruitment of M2 macrophages³¹⁵. In cervical mouse models, C5a acts as a chemoattractant for MDSCs that expressed C5aR and enhances their infiltration (Table 12). C5a also modulates the functionality of MDSC by promoting ROS and RNS production as well as integrin expression leading to an altered CD8+ T cell response³⁰⁴ (Table 12). Moreover, the blockade of C5aR1 in a mouse model of lung cancer is associated with the expression of immunomodulators such as arginase-1, CTLA4, IL-6, IL-10, LAG3 and PD-L1²⁵⁷ (Table 12). Besides C5a, C5aR1 expressed on MDSCs can bind to other ligands such as ribosomal protein S19 (RSPS19) which is released from apoptotic cells in a model of breast cancer (Table 12). The binding of RSP19 can indirectly affect T cell response through an increase release of TGF- β that promotes a Th2 phenotype³¹⁶.

Complement also interferes with other innate immune cells to promote an immunosuppressive environment such as neutrophils, DCs or NK cells. In a mouse model of melanoma, C3 depletion induced by cobra venom factor treatment resulted in an enhancement of antitumor

response through a cytotoxic actions of NK cells³¹⁷ (Table 12). In melanoma, C3aR deficient mice display an increase of tumor neutrophils. The depletion of these cells is associated with a reversion of the antitumor effect of C3aR deficiency underlying its central role in the pro-tumoral effect of C3aR¹¹⁶ (Table 12). In ovarian cancer mouse model, C3b and CR3 are involved in neutrophils suppressor phenotype³¹⁸ (Table 12). In a model of spontaneous intestinal tumorigenesis, C3aR triggers neutrophils extracellular traps (NETs) release and polarization of neutrophils towards a pro-tumorigenic phenotype³¹⁹ (Table 12). Opsonization of tumor cells by iC3b can induce a tolerogenic immune response that allows tumor growth in melanoma and pancreatic carcinoma through engagement of CR3 on NK³²⁰ cells and dendritic cells respectively³²¹ (Table 12).

Moreover, CD8+ T cells produce C3 which can act through an autocrine manner on C3aR resulting in IL-10 inhibition, a cytokine mandatory for maintaining their antitumor phenotype¹¹⁷.

In discordance with previous studies, C3 deficient Her2/neu transgenic mice spontaneously develop more tumors and have an accelerated tumor onset and metastasis (Table 12). This effect results from an enhance immunosuppression and an increase blood permeability in C3 deficient mice³²². Moreover, C3a and C5a can reverse the endothelial quiescence allowing T-cell homing to tumor associated with an effective tumor control in a cervical mouse model (Table 12)³²³. Finally, C3d by binding to CR2 can specifically modulate the immune microenvironment of the lymphoma tumors by increasing CD8+ T cells, suppressing expression of PD-1 and depleting Tregs³²⁴(Table 12).

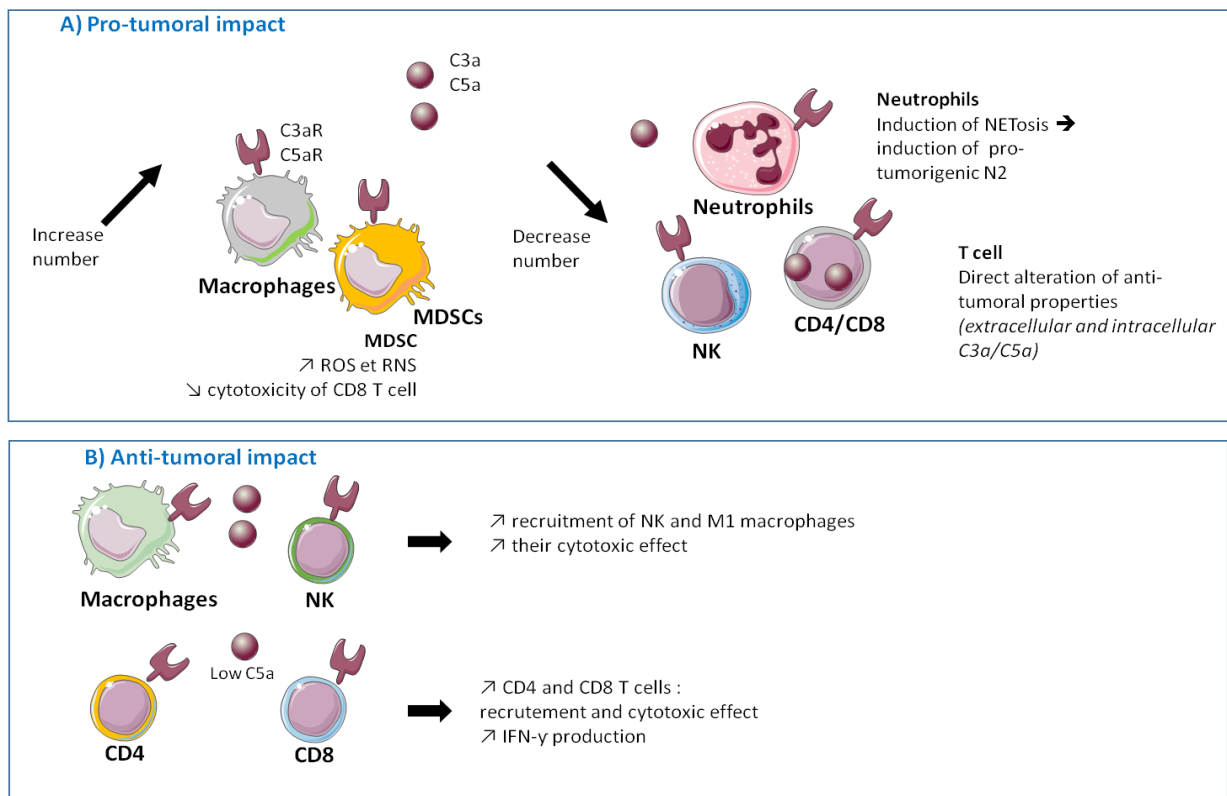


Figure 16: Effect of complement system in immune microenvironment. Depending on cancer types and local concentration, the complement system can exert both pro (A) and anti-tumoral effects (B).

C1s is also a potential modulator of tumor immune microenvironment. In colorectal cancer and melanoma, C1s is expressed by a specific subtype of fibroblast and its expression correlates with T cell infiltration³²⁵⁻³²⁶. In breast cancer, C1s is highly expressed by a subtype of fibroblasts associated with an immunosuppressive environment³²⁷.

The apparent discrepancy of complement effects can be linked to some specific characteristics of the TME. For example, the local concentration of C5a is a key factor that controls C5a effects on immune microenvironment. Indeed, in mouse lymphoma, while high level of C5a promote tumor growth and an immunosuppressive phenotype, a low level of C5a slower tumor growth through the induction of an anti-tumoral T cell response³²⁸ (Table 12). This dose-dependent effect of C5a is also observed *in vitro* on T cell polarization; high level of C5a (500ng/mL) promotes Treg differentiation whereas low level of C5a favors Th1 phenotype³²⁸. Hypoxia can also modulate the expression of complement proteins³²⁹ and susceptibility³³⁰ or resistance³³¹ to complement attack depending to the model. Furthermore, specific mutations also affect local complement activation. For example, isocitrate dehydrogenase mutation in glioma is associated with a significant reduced complement activation³³². Moreover, in mouse model for neuroendocrine tumors of the pancreas, a

significant difference in plasmatic and intratumoral level of C5 is observed between sex with a higher level in males that correlates with the frequency of liver metastasis³³³.

III.4.2 Effect on angiogenesis

In different mouse model, complement activation is associated with a modulation of the tumor vasculature^{334,335}. C3a and C5a can affect angiogenesis by a direct or indirect manner. By modulating the functionality of macrophages, C3a and C5a favor immunosuppression and pro-angiogenic properties^{309,336}. C5a also interacts directly with *in vitro* endothelial cells and promotes their migration, proliferation and vessel formation capacity¹¹¹. In a mice model of ovarian cancer, C3 and C5aR deficient mice have a significant impairment of angiogenesis resulting in a decrease tumor growth³³⁴(Table 12). This effect results from an alteration of endothelial cell function possibly through interaction with VEGF.

C1q can also promote tumor growth by acting on angiogenesis independently of complement activation and anaphylatoxins generation. In cervical and melanoma mouse cancer models C1q exert non-canonical effects by promoting tumor growth through modulation of neoangiogenesis^{167,170}(Table 12). Indeed, we found that C1q deficient mice display an alteration in vascular density and organization associated with a decrease expression of VEGF and VEGFR. These effects of C1q are independent of classical pathway activation since the lack of C4 doesn't alter vascular functions. However, this pro-angiogenic action is tumor-type dependent since C1q has the opposite effects in Her2/neu breast cancer model³³⁷(Table 12).

Recent data show that C1s can also be implicated angiogenic process since its knockdown significantly suppress vascularization of human cSCC xenograft tumors *in vivo*¹⁹⁸(Table 12).

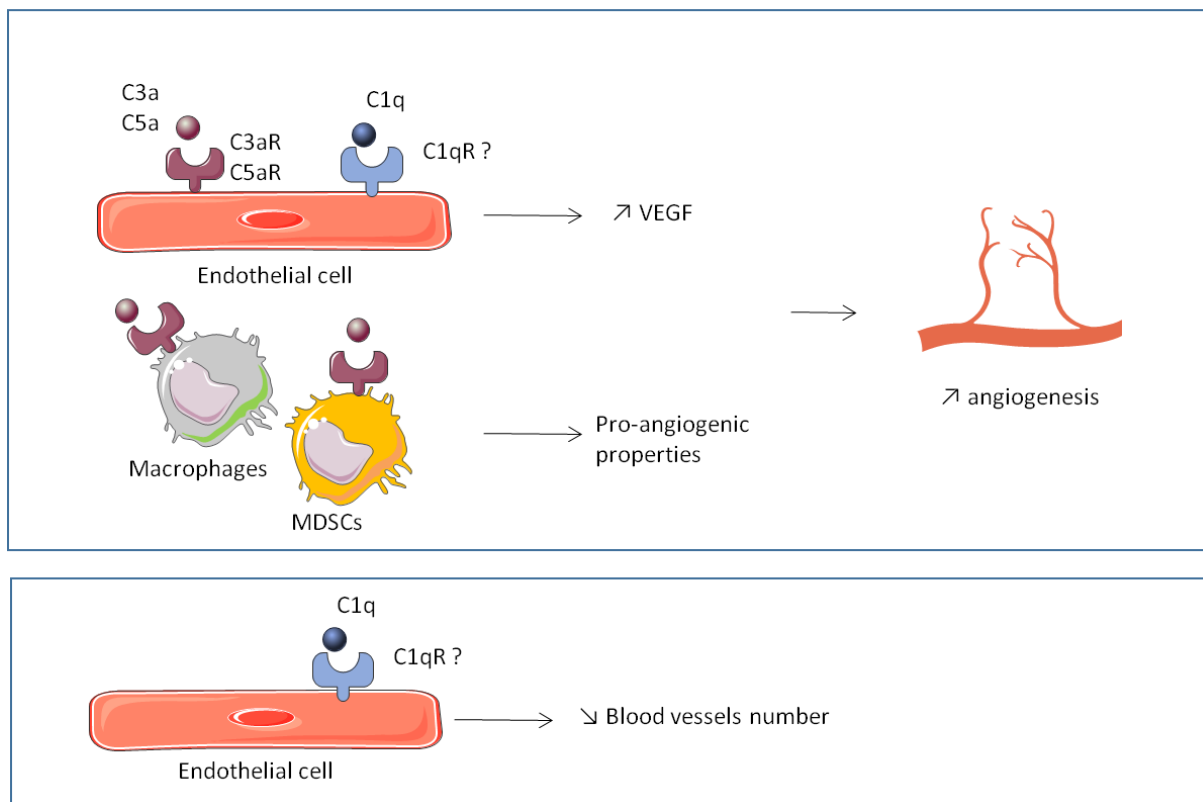


Figure 17: Effect of complement system on angiogenesis. Depending on cancer types, the complement system can exert both pro and anti-tumoral effects

III.4.3 Effect on tumor cells

In addition to fueling inflammation, C3a and C5a axis can affect tumor cell behavior by modulating proliferation, migration, survival, stemness^{258,338–340} (Figure 18). For example, in cutaneous squamous cell carcinoma, C3a promote proliferation, migration and stemness as well as activation of the Wnt and β -catenin pathway³³⁸. The effects of C3a and C5a can be related to an autocrine stimulation of C3aR/C5aR1 on tumor cell surface which induces PI3K signaling pathway and promotes tumor cell proliferation, migration and invasion^{283,309} as reported in bile duct, colon and ovarian cancer cells. On the contrary, the expression of C5a by murine mammary sarcoma induces a reduced tumor growth by blocking cell cycle progression and high level of apoptosis³⁴¹(Table 12).

Besides its effect as classical pathway activator, C1q exerts non-canonical functions by acting directly on tumor cell behavior¹⁶⁷. In malignant pleural mesothelioma, C1q promote tumor proliferation by interacting with hyaluronic acid in the TME³⁴² without activating the complement cascade. The proliferative effect of C1q occurs after enhancement of Erk1/2, SAPK/JNK and p38 phosphorylation. In HCC, C1q stimulates migration and invasion of

tumor cells through activation of discoidin domain receptor 1 and promotion of MAPK and PI3K/Akt signaling pathway as well as expression of matrix metalloproteinases (MMP2 and MMP9)¹⁶⁹. A potential implication of gC1qR in this invasive process is feasible because of the properties of gC1qR to regulate lamellipodia formation and metastasis in human lung carcinoma cells³⁴³.

In addition to C1q, C3a and C5a, various complement proteins produced by tumor cells can support their proliferation. C1r, C1s, FB, FH and FI promote squamous cell tumor proliferation upon the activation of Erk1/2^{198,261,339,344}. C7, FH and CD59 are also required for stemness^{295,345,346}. Moreover, a sublytic level of C5b9 can induce cell cycle progression by activating PI3K/Akt/FOXO1 and ERK1 signaling pathways³⁴⁷ and protection from cell death^{348,349}.

A potential role of C1s in carcinogenesis has been described. Transfection of mouse fibroblasts with C1s give them tumorigenic properties since they were able to form invasive tumor when they were injected in immunodeficient mice³⁵⁰. This effect on tumor growth is supporting by other studies showing C1s is associated with increase tumor proliferation¹⁹⁸ and invasion³⁵¹ and may results from its enzymatic activity³⁵².

On the contrary, complement proteins can also exert anti-tumoral effects in some cancers. By activating the tumor suppressor WWOX, C1q promotes the apoptosis of prostate and breast cancer cells^{177,337}. C1q also induces apoptosis of ovarian cancer cells through activation of TNF- α and upregulation of the pro-apoptotic Bax and Fas³⁵³. In Her2/neu mouse model, C1q deficient mice have an accelerated tumor progression as well as a spontaneous increase of lung metastases occurrence (Table 12). In this model, the anti-tumoral effect of C1q is independent from complement activation and results from an increase number of intratumoral blood vessels and a decrease activation of WWOX. Properdin also displays some antitumor effects by promoting intracellular upregulation of the pro-apoptotic factor DDIT3 which is associated with endoplasmic reticulum-stress response in breast cancer³⁵⁴(Table 12).

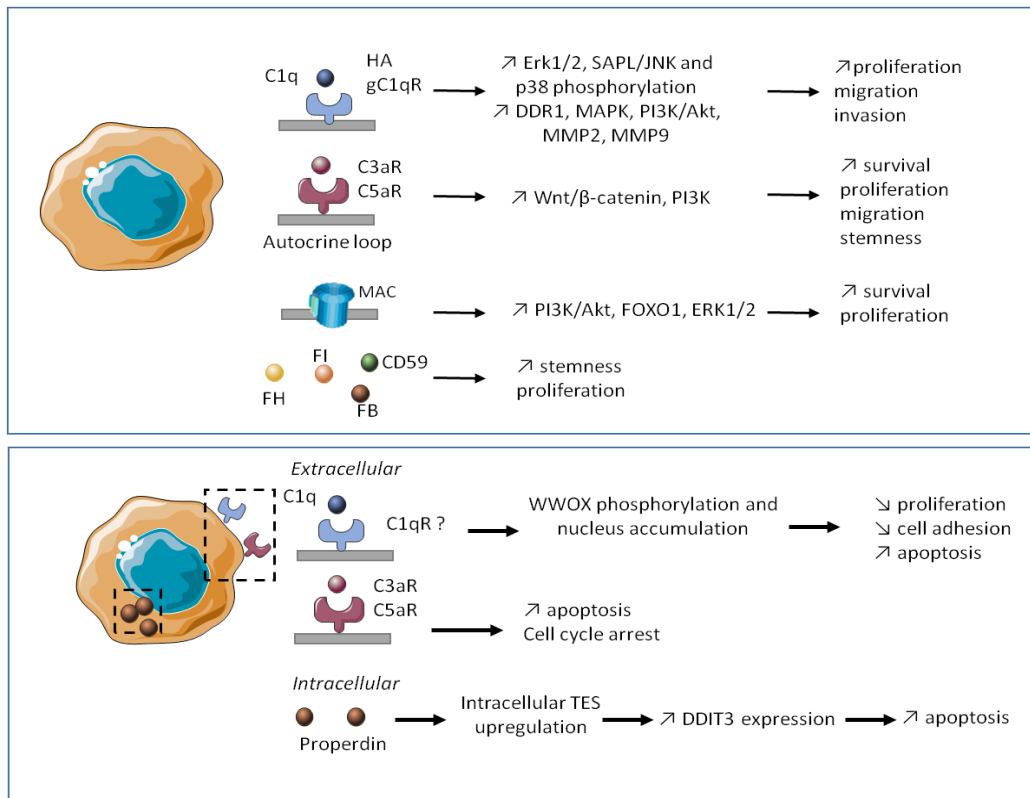


Figure 18: Effect of complement system on tumor cell behavior. Depending on cancer types, the complement system can exert both pro (top) and anti-tumoral (bottom) effects by acting through an extra- or intracellular manner. Akt: protein kinase B, HA: hyaluronic acid, DDIT3: DNA Damage Inducible Transcript 3, DDR1: discoidin domain receptor 1, Erk: extracellular signal-regulated kinases, FOXO1: Forkhead box protein O1, gC1qR: receptor for the globular heads of C1q, JNK: Jun amino-terminal kinase, MAPK: mitogen-activated protein kinase, MMP: matrix metalloproteinase, PI3K: phosphoinositide 3-kinase, SAPK: Stress-activated protein kinases, WWOX: WW Domain Containing Oxidoreductase.

III.4.4 Effect on metastasis

Besides promoting primary tumor growth, the complement system also enhances metastasis formation³⁵⁵ (Figure 19). In a mouse melanoma model, C1q promotes lung metastasis¹⁶⁷ (Table 12). In ovarian cancer, C3 is upregulated by the transcription factor TWIST1 and is involved in EMT and associated with a decrease of E-cadherin via Krüppel-like factor 5–dependent mechanism³⁵⁶. Moreover, the activation of C3a/C3aR axis on the choroid plexus epithelium can disrupt the blood-cerebrospinal fluid barrier and permit dissemination in the nervous system³⁵⁷ (Table 12).

C5a is a multifaceted effector that can also promote metastasis. Indeed, a reduced number of lung metastasis occurs after C5aR blocking due to a decrease production of IL-10 and TGF- β by MDSCs which are implicated in Tregs generation and Th2 response in the premetastatic

niche³⁵⁸ in mouse breast model (Table 12). Equivalent findings have been shown in hepatic metastases of colon cancer³⁰⁸ (Table 12). In this model, C5a induces the expression of chemokines monocytes chemoattractant protein-1 that allows an immunosuppressive microenvironment which supports hepatic metastasis through NF- κ B signaling. Pharmacological blockade of C5aR impairs lung and liver metastasis associated with an increase infiltration of CD8+ T cells in metastatic nodules³⁵⁸ in mouse breast model (Table 12). C5a also acts directly on tumor cells to promote metastasis by enhancing their invasiveness. In lung mouse cancer model, the blockade of C5aR1 abrogates the tumor-associated osteoclastogenic activity and impaired bone metastasis through a CXCL16-mediated effects²⁸⁶ (Table 12). In HCC and renal cancer cells, C5aR1 mediates EMT, migration and invasion of tumor cells by activating the Erk1/2 signaling pathway^{359,360}. In addition, C5a enhances invasiveness through secretion of metalloproteinase and degradation of extracellular matrix³⁰⁹. C5a also promotes the invasive ability of gastric cancer cell lines via RhoA activation and causes morphological changes such as expression of stress fibers and filopodia²⁸⁷. C3a and C5a in leukemic cells enhances the motility of tumor cells and their dissemination through upregulation of p38 MAPK and downregulation of heme oxygenase 1³⁶¹.

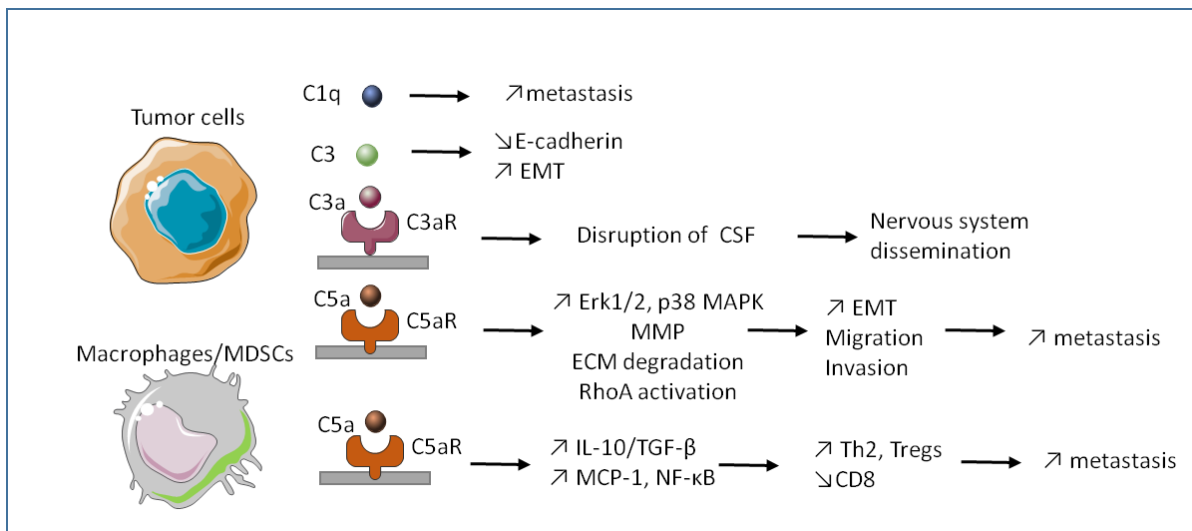


Figure 18: Pro-metastatic effect of complement system. CSF: blood-cerebrospinal fluid barrier, ECM: extracellular matrix, EMT: epithelial to mesenchymal transition, Erk: extracellular signal-regulated kinases, MAPK: mitogen-activated protein kinase, MCP-1: monocytes chemoattractant protein-1, MMP: metalloproteinase, NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells, RhoA: Ras homolog gene family, member, Th: T lymphocyte helper, Tregs: Regulatory T lymphocyte.

Emerging studies allow the repositioning of the complement system as a key effector that acts at different levels to support or inhibit tumor progression depending on tumor types and thus, can be considered as a powerful prognostic biomarker. In order to explain this context-dependent effect of complement, many mouse and *in vitro* studies have focused on its mechanism of action and highlight that complement is a multimodal effector that modulates tumor progression by acting on tumor cells, angiogenesis as well as immune microenvironment. These effects are either linked to complement activation and especially to the generation of anaphylatoxins or to cascade-independent functions of complement proteins. Interestingly, these non-canonical complement functions related or not to immune system are described for a large amount of complement proteins. To make the situation more complex, complement proteins exert their functions through an extracellular action but also to an unexpected intracellular mode of action. Unfortunately, analyses of human cancer are scarce and considering that substantial differences exist between mouse and human complement system, mouse data should be interpreted with caution. Taking together, these novel findings sustain the scientific attractiveness of the complement system and additional investigation especially in humans are needed to gain a holistic view of the role and mode of action of all complement proteins next to new features and re-evaluate its potential as therapeutic target in a context-dependent manner.

Cancer model	Target protein	Effects on tumor growth	Mechanism	Ref
Cervical (Lung): syngeneic model (TC-1 cells)	C4, C3, FB, C5aR1	C4 ^{-/-} , C3 ^{-/-} and C5aR ^{-/-} mice but not FB ^{-/-} have impaired tumor growth. C5aR1 blockade slower tumor growth	Modulation of migration and production of ROS and RNS by MDSC.	304
Cervical (Lung): Syngeneic model (TC-1)	C1q, C3, C4	C1q ^{-/-} , C4 ^{-/-} and C3 ^{-/-} mice have an impaired tumor growth.	Classical complement activation promotes angiogenesis immunosuppressive environment.	170
Lung: Syngeneic (LLC)	C1q	C1q ^{-/-} but not C5 ^{-/-} mice have an impaired tumor growth.	C1q induced angiogenesis independently of complement activation	167
Lung: Syngeneic, Orthotopic, CMT167-luc, EML4-ALK, LLC-luc	C3	C3 ^{-/-} mice have impaired tumor growth and a decreased number of lung metastases. Blockade of C3aR or C5aR results in an inhibition of tumor growth.	C3 signaling inhibits the production of multiple cytokine by CD4 ⁺ T cells	115
Lung: syngeneic model (393P or LLC cells)	C5aR1	Combination of C5a/C5aR inhibition and PD1	Increase frequency of CD8 T cells and decrease frequency of	362

in <i>Kras</i> ^{LSL-G12D/+} mice		blockade results in a decrease tumor growth and number of metastasis.	myeloid-derived suppressor cells within tumors	
Lung metastasis: Xenograft model, A549M1, H460M5	C5aR1	Decrease tumor burden when mice are injected with A549 silenced for C5aR1. Decreased number of bone metastasis	C5aR1 silenced tumor cell have a decreased motility and a lower metalloproteolytic activity.	286
Lung: Syngeneic (3LL)	C5aR	Pharmacological blockade of C5aR results in an inhibition of tumor growth.	Decreased expression of bFGF and number of MDSCs in tumor treated with C5aR antagonist	257
Lung : Xenograft model (A549) in athymic nude mice	FH	Tumor cells silenced for FH have a slower tumor growth in vivo	FH deficiency sensitizes tumor cells to complement mediated attack	363
Lymphoma : syngeneic (RMA)	C5a	Accelerated tumor growth when mice are injected with high C5a producing lymphoma cells	C5a decrease CD4 and CD8 T cells infiltration in tumor	328
Ovarian: syngeneic model (ID8-VEGF)	C3	Mice injected with C3 shRNA have a slower tumor growth	C3 enhances EMT	356
Ovarian: syngeneic model (ID8-VEGF) Ovarian: xenograft model (SKOV3ip1 cells)	C3, C5aR1	C5aR ^{-/-} mice have impaired tumor growth.	Autocrine effect of C3a, C5a on their receptors on cancer cells and induced proliferation through PI3K/AKT pathway	283
Ovarian: xenograft model (RMA-3CF4 cells) Lymphoma: syngeneic model (RMA-1474 cells)	C5aR1	Correlation between tumor burden and expression of C5a.	Correlation between C5a and decreased number of effector T cells	328
Ovarian: genetic model (TgMISIIR-Tag)	C3, C5aR1	Deficiency of C3 or C5aR1 attenuate tumorigenesis. Pharmacological inhibition of C5aR1 impaired tumor growth	C3 induced production of pro-inflammatory cytokines. C5a induced the production of pro-angiogenic factors.	334
Breast: syngeneic, metastatic (4T1 cells)	C5aR1	C5aR1 ^{-/-} mice or pharmacological inhibition of C5aR1 decreased metastasis	C5aR1 facilitates metastasis by suppressing effector T cells through recruitment of immature dendritic cells that produced TGFb and IL10.	358
Breast: Syngeneic model (4T1)	C5aR1	C5aR1 deficiency or C5aR1 blockade decreased lung metastasis	Reduced recruitment of MDSCs	364

Colon: syngeneic model (MC38 cells)	C3	C3 depleted mice (using CVF) have an impaired tumor growth.	Increased expression of CCL5, CXCL10 and CXCL11 and migration of CD8 ⁺ T cells.	365
Intestine: genetic model (<i>Apc^{Min/+}</i>)	C3aR	C3aR ^{-/-} mice have impaired tumor growth.	C3a induced polarization of neutrophils toward a pro-tumorigenic phenotype	319
Colon: syngeneic, metastatic (SL4, CT26 cells) Colon: xenograft model (HCT116, SW116 cells)	C5aR1	C5aR1 ^{-/-} mice have a decreased number of metastasis in the liver	Infiltration of inflammatory cells and production of CCL2 by macrophages via AKT pathway	308
Colon metastasis: SL4-luc	C5aR	C5aR ^{-/-} mice have a decreased number of hepatic metastasis	C5a/C5aR regulates the M2 phenotype of TAMs in tumor metastases through NF-κB signaling	366
Colitis-induced colorectal cancer (azoxymethane plus dextran sulfate sodium)	C3, C5, C5aR1	C3, C5 and C5aR ^{-/-} mice have impaired tumor growth.	C5a promotes production of IL-1b on neutrophils.	367
Bile duct: Xenograft model (HuCCT1)	C5aR	C5a-treated HuCCT1/C5aR cells spread more broadly than C5a-treated HuCCT1/mock cells in nude mice skin tissue	C5a enhances invasion of C5aR-expressing cancer cells	368
Sarcoma: induced by carcinogen (3-MCA)	PTX3, FH, C5a	PTX3 ^{-/-} mice have an increased susceptibility to carcinogenesis	Factor H is recruited by PTX3 and controls C5a levels and recruitment of M2 macrophages	315
Melanoma: syngeneic model (B16F10 cells)	C1q	C1q ^{-/-} (but not C5 ^{-/-}) mice have impaired tumor growth and a decreased number of metastasis	C1q induced angiogenesis, cell adhesion and proliferation independently of complement activation	167
Melanoma: syngeneic model (B16F10 cells transfected with gp33)	C3	C3 depleted mice (using CVF) have an impaired tumor growth.	NK-mediated cytotoxic T-cell-dependent antitumoral immune responses	317
Melanoma: syngeneic model (B16F10 cells, SM1WT1) Colon: Syngeneic model (MC38 cells)	C3aR	C3aR ^{-/-} mice have impaired tumor growth.	C3aR ^{-/-} mice have an increased neutrophils, CD4 T infiltration and a decreased numbers of macrophages	116
Melanoma: syngeneic model (B16F10 cells) Breast: syngeneic model (E0771 cells)	C3, C3aR, C5aR1	C3, C5 and C5aR ^{-/-} mice have impaired tumor growth.	C5a induces IL-10 secretion by effector CD8 T cells. Enhanced antitumor response by combination of C3aR or	117

			C5aR antagonism and anti PD-L1	
Leptomeningeal metastasis: Breast cancer cell lines (MDA231-LeptoM, HCC1954-LeptoM) and lung cancer cell lines (PC9-LeptoM LLC-LeptoM)	C3 C3aR	MDA231-LeptoM cells silenced for C3 injected in mice have a slower tumor growth C3aR ^{-/-} mice injected with LLC-LeptoM in the leptomeningeal space have impaired tumor growth. Pharmacological inhibition of C3aR decreased metastasis	Leptomeningeal metastatic cells produce C3 that disrupts the choroidal blood-CSF barrier to adapt the CSF for cancer cell growth	357
Gastric: Syngeneic, MFC in nude mice	C5aR	Pharmacological blockade of C5aR induces inhibition of tumor growth.	C5aR blockade inhibits p-PI3K/p-Akt and increased level of p21/p-p21	258
Leukemia: Xenograft model (U937) in SCID mice	C3a, C5a	Tumor cell treated before inoculation with C3a or C5a have an enhance spread to murine organs	C3a and C5a activates p38 MAPK which downregulates HO-1 expression and enhance motility.	361
Pancreas neuroendocrine : Genetic model (BT2 B6)	C5aR	Pharmacological blockade of C5aR induces inhibition of tumor growth.	C5a/C5aR increases invasiveness and macrophage recruitment	333
Cutaneous squamous cell carcinoma: Xenograft model (UT-SCC-7) in SCID mice	FI	Tumor cells silenced for FI have a slower tumor growth in vivo	FI promotes proliferation and migration	270
Squamous cells: Genetic (K14-HPV16)	C5aR1	C5aR ^{-/-} mice have impaired tumor growth. Synergic effect of the combination of C5aR1 blockade and chemotherapy	C5aR1 deficient tumors have an impaired angiogenesis and a decreased number of mast cells and macrophages. C5 is cleaved by uPA	96
Squamous cells: xenograft model (cSCCIS cells)	FB, C3	Silencing of FB or C3 in tumor cells inhibited growth of human xenograft tumors in vivo.	Silencing of FB or C3 in tumor cells decreases their migration and proliferation and resulted in Erk1/2 inhibition.	339
Squamous cells: xenograft model (UT-SCC-91cells) in SCID mice	C1r, C1s	Silencing of C1r or C1s in tumor cells inhibited growth of human xenograft tumors in vivo.	Silencing of C1r or C1s in tumor cells decreases their proliferation capacity and increase apoptosis associated with a decrease of Akt and Erk1/2 phosphorylation. A lower density of blood vessels was also observed in C1r and C1s deficient tumors.	

Table 12: Pro-tumoral effects of complement in mouse models.

Cancer model	Target protein	Effects on tumor growth	Mechanism	Ref
Breast: Xenograft model (MCF7) In NOD-SCID mice	CFP	CFP overexpressing tumor cells have an impaired tumor growth.	CFP induces ER stress, TES transcription and increase of DDIT3	³⁵⁴
Breast: Syngeneic neuT-C3-/- mice	C3	NeuT-C3-/- mice have an accelerated tumor growth and lung metastases number	NeuT-C3-/- mice have an increase in Treg frequency and a modification of vascular architecture	³²²
Breast: genetic, neuT-C1q-/- mice	C1q	NeuT-C1q-/- mice have an accelerated tumor growth and lung metastases number	Higher number of intratumor blood vessels and a decrease in the activation of the tumor suppressor WWOX. No difference in the tumor infiltrating immune cell or local complement activation.	³³⁷
Lymphoma : syngeneic (RMA)	C5a	Decrease tumor growth when mice are injected with low C5a producing lymphoma cells	Low C5a stimulate antitumor immune response	³²⁸
Melanoma: Syngeneic (B16F10-OVA)	C3 C3aR, C5aR1	Tumors in C3-/- mice grow slower but are resistant to radiotherapy. C3aR and C5aR1 inhibition results in resistance to radiotherapy	The local production of C3a and C5a was necessary for the tumor response to radiotherapy and for the stimulation of tumor-specific immunity.	³⁶⁹
Colon: Syngeneic (CT26)	C3aR, C5aR1	C3aR-/- and C5aR1-/- results in resistance to radiotherapy	The local production of C3a and C5a was necessary for the tumor response to radiotherapy and for the stimulation of tumor-specific immunity.	³⁶⁹
Cervical (Lung): Syngeneic model (TC-1), adoptive T cell therapy	C3, C5aR1	C3-/- or C5aR1-/-, and pharmacological blockade of C5aR1, impaired the ability of T cells to overcome the endothelial barrier, infiltrate tumors, and control tumor progression.	C3 and C5aR1 expression by tumor stroma, and not leukocytes, governs T cell homing, acting on the local endothelium.	³²³

Table 13: Anti-tumoral effects of complement in mouse models.

III.5 Complement in the anti-cancer therapy

From a therapeutic point of view, the complement system has been used as an adjunctive component that promotes the effects of anti-cancer monoclonal antibodies. However, taking into account the emerging pro-tumoral actions of the complement system, complement inhibitors might have a place in the therapeutic armamentarium against cancer.

III.5.1 Complement inhibitors in cancer

Despite some contradictory data depending on cancer types, accumulating evidences are in favor of a pro-tumoral role of C3a and C5a in cancer. Thus, C3a/C3aR and C5a/C5aR axis are considered as a novel class of immune checkpoint that can be targeted.

In mouse models, the treatment with an antagonist of C3aR called SB290157 is associated with an impaired tumor growth¹¹⁶. Moreover, the treatment with a C5aR1 antagonist alone or in combination with a checkpoint inhibitor (anti PD-L1 and anti-PD-1) is associated with a reduce tumor growth and metastasis^{117,304,334,362,370}. In addition, clinical phase I testing the combination of an anti-PD-L1, durvalumab, with an anti-C5aR1 monoclonal antibody (IPH5401) is currently on-going in patient with advanced solid tumors (STELLAR-001, NCT03665129)³⁷¹.

The advantage of targeting C5aR1 instead of C5a is the intact capacity of the complement to opsonize pathogens and thus limit adverse effects like infections. Indeed, the major side effect of eculizumab, an anti-C5 antibody is the development of severe infections such as meningococcal meningitis.

Moreover, the targeting of C5a/C5aR1 axis can improve the efficacy of other anti-cancer therapy suggesting the interest of drug combinations. Indeed, C5aR1 blockade improve the efficacy of paclitaxel chemotherapy by promoting antitumor T cell response⁹⁶. The rational of drug combinations with complement inhibitors must be clearly evaluated. Indeed, even if the combination can bring synergic effects in some cases it can also have no interest or even detrimental effects. For example, radiotherapy induces a release of C3a and C5a in the TME associated with an anti-tumor response through DC stimulation and CD8+ T cell response³⁶⁹. In this case, complement blockade can reverse the anti-tumor effects of radiotherapy. However, the relation between complement and radiotherapy seems to be controversial since other studies show that co-treatment of radiotherapy with C3 inhibition enhance antitumor response³⁷². Moreover, in vitro, the treatment of tumor cells with paclitaxel induces a downregulation of complement gene expression such as C3, C1r, C1s and FB³⁷³. In addition to C5a blockade, recent studies have highlighted a non-canonical role of many complement

proteins that can represent good therapeutic targets. Thus, many complement-targeting molecules that are in the pipeline for variety of disease indications and can be adapted for cancer therapy, acting at different steps of the cascade⁶⁷. Nonetheless, considering the context-dependent effect of complement, a better understanding of the mechanisms that govern its effects on tumor progression is required in order to design the most efficient complement targeted therapeutics. In this way, a personalized approach with either activation or neutralization of complement could be made to tilt the finely-tuned balance of complement reaction towards tumor rejection. Besides its role as therapeutic targets, the complement system can be also used as a predictive biomarker for response to anti-cancer therapy. In malignant pleural mesothelioma patients, the plasmatic level of C4d can help to follow chemotherapy efficacy. Indeed, plasma C4d level after chemotherapy is significantly higher in patients stable/progressive disease compared to those with partial/major response²⁷⁹.

III.5.2 Complement activators in cancer

III.5.2.1 Complement dependent cytotoxicity

Complement activation by monoclonal antibody can be detrimental for cancer cells through different mechanisms: opsonization of cancer cells which will be then phagocytes (complement dependent cell-mediated cytotoxicity, CDCC) and a direct induction of cell lysis due to MAC accumulation (complement-dependent cytotoxicity, CDC)³⁷⁴⁻³⁷⁵ (Figure 19).

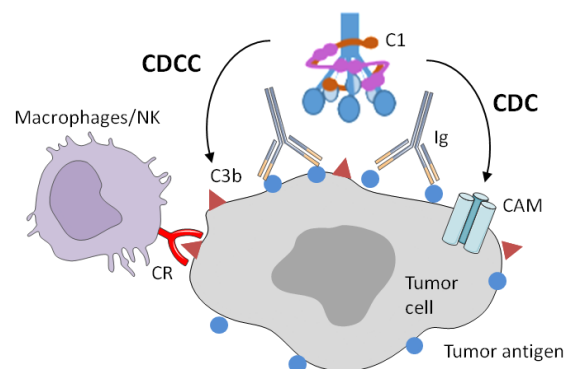


Figure 20: Complement cytotoxicity mediated by monoclonal antibody (adapted from Imai et al., Nat Rev Can, 2006)

CDCC. Complement activation leads to the generation of C3 fragments (C3b, iC3b) that can opsonize tumor cells. These fragments are recognized by immune cells (macrophages, NK) and phagocyte through the expression complement receptors (CRs) at cell surface.

CDC. The effect of MAC in tumor progression can be versatile depending on MAC concentration. While sublytic MAC can promote cancer cell proliferation and accumulation of enough MAC at the surface of tumor cells can lead to cancer cells lysis.

III.5.2.2 Monoclonal antibody

This cytotoxic effect of complement is exploited in the context of monoclonal antibody therapy. Indeed, some antibodies are designed to activate complement in order to induce tumor cell death (Table 14).

Drug	Antigen	CDC
Rituximab (IgG1)	CD20	Yes (weak)
Ofatumumab (IgG1)	CD20	Yes (strong)
Obinutuzumab (IgG1)	CD20	No
⁹⁰ Y-labeled ibritumomab tiuxetan (IgG1)	CD20	No
Trastuzumab (IgG1)	HER2	Yes (weak)
Pertuzumab (IgG1)	HER2	Yes (weak)
Trastuzumab emtansine (IgG1)	HER2	Yes (weak)
Cetuximab (IgG1)	EGFR	Yes (weak)
Panitumumab (IgG2)	EGFR	No
Bevacizumab (IgG1)	VEGF	No
Alemtuzumab (IgG1)	CD52	Yes (strong)
Brentuximab vedotin (IgG1)	CD30	No
Nivolumab (IgG4)	PD-1	No
Pembrolizumab (IgG4)	PD-1	No
Atezolizumab (IgG1)	PD-L1	No
Durvalumab (IgG1)	PD-L1	No
Avelumab (IgG1)	PD-L1	No
Ipilimumab (IgG1)	CTLA4	No
Elotuzumab (IgG1)	SLAMF7	No
Daratumumab (IgG1)	CD38	Yes (strong)

Table 14: Anti-cancer monoclonal antibodies used in clinic

However, cancer cells expressed high level of regulators such as CD46, CD55 and CD59 (CRPs) that allow their escape from complement attack^{376,377}. This resistance mechanism is responsible for inconclusive data in patients and questions the effectiveness of using complement cytotoxicity to induce cell death. To overcome this resistance, big efforts are made to design anti-cancer monoclonal antibodies that can efficiently kill tumor cells. Two strategies can be used to optimize the CDC: antibody engineering and modulation of complement regulators (Figure 20).

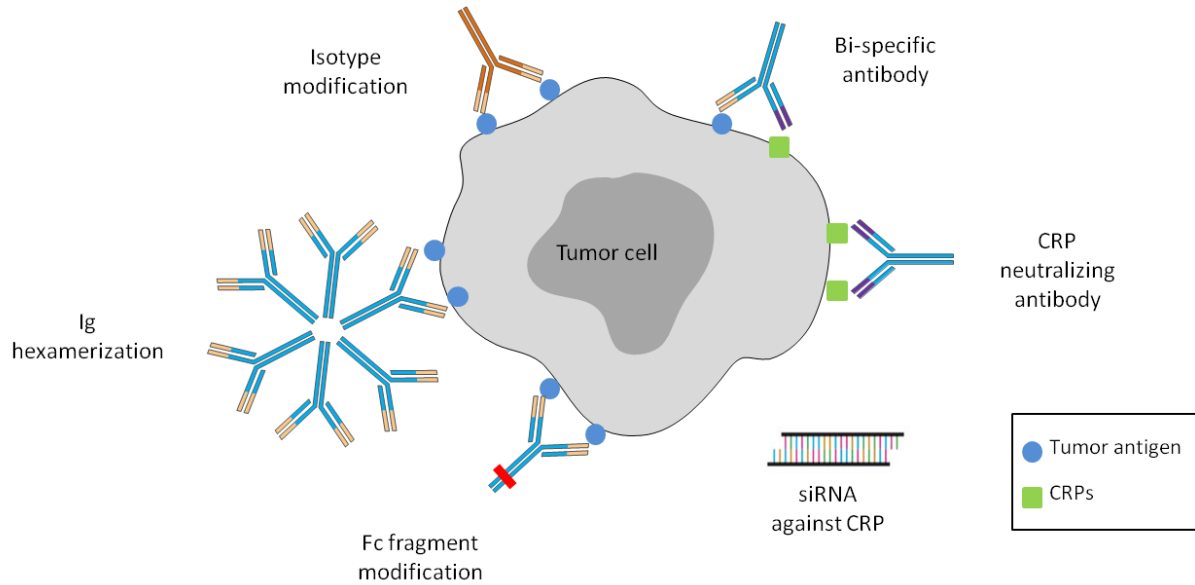


Figure 21: Optimization strategies to enhance complement cytotoxicity mediated by monoclonal antibodies

The activation of complement is very dependent of the antigen density. Thus, a resistance to CDC is observed in patients treated by rituximab that expressed low level of CD20 on tumor cell surface. The choice of isotype is very important because it conditions the efficacy of complement activation. Indeed, to allow C1 binding is necessary to have at least two Fc fragments in close proximity. Thus, IgM will be more efficient to activate complement than IgG. Moreover, heterogeneity of the ability to induce complement activation is observed between IgG subclasses. IgG3 can activate complement more efficiently than IgG1 or IgG2. On contrary, IgG4, IgA, IgD or IgE are not able to induce complement activation. In this context, an IgG3 variant of rituximab has been developed and shows a better induction of CDC³⁷⁸. In the same line, IgG hexamerization increases C1q binding and CDC³⁷⁹. Finally, the introduction of mutation in the Fc domain can promote C1q binding and increase complement activation³⁸⁰. For example, aa modification K326W into IgG1 lead to 2-fold increase of CDC. Moreover, a triple mutant S267E-H268T-S324T has a 47-time superior C1q affinity and a 7-fold increase in CDC induction.

Another strategy to increase CDC efficacy is to neutralize or decrease complement regulators. The development of a strategy based on CD46 internalization on tumor cells is associated with an increase efficacy of rituximab, alemtuzumab and trastuzumab in mouse models and primates³⁸¹. The use of siRNA against CRPs in combination with trastuzumab also increase CDC and CDCC on human breast, ovarian and lung cancer cell lines³⁸²⁻³⁸⁴. Moreover,

neutralizing CRP antibodies are able to increase CDC of rituximab in vitro and in vivo^{385,386}. To avoid adverse effects linked to complement overactivation, the modulation of CRPs on tumor cells needs to be specific. To address this issue, bi-specific antibodies against CD55 and the tumor antigen EpCAM are in development^{387,388}.

Unfortunately, there are some limitations in using CDC to induce tumor cell death. Despite complement activation can induce tumor cell lysis, concomitant studies have shown that C3b opsonization results in decrease antibody dependent cell cytotoxicity (ADCC) by inhibiting rituximab interaction with Fc receptors on NK cells^{389,390}. In addition, considering that complement activation can exert pro-tumoral effect, the potential benefit of using monoclonal antibody has to be evaluated. Indeed, the complement activation needs to be intense to overcome tumor cell resistance to complement attack and induce cell death otherwise, complement can induce a potential enhancement of tumor growth³⁹¹.

OBJECTIVES AND STRATEGIES

Tumors are a complement rich environment resulting from a systemic and a local production of the complement proteins by various cell types. Recent studies have highlighted a new role of these complement proteins on tumor progression. Indeed, besides the well established cytotoxic effect of the complement system through opsonization and MAC formation, complement proteins can also modulate key features of the tumors such as tumor cell behavior, angiogenesis and immune microenvironment. Nevertheless, the analyses of the literature clearly demonstrate a duality in the effect of complement in tumor growth ranging from anti-tumor defense to potent tumor promotion. Moreover, the majority of these studies are performed *in vitro* or by using mouse models but very few data are available in patients. Thus, additional studies are needed to elucidate the role of complement system as well as its mechanism of action especially in human in the different tumor types in order to further consider its use as therapeutic targets.

To shed light on complement effects on tumor progression, we set up following objectives:

- The first objective of this work was to establish an overview of complement landscape in different tumor types.
- The second objective was to understand how complement can be activated within the tumor in clear cell renal cell carcinoma, a tumor type where complement is highly expressed and associated with poor prognosis.
- The third objective was to study the role of the complement molecules independently from complement activation with a specific focus on factor H (FH) and C1s.

In order to respond to these objectives, bioinformatic studies, *in vitro* and *in vivo* experiments as well as different cohorts of patients were exploiting.

To address the first objective, a deep analysis of the transcriptomic data of cancer patients was performed to determine the expression as well as the prognostic impact of 46 complement proteins in more than 30 cancer types (article 1).

To answer the second objective, we combine data from *in vitro*, *in vivo* experiment and *in situ* analysis of ccRCC human tumors to understand the impact of complement classical pathway and how it is initiated locally. Human ccRCC tumors were used to identify the local complement producing cells and to investigate prognostic impact on patient survival. Moreover, plasma from ccRCC patients was used to search for novel biomarkers, capable to predict patients' survival. Human cancer cell lines as well as C1q, C4 and C3 deficient mice provide essential source of data to understand the functional consequences of classical complement proteins on tumor growth, angiogenesis and immune microenvironment (article 2 and 3).

To address the third objective, cohorts of ccRCC and non small cell lung cancer (NSCLC) patients were used to establish the specific role of FH and C1s in tumor progression as well as

the correlation with immune or other complement marker. *In vitro* experiments with purified proteins and generation of RCC and NSCLC cell lines silenced for either FH or C1s were used to study the mechanism of action of these two proteins. The modulation of key characteristics of tumor cells was assessed by using RNAseq as well as functional experiment on proliferation, migration, survival and metabolism. To decipher the role of local production vs. plasmatic FH, a mouse model specifically deficient for hepatic FH production resulting in 80% FH decrease in the plasma was used (article 4 and 5).

RESULTS

ARTICLE 1: Roumenina, L.*, Daugan, M. V.*, Petitprez, F., Sautès-Fridman, C. & Fridman, W. H. Context-dependent roles of complement in cancer. Nat. Rev. Cancer (2019).
Accepted

ARTICLE 2: Marie V. Daugan, Laetitia Lacroix, Catherine Sautès-Fridman, Wolf H. Fridman, Lubka T. Roumenina, Complement detection in human tumors by immunohistochemistry and immunofluorescence, Book Chapter, "The complement system - innovative diagnostic and research protocols" – *Accepted*

ARTICLE 3: Roumenina, L.T.*, Daugan, M.V.*, Noé, R.*, Petitprez, F., Vano, Y.A., Sanchez-Salas, R., Becht, E., Meilleroux, J., Clec'h, B.L., Giraldo, N.A., Merle, N.S., Sun, C.-M., Verkarre, V., Validire, P., Selves, J., Lacroix, L., Delfour, O., Vandenberghe, I., Thuilliez, C., Keddani, S., Sakhi, I.B., Barret, E., Ferré, P., Corvaia, N., Passiukov, A., Chetaille, E., Botto, M., Reynies, A. de, Oudard, S.M., Mejean, A., Cathelineau, X., Sautès-Fridman, C., Fridman, W.H., 2019. Tumor Cells Hijack Macrophage-Produced Complement C1q to Promote Tumor Growth. Cancer Immunol. Res. 2018 <https://doi.org/10.1158/2326-6066.CIR-18-0891> - *Accepted*

MANUSCRIPT 4: Marie V. Daugan, Margot Revel, Romane Thouenon, Tania Rybkine, Carine Torset, Nicolas S. Merle, Rémi Noé, Virginie Verkarre, Stephane Marie Oudard, Arnaud Mejean, Pierre Validire, Xavier Cathelineau, Raphael Sanchez-Salas, Mathew Pickering, Marie-Agnès Dragon-Durey, Isabelle Cremer, Audrey Lupo, Marco Alifano, Catherine Sautés-Fridman, Diane Damotte, Wolf H. Fridman, Lubka T. Roumenina, "Implication of intracellular FH as a novel modulator of tumor cell biology " – *In preparation*

MANUSCRIPT 5: Marie V. Daugan, Margot Revel, ... , Oliver Kepp, Allan Sauvat, Virginie Verkarre, Stephane Marie Oudard, Arnaud Mejean, Pierre Validire, Xavier Cathelineau, Raphael Sanchez-Salas, Diane Damotte, Isabelle Cremer, Catherine Sautès-Fridman, Wolf H. Fridman, Lubka T. Roumenina' " Complement C1s promotes clear cell renal cell cancer progression through classical pathway and non-canonical action on tumor cells " – *In preparation*

ARTICLE 1: Roumenina, L.*, Daugan, M. V.*, Petitprez, F., Sautès-Fridman, C. & Fridman, W. H. Context-dependent roles of complement in cancer. *Nat. Rev. Cancer* (2019). *Accepted*

* contributed equally to this article

Summary and novelty: Despite a protective role of the complement system to control pathogens infections and maintain homeostasis, it has been reported that either a deficient or excessive activation of this system can be involved in the physiopathology of many diseases including cancer. Indeed, a growing number of studies especially in mice have reported that complement proteins and activation can modulate hallmarks of cancer such as tumor cell proliferation and survival, angiogenesis or immune microenvironment. However, the results are controversial depending on the model used. In this context, the goal of this analysis type of review article is to reposition the pro and anti-tumoral effect of the complement system in a context dependent manner. We have performed an original bioinformatic analysis of the complement transcriptome in human tumors to give an overview of complement genes expression landscape and prognostic value in 30 different tumor types. This allowed me to distinguish a group of cancers, in which complement classical and alternative pathways could be implicated in the progression and to stratify the impact of complement as “protective” or “aggressive”. Upon validation, this classification could guide the therapeutic strategies, tackling complement in cancer.

Context-dependent roles of complement in cancer

Lubka T. Roumenina^{1,3*}, Marie V. Daugan^{1,3}, Florent Petitprez^{1,2}, Catherine Sautès-Fridman¹ and Wolf Herman Fridman^{1*}

Abstract | The tumour microenvironment (TME) highly influences the growth and spread of tumours, thus impacting the patient's clinical outcome. In this context, the complement system plays a major and complex role. It may either act to kill antibody-coated tumour cells, support local chronic inflammation or hamper antitumour T cell responses favouring tumour progression. Recent studies demonstrate that these opposing effects are dependent upon the sites of complement activation, the composition of the TME and the tumour cell sensitivity to complement attack. In this Review, we present the evidence that has so far accrued showing a role for complement activation and its effects on cancer control and clinical outcome under different TME contexts. We also include a new analysis of the publicly available transcriptomic data to provide an overview of the prognostic value of complement gene expression in 30 cancer types. We argue that the interplay of complement components within each cancer type is unique, governed by the properties of the tumour cells and the TME. This concept is of critical importance for the design of efficient therapeutic strategies aimed at targeting complement components and their signalling.

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 into the tumour microenvironment (TME)¹. Frequently neglected elements of the TME are the components of the complement system, produced by the tumour and infiltrating cells or originating from the circulation². Complement is a key player in the innate immune defence 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 into the circulation as well as receptors expressed on the membranes of different cell types. The individual components interact with each other in the extracellular space³ (FIG. 1). Recent discoveries have made clear that complement effectors can also be generated intracellularly, leading to locally occurring complement activation, and that complement proteins have non-canonical functions, which are independent of the plasmatic cascade^{4,5}. Cumulative evidence over the past 10 years has proved that complement proteins are present in the TME and that malignant and infiltrating cells have the capacity to produce in situ a large spectrum of these components⁶.

Complement and cancer is an emerging field and most of the phenomena have been described in a single study or for a single type of cancer. Nevertheless, a solid body of evidence has accumulated to demonstrate that

the functionality and level of expression of complement proteins by malignant cells or in the TME can modulate the fate of the tumour. In cancer, the impact of complement is diverse, ranging from antitumour defence to potent tumour promotion. The data in the literature, mostly focused on animal models and in vitro studies, have yielded mixed 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 patients with cancer. In this Review, we present the evidence showing the high diversity of actions of complement components in cancer and the heterogeneity of their production and activation pathways. Using data from human cancers and mouse models, mechanisms of tumour control and tumour promotion are discussed. We also compare the expression of complement genes and their clinical impact in different cancer types, using publicly available data sets, which highlights the context-dependent effects of complement across cancer types. Finally, we argue that the most appropriate therapeutic approaches to activate or neutralize complement will be dependent on the tumour context and, therefore, will require a personalized approach.

The complement system

Complement is a central part of immunity that serves as a first line of defence against pathogens and stressed host cells³. The complement system is composed of

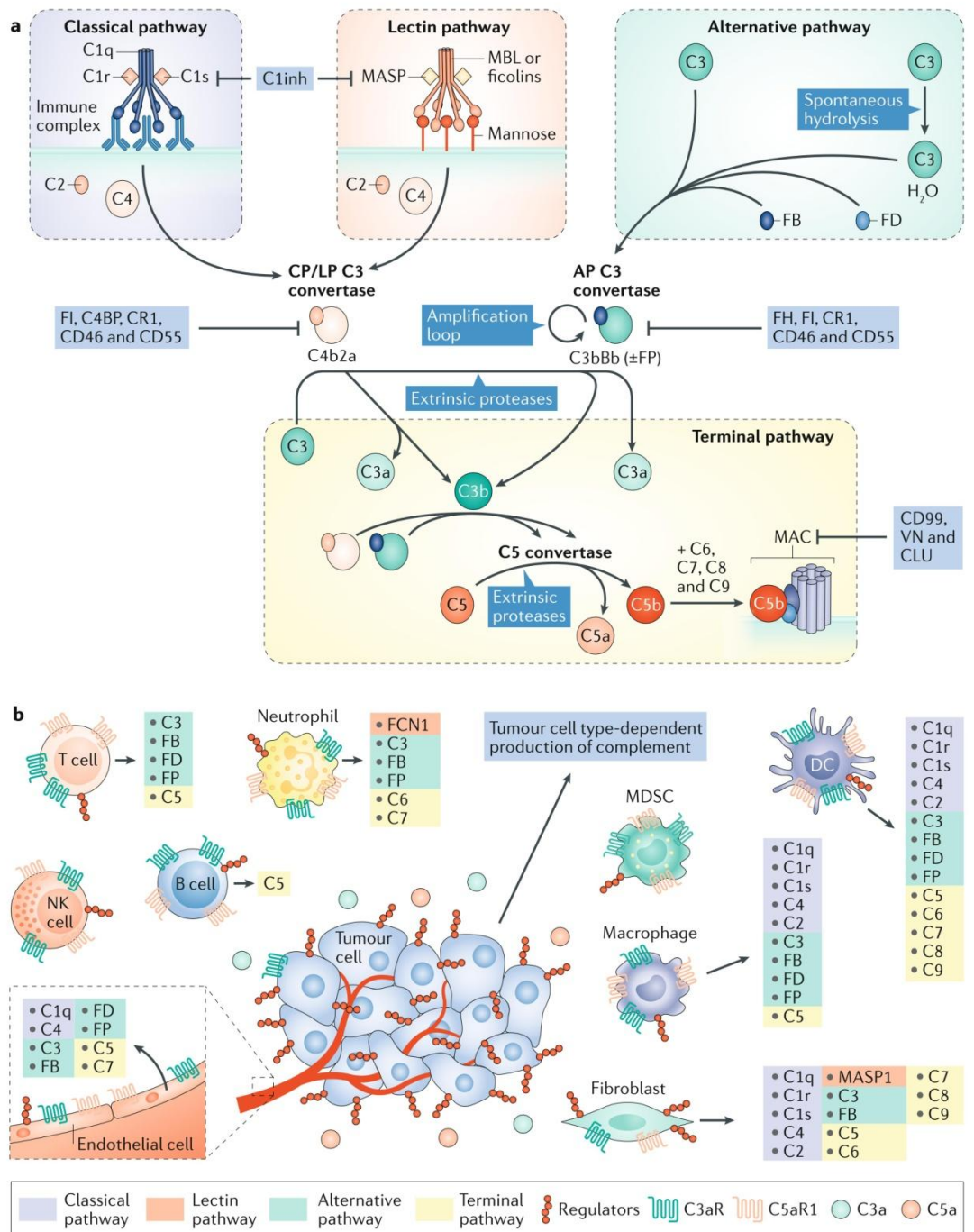
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<https://doi.org/10.1038/s41568-019-0210-0>



plasma proteins that react with one another to opsonize pathogens, inducing a series of inflammatory responses that concomitantly help immune cells to fight against infections and to maintain homeostasis². The initiation of the complement cascade is dependent both on the context (for example, the nature of the trigger or the type of antigen) and on the tissue location (FIG. 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 motifs, foreign to a healthy tissue. The alternative pathway is constitutively active at low levels, serving as a sentinel to attack any surface which is not specifically protected by complement regulators.

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. This is achieved by enzymatic complexes — C3 convertases of the classical, lectin and alternative pathways. The downstream component C5 is

◀ **Fig. 1 | The complement system in the tumour microenvironment. a** | This schematic shows the mechanisms of activation and regulation of the complement cascade. The complement system can be activated by three pathways: the classical, lectin and alternative pathways leading to the generation of C3 convertase. The classical pathway is activated after recognition by the C1 complex (composed of C1q, C1r and C1s) of immune complexes and/or apoptotic cells; the lectin pathway is initiated after fixation of the complex comprising mannose-binding lectin (MBL) or ficolins with mannose-binding lectin-associated serine proteases (MASPs) to terminal mannose residues on cell surfaces. The alternative pathway is constitutively activated at low levels 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 and C3b. C3a is an anaphylatoxin and C3b can opsonize cells. The alternative pathway C3 convertase is composed of the activation fragments of C3 and factor B (FB) (C3bBb). The classical and lectin pathway convertase consists of the activation fragments of C2 and C4. A recent effort to harmonize the complement nomenclature postulated that it should be denoted C4b2b¹⁴¹, but in the previous literature was indicated as C4b2a. C3b binds close to or on the C3 convertases, allowing formation of the C5 convertases, which cleave C5 into C5a (an anaphylatoxin) and C5bC5b that initiates the terminal pathway of the complement system, leading to the formation of the membrane attack complex (MAC). Some proteases, extrinsic to the cascade (such as plasmin), can cleave C5 in a non-canonical way, independently of convertase formation, in the circulation or within the tissues. An intracellular cleavage of C3 by cathepsin L also occurs independently of the cascade. To avoid host tissue damage, this system is tightly regulated by soluble or membranous proteins at different levels of the cascade. **b** | This schematic shows the composition of the tumour microenvironment (TME) and the complement proteins produced by different non-malignant host cell types. The tumour has a rich complement environment. All stromal and tumour cells participate in the local production of complement proteins. The immune cells, especially myeloid cells (such as macrophages, neutrophils, myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs)), can produce complement components, especially those of the classical and alternative pathways, as well as express high levels of complement receptors and multiple regulators. The endothelial cells and fibroblasts are also key players in the TME and produce complement proteins, express regulators and a lower level of complement receptors. Finally, the participation of tumour cells in the complement cascade is dependent upon the cancer type but a key feature is the high expression of complement regulators to protect against complement-dependent cytotoxicity. Of note, this is a qualitative representation. Each cell type produces different amounts of each of the complement proteins. AP, alternative pathway; C1inh, C1 inhibitor; C3aR, C3a receptor; C4BP, C4b-binding protein; C5aR1, C5a receptor 1; CLU, clusterin; CP, classical pathway; CR1, complement receptor 1; FCN1, ficolin 1; LP, lectin pathway; NK cell, natural killer cell; VN, vitronectin.

cleaved by C5 convertases, thus triggering the terminal pathway, generating C5a and the terminal complement complex C5b-9, known as the membrane attack complex (MAC)^{2,7}. The anaphylatoxins C3a and C5a, the opsonizing C3 activation fragments C3b, iC3b and C3d, and the MAC are the canonical effectors of the complement system. Complement is also tightly controlled by negative regulators, such as factor H (FH), FI, CD35, CD46, CD55, CD59, C4BP and C1 inhibitor (C1inh), to avoid damage to healthy cells.

C3a and C5a bind to their respective receptors, C3a receptor (C3aR) and C5aR1 or C5aR2, and play a critical role in inducing inflammation and activation of immune cells as well as endothelial cells, epithelial cells, fibroblasts and certain malignant cells, which express the anaphylatoxin receptors³. Anaphylatoxins induce the oxidative burst in macrophages, eosinophils and neutrophils, which supports inflammation³. In a physiological context, these events contribute to acute inflammation, and eradication of pathogens. Within tissues, locally produced C3a and C5a activate C3aR and C5aR1, respectively, on antigen-presenting cells and T cells, and consequently control proliferation, differentiation, expansion and viability^{8,9}. In the context of cancer,

complement anaphylatoxins are continually generated and in the majority of the studied mouse models lead to tumour-promoting chronic inflammation⁶.

MAC assembly creates a transmembrane pore that causes prompt osmotic lysis of certain bacteria and metabolically inert targets (such as erythrocytes and liposomes). Nucleated host cells often resist lytic killing by MAC owing to a high expression of membrane regulators. Nevertheless, when formed, the C5b-9 complex can have profound effects on cell functions, leading to activation and adaptation or cell death depending on the context¹⁰. To avoid inadvertent healthy host tissue damage, complement is a tightly regulated cascade, constantly kept in check. However, cancer cells acquire escape mechanisms to protect against MAC activity, such as overexpression of complement regulators^{6,11}. Furthermore, one study suggests that heat shock protein 90 (HSP90) protects tumour cells from complement-dependent cytotoxicity by inhibiting, together with mortalin (also known as mitochondrial stress 70 protein or GRP75), C5b-9 assembly and/or stability at the plasma membrane¹².

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 tumours have evolved mechanisms to adapt to its presence and to subvert it for their benefit.

Non-canonical and intracellular complement initiation.

Complement can also be activated by an unconventional, convertase-independent pathway, through other enzymes cleaving C3 and C5, such as cathepsin L, renin, thrombin and plasmin¹³⁻¹⁷. Although generated by non-canonical mechanisms, the C3a and C5a as well as C3b and C5b components produced are often identical in sequence to the convertase-generated anaphylatoxins. Therefore, they are canonical effectors but are generated in a non-canonical manner. This cleavage can occur in the circulation, within the tissues but also intracellularly. Interestingly, thrombin can cleave C5 at a different site from that of C5 convertases, generating an even more potent equivalent of C5b, with higher lytic activity¹⁵. In addition, C3a generated by mouse tumour cell lines promoted an immunosuppressive TME by acting on the tumour-associated macrophage (TAM) phenotype in such a way that could not be replicated by C3a generated exogenously¹⁸. The mechanism for this differential activity is still not well defined, but this study shows that local or intracellularly generated anaphylatoxins may have properties that are different from their plasmatic counterparts.

Complement activation inside cells has been described for T cells and exerts homeostatic and immunological functions^{19,20}. Through their non-canonical functions, complement components modulate the fundamental processes of immune cells, including immune cell proliferation, migration, metabolism and even transcriptional activity²⁰⁻²⁴. The autocrine complement activation and the activity of the complement regulator CD46 are indispensable for the functioning of human CD4⁺ and cytotoxic CD8⁺ T cells in physiological conditions²⁵⁻²⁷. It should be noted that the absence of CD46 on mouse immune cells renders these phenomena human specific,

Anaphylatoxins

The collective name for the complement activation fragments C3a, C4a and C5a.

Oxidative burst

A rapid release of reactive oxygen species (superoxide radicals and hydrogen peroxide) from different types of cells.

Mortalin

A highly conserved heat shock protein implicated in functions ranging from the stress response to control of cell proliferation and inhibition of apoptosis.

emphasizing the divergent roles of innate immune sensors between mice and humans. Studies on human T cells also show unconventional intracellular C3 cleavage by cathepsin L²⁷. The 'tonic' intracellular C3a that is generated is required for homeostatic T cell survival. Although it is generated intracellularly, C3a could be released locally to act in an outside-in, autocrine manner. At least in part, intracellular C3 stores derive from the internalization of the hydrolytic product of C3, C3(H₂O), from the extracellular milieu²⁸. It was suggested that the cleavage of C3 by cathepsin L is species specific, described only in human T cells, and does not operate in mice²⁷. Nevertheless, another study described cathepsin L-mediated C3 cleavage in a mouse model of ischaemia or reperfusion²⁹. These potential species differences should be taken into account if and when this mechanism is studied in animal models of cancer. In addition, in human T helper 1 (T_H1) cells, intracellular C5 activation and subsequent C5a stimulation of intracellular C5aR1 results in the assembly of the NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome, needed for the optimal production of interferon- γ (IFN γ)²⁶. Moreover, novel functions of intracellular C3 have emerged, such as its implication in immune cell gene transcription²³ or regulation of autophagy³⁰. Soluble C1q was shown to be internalized by CD8⁺ T cells and to modulate their metabolism in the context of autoimmunity and viral infections²². All of these processes have not yet been studied in the context of cancer. Nevertheless, it is tempting to speculate that the non-canonical functions of complement will shape the immune TME and play a key role in antitumour immunity.

Functions of complement in cancer

Intratumoural initiation of the complement cascade.

Despite strong evidence for complement activation in human tumours and mouse models (TABLES 1, 2), very few studies have addressed the specific pathway by which these anaphylatoxins are generated.

The TC-1 mouse tumour cell line used to form syngeneic tumours in mice is probably the best-characterized model for classical pathway complement activation. This cell line is derived from primary lung epithelial cells, and expresses the human papilloma virus 16 (HPV16) oncoproteins E6 and E7; it serves as a model of human tumours infected with HPV16, such as lung or cervical cancer, depending on the study. Indeed, in the seminal paper describing the pro-tumoural role of complement, the TC-1 cell line was used to demonstrate that complement activation occurred via either the C4-dependent classical or lectin pathway³¹. Recently, we established using this same model that it is in fact the classical pathway which is activated³². In another syngeneic lung cancer mouse model using the KRAS-mutant CMT167 lung cancer cell line, the classical pathway has also been shown to be activated, likely by intratumoural immunoglobulins³³. Although not necessarily implicated in complement initiation, the alternative pathway can amplify the C3 activation fragment deposits, thus perpetuating intratumoural complement activation. Indeed, C3^{-/-} mice are protected against tumour progression in nearly all of the tested mouse tumour models (TABLES 1, 2).

However, little evidence is available implicating the lectin pathway in tumour progression^{34,35}.

In cancer models, C3 and C5 can also be cleaved by complement cascade-independent proteases, thus bypassing the initial recognition events¹⁵⁻¹⁷. Early studies from the 1990s showed that (pro)cathepsin L, released by tumour cells (mouse and human cell lines), can cleave C3 (REFS^{36,37}). Knock-in of cathepsin L into a mouse melanoma cell line makes it capable of cleaving exogenous C3, promoting its tumorigenic capacity and endowing it with metastatic potential. Although the cleavage pattern reported in these papers is not consistent with the recent findings for C3a generation²⁷, these studies suggest that the capacity of tumour cells to cleave C3 promotes tumour progression. Furthermore, in 2019, a study demonstrated acquisition of a tumour-promoting phenotype upon knock-in of C3 into various tumour cell lines with consequent intracellular activation of C3, potentially by cathepsin L¹⁸.

C5 is cleaved by plasmin, activated by urokinase plasminogen activator (uPA)-expressing macrophages in mouse models of squamous carcinogenesis, leading to C3-independent release of C5a¹⁶. Using C5-producing tumour cell lines, it was shown that C5a can be generated by a, hitherto unidentified, cell membrane-bound serine protease¹⁷. Thrombin is produced in tumours and has potent pro-tumoural activity³⁸. Therefore, it is tempting to speculate that the cleavage of C5 will be yet another mechanism contributing to its pro-tumoural activity in situ.

Certain tumour cells contain intracellular pools of C3 and C5 (REFS^{32,39-41}). Although poorly studied currently, we postulate that the intracellular cleavage of C3 and C5 recently found to occur in T cells^{26,27} will not be restricted to this immune cell population and it can be expected that the intracellular generation of C3a and C5a will have a major role in the function of tumour cells, as well as non-immune constituents of the TME, such as endothelial cells and fibroblasts.

Complement effectors and the immune contexture of the tumour.

The immune contexture of tumours, which is the immune profile determined by the density, composition, functional state and organization of the leukocyte infiltrate, is a key determinant of tumour progression¹. Complement receptors are expressed on the surface of immune cells. C3a and C5a, generated locally within the tumour, promote leukocyte attraction and impact their phenotype (FIG. 2). Following the discovery that C5a recruits myeloid-derived suppressor cells (MDSCs) to the TME, which in turn suppress effector T cells³¹, it was found that C3a and/or C5a exerts a profound influence on the TME by inducing a series of context-dependent changes, including: the recruitment of tumour-promoting macrophages and CC-chemokine ligand 2 (CCL2) production (in a PTX3 and FH-dependent manner)⁴²; a decrease in recruitment of CD4⁺ T cells and neutrophils⁴³; a decrease in recruitment of natural killer (NK) cells⁴⁴; stimulation of a pro-tumoural phenotype for CD4⁺ T cells³³; inhibition of interleukin 10 (IL-10) expression by intratumoural CD8⁺ T cells⁴⁵; stimulation of the pro-tumorigenic properties of mast cells and macrophages, including suppression of CD8⁺ T cell

Autophagy

A cellular stress response in which cellular proteins and organelles are digested and recycled by lysosomes in order to maintain active metabolism.

Table 1 | Pro-tumoural role of complement in mouse models of cancer

Cancer mouse model	Complement component studied	Effects on tumour growth	Mechanism	Ref.
Syngeneic (using TC-1 cells)	C4, C3, C5aR1, factor B	<i>C4^{-/-}</i> , <i>C3^{-/-}</i> and <i>C5ar1^{-/-}</i> mice, but not <i>Cfb^{-/-}</i> mice, have impaired tumour growth; pharmacological inhibition of C5aR1 impaired tumour growth	Modulation of migration and production of ROS and RNS by MDSCs	31
Syngeneic (using TC-1 cells)	C1q, C3, C4	<i>C1q^{-/-}</i> , <i>C4^{-/-}</i> and <i>C3^{-/-}</i> mice have impaired tumour growth	Classical complement pathway activation induced pro-tumoural effects on angiogenesis and the immune environment	32
Lung syngeneic (LLC)	C1q, C5	<i>C1q^{-/-}</i> but not <i>C5^{-/-}</i> mice have impaired tumour growth	C1q induced angiogenesis independently of complement activation	58
Lung syngeneic and orthotopic (CMT167-luc, EML4-ALK, LLC-luc)	C3, C3aR1, C5aR1	<i>C3^{-/-}</i> mice have impaired tumour growth and a decreased number of metastases in other lobes of the lung; pharmacological blockade of C3aR or C5aR1 results in inhibition of tumour growth	C3 signalling inhibits the production of multiple cytokines from CD4 ⁺ T cells	33
Lung syngeneic in <i>Kras^{LSL-G12D/+}</i> mice	C5aR1	Combination of C5a and C5aR1 inhibition and PD1 blockade results in decreased tumour growth and metastasis	Increased frequency of CD8 ⁺ T cells and decreased frequency of MDSCs within tumours	132
Experimental lung metastasis and lung xenograft (A549M1, H460M5)	C5aR1	Decreased tumour burden when mice are intravenously or intracardially injected with A549M1 cells silenced for <i>C5ar1</i> ; decreased number of bone metastases	<i>C5ar1</i> -silenced tumour cells have decreased motility and a lower metalloproteolytic activity	70
Lung syngeneic (3LL)	C5aR1	Pharmacological blockade of C5aR1 results in inhibition of tumour growth	Decreased expression of bFGF and decreased numbers of MDSCs in tumours	55
Lung xenograft (A549)	Factor H	Tumour cells silenced for <i>Cfh</i> have slower tumour growth	Factor H deficiency sensitizes tumour cells to complement-mediated attack	82
Ovarian syngeneic (ID8-VEGF)	C3	Mice injected with C3 shRNA into the peritoneum have slower tumour growth	C3 enhances EMT	39
Ovarian syngeneic (ID8-VEGF) and xenograft (SKOV3ip1)	C3, C5aR1	<i>C5ar1^{-/-}</i> mice have impaired tumour growth	Autocrine effect of C3a and C5a on their receptors on cancer cells and induction of proliferation through the PI3K–AKT pathway	40
Ovarian xenograft (RMA-3CF4 cells) and lymphoma syngeneic (RMA-1474)	C5aR1	Correlation between tumour burden, expression of C5a and C5aR1 signalling	Correlation between C5a and decreased number of effector T cells	47
Ovarian transgenic (TgMISIIR-TAg)	C3, C5aR1	C3 or C5aR1 deficiency attenuates tumorigenesis; pharmacological inhibition of C5aR1 impaired tumour growth	C3 induced production of pro-inflammatory cytokines; C5a induced the production of pro-angiogenic factors	56
Breast syngeneic metastatic (4T1)	C5aR1	<i>C5ar1^{-/-}</i> mice or pharmacological inhibition of C5aR1 decreased metastasis	C5aR1 facilitates metastasis by suppressing effector T cells through recruitment of immature dendritic cells that produce TGFβ and IL-10	9
Breast syngeneic metastatic (4T1)	C5aR1	C5aR1 deficiency or C5aR1 blockade decreased lung metastasis	Reduced recruitment of MDSCs	8
Colon syngeneic (MC38)	C3	C3-depleted mice (using CVF) have impaired tumour growth	Increased expression of CCL5, CXCL10 and CXCL11, and migration of CD8 ⁺ T cells	24
Intestine transgenic (<i>Apc^{Min/+}</i>)	C3aR	<i>C3ar^{-/-}</i> mice have impaired tumour growth	C3a induced polarization of neutrophils via C3aR towards a pro-tumorigenic phenotype	46
Colon syngeneic metastatic (SL4, CT26) and colon xenograft (HCT116, SW116)	C5aR1	<i>C5ar1^{-/-}</i> mice have decreased numbers of metastases in the liver	Infiltration of inflammatory cells and production of CCL2 by macrophages via the AKT pathway	135
Experimental colon metastasis (SL4-luc)	C5aR1	<i>C5ar1^{-/-}</i> mice have a decreased number of hepatic metastases	C5a–C5aR1 pathway regulates the M2 phenotype of TAMs in metastases through NF-κB signalling	145

Table 1 (cont.) | Pro-tumoural role of complement in mouse models of cancer

Cancer mouse model	Complement component studied	Effects on tumour growth	Mechanism	Ref.
Colitis-induced colorectal cancer (azoxymethane plus dextran sulfate sodium)	C3, C5, C5aR1	<i>C3^{-/-}</i> , <i>C5^{-/-}</i> or <i>C5aR1^{-/-}</i> mice have impaired tumour growth	C5-derived C5a promotes production of IL-1 β from neutrophils via C5aR1	78
Bile duct xenograft (HuCCT1)	C5aR1	C5a-treated HuCCT1 cells overexpressing C5aR1 spread more broadly than C5a-treated HuCCT1 control cells in nude mice skin tissue	C5a enhances invasion of C5aR1-expressing cancer cells	80
Sarcoma induced by a carcinogen (3-MCA)	PTX3, factor H, C5a	<i>Ptx3^{-/-}</i> mice have an increased susceptibility to carcinogenesis	Local complement activation under <i>Ptx3</i> silencing promotes M2-like macrophage infiltration; factor H is recruited by PTX3 and controls C5a levels	42
Sarcoma induced by a carcinogen (3-MCA)	C3, C3aR1	<i>C3^{-/-}</i> and <i>C3aR1^{-/-}</i> mice have reduced tumour growth	Deficiency of the C3–C3aR axis induces protection by reduction of macrophage recruitment and skewing of their phenotype towards M1 as well as induction of antitumour CD8 ⁺ T cell immune responses	146 (conference abstract)
Melanoma syngeneic (B16F10)	C1q	<i>C1q^{-/-}</i> (but not <i>C5^{-/-}</i>) mice have impaired tumour growth and a decreased number of metastases	C1q induced angiogenesis and tumour cell adhesion and proliferation independently of complement activation	58
Melanoma syngeneic (B16F10 cells transfected with gp33)	C3	C3-depleted mice (using CVF) have impaired tumour growth	NK cell-mediated cytotoxic T cell-dependent antitumoural immune responses	44
Melanoma and colon syngeneic (B16F10, SM1WT1) and colon syngeneic (MC38)	C3aR	<i>C3aR^{-/-}</i> mice have impaired tumour growth	<i>C3aR^{-/-}</i> mice have increased infiltration of neutrophils and CD4 ⁺ T cells, and decreased numbers of macrophages	43
Melanoma and breast syngeneic (B16F10) and breast syngeneic (E0771)	C3, C3aR, C5aR1	<i>C3^{-/-}</i> mice have impaired tumour growth; pharmacological blockade of C3aR or C5aR1 suppresses tumour growth	C3 inhibits IL-10 secretion by effector CD8 ⁺ T cells; enhanced antitumour response with combination of C3aR or C5aR1 antagonism and anti-PDL1	45
Experimental breast cancer (MDA231-LeptoM, HCC1954-LeptoM) and lung cancer (PC9-LeptoM LLC-LeptoM) leptomeningeal metastasis	C3, C3aR	MDA231-LeptoM cells silenced for C3 injected into mice have slower tumour growth; <i>C3aR^{-/-}</i> mice injected with LLC-LeptoM cells in the leptomeningeal space have impaired tumour growth; pharmacological inhibition of C3aR decreased metastasis	Leptomeningeal metastatic cells produce C3 that disrupts the choroidal blood–CSF barrier to adapt the CSF for cancer cell growth	73
Gastric syngeneic (MFC)	C5aR1	Pharmacological blockade of C5aR1 inhibits tumour growth	C5aR1 blockade inhibits phosphorylation of PI3K–AKT and increases levels of p21 and phosphorylated p21	81
Leukaemia xenograft (U937)	C3a, C5a	Tumour cells treated with C3a or C5a before subcutaneous inoculation have an increased capacity to spread to organs	C3a and C5a activates p38 MAPK, which downregulates HO1 expression and enhances motility	79
Pancreatic neuroendocrine transgenic (BT2 B6)	C5aR1	Pharmacological blockade of C5aR1 inhibits tumour growth	C5a–C5aR1 pathway increases invasiveness and macrophage recruitment	85
Cutaneous squamous cell xenograft (UT-SCC-7)	Factor I	Tumour cells silenced for <i>CFI</i> have slower tumour growth	Factor I promotes proliferation and migration	76
Squamous cell transgenic (K14-HPV16)	C5aR1	<i>C5aR1^{-/-}</i> mice have impaired tumour growth; synergic effect with the combination of C5aR1 blockade and chemotherapy	C5aR1-deficient tumours have impaired angiogenesis and a decreased number of mast cells and macrophages; C5 is cleaved by plasmin	16
Squamous cell xenograft (cSCCIS)	Factor B, C3	Silencing of <i>CFB</i> or C3 in tumour cells inhibited tumour growth	Silencing of <i>CFB</i> or C3 in tumour cells decreased their migration and proliferation and resulted in inhibition of ERK1 and/or ERK2 signalling	147

bFGF, basic fibroblast growth factor; C3aR, C3a receptor; C5aR1, C5a receptor 1; CCL, CC-chemokine ligand; CF, complement factor; CSF, cerebrospinal fluid; CVF, cobra venom factor; CXCL, CXC-chemokine ligand; EMT, epithelial-to-mesenchymal transition; HO1, haem oxygenase 1; HPV16, human papillomavirus 16; IL, interleukin; MDSC, myeloid-derived suppressor cell; NF- κ B, nuclear factor- κ B; NK cell, natural killer cell; PD1, programmed cell death 1; PDL1, programmed cell death 1 ligand 1; PTX3, pentraxin-related protein 3; RNS, reactive nitrogen species; ROS, reactive oxygen species; shRNA, short hairpin RNA; TAM, tumour-associated macrophage; TGF β , transforming growth factor β ; VEGF, vascular endothelial growth factor.

NETosis

A process of release of neutrophil extracellular traps (NETs) from overactivated neutrophils. NETs are defensive networks of extracellular fibres, primarily composed of DNA and histones.

cytotoxicity¹⁶; and promotion of pro-tumoural neutrophil extracellular trap (NET) formation (known as NETosis)⁴⁶.

The level of intratumoural C5a may be a key determinant of the composition of the immune TME. A mouse lymphoma cell line engineered to produce low levels of C5a grew more slowly in mice, resulting in increased IFN γ -producing T cells in the spleen and tumour-draining lymph nodes⁴⁷. Conversely, mice engrafted with a high C5a-producing tumour cell line had accelerated tumour progression with more Gr-1⁺CD11b⁺ myeloid cells in the spleen and overall decreased numbers of T cells in the tumour, tumour-draining lymph nodes and spleen in this experimental model.

The C3 activation fragments are potent effectors, modulating the immune response³. In patients with ovarian cancer, mature neutrophils acquire a suppressive phenotype that is linked to complement C3 activation⁴⁸. These immunosuppressive neutrophils completely suppressed the proliferation of naive, central memory and effector memory T cells, hampering the antitumour immune response. This process requires T cell contact and interaction between iC3b and complement receptor 3 (CR3) on neutrophils. Chemotherapeutic as well as immunotherapeutic approaches induce apoptosis in tumour cells and may induce immunogenicity^{49,50}. Opsonization of apoptotic tumour cells with iC3b prevents the maturation of dendritic cells via interaction with CR3 and contributes to the induction of antigen-specific silencing and tolerance⁵¹. Moreover, the iC3b–CR3 interaction results in dysregulation of NK cell-dependent tumour surveillance⁵².

Although the majority of the experimental models agree on the pro-tumoural role of C3a and especially C5a, the mechanisms described above are context specific. Rarely is the same mode of action of the anaphylatoxins found across different cancer models. This could reflect

differences in the composition of the immune microenvironment between cancer types. Indeed, in mice, as in humans, the immune infiltration is largely controlled by the properties of the tumour cells themselves^{1,53}.

Neoangiogenesis and complement. Neovascularization is critical for the supply of oxygen and nutrients to the tumour. 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 (FIG. 3a).

For around 10 years, it has been known that C5a promotes migration and tube formation of endothelial cells in vitro^{54,55}. In addition, C3^{-/-} and C5ar^{-/-} endothelial cells have impaired angiogenesis capacity⁵⁶. Nevertheless, the impact of C3a and C5a in mouse tumours seems to be model dependent. C3^{-/-}, C3ar^{-/-} or C5ar^{-/-} showed either impaired tumour angiogenesis^{16,56}, increased blood vessel permeability without an effect on microvascular density⁵⁷ or no effect⁵⁵. Therefore, the context-dependent impact of the anaphylatoxins on neoangiogenesis requires further investigation.

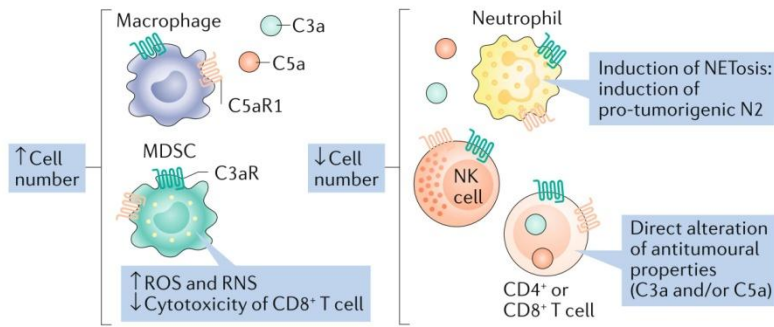
Recent evidence points towards a major role of C1q in cancer neoangiogenesis via a non-canonical, cascade-independent mechanism. A fraction of tumour vessel endothelial cells produce C1q in tumour mouse models and in human tumours^{32,58}. The microvascular density was either decreased or the vascular network was disorganized in tumours growing in C1q^{-/-} mice^{32,58}. This could be explained by alteration of C1q-mediated expression of vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFRs), as shown in tumour mouse models³² and in studies of pregnancy complications of mice⁵⁹. The pro-angiogenic effect of C1q is again context dependent, as neoangiogenesis was enhanced in the human epidermal growth factor receptor 2 (HER2;

Table 2 | Antitumoural role of complement in mouse models of cancer

Cancer mouse model	Complement component studied	Effects on tumour growth	Mechanism	Ref.
Breast xenograft (MCF7)	Factor P	Factor P-overexpressing tumour cells have impaired tumour growth	Factor P induces ER stress, TES transcription and increased expression of DDIT3	84
Breast transgenic (<i>neuT⁺-C3^{-/-}</i>)	C3	<i>neuT⁺-C3^{-/-}</i> mice have accelerated tumour growth and an increased number of lung metastases	Tumours in <i>neuT⁺-C3^{-/-}</i> mice had an increased number of T _{reg} cells and a modified vascular architecture	57
Breast transgenic (<i>neuT⁺-C1q^{-/-}</i>)	C1q	<i>neuT⁺-C1q^{-/-}</i> mice have accelerated tumour growth and an increased number of lung metastases	A higher number of intratumour blood vessels and a decrease in the activation of the tumour suppressor WWOX within tumour cells; no difference in the numbers of tumour infiltrating immune cells or local complement activation	60
Melanoma syngeneic (B16F10-OVA)	C3, C3aR, C5aR1	Tumours in C3 ^{-/-} mice grow slower but are resistant to radiotherapy; C3aR and C5aR1 inhibition results in resistance to radiotherapy	Local production of C3a and C5a was necessary for the tumour response to radiotherapy and for the stimulation of tumour-specific immunity	136
Colon syngeneic (CT26)	C3aR, C5aR1	C3ar ^{-/-} and C5ar1 ^{-/-} results in resistance to radiotherapy	Local production of C3a and C5a was necessary for the tumour response to radiotherapy and for the stimulation of tumour-specific immunity	136
Syngeneic (using TC-1 cells) in combination with adoptive T cell therapy	C3, C5aR1	C3 ^{-/-} or C5ar1 ^{-/-} , and pharmacological blockade of C5aR1 impaired the ability of T cells to overcome the endothelial barrier, infiltrate tumours and control tumour progression	C3 and C5aR1 expression by tumour stroma, and not leukocytes, governs T cell homing, acting on the local endothelium	137

C3aR, C3a receptor; C5aR1, C5a receptor 1; DDIT3, DNA damage-inducible transcript 3; ER, endoplasmic reticulum; TES, testin; T_{reg} cell, regulatory T cell; WWOX, WW domain-containing oxidoreductase.

a Pro-tumoural impact on the immune microenvironment



b Antitumoural impact on the immune microenvironment

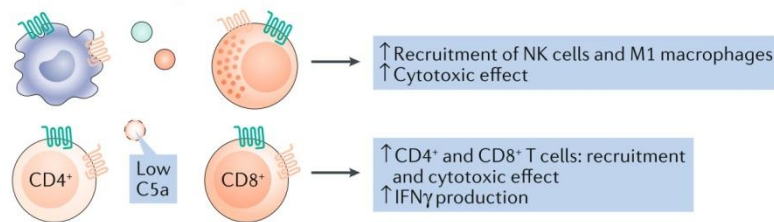


Fig. 2 | The pro-tumoural and antitumoural impact of complement on the immune contexture. C3a and C5a can modulate the immune microenvironment towards a pro-tumour or antitumour response depending on the tumour type and local concentrations of the anaphylatoxins. **a** | This schematic shows the pro-tumoural actions of complement effectors on immune cells. In the majority of cancer types, the anaphylatoxins can change the inflammatory milieu by increasing the recruitment of immunosuppressive cells and decreasing the number of pro-inflammatory cells through activation of their receptors. C5a induces the recruitment of myeloid-derived suppressor cells (MDSCs) and promotes their immunosuppressive functions by increasing the amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that in turn contribute to the suppression of antitumour T cell responses. C3a and C5a can induce CC-chemokine ligand 2 (CCL2) production, which enhances recruitment of tumour-promoting macrophages (M2-like). The C3a–C3a receptor (C3aR) axis is also involved in the recruitment of neutrophils, induction of a polymorphonuclear suppressor phenotype (known as N2) and neutrophil extracellular trap (NET) formation that promotes tumorigenesis. Indeed, all of these functions are impaired in the absence of C3aR. Complement activation blockade by cobra venom factor (CVF) resulted in an increase in the infiltration of natural killer (NK) cells in mouse models of cancer⁴⁴. Overall, modulation of the myeloid compartment towards an immunosuppressive phenotype can lead to an ineffective T cell response. The C3a–C3aR axis is also involved in decreasing cytokine production by CD4⁺ T cells. Moreover, this axis inhibits IL-10 production by CD8⁺ T cells resulting in an ineffective T cell response. In addition, C3a can be generated intracellularly by cathepsin L cleavage and can interact with its receptor present at the surface of lysosomes to modulate T cell activity. **b** | This schematic shows the antitumoural actions of complement effectors on immune cells. In contrast to the pro-tumoural roles described above, in some cancer types the anaphylatoxins can play a protective role against tumour progression by promoting the recruitment of pro-inflammatory cells. In the case of an ovarian cancer mouse model, a low level of C5a is associated with recruitment of antitumoural macrophages (M1-like) and NK cells that display cytotoxic effects in contrast to high C5a levels. C5aR1, C5a receptor 1; IFN γ , interferon- γ .

also known as neu or ERBB2) transgenic *neuT⁺-C1q^{-/-}* breast cancer mouse model⁵⁰.

Although mouse models provide insights into the role of the complement cascade and its individual components in tumour neoangiogenesis, most of the data are generated with subcutaneously implanted tumour cell lines. Endothelial cells from different organs have unique properties, including a different spectrum of expressed complement proteins^{61,62}, which may differentially impact upon neoangiogenesis in each cancer type.

Leptomeningeal metastasis
A complication occurring when tumour cells spread to the leptomeninges, membranes lining the brain and spinal cord, enclosing the cerebrospinal fluid.

Therefore, further studies are needed to determine the relative impact of complement on neoangiogenesis of human tumours.

Direct impact of complement effectors on tumour cells.

In addition to promoting inflammation, C3a and C5a could directly affect fundamental processes of tumour cells, such as survival, proliferation, migration and stemness (FIG. 3b,c). Anaphylatoxin receptors are expressed on certain cancer cells⁶. Multiple reports show that these tumour cells also express C3 and/or C5 and generate C3a and C5a, acting in an autocrine manner. The impact of this signalling on tumour cells ranges from stimulation of proliferation^{40,63} to maintenance of a multipotent state of glioblastoma stem-like cells⁶⁴, induction of the epithelial-to-mesenchymal transition (EMT)^{39,65}, changes in invasiveness and morphology⁶⁶, and promoting stemness⁶⁷. Specifically, C3a enhances tumour cell proliferation, migration and stemness in mouse cutaneous squamous cell carcinoma and this activity was correlated with activation of the WNT– β -catenin pathway⁶⁷. If the cascade proceeds to terminal MAC formation, the sublytic levels of C5b-9 mediate signalling, promoting cancer cell cycle progression⁶⁸.

Complement also promotes metastasis, as reviewed by Ajona et al.⁶⁹. Briefly, in tumour cells, C5a triggers expression of matrix metalloproteinases (MMPs), increases tumour cell migration and invasiveness, enhances the release of pro-angiogenic factors and induces EMT. Anaphylatoxins also facilitate tumour dissemination by stimulating a hypercoagulable state (an increased predisposition to form blood clots) and NETs, and adapt specific organ environments to metastatic spread⁶⁹. In addition, C5a induces CXC-chemokine ligand 16 (CXCL16)-mediated osteoclastogenesis and the generation of an immunosuppressive microenvironment in a mouse model of lung cancer bone metastasis⁷⁰. Pharmacological blockade or genetic deficiency of C5aR1 was sufficient to reduce lung metastases in a breast cancer mouse model⁷¹. Specifically, C5aR1 signalling promoted regulatory T (T_{reg}) cell generation and suppressed T cell responses in the lungs in this context. In addition, C5aR1 expression in patients with gastric cancer is associated with cancer progression, liver metastasis and poor prognosis⁷². Cancer cell-derived C3a also promotes leptomeningeal metastasis by activation of C3aR on the choroid plexus epithelium, thus disrupting the blood–cerebrospinal fluid barrier in vivo⁷³.

In theory, the complement cascade may lead to tumour cell killing within primary tumours or metastases, if sufficiently strongly activated by host antitumoural immunoglobulin M (IgM) or IgG, or by therapeutic antibodies, and if abundant MACs are inserted into the cell membrane. Yet, in the context of cancer, current evidence suggests that complement can only proceed to cell-killing MAC assembly following treatment with targeted therapeutics (such as monoclonal antibodies that target tumour cells⁷⁴). A large body of evidence has demonstrated that this escape from complement killing is in part linked to a high expression of complement regulators at the tumour cell surface^{6,11,75–82}.

Many complement components, such as C1q, C1s, C3, FP (also known as properdin), FH and FI, have

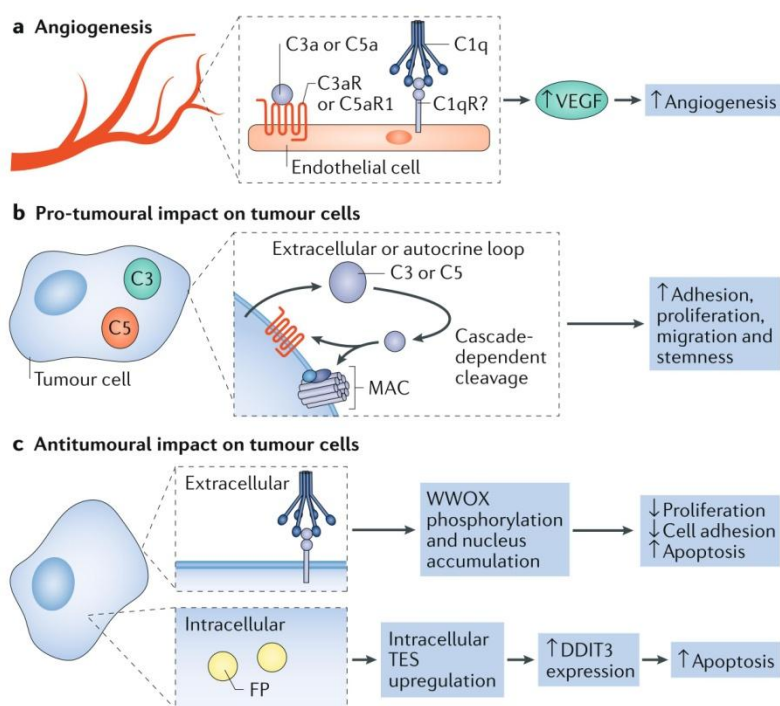


Fig. 3 | The pro-tumoural and antitumoural impact of complement on neoangiogenesis and the biology of tumour cells. **a** | This schematic shows the effects of the complement system on angiogenesis. C3a and C5a can interact with their receptors to promote angiogenesis through an upregulation of growth factor expression, such as basic fibroblast growth factor (bFGF) and vascular epithelial growth factor (VEGF), and enhancement of endothelial cell proliferation. C1q can also modulate angiogenesis in a non-canonical way through the expression of VEGFs and VEGF receptors (VEGFRs) via an unidentified C1q receptor (C1qR). **b** | This schematic shows the pro-tumoural effects of the complement system on key characteristics of tumour cells. Cancer cells can produce C3 and C5 that can be cleaved extracellularly after complement activation or potentially intracellularly through proteases. This cleavage leads to the generation of C3a and C5a that can act in an autocrine manner on tumour cells. Extracellular C3a and C5a that are generated in a canonical manner can act on their receptors at the surface of tumour cells and induce different signalling pathways such as PI3K–AKT, WNT and β -catenin, which promote tumour cell adhesion, proliferation, migration and stemness. Complement activation can also lead to sublytic membrane attack complex (MAC) formation at the surface of tumour cells. MAC formation can activate signalling pathways like ERK1 and/or ERK2, PI3K–AKT and p70 S6 kinase, which promote tumour growth. **c** | This schematic shows the antitumoural effects of the complement system on key characteristics of tumour cells. Intracellular factor P (FP; also known as properdin) induces endoplasmic reticulum stress and expression of the tumour suppressor testin (TES, a LIM domain protein), which upregulates the expression of pro-apoptotic transcription factor DNA damage-inducible transcript 3 (DDIT3) and suppresses tumour growth independently of complement activation in breast cancer cells. Extracellular C1q activates the tumour suppressor WW domain-containing oxidoreductase (WWOX), in a non-canonical manner, to induce apoptosis of prostate and breast cancer cells. C3aR, C3a receptor; C5aR1, C5a receptor 1.

non-canonical, extracellular and intracellular functions, modulating the fundamental processes of tumour cells and promoting proliferation and tumour progression in selected tumour mouse models^{58,76,77,83} (TABLES 1, 2). In embryonic development and in cancer models, intracellular C3 impacts EMT³⁹. FP, which is the only positive regulator of the cascade, has non-canonical functions, which oppose the majority of the other tested complement proteins. For example, FP suppresses breast cancer cell growth in vitro by control of transcription⁸⁴. Other in vitro studies show that C1q can also exert antitumoural effects

by induction of apoptosis in an ovarian cancer cell line by stimulation of the tumour necrosis factor (TNF) pathway⁸⁵ or by activation of the tumour suppressor WW domain-containing oxidoreductase (WWOX) to induce apoptosis in prostate and breast cancer cell lines^{60,86}. WWOX is only weakly expressed in the majority of tumour types, suggesting that this effect of C1q is context dependent, and is particularly relevant in cancers arising in hormone-regulated tissues (such as the breast, ovary and prostate). Indeed, *CIQA*, *CIQB* and *CIQC* gene expression is associated with better prognosis in basal-like breast cancer with C1q protein expression detected by immunohistochemical staining in macrophage-like cells in the stroma⁸⁷.

Complement in human tumours

The potential role of complement in the interplay between malignant cells and the TME in human cancer has begun to be uncovered. Although individual studies are quite fragmented, the accumulated literature shows that tumours develop in a complement-rich milieu. Many of the cells present in the TME produce complement components and/or bear complement receptors and complement regulators⁸⁸, suggesting a potential in situ activation of the complement pathways (FIG. 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 tumour, 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 observation is the presence of complement receptors, particularly C3aR and C5aR1, on most of the cell types in the TME, suggesting that the activity and functions of T and B lymphocytes, neutrophils, macrophages, MDSCs, dendritic cells, endothelial cells and fibroblasts can be modulated by the activation fragments of C3 and C5. The second observation is the presence of complement regulators at high levels, capable of inhibiting complement activation, particularly the terminal pathway, on malignant cells. This is an underappreciated mechanism of escape for tumour cells from the attack of complement, which could contribute to tumour immune escape mechanisms in general as well as to guide novel cancer immunotherapy development^{89,90}. This may also explain why intratumoural C5b-9 staining has not been frequently reported⁹¹. Unfortunately, only a very limited number of studies address the expression and activation of complement in large cohorts of patients with different cancers. This problem can now be resolved, at least in part, owing to the data-mining and bioinformatics analyses of publicly available databases such as The Cancer Genome Atlas (TCGA) or the Pathology Atlas of the human cancer transcriptome (Human Protein Atlas)^{92,93}.

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 30 tumour types, using publicly available data sets^{92,93}. FIGURE 4 shows an unsupervised

hierarchical clustering of the expression of 50 complement-related genes in solid tumours, encompassing 30 cancer types. Several conclusions can be drawn from this analysis. Overall, there is strong heterogeneity in expression among genes but, surprisingly, not much heterogeneity between cancer types. The gene encoding C3, the pivotal complement component, is strongly expressed in all cancer types together with genes of the components of the classical pathway (*CIQA*, *CIQB*, *CIQC*, *C1R*, *C1S*, *C4A* and *C2*). In contrast, genes encoding components of the lectin pathway are either poorly expressed in most tumour types (mannose-binding lectin 2 (*MBL2*), mannose-binding lectin-associated serine protease 2 (*MASP2*) and ficolin 2 (*FCN2*)) or heterogeneously expressed with an overall low expression in the majority of the cancers (*MASP1*, *FCN1* and *FCN3*), arguing against a major contribution of this pathway to in situ activation of the complement cascade. As regards the alternative pathway, complement factor B (*CFB*) and *CFD* are heterogeneously expressed with a particular low expression in kidney chromophobe (KICH; also known as chromophobe renal cell cancer), uveal melanoma (UVM) and prostate adenocarcinoma (PRAD). All other tumour types exhibit higher expression levels 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 *C8A*, *C8B* and *C9* genes in all of the tumour types (with the exception of cholangiocarcinoma (CHOL; also known as bile duct cancer)), which suggests that the terminal pathway is unlikely to be activated via components produced in situ. Moreover, the genes encoding complement regulators acting at the level of C1 (*SERPING1*, which encodes C1inh) and at the level of the C3 convertases (*CFH*, *CFI*, *CD46* and *CD55*) are highly expressed in most cancers. The gene encoding the terminal pathway negative regulator, *CD59* is among the most highly expressed of all complement genes in the analysed tumour types, suggesting efficient protection of malignant cells from complement-mediated killing. This pattern of gene expression is entirely in line with specific examples from the literature, demonstrating high expression of these regulators in different types of cancer^{75,91,94–98}. Complement-mediated cytotoxicity may act as a selective pressure for tumour-intrinsic overexpression of complement regulators. Indeed, hypoxic colorectal cancer cells in vitro are resistant to complement-mediated cytotoxicity owing, in part, to hypoxia-induced expression of the complement regulator CD55 (REF.⁹⁹). This, again, is context dependent, as the effect of hypoxia on a non-small-cell lung cancer (NSCLC) cell line in vitro was decreased expression of complement regulators and increased susceptibility of the tumour cells to complement attack¹⁰⁰.

The low expression levels of terminal pathway genes together with the high expression of genes encoding complement regulators reinforces the hypothesis that malignant cells evolve and adapt to avoid potential cell lytic MAC formation. Instead, intratumoural complement activation can be sustained via locally expressed classical and alternative pathway components, thus generating the largely pro-tumoural anaphylatoxins C3a and C5a.

Furthermore, we analysed gene expression in the same tumour types normalized to expression in the corresponding normal tissues (Supplementary Figure 1). The pattern which emerged is cancer dependent. Nearly half (11/26) of the analysed cancer types upregulated the majority of the complement genes relative to the corresponding normal tissue and clustered in an 'upregulated complement' group. These included kidney renal papillary cell carcinoma (KIRP), kidney renal clear cell carcinoma (KIRC), head and neck squamous cell carcinoma (HNSC), oesophageal carcinoma (ESCA), stomach adenocarcinoma (STAD), lower grade glioma (LGG), glioblastoma (GBM), pancreatic adenocarcinoma (PAAD), testicular germ cell tumours (TGCT), skin cutaneous melanoma (SKCM) and ovarian serous cystadenocarcinoma (OV). The other half (13/26) of the cancer types had 'downregulated complement', expressing less complement genes compared with the corresponding normal tissue, such as lung squamous cell carcinoma (LUSC), lung adenocarcinoma (LUAD), adrenocortical carcinoma (ACC), KICH, rectum adenocarcinoma (READ), colon adenocarcinoma (COAD), bladder urothelial carcinoma (BLCA), PRAD, breast invasive carcinoma (BRCA), thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), and uterine carcinosarcoma (UCS). Two cancers, thymoma (THYM) and diffuse large B cell lymphoma (DLBC), similarly show a mixed profile, strongly upregulating more than half of the complement genes but downregulating the rest. These analyses also show that the majority of the cancer types (but not all) overexpress the complement regulators, supporting the notion that this is an escape mechanism to counteract complement attack. The C1q genes are overexpressed in the majority of the tumour types, which might correlate with the infiltration of immunosuppressive macrophages in the TME³².

The prognostic impact of complement expression in patients with cancer. Next, 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 database of TCGA⁹² (FIG. 5). Four groups of cancers could be defined. The first contains tumour types for which stronger expression of genes encoding components of the classical and alternative pathways are associated with good prognosis, that is, with longer overall survival. This group of cancers with 'protective complement' includes PRAD, mesothelioma (MESO), sarcoma (SARC) and SKCM. Hepatocellular carcinoma (HCC) belongs to this group as well, but is not presented in FIG. 5 to be consistent with FIG. 4. A second group in which C3 expression correlates with longer overall survival ('protective C3') comprises KICH, ACC and 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' tumours includes UVM, LGG, GBM, KIRC and LUSC as the tumour types most significantly

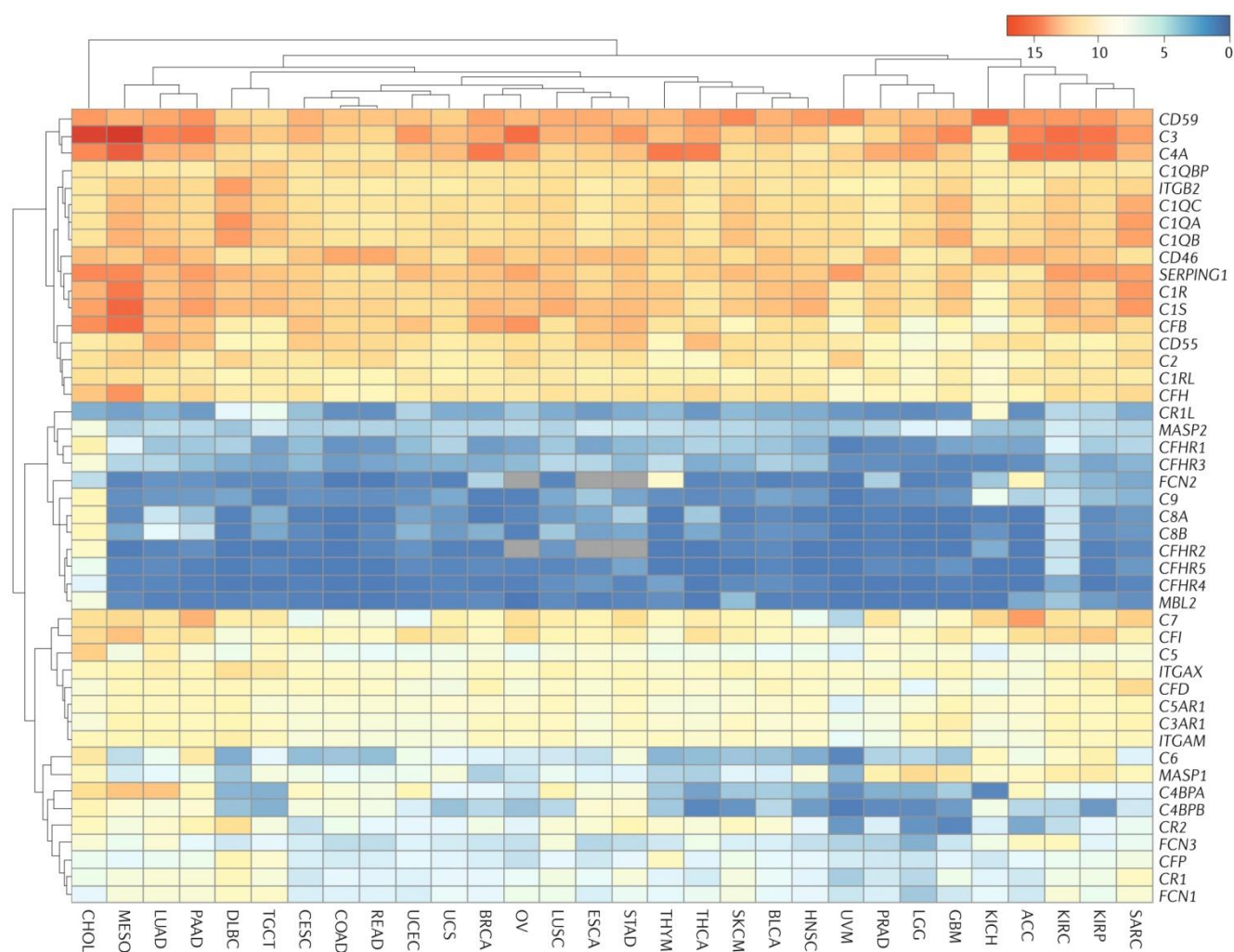


Fig. 4 | Expression of complement genes in human cancers. The Cancer Genome Atlas (TCGA) PanCanAtlas¹⁴² data used in this analysis of the expression of complement genes in human cancers were downloaded through cBioPortal¹⁴³ and come from TCGA Data Coordinating Center (DCC). This heatmap shows the expression of complement genes in different cancer types. RNA sequencing (RNA-Seq) expression data, RNASeqV2, from TCGA was processed and normalized using the RSEM (RNA-Seq by Expectation Maximization) algorithm to generate transcripts per million (TPM). This method uses raw counts to quantify the abundance of transcripts and provided estimated counts for this analysis. Specifically, the data_RNA_Seq_v2_expression_median file in cBioPortal corresponds to the rsem.genes.normalized_results file from TCGA. Thirty solid tumour types were 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 levels of complement genes. The mean TPM of each complement gene for the patients of different TCGA cohorts was calculated and then converted into $\log_2(1 + \text{TPM})$ values. Using the R package 'pheatmap' and the clustering method 'complete', the heatmap was generated to enable visualization of the mean TPM of each complement gene for the different tumour types. Blue colours correspond to low expression, and red and orange colours to high expression. Overall, strong heterogeneity in expression among genes was revealed but not as much between cancer types. The genes of the classical pathway, C3 and complement factor B (CFB), as well as genes encoding the complement regulators, were strongly expressed in the majority of the cancer types. On the contrary, the expression of the lectin and terminal pathway genes showed very low expression. ACC, adrenocortical carcinoma; BLCA, bladder carcinoma; BRCA, invasive breast carcinoma; C1QBP, C1q subcomponent-binding protein; C1RL, C1r subcomponent-like protein; C3AR1, C3a receptor 1; C4BP, C4b-binding protein; C5AR1, C5a receptor 1; CESC, cervical squamous carcinoma; CFHR, complement factor H-related protein; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; CR, complement receptor; DLBC, diffuse large B cell lymphoma; ESCA, oesophageal carcinoma; FCN, ficolin; GBM, glioblastoma; HNSC, head and neck squamous cell carcinoma; ITG, integrin; 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; MASP, mannose-binding lectin-associated serine protease; MBL2, mannose-binding lectin 2; 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 tumours; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

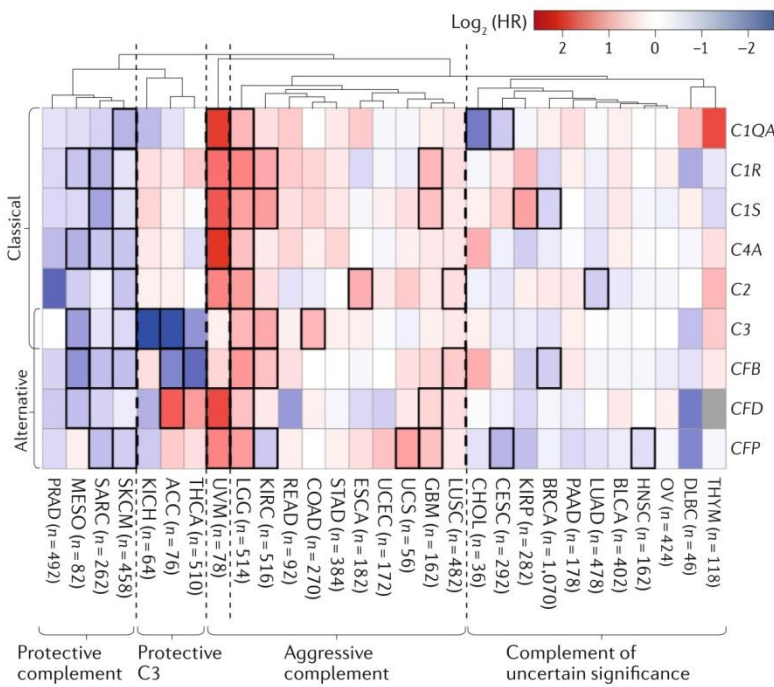


Fig. 5 | Impact of the expression level of complement genes on the survival of patients with cancer. Survival analysis was performed using the Gene Expression Profiling Interactive Analysis (GEPIA) tool¹⁴⁴. Overall survival analysis based on gene expression with a median cut-off was used to calculate hazards ratios (HRs) based on the Cox proportional-hazards model and log-rank *P* value. The median cut-off was used to avoid any prior knowledge of the distributions of gene expression profiles. Given the very low number of events, the testicular germ cell tumours (TGCT) cohort was excluded from the survival analysis. The heatmap representing the log₂ HR was generated using the R package 'pheatmap' and the clustering was done with the Euclidian distance and 'ward.D2' linkage criterion. The heatmap enables the visualization of the log₂ HR with a scale centred at 0 for each complement gene in the different tumour types. Blue colours correspond to a protective effect of complement gene expression, and red colours to an increased risk for tumour progression when the complement gene is overexpressed. The bold outlined boxes correspond to log-rank *P* < 0.05. Four groups of cancers could be defined. The 'protective complement' group contains tumour types for which stronger expression of genes encoding components of the classical and alternative pathways are associated with favourable prognosis. The 'protective C3' group comprises cancer types for which only the C3 overexpression was associated with good prognosis. The 'aggressive complement' group encompasses cancers for which the high expression of complement genes indicates negative prognostic impact. The fourth group most often shows a lack of significant prognostic impact of the complement genes and is hence named 'complement of uncertain significance'. ACC, adrenocortical carcinoma; BLCA, bladder carcinoma; BRCA, invasive breast carcinoma; CESC, cervical squamous carcinoma; CF, complement factor; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B cell lymphoma; ESCA, oesophageal carcinoma; GBM, glioblastoma; HNSC, head and neck squamous cell 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; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

impacted. Although significance was not reached for most of the genes, the group also includes digestive tract cancers such as READ, COAD, STAD and ESCA, as well as uterine cancers, such as UCEC and UCS. The fourth group encompasses a large number of tumour types in which the gene expression analysis did not reveal any

clear clinical impact; therefore, this group has been named 'complement of uncertain significance'. This group comprises CHOL, CESC, KIRP, BRCA, PAAD, LUAD, BLCA, HNSC, OV, DLBC and THYM.

It is striking that SARC is one of the cancer types within the 'protective complement' group, as the density of B cells and expression of B cell-associated transcriptional signatures have been associated with longer survival and response to immune checkpoint blockade in soft tissue sarcoma (W.H.F., unpublished results). Hence, it is tempting to speculate that, in this case, complement is involved in the antitumoural effect of B lymphocytes via its activation on intratumoural immune complexes. Indeed, early studies from the 1960s had already noted a potential link between the presence of sarcoma antibodies and an antitumoural effect of complement in mice¹⁰¹ and, later, that immune complexes could be detected in sera from patients with sarcoma¹⁰². Furthermore, high expression of C5, produced mainly by tumour cells, correlated with better event-free and overall survival in Ewing sarcoma¹⁰³ (not included in the data set of TCGA). In this case, C5aR1 was predominantly detected on tumour cells in situ.

Despite abundant data related to the role of complement in mice grafted with melanoma cell lines (yielding often contradictory conclusions)^{43–45,58}, little is known about complement activation in human melanoma and its association with clinical parameters in patients' cohorts.

In addition, very limited data related to complement and human prostate cancer are available. Sublytic complement C5b-9 protects prostate cancer cell lines from TNF-induced cell death in vitro¹⁰⁴. Interestingly, proteolysis of iC3b and C5 by the serine protease prostate-specific antigen (PSA) in prostatic fluid of patients with prostate cancer was detected, which would serve to inhibit the terminal pathway¹⁰⁵.

MESO offers an interesting case. The analysis presented here includes only 82 patients with heart, mediastinum and pleural MESO, and shows an overall tendency for good prognosis in association with high expression of classical and alternative complement pathway genes. Experimental evidence for the role of complement is available only for pleural MESO. Evaluation of C4d and C1q immunohistochemical staining in a patient cohort with malignant pleural MESO revealed an absence of C1q expression in the majority of tumours (only a few infiltrating immune cells stained positively) and an absence of C4d deposits on malignant cells¹⁰⁶. Membranous C4d deposits were found only in tertiary lymphoid structures and this staining was associated with poor prognosis. Patients with low C4d plasma levels at diagnosis had a significantly better overall survival. In another patient cohort, C1q staining of tumour cells and infiltrating myeloid cells was strong¹⁰⁷. In this context, C1q was shown to bind to hyaluronic acid and promote human pleural MESO cell adhesion and proliferation in a complement cascade-independent manner in vitro.

Further studies are needed to clarify the potential link between complement and cancer progression for the tumour types within the 'protective complement' group.

Tertiary lymphoid structures
Ectopic lymphoid aggregates that reflect lymphoid neogenesis occurring in tissues at sites of inflammation. These structures are detected in tumours where they orchestrate local and systemic antitumour responses.

These will be needed to provide experimental validation of whether the concerted expression of these proteins indeed occurs and whether complement is activated in situ. Immunohistochemical staining of large cohorts of patients for C1q, C4d, C3d and C5b-9 is necessary to determine the real impact of complement on cancer progression and prognosis.

With respect to the 'protective C3' group, cellular deposits of IgG and complement factors C3d, C4d and C5 were previously shown in up to 80% of patients with THCA (in a cohort of 59 patients), but data relating to prognostic impact were not reported¹⁰⁸. This cancer type, in general, has a particularly good prognosis, which might account for the lack of prognostic impact of complement found in our analysis of THCA. No data from the literature are available to verify the impact of in situ complement in KICH and ACC, for which high expression of the C3 gene is associated with particularly good prognosis in our analysis. In these cancer types, the protective role of C3 could potentially be driven by non-canonical functions.

Of the cancer types within the 'aggressive complement' group, our analysis reveals a remarkably strong negative prognostic impact of the complement genes in UVM. A high expression level of complement regulators has been reported for this cancer type¹⁰⁹, but no data are available for the status of complement activation in situ. Further studies are warranted to test whether complement expression and/or deposits do indeed impact prognosis in UVM and to evaluate whether patients with this cancer type are likely to benefit from complement blocking therapy.

We propose that gliomas should be classified as cancer types with 'aggressive complement'. In a previous report, the complement components *C1QA*, *C1S*, *C2* and *C7* were found to be upregulated in patients with high-risk relative to low-risk glioma (a glioma cohort comprising GBM and LGG)¹¹⁰. The activation of B cells around high-risk gliomas is also likely, as indicated by the enrichment of a B cell-related gene set in high-risk compared with low-risk glioma¹¹⁰. Other studies also suggest that complement is activated in GBM, but the deleterious impact on prognosis remains to be proved by in situ analyses^{64,111}.

To date, the best examples of 'aggressive complement' tumour types for which gene expression and bioinformatics analyses are in agreement with the assessment of complement components at the protein level in situ are KIRC and NSCLC. 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 tumour types such as NSCLC¹¹², ovarian cancer^{113,114}, KIRC³² and breast cancer¹¹⁵. These antibodies may come from the circulation or be produced at the tumour site^{69,112,114}, potentially initiating the complement cascade. The recognized antigens are frequently unknown. Further studies are needed to identify them and to determine whether they are recognized through shaping of the antigen-binding site by genetic processes of recombination and mutation or through unconventional strategies for diversification of the repertoire of

antigen specificities¹¹⁶. In KIRC³² and NSCLC¹¹⁷, C1q-mediated classical pathway activation has been detected, leading to C4d deposits. In both cases, high levels of intratumoural C4d staining were associated with poor prognosis^{32,117}. In addition, C4d levels in plasma were increased in patients with NSCLC and associated with poor prognosis^{117,118}. Data in the literature do not discriminate between the two subtypes of NSCLC: LUSC and LUAD. In our analysis, although LUSC falls into the 'aggressive complement' group, for LUAD the significance of complement expression is uncertain.

The assessment of complement gene expression in different tumours strongly suggests that when complement is activated in situ, this occurs owing to locally produced complement proteins. Whilst data are lacking for the majority of cancer types, we evaluated the complement status at the protein level in patients with KIRC³², a cancer type which according to our analysis is part of the 'aggressive complement' group. We found that there is an in situ orchestrated production of C1q by TAMs and of C1r, C1s, C4 and C3 by tumour cells, concomitant with IgG deposits. This enables C1 complex assembly and complement activation. Interestingly, what conferred the poor prognosis in patients with KIRC was the presence of intratumoural C1q-producing TAMs, as well as the concomitant local production and deposition of C4 activation fragments on the tumour cells. A surprising finding was that the local production of C3 by tumour cells indicated poor prognosis, whereas C3d-positive deposits were not associated with prognostic impact. This, together with the clear context-dependent impact of C3 in different tumour mouse models (TABLES 1,2), suggests it will be important to evaluate the full spectrum of deposits of C3 activation fragments in human tumours. Indeed, the antibodies used in our study to evaluate protein expression lacked specificity, with the C3d antibody recognizing C3, C3b and iC3b as well as the C3dg and C3d fragments, each of which have different functions^{2,32}. However, in another patient cohort, positive C5a (indistinguishably marking the presence of the anaphylatoxin C5a and the intact C5) and C5aR1 staining was correlated with poor prognosis^{41,119}. Therefore, it appears that the malignant cells in KIRC hijack macrophage-produced C1q to supplement the remaining components made by the cancer cells themselves, to promote tumour growth. Moreover, the production of C1q by TAMs and its negative prognostic impact in KIRC was confirmed by another study⁸⁷. The proposed mechanism of action in tumours with 'aggressive complement' is depicted in FIG. 6, based largely on the data from patients with KIRC and NSCLC as well as in vivo and in vitro data. Further studies are needed to evaluate to what extent this mode of action of complement is valid for other cancer types.

The 'complement of uncertain significance' group encompasses a large number of different cancer types, for which either significance associated with prognostic impact was achieved for some complement genes and not others or no significance was found at all, using the median cut-off. Interestingly, in PAAD, tumour cells release exosomes which harbour B cell targets and bind antitumoural IgG to exert decoy function against potential

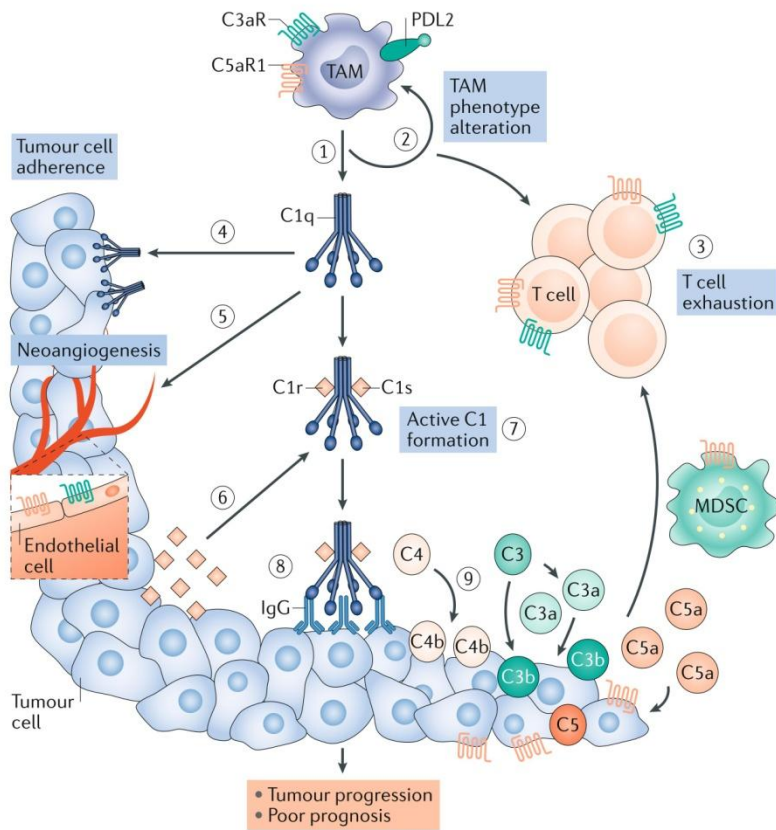


Fig. 6 | Proposed mechanism of classical complement pathway activation and its consequences on tumour progression in tumours with ‘aggressive complement’. C1q is produced by tumour-associated macrophages (TAMs) (step 1) and contributes to a tumour-promoting phenotype of these cells (step 2) and T cell exhaustion (step 3). Secreted C1q promotes adherence of tumour cells to the extracellular matrix (step 4) and neoangiogenesis (step 5). A particular feature of clear cell renal cell carcinoma (also known as kidney renal clear cell carcinoma (KIRC)) is that the tumour cells produce C1r and C1s (step 6) and allow formation of a functionally active C1 complex (step 7), capable of activating the classical pathway. Moreover, immunoglobulin G (IgG) deposits on tumour cells serve as C1 ligands (step 8) to initiate the cascade. The tumour cells also produce the subsequent components of the complement cascade, which enable C4 and C3 activation fragment deposition (C4b, C4d and C3b, iC3b, C3d; note that not all of these are shown on the schematic for simplicity) (step 9). Anaphylatoxins C3a and C5a are released, exerting their action on the tumour cells and on the microenvironment. The ensemble of these processes contributes to tumour progression and poor prognosis for patients with cancer. This model is based on the data for KIRC and is potentially applicable to other tumour types within the ‘aggressive complement’ group. C3aR, C3a receptor; C5aR1, C5a receptor 1; MDSC, myeloid-derived suppressor cell; PDL2, programmed cell death 1 ligand 2.

complement-mediated cytotoxicity¹²⁰. This could explain why classical and alternative pathway genes and proteins in PAAD do not seem to be associated with prognosis, despite their high expression¹²¹. Complement may not be efficiently activated in situ, when IgG is subverted from the tumour cells and targeted to exosomes.

Cascade-independent impact of complement in human cancers. As evidenced from 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 tumour groups described herein. An indication of such phenomena could be that one gene or expressed and/or deposited protein is

associated with either good or poor prognosis, whereas the remaining members of the same pathway are not. Another indication might be that the protein present within the cell is associated with a prognostic impact, whereas its deposits are not.

The cascade-independent impact has been illustrated with C1q-producing TAMs in patients with KIRC. We found that these TAMs were a robust marker for poor prognosis in three independent cohorts. As C1q was produced by an M2-like subtype of TAMs, the question arises of whether in addition to its role in complement activation, the negative impact of this TAM subtype was a reflection of the well-known pro-tumoural function of the immunosuppressive and pro-angiogenic M2 TAMs^{122–124} with C1q being an additional biomarker for this population or whether C1q itself plays a role in the balance between TAM phenotypes, influencing their mode of action. Interestingly, a recent in-depth immune profiling study revealed that TAMs in human KIRC (but likely in other cancers as well) represent a heterogeneous cell population¹²⁵. In particular, a subset called M-5 was shown to be associated with T cell exhaustion¹²⁵. 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 C3a³². In addition, they overexpress programmed cell death 1 ligand 2 (PDL2). Both M-5 and C1q⁺ TAMs were associated with T cell exhaustion^{32,125}. As the immunosuppressive action of C1q on T cells has already been described^{22,126,127}, it is tempting to speculate that M-5 macrophages exert their immunosuppressive activity at least in part via C1q³².

Another example is the expression of C1r and C1s in human cutaneous squamous cell carcinoma cells, which promote tumour growth in mice as well as ERK1 or ERK2–AKT signal transduction in vitro in the absence of C1q¹²⁸. FH and FI are also produced by this cancer type and promote tumour growth in vivo without evidence of complement activation^{76,77}.

Caveats associated with transcriptomic data to predict patient outcome. Gene expression analyses may inform on the potential production level of encoded proteins. However, in the complement cascade the generation of effectors is attributed to protein activation and cleavage, which are not detectable at the transcript level. Although transcriptomic analyses are useful to pinpoint associations between complement components and clinical outcome, only in situ analyses of the complement proteins, their activation fragment deposits (C4d, C3b, iC3b and C3d), the anaphylatoxins (C3a and C5a), their receptors (C3aR, C5aR1 and C5aR2) and their regulators will enable us to fully understand how complement modulates tumour cells and the TME, resulting in control or progression of cancers.

A clear example of such discrepancy is the modest prognostic impact of C3AR and C5A1 gene expression, which reaches statistical significance at the median cut-off only for four malignancies each (data not shown). Nevertheless, C5aR1 seems to be a key effector of the pro-tumoural impact of complement, even when C5a is generated by cascade-independent proteases^{16,17,31,42,45,129}. Another example relates to C1S. A clear negative

prognostic impact was demonstrated for C1s over-expression in BLCA at the protein level¹³⁰. Detailed gene expression analyses of the cohort for TCGA revealed that *C1S* was one of the most significantly upregulated genes both in urothelial carcinoma of the upper tract and in urinary bladder cancer¹³⁰. This upregulation correlated with a panel of disease markers, but the gene expression failed to reach significance in terms of prognostic impact, contrary to the immunohistochemical staining. This case illustrates the importance of performing immunohistochemical staining to determine the prognostic impact of a given protein.

Another hurdle with transcriptomic data 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 routinely in pathology laboratories and the difficulties of working with paraffin-embedded tissues are well documented¹³¹. Nevertheless, reliable protocols nowadays exist for immunohistochemical staining of complement components and activation fragments in paraffin-embedded tumour tissue³², with antibodies validated by competition tests with purified intact proteins or fragments. With the current state of imaging analyses, distinction between intracellular production and deposits with automated algorithms is tricky. This therefore requires experienced observers to stratify the patients. Moreover, proteomic analyses (such as high spatial resolution mass spectrometry imaging or cytometry by time of flight (CyTOF)) enable spatially resolved profiling of the proteins within different tumour regions. Exploration of the role of complement in cancer will benefit from the advent of these technologies, which will ultimately enable better evaluation of complement production and deposits in situ. In turn, this will leverage complement biomarker discovery and validation in patients with cancer. Furthermore, this could open up avenues for personalized therapeutic approaches, selecting for patients who might benefit most from complement-targeting therapy.

A further challenge, when a complement protein is produced both by malignant cells and infiltrating host cells, is to ascribe the pro-tumoural or antitumoural effect to this particular complement protein and its canonical and/or non-canonical functions, or to the presence of the cell itself, as in the case for C1q⁺ TAMs³².

Indeed, our prognostic data presented here need to be interpreted within the limits of using median gene expression as a cut-off to stratify patients. Even if the median cut-off did not reveal significant prognostic impact of complement genes in the 'complement of uncertain significance' group of patients, use of statistically defined optimal cut-offs may have revealed significant correlations.

Therapeutic perspectives

Our analysis of a large panel of cancers (FIG. 4) revealed simultaneous intratumoural expression of genes coding for proteins involved in complement activation (C1q, C1s, C1r and C3) or sensing activation products (C3aR

and C5aR) as well as in complement regulation (C1inh, FH, FI and CD59). Taken together, the local production and increased activation of complement in the TME is associated with dampening of the antitumour immune responses and promotion of cancer progression in a large number of different cancer types. Although further studies are needed, current evidence suggests that liver-derived complement seems to be less important in tumour biology compared with locally and/or intracellularly derived complement. It is now important to define which cells produce which components at what time points and in which cancer types. This will guide the design of future therapeutic strategies, targeting the right complement component spatially and temporally in the appropriate type of cancer.

Despite the heterogeneity of the data reported in the literature, it can be concluded that, in the majority of tumour types, the anaphylatoxin receptors C3aR and C5aR1 can be considered a novel class of immune checkpoints that could be targeted for cancer immunotherapy. Indeed, C5aR1 blockade by small-molecule inhibitor or antibody led to decreased tumour growth in various tumour mouse models either alone⁶ or in combination with immune checkpoint inhibitors (anti-programmed cell death 1 (PD1) or anti-PDL1)^{18,45,132}. Nevertheless, mouse data should be interpreted with caution, as substantial differences exist between rodent and human complement regulators and receptors. However, the *in vitro* experiments with human cells validated the protumoural role of the C5a–C5aR1 axis identified in mouse models. This subsequently promoted a phase I clinical trial for administration of a C5aR1 monoclonal antibody (IPH5401) in combination with the anti-PDL1 therapy durvalumab in patients with advanced solid tumours, which is now recruiting (STELLAR-001, NCT03665129)¹³³. C5aR1 blockade has also been shown to improve the efficacy of chemotherapy. Indeed, C5aR1 targeting with the small-molecule inhibitor PMX-53 improved the efficacy of paclitaxel chemotherapy, by promoting an antitumoural T cell response in a mouse model of squamous carcinogenesis¹⁶. Recent evidence for the role of the interaction of iC3b with CR3 in generating an immunosuppressive phenotype of intratumoural neutrophils suggests that blockade at the level of C3 might be another promising target, at least for ovarian cancer¹⁸.

Many complement-targeting molecules are in the pipeline for various disease indications^{134,135} and could be adapted for cancer therapy, acting at different steps of the cascade¹⁹. Nevertheless, any therapeutic combinations with standard-of-care therapies 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 antitumour immune response¹³⁶. Moreover, newly described connections between intracellular complement and cellular metabolism, discovered in T cells¹⁹, will likely turn out to shape the TME, affecting therapeutic outcome.

Cancer vaccines are a promising approach to stimulate the immune system to efficiently recognize and kill tumour cells. Endothelial quiescence prevents tumour-specific T cell homing. This endothelial quiescence was

Endothelial quiescence

The resting state of the endothelium, enabling it to exert its barrier functions, preventing thrombosis and inflammation. In the context of cancer, quiescent endothelium establishes a barrier that prevents T cells from efficiently penetrating the tumour.

reversed by cytokine-mediated activation of the tumour vasculature followed by upregulation of C3 and local generation of C5a in the TC-1 mouse model, inoculated with primed T cells. These T cells were derived from mice and primed with human papilloma virus (HPV) antigens expressed by the TC-1 cell line¹³⁷. The C5a-dependent upregulation of endothelial adhesion molecules resulted in efficient T cell extravasation, infiltration into the tumour and malignant cell killing. These results highlight once again the context-dependent action of complement. In the same model, complement activation is pro-tumoural^{31,32}, but it becomes antitumoural during therapy to elicit a robust antitumour immune response¹³⁷. Indeed, these data suggest that when effector T cells are present, complement facilitates tumour rejection, whereas it may promote inflammation and tumorigenesis when other immune cell types predominate over antitumour T cells¹³⁷.

Big efforts are now focused on the design of anticancer monoclonal antibodies with enhanced complement-mediated cytotoxicity in order to kill the tumour cells to which they are directed. The recent discovery of the potentiation of the C1q binding and complement activation by IgG hexamerization prompted the development of a new generation of therapeutic antibodies^{138–140}. If given in the right context, these antibodies might provide benefit in the eradication of tumours. Although limited, the data from the literature suggest that the impact of tumour cell-binding IgG on tumour growth is context dependent. In an immunostimulatory context, such antibodies can induce powerful antitumour immunity that can potentially be harnessed for the treatment of patients with cancer. The potential benefit-to-harm

ratio has to be evaluated for each subset of patients even within one tumour type to avoid potential enhancement of the pro-tumoural impact of complement. Intervention strategies¹¹, such as blocking or silencing the membrane complement regulatory proteins, inhibiting the extracellular enzymes that interfere with complement activation or inhibiting the intracellular pathways that support tumour cell resistance and recovery, have to be established to overcome the resistance of tumour cells to complement-mediated killing, which will improve the efficacy of these therapeutic antibodies.

Conclusions

In order to design efficient complement-targeted therapeutics for cancer we have to better understand the mechanisms by which these complement proteins contribute to tumour development. This will enable us to tip the finely tuned balance of the complement reaction to favour tumour rejection and to decide whether the best strategy going forward would be to block the activation, prevent the regulation and/or act on the functions of these proteins of the complement system to treat patients with cancer.

Note added in proof

While this review was in proof, Aykut et al. reported that the binding of MBL to glycans of fungal walls and lectin pathway activation was required for oncogenic progression in pancreatic cancer. Deletion of MBL or C3 in the extratumoural compartment or knockdown of *C3ar* in tumour cells were both protective against tumour growth in mouse models¹⁴⁸.

Published online: 30 October 2019

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Acknowledgements

This work was supported by grants from Pierre Fabre Research Institute (to W.H.F. and L.T.R.); the Association pour la Recherche sur le Cancer (ARC), the Cartes d'Identité des Tumeurs (CIT) programme from the Ligue Nationale Contre le Cancer (RS19/75-111) and Cancer Research for Personalized Medicine (CARPEM) (to L.T.R.); and the Institut National du Cancer (INCa) (HTE Plan Cancer (C1608DS) and PRTK G26 NIVOREN) and the Association pour la recherche de thérapies innovantes en cancérologie (ARTIC, BioniKK programme) (to C.S.-F.). This work was also supported by INSERM, University of Paris, Sorbonne University, CARPEM T8 and the Labex Immuno-Oncology Excellence Program. M.V.D. received a PhD fellowship from ARC, and F.P. received a doctoral fellowship from CARPEM.

Author contributions

L.T.R., M.V.D. and F.P. researched data for the article. M.V.D. performed the transcriptomic analyses and designed the figures. L.T.R., M.V.D., C.S.-F. and W.H.F. provided a substantial contribution to discussions of the content. L.T.R., M.V.D. and W.H.F. contributed to writing the article and all authors contributed to reviewing and editing the manuscript before submission.

Competing interests

L.T.R. and W.H.F. received research funding from the Research Institute of Pierre Fabre. M.V.D., F.P. and C.S.-F. declare no competing interests.

Peer review information

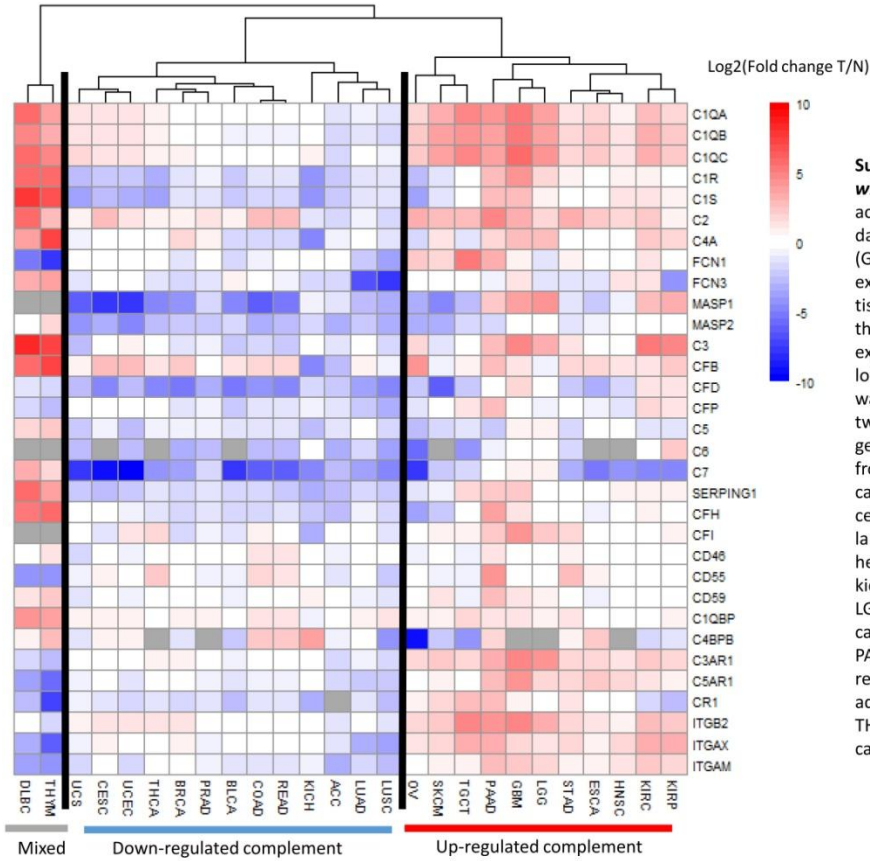
Nature Reviews Cancer thanks V. Afshar-Khargan, C. Kemper and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Publisher's note

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Supplementary information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41568-019-0210-0>.



Supplementary Figure 1: Complement gene expression in tumors compared with normal tissue. Heatmap representing the complement gene expression across multiple cancer types normalized to paired normal tissue samples. The data are extracted from Gene Expression Profiling Interactive Analysis (GEPIA)¹⁴⁶. The color represents the log₂ fold change of complement gene expression (transcripts per million (TPM)) in tumors versus paired normal tissues. The normal samples come from the cancer genome atlas (TCGA) and the genotype-tissue expression (GTEx) database. A red box means a higher expression in tumors compared with the paired normal sample and a blue box lower expression in tumors compared with the paired normal sample. As there was a lack of data related to normal tissue samples for SARC and CHOL, these two cancer types were excluded from the analysis. In addition, the following genes: *MBL2*, *CFHR1-5*, *FCN2*, *C8A*, *C8B*, *C9*, *CR2* and *C4BPA* were also excluded from the analysis because of their very low expression. ACC, adenocortical carcinoma; BLCA, bladder carcinoma; BRCA, invasive breast carcinoma; CESC, cervical squamous carcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma; HNSC, head and neck squamous cell 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; 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.

ARTICLE 2: Marie V. Dugan, Laetitia Lacroix, Catherine Sautès-Fridman, Wolf H. Fridman, Lubka T. Roumenina, Complement detection in human tumors by immunohistochemistry and immunofluorescence, Book Chapter, "The complement system - innovative diagnostic and research protocols" – *Accepted*

Summary and novelty: The complement cascade consists of a succession of proteolytic cleavage allowing the generation of bioactive fragments. While transcriptomic analysis gives some information on complement gene expression, it does not offer the possibility to study the activation/inactivation status of complement proteins. In this context, techniques that detect specifically bioactive fragments at the protein level are of critical importance. Moreover, contrary to other protein detection methods, immunohistochemistry (IHC) and immunofluorescence (IF) have the advantages to keep the crucial information of complement proteins localization. This methodological book chapter described the IHC/IF procedures that we have developed and validated to detect complement proteins in their full-length forms or bioactive fragments *in situ* in tumors of patients.

Complement detection in human tumors by immunohistochemistry and immunofluorescence

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Abstract

Tumors contain a complement rich microenvironment in which all cell types (e.g tumor cells and stromal cells) are able to produce different proteins¹. We developed immunohistochemistry (IHC) assays allowing to identify on paraffin embedded tumor sections, not only the complement producing cells but also the complement activation fragments which result from activation of complement cascade within the tumor. The local production of complement can be detected by cytoplasmic staining whereas the activation fragments are localized at the surface of the cells. There is a high heterogeneity of the staining within tumors but also between patients' tumors. Semi-quantification of the staining in large cohorts of patients allows to investigate the prognostic impact of the local complement production and activation. Here we explain the staining process for C1q, C4 and C3 in human paraffin-embedded tumor sections by immunofluorescence and immunohistochemistry.

Key Words

Complement, immunohistochemistry (IHC), immunofluorescence (IF), tumors, production, activation, prognostic

1. Introduction

Tumors contain a complement rich environment including a series of complement proteins, regulators and receptors². Numerous studies show that complement plays a key role in cancer progression in animal models and patients cohorts³. Negative prognostic impact of the positive staining for C1q and/or C4d were detected by our group in clear cell renal cell carcinoma (ccRCC) and in non-small cell lung cancer^{4,5}.

Immunohistochemistry is a robust method to study the localization (adjacent healthy tissue, tumor core, invasion margin, necrotic areas) and the density of the complement proteins within tumors. For C1q, C4 and C3, two types of staining are observed in FFPE sections; a cytoplasmic one showing the local production and a membranous one reflecting complement activation [4]. A high heterogeneity of complement staining is observed between tumors of patients but also within the same tumor, some areas being strongly positive others completely negative. This difference can be used to perform semi-quantification and classify the patients into groups according to the density of positive cells. Ensuing survival analyses allow to evaluate the prognostic value of these proteins and may be useful to identify new biomarkers and targets in cancer.

Within the TME, complement proteins can be produced by various cell types including immune cells, endothelial cells, fibroblasts and tumor cells. The identification of complement producing cells can be performed by multiplex-immunofluorescence which provides sensible resolution, spatial information and information about staining intensity. The complement cell map that is generated is very informative to identify the keys cell actors within the complement tumor microenvironment.

2. Materials

All reagents must be handled with care. Wear protective gloves/protective clothing. Refer to safety datasheet to have more details on specific hazards.

2.1 Buffers

Ethanol solution: Ethanol absolute (VWR, ref 20821). Dilute ethanol absolute in distilled water to make 90%, 70% and 50% final solutions.

Clearene: Leica, ref-3803600

Target retrieval solution: Dako Target Retrieval Solution pH 6, Citrate buffer (Dako, S236984-2) or pH 9, Tris/EDTA buffer (Dakio, K800421-2)

Blocking buffer: Dako protein block, serum free (Dako, X0909). 0.25% casein in PBS, containing stabilizing protein and 0.015 mol/L sodium azide (NaN₃).

Antibody diluent: Antibody diluent, Dako real (Dako, S2022). Tris buffer, pH 7.2, containing 15 mmol/L NaN₃, and protein

TBS buffer: 140mM Sodium Chloride, 25mM Tris, 3mM Potassium Chloride, pH 7.4 in water

TBS buffer Tween 0.04%: 140mM Sodium Chloride, 25mM Tris, 3mM Potassium Chloride, Tween 20 0.04%, pH 7.4 in water

2.2 Antibodies

Antibody	References	Species	Clone	Supplier	Concentration (µg/mL)	Antigen Retrieval
C1q	A0136	Rabbit IgG	Polyclonal	Dako	7,90	pH High
C3d	A0063	Rabbit IgG	Polyclonal	Dako	6,2	pH Low
C4d	DB107	Rabbit IgG	A24-T	DB-biotech	4,00	pH High
CD31	NCL-CD31-1A10	Mouse IgG1	1A10	Leica	1,14	pH High

The sensibility and specificity of the staining has been evaluated carefully for these antibodies. If other antibodies than those referenced here are used, the quality of the staining is not guaranteed (**see Note 1**)

2.3 Common for IHC and IF

- Slides with formalin fixed, paraffin embedded tumor sections, 3µm thick
- Timer
- Oven at 37°C
- Glass staining dishes with glass slide rack
- Clearene
- Ethanol 100%, 90%, 70%, 50%
- Distilled water
- Dako PT link or Water bath capable of sustaining 95° C for 30 minutes.
- Target retrieval solution pH 9
- Target retrieval solution pH 6
- TBS 1X
- TBS Tween 0.04%
- Humidified black chamber with black lid
- Oxygenated water 30 volumes (9%)
- Blocking solution
- Antibody diluent
- Primary antibody: rabbit polyclonal anti human C1q (Dako), rabbit monoclonal anti human C4d (DB-biotech, Clone A24-T), rabbit polyclonal anti human C3d (Dako), mouse monoclonal anti human CD31 (Leica, Clone AA10)
- Microscope cover glasses 40 x64 mm
- Slide scanner

2.4 Specific for IHC

- Secondary antibodies: Polymer HRP Rabbit (Peroxidase labelled polymer conjugated to goat anti-rabbit immunoglobulins in TBS 1X buffer containing (see **Note 2**)
- AEC (3-amino-9-ethylcarbazole)
- Hemotoxyline (see **Note 3**)
- Aqueous based mounting medium
- Hot plate

2.5 Specific for IF

- Secondary antibodies:
 - o Classical immunofluorescence: Goat anti rabbit Cy5, Goat anti mouse Cy3 IgG1
 - o Tyramide immunofluorescence: AF647 tyramide reagent, AF546 tyramide reagent
- DAPI (4',6-diamidino-2-phenylindole)
- Mounting medium that preserves fluorescence signal across the entire visible spectrum and causes little or no quenching of the initial signal.

3. Methods

3.1 Heating

- Put the slides in the oven at 37°C for at least 30 minutes

3.2 Deparaffinization and rehydration

- Place 8 clean glass staining dishes under a chemical fume hood. Fill 3 of them with Clearene solution (see **Note 4**) in order to recover the slide rack. The next 4 clean glass staining dishes are filled with Ethanol gradient (100%, 90%, 70%, 50%). The last one is filled with distilled water. Pre-set timer for 5 minutes.
- Place slide(s) in the glass slide rack and put it in the different clean glass staining dishes in order to submerge all tissue sections for 5 minutes in the following order: Clearene #1, Clearene #2, Clearene #3, Ethanol 100%, Ethanol 90%, Ethanol 70%, Ethanol 50%, distilled water.

3.3 Antigen retrieval

- Pre-warmed the water bath containing the antigen retrieval solution (pH high or Low) at 65°C (see **Note 5**). Put the rack containing the slide in the corresponding bath (High or Low pH). Warm to reach 97°C and leave the slide for 30 minutes. Let the bath cool to minimum 60°C before remove the slide rack.
- Rinse the slide for 5 minutes with the Dako Wash solution (0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.05% Tween 20, pH=7.6)

4. Staining

For all the solutions used, prepare 200µl of solution by slide.

4.1 Immunohistochemistry staining : C1q, C4d, C3d (Figure1-3)

- Prepare a black humidified chamber by adding a layer of water. Gently place slides on the plastic rails and put the lid.
- Prepare a solution of 3% oxygenated water by diluting 1:3 the 9% stock solution with distilled water.
- Dry the tissue by carefully absorbing the excess of water with absorbent paper towels (see **Note 6**).

- Without waiting, add carefully 200 μ L of % oxygenated water 3 on each the tissue section for 15 minutes at room temperature.
- Put the slide in a glass staining dish containing a slide rack, a bar magnet and filled with TBS Tween 0.04% in order to wash. Repeat this step with new TBS Tween 0.04% bath two times. Remove the slide and dry them as before and put it back to the humidified chamber.
- Without waiting, add carefully 3-4 drops of ready to use blocking solution and wait for 30 minutes
- During the incubation period, prepare the primary antibodies by diluting the antibody stock solution in the antibody diluent buffer (see **Note 7**), vortex the solution and stock at 4°C until ready to apply to tissues
- At the end of the incubation time, dry the slides as before and put it back to the humidified chamber
- Apply 200 μ l of the primary antibody and making sure to cover the entire tissue surface. Incubate at room temperature for 30 minutes.
- Repeat step 5
- Without waiting, add carefully 3-4 drops of ready to use rabbit polymer HRP solution and wait for 30 minutes
- Repeat step 5
- During the wash step, prepare AEC solution by adding 2 drops of buffer stock solution (approximately 72 μ L), 3 drops of AEC stock solution (approximately 90 μ L) and 2 drops of Hydrogen Peroxide solution (approximately 80 μ L) and mix well before use.
- Remove the slide and dry them as before and put it in a white paper towel on the bench.
- Add 200 μ l of AEC solution on each tissue and wait for 30 minutes. Watch carefully the staining appearance under a microscope (see **Note 8**).

- Wash two times in TBS tween 0.04% and one time in distilled water for 5 minutes each.
- Dry the slides and put 200 μ L of hematoxylin counter stain solution for 1-10 minutes (see **Note 9**)
- Wash the slides in distilled water 5 minutes two times

4.2 Classical double immunofluorescence staining : C1q/CD31 (Figure 4)

1. Perform the steps 1-6 described above to achieve slides blocking.
2. During the incubation period, prepare the mix of the primary antibodies (C1q, CD31) by diluting the antibody stock solution in the antibody diluent buffer (see **Note 10**), vortex the solution and stock at 4°C until ready to apply to tissues
3. At the end of the incubation time, dry the slides as before and put it back to the humidified chamber
4. Apply 200 μ l of the primary antibody mix and making sure to cover the entire tissue surface. Incubate at room temperature for 1 hour.
5. Put the slide in a glass staining dish containing a slide rack, a bar magnet and filled with TBS Tween 0.04% in order to wash. Repeat this step with new TBS Tween 0.04% bath two times. Remove the slide and dry them as before and put it back to the humidified chamber
6. During the washing steps, prepare the secondary antibody by diluting the secondary Goat anti rabbit Cy5 and Goat anti mouse Cy3 (see **Note 11**).
7. Add 200 μ L of the secondary antibody solution freshly prepare to the slide in the humidified black chamber. Put the black lid and incubate 30 minutes.
8. Repeat step 5. Cover the glass staining dish aluminum to avoid light exposure.
9. During the washing steps, prepare the DAPI solution by diluted DAPI stock solution at 1 μ g/mL in distilled water.

10. Add 200 μ L of DAPI solution freshly prepare to the slide in the humidified black chamber. Put the black lid and incubate 5min.
11. Wash 3 times in distilled water. Cover the glass staining dish aluminium to avoid light exposure.

4.3 Tyramide Signal Amplification multiplex immunofluorescence staining : C1q/C4d (Figure 5)

- Pre-warmed the water bath containing antigen retrieval solution pH High or Low at 65°C
- Perform the steps 1-12 of the immunohistochemistry staining method (see **Note 12**).
- Prepare the first tyramide solution AF546, by first diluted oxygenated water 1:20 in distilled water (solution A). Then, diluted in TBS 1X tyramide stock solution 1:200 and solution A 1:200.
- Add 200 μ L of the tyramide solution freshly prepare to the slide in the humidified black chamber. Put the black lid.
- Wash 3 times in TBS tween 0.04%. Cover the glass staining dish aluminium to avoid light exposure.
- Put the rack containing the slide in the corresponding bath (High or Low pH). Warm to reach 97°C and leave the slide for 10 minutes. Let the bath cool to minimum 60°C before remove the slide rack.
- Perform the steps 6-12 of the immunohistochemistry staining method
- Prepare the second tyramide solution AF647, by first diluted oxygenated water 1:20 in distilled water (solution A). Then, diluted in TBS 1X tyramide stock solution 1:200 and solution A 1:200.
- Add 200 μ L of the tyramide solution freshly prepare to the slide in the humidified black chamber. Put the black lid.

- Wash 3 times in TBS tween 0.04%. Cover the glass staining dish aluminium to avoid light exposure.
- During the washing steps, prepare the DAPI solution by diluted DAPI stock solution at 1µg/mL in distilled water
- Add 200µL of DAPI solution freshly prepare to the slide in the humidified black chamber. Put the black lid and incubate 5min.
- Wash 3 times in distilled water. Cover the glass staining dish aluminium to avoid light exposure.

5. Mounting

1. If needed, warm the mounting medium in hot water at 150°C on a hot plate
2. Dry the slide as explained before
3. Put the 24x40 mm cover glasses (see **Note 13**)
4. Add one drop of mounting medium in the center of the cover glass
5. Carefully bring the slide more and more close from the drop of mounting medium until the slides are in contact. Let the mounting medium diffuse by capillarity in the all slide and recover all the tissue.
6. Leave to dry for 1 hour

6. Scan

1. Clean the slide with ethanol
2. Scan the entire slide at 20x

7. Notes

1. Of note, we have tested anti C4d (Cell marque, 04A-16) but no staining was detected.
2. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The rabbit polymer HRP is an extremely sensitive reagent and, as a result, optimal dilutions of the primary antibody are higher than those used for the traditional peroxidase anti-peroxidase method technique (PAP). This protocol offers an enhanced signal generating system for the detection of antigens present in low concentrations or for low titer primary antibodies.
3. AEC reaction product is soluble in alcohols and other solvent. Use a hematoxylin solution without any solvents. Hematoxylin (Dako, S3301) is good to stain nucleus when a AEC revelation is used.
4. Clearene is a mix of selected terpenes and must be handle cautiously. It replaces Xylene and Toluene. It is less oily than the others solvents and dry faster without leaving residues.
5. The pH of the antigen retrieval solution depends on the antibody used. For anti-C1q antibody the pH is High, for anti-C4d antibody the pH is High, for anti-C3d antibody the pH is Low.
6. Be very careful when absorbing the excess of water. Take an absorbent towel paper, make a triangle pointing and circumvent the tissue with precautions. Do not let the slide dry completely because it can cause background.
7. The optimal final concentration for the antibodies are indicated below:
 - anti-C1q antibody (Dako, A0136): 7.9 µg/mL
 - anti-C4d antibody (DB-biotech, DB107): 4 µg/mL
 - anti-C3d antibody (Dako, A0063): 6.2 µg/mL

8. The incubation time for AEC is variable between experiments. Please check, the appearances of the staining on microscope and stop the reaction before 30 minutes if some background begins to appear.
9. The efficacy of hematoxylin staining decreases in time after opening. Make a regular test to determine the best incubation time to stain the nucleus.
10. In the case of classical double immunofluorescence staining, the two primary antibody used must be a different species. In the case of C1q/CD31, C1q is a rabbit polyclonal antibody and CD31 a monoclonal mouse antibody. The 2 primary antibodies are mix together in the antibody diluent buffer. The optimal final concentration for CD31 is 1.15µg/mL and the pH for the antigen retrieval solution is High.
11. The 2 secondary antibodies must be coupled with a different fluorochrome and mix together in the antibody diluent buffer. If possible, FITC should be avoided since it causes a lot of auto-fluorescence. For C1q/CD31 staining, the 2 secondary antibodies are a goat anti rabbit Cy5 conjugated and a goat anti mouse Cy3 conjugated.
12. Tyramide Signal Amplification amplify the fluorescence signal and allow high resolution images and the optimal concentration of the antibodies must be adjusted (see below).

- Anti-C1q antibody (Dako, A0136): 2 µg/mL
- Anti-C4d antibody (DB-biotech, DB107): 2 µg/mL

In the case of Tyramide Signal Amplification multiplex immunofluorescence staining, the two staining must be performed one after one. The two primary antibody used can be from the same species. In the case of C1q/C4d, both are rabbit antibodies.

13. The length of the cover glass depends of the size of your tissue.
14. The quantification of the deposits and the cytoplasmic staining has to be done in two separate categories, because it brings different information. If a cohort of patients is studied, it is recommended to quantify the staining on the tumor sections in 3 categories: very low

(below 5% of total number of infiltrating or tumor cells respectively for C1q+ or C4d+ and C3d+), low (5-30%), and high (over 30%) stainings. A survival analysis should be carried out for the 3 groups. If two of the groups show similar survival, they should be pooled and the analysis – carried out with only 2 groups.

15. The cytoplasmic staining reflects the production of the protein by the cells, while the membranous one – the deposits. The two are not necessarily present on the same cells. The deposits reflect the activity of the complement cascade.

8. Figures

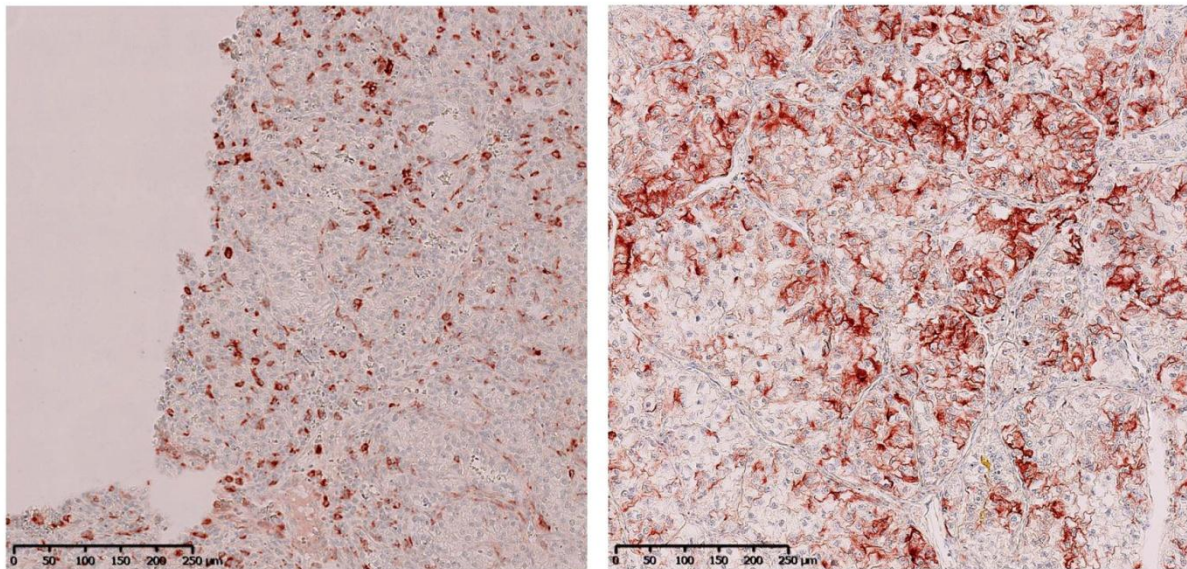


Figure 1: C1q staining on ccRCC sections. Two types of staining are detected; a cytoplasmic (left) and an extracellular as the surface of tumor cells (right)

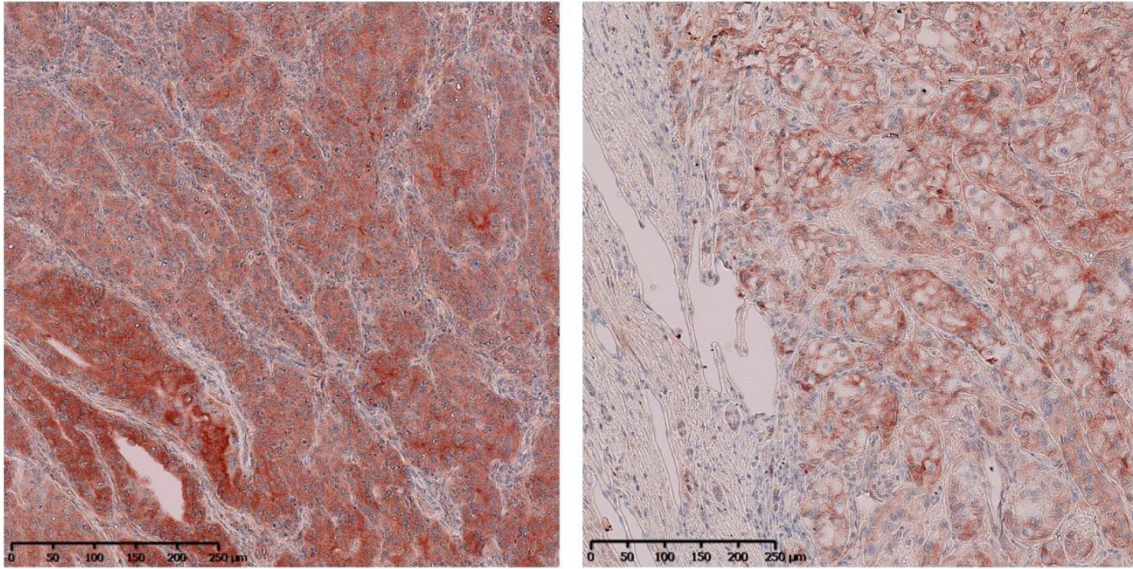


Figure 2: C4d staining on ccRCC sections. Two types of staining are detected; a cytoplasmic reflecting C4 production by tumor cells (left) and an extracellular as the surface of tumor cells reflecting complement activation (right)

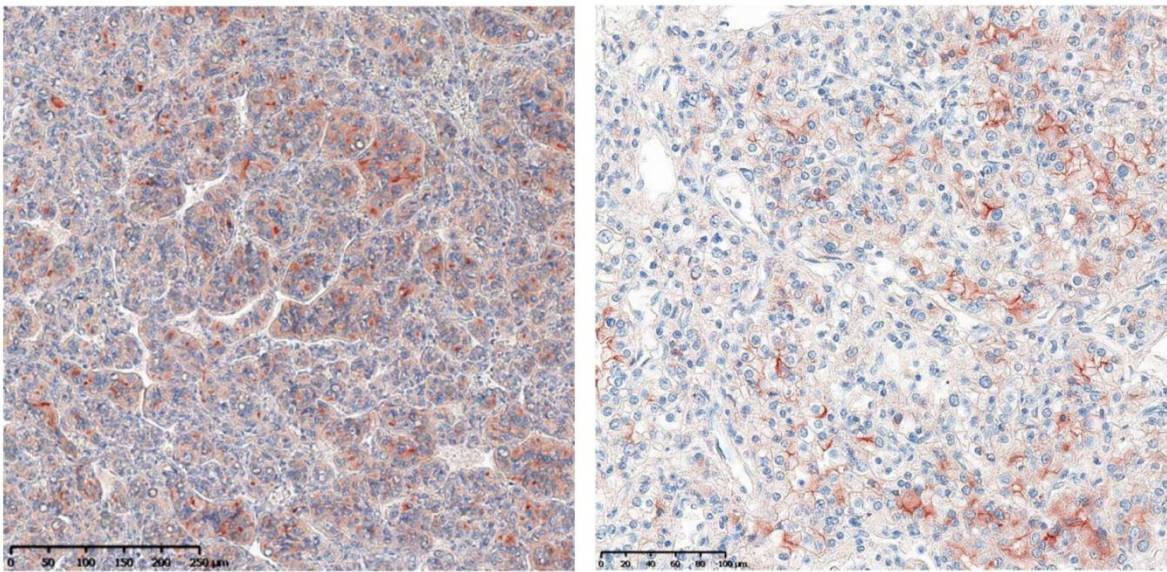


Figure 3: C3d staining on ccRCC sections. Two types of staining are detected; a cytoplasmic reflecting C3 production by tumor cells (left) and an extracellular as the surface of tumor cells reflecting complement activation (right)

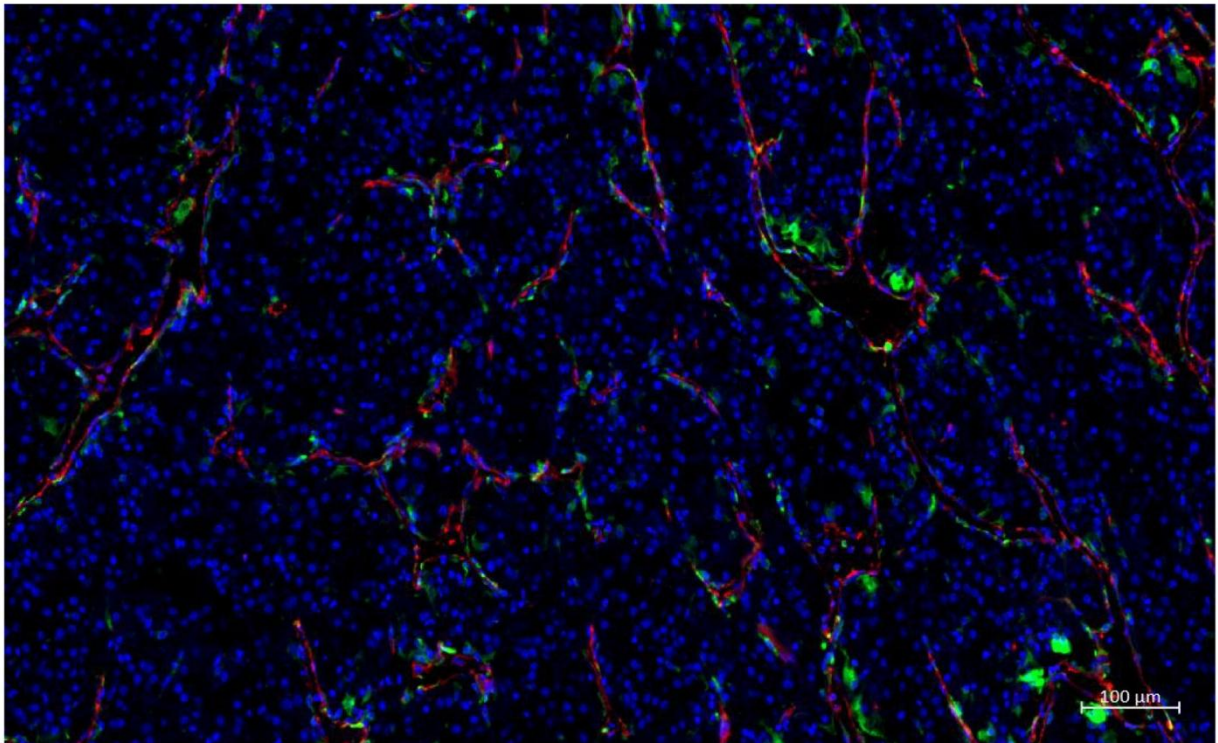


Figure 4: Double staining of C1q(green)/CD3(red). Some endothelial cells are positives for C1q staining.

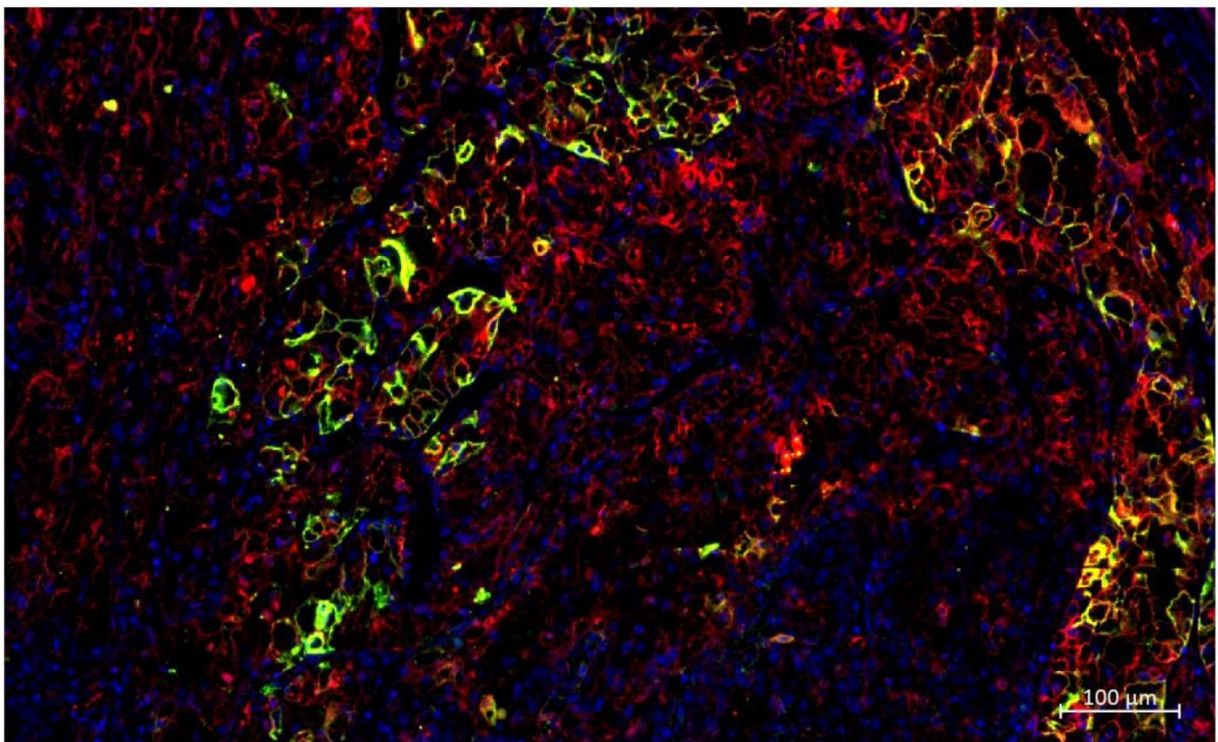


Figure 5: Double staining of C1q(green)/C4d(red). C1q deposits colocalized partially with C4d deposits.

Acknowledgements

This work was supported by a grant from Association pour la Recherche sur le Cancer (ARC); Cancer Research for Personalized Medicine (CARPEM) (to LTR) and La Ligue contre le cancer (RS19/75-111 to LTR). This work was also supported by INSERM, University Paris Descartes, Sorbonne University, CARPEM, and the Labex Immuno-Oncology Excellence Program. MVD received a PhD fellowship from ARC.

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ARTICLE 3: Roumenina, L.T.*, Daugan, M.V.*, Noé, R.*, Petitprez, F., Vano, Y.A., Sanchez-Salas, R., Becht, E., Meilleroux, J., Clec'h, B.L., Giraldo, N.A., Merle, N.S., Sun, C.-M., Verkarre, V., Validire, P., Selves, J., Lacroix, L., Delfour, O., Vandenberghe, I., Thuilliez, C., Keddani, S., Sakhi, I.B., Barret, E., Ferré, P., Corvaia, N., Passiukov, A., Chetaille, E., Botto, M., Reynies, A. de, Oudard, S.M., Mejean, A., Cathelineau, X., Sautès-Fridman, C., Fridman, W.H., 2019. **Tumor Cells Hijack Macrophage-Produced Complement C1q to Promote Tumor Growth.** *Cancer Immunol. Res.* 2018 <https://doi.org/10.1158/2326-6066.CIR-18-0891> - *Accepted*

* contributed equally to this article

Summary and novelty: It is now considered that the anaphylatoxins C3a and C5a play a major role in the establishment of chronic inflammation that support tumor growth in many cancer types. Indeed, in many mouse models, C3a/C5a and their respective receptors are involved in the induction of an immunosuppressive microenvironment, an enhancement of angiogenesis and a promotion of tumor cells growth. However, very few studies have been performed in human context and the way these anaphylatoxins are generated remains unclear. In this article, we described that in human clear cell renal cell carcinoma; an activation of the classical pathway occurred and was associated with poor prognosis for the patients. Interestingly, we have discovered that local classical pathway activation resulted from a dynamic interaction between macrophages that produce C1q and tumor cells that produced the other complement proteins (C1r, C1s, C4, C2, C3) in presence of IgG deposition at the surface of tumor cells.

Tumor Cells Hijack Macrophage-Produced Complement C1q to Promote Tumor Growth

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Abstract

Clear-cell renal cell carcinoma (ccRCC) possesses an unmet medical need, particularly at the metastatic stage, when surgery is ineffective. Complement is a key factor in tissue inflammation, favoring cancer progression through the production of complement component 5a (C5a). However, the activation pathways that generate C5a in tumors remain obscure. By data mining, we identified ccRCC as a cancer type expressing concomitantly high expression of the components that are part of the classical complement pathway. To understand how the complement cascade is activated in ccRCC and impacts patients' clinical outcome, primary tumors from three patient cohorts ($n = 106$, 154, and 43), ccRCC cell lines, and tumor models in complement-deficient mice were used. High densities of cells producing classical complement pathway components C1q and C4 and the presence of C4 activation fragment

deposits in primary tumors correlated with poor prognosis. The *in situ* orchestrated production of C1q by tumor-associated macrophages (TAM) and C1r, C1s, C4, and C3 by tumor cells associated with IgG deposits, led to C1 complex assembly, and complement activation. Accordingly, mice deficient in C1q, C4, or C3 displayed decreased tumor growth. However, the ccRCC tumors infiltrated with high densities of C1q-producing TAMs exhibited an immunosuppressed microenvironment, characterized by high expression of immune checkpoints (i.e., PD-1, Lag-3, PD-L1, and PD-L2). Our data have identified the classical complement pathway as a key inflammatory mechanism activated by the cooperation between tumor cells and TAMs, favoring cancer progression, and highlight potential therapeutic targets to restore an efficient immune reaction to cancer.

Introduction

Renal cell cancer (RCC) is the cause of over 140,000 deaths per year (1). RCC encompasses different histologic subtypes, with clear-cell RCC (ccRCC) representing 75% of the cases. ccRCC is still a clinical challenge, particularly at the metastatic stage when surgery has limited efficacy. In addition to high vascularization

(due, in part, to the Von Hippel-Lindau, VHL mutations), many ccRCC tumors have an immune and inflammatory cell infiltrate. These tumors display a disorganized tumor microenvironment (TME), where a high density of CD8⁺ T cells, showing an exhausted phenotype, correlates with shorter survival (2). Tumor-associated macrophages (TAM) exhibit M2-like functions,

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

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Cancer Immunol Res 2019;7:1091-105

doi: 10.1158/2326-6066.CIR-18-0891

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favoring cancer growth, neovascularization, and invasion (3–5). In fact, in-depth immune profiling reveals that TAMs represent a heterogeneous cell population in ccRCC tumors, with one population, annotated M5, being associated with T-cell exhaustion (6). Understanding how local inflammation modulates T-cell function and impacts patients' prognosis are indispensable to define novel targets for immunotherapy to restore an efficient immune reaction in ccRCC (7–9).

The complement system is one of the key factors in tissue inflammation (10), and animal models demonstrate that complement component 5a (C5a), generated within the TME, promotes cancer progression by activating angiogenesis and driving immunosuppression (11–13). C5a production can be generated through: (i) the classical pathway starting with C1 activation, (ii) the alternative pathway starting with direct activation of C3, or (iii) the lectin pathway (13). A study has also demonstrated that C5a can be generated in a cascade-independent manner in a mouse model of squamous carcinogenesis (14).

Kidneys produce a large spectrum of complement proteins allowing *in situ* cascade activation, leading to a variety of inflammatory diseases due to complement activation or dysregulation (14–16). Therefore, we investigated the mechanisms of complement cascade activation in ccRCC tumors and their consequences on the TME and patients' prognosis. Our data show that tumor cells produce C1r, C1s, C4, and C3 *in situ*, and C1r and C1s hijack TAM-produced C1q for *in situ* formation of the initiating C1 complex and activation of the classical pathway on intratumoral immune complexes. The expression of C1q and the density of the C1q⁺ TAM subset correlated with an exhausted T-cell phenotype and poor clinical outcome. The production of C4 and C3, as well as the deposition of C4 fragments, was also associated with poor prognosis. Collectively, our data provide evidence for activation of the classical pathway in ccRCC by cooperation between tumor cells and TAMs, causing immune modulation and increasing the risk of cancer progression.

Materials and Methods

Transcriptomic analyses

The gene expression for *C1QA*, *C1QB*, *C1QC*, *C1R*, *C1S*, *C2*, *C3*, *C4*, and *C3AR1* were assessed using the average Fragments Per Kilobase Million (FPKM) values from Human Protein Atlas in 20 different cancer cohorts available (17). Liver cancer was excluded from the analyses, because the liver tissue is the major production site for complement. The heatmap is generated using R software 3.4.2 with heatmap.2 package. The correlation between the *C1QA* gene and the endothelial cell gene signature was evaluated in the transcriptomic data of The Cancer Genome Atlas (TCGA) cohort and our Cohort 3 using the Microenvironment Cell Populations-counter (MCP-counter) software as described previously (18). No correlation was found. Normalized RNA-sequencing (RNA-Seq) data were downloaded from the GDC data portal (TCGA-KIRC cohort) and log₂-transformed. The correlations between gene expression were computed using Pearson coefficient and a subsequent non-nullity test using R software version 3.4.2. Processed RNA-Seq data from the study by Chevrier and colleagues (6) were downloaded from ArrayExpress (accession code: E-MTAB-5640). Differential gene expression between M5 and control macrophages was estimated using Mann–Whitney tests with R software version 3.4.2.

Patients

Study approval. All the included patients signed an informed consent form prior to inclusion in the study, and the research was approved by the medical ethics board of all participating institutions (no. CEPAR-2014-001). The study was conducted according to the recommendations in the Helsinki Declaration.

Cohort descriptions. Primary ccRCC tumor specimens were collected from three retrospective cohorts. Inclusion criteria for the study were: histology type ccRCC, all tumor–node–metastasis (TNM) stages (except cohort 3, which included only stage IV). The patients lacking clinical data and slides with poor quality tissue were excluded. Tumor specimens were included in paraffin and stored at 4°C. Slides with 3- μ m sections were kept at 4°C until use.

Cohort 1 included 106 patients undergoing nephrectomy at Necker-Enfants Malades Hospital (Paris, France) between 1999 and 2003 (2). Cohort 2 was comprised of 154 patients operated on at the Institut Mutualiste Montsouris (IMM; Paris, France) between 2002 and 2010. Cohort 3 included 43 metastatic patients receiving surgery at one Belgian and three French hospitals from 1994 to 2011 (19). A prospective cohort composed of seven randomly selected patients recruited in 2017 at IMM was also used. Histopathologic features, such as histologic subtype, tumor size, Fuhrman nuclear grade, and TNM stage were available for the majority of the patients (Table 1), and the duration of follow-up was calculated from the date of surgery to the date of cancer progression, last follow-up, or death. A TCGA-KIRC (kidney clear cell carcinoma) cohort composed of 537 primary ccRCC samples with clinical and transcriptomic data was also used in this study. All available data were used, expressed as average FPKM.

IHC and immunofluorescence for complement detection

Human tissues. Formalin-fixed paraffin-embedded (FFPE) human tumor specimens were cut into 3- μ m-thick sections and stained for C1q, C4d, C3d, IgG, CD163, LAG3, and PD-1 Supplementary Table S1. Human FFPE tonsil sections were used as a positive control for C1q, C4d, CD163; liver sections as positive controls for C3, C4; and mannan-binding lectin (MBL) and sections from skin of the patients with pemphigus vulgaris (Geneticist) for C3d (Supplementary Fig. S1). For each stain, an isotype control was also used. The specificity of the anti-C1q, anti-C3d, and anti-C4d was verified by a competition test (Supplementary Fig. S1A–S1C).

The antigen retrieval was carried out on a PT-link (Dako) using the EnVision FLEX Target Retrieval Solutions (Dako) with low or high pH for the detection of C1q, C4d, C3d, CD163, LAG3, and PD-1 or with Proteinase K (Dako, S3020) for IgG staining. Endogenous peroxidase and nonspecific staining were blocked with 3% H₂O₂ (Gifrer, 10603051) and protein block (Dako, X0909), respectively. The primary and secondary antibodies used for IHC and IF are summarized in Supplementary Table S3. For IHC studies, staining was revealed with 3-amino-9-ethylcarbazole substrate (Vector Laboratories, SK-4200). After mounting either with glycergel (Dako, C056330-2) for IHC or ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific, P36935) for IF, the slides were scanned with Nanozoomer (Hamamatsu) for IHC or Axio Scan (Zeiss) for IF. Stained slides were analyzed by Calopix software (Tribvn). For CD163, LAG3, and PD-1 markers, the density of positive cells was quantified in the tumor core and in the invasive margin. The percent colocalization between

Table 1. Demographic and clinical characteristics of the analyzed patients in the 4 ccRCC cohorts

ccRCC Retrospective cohort 1		ccRCC Retrospective cohort 2		ccRCC Retrospective cohort 3		ccRCC Prospective cohort	
Number of patients	106	Number of patients	154	Number of patients	43	Number of patients	7
Males, <i>n</i> (%)	80 (75%)	Males, <i>n</i> (%)	104 (68%)	Males, <i>n</i> (%)	31 (74%)	Males, <i>n</i> (%)	NA
Age (years)	63	Age (years)	62	Age (years)	56	Age (years)	59
OS time (days)	2,107	OS time (days)	NA	OS time (days)	1,220	OS time (days)	NA
Progression-free survival (days)	2,094	Progression-free survival (days)	1,179	Progression-free survival (days)	877	Progression-free survival (days)	NA
Tumor size major axis (cm)	5.25	Tumor size major axis (cm)	NA	Tumor size major axis (cm)	NA	Tumor size major axis (cm)	NA
Sarcomatoid variant	12 (11%)	Sarcomatoid variant	2 (1%)	Sarcomatoid variant	13 (31%)	Sarcomatoid variant	NA
TNM Stage		TNM Stage		TNM Stage		TNM Stage	
I	42 (40%)	I	61 (40%)	I	0	I	3 (43%)
II	6 (6%)	II	7 (5%)	II	0	II	1 (14%)
III	43 (41%)	III	83 (54%)	III	0	III	2 (29%)
IV	15 (14%)	IV	3 (2%)	IV	43 (100%)	IV	1 (14%)
Fuhrman grade		Fuhrman grade		Fuhrman grade		Fuhrman grade	
I	5 (5%)	I	1 (1%)	I	0 (0%)	I	0 (0%)
II	23 (22%)	II	32 (21%)	II	0 (0%)	II	4 (57%)
III	62 (58%)	III	102 (66%)	III	19 (44%)	III	2 (29%)
IV	15 (14%)	IV	19 (12%)	IV	23 (53%)	IV	1 (14%)
NA	1 (1%)	NA	0 (0%)	NA	1 (2%)	NA	0 (0%)

NOTE: Cohort 1 comprises (in part) patients published in ref. 2 and cohort 3 is published in ref. 19.

different staining patterns revealed by immunofluorescence (IF) was calculated using HALO Image Analysis Software (Indica Labs) in selected sections, rich of C1q-positive infiltrating cells or C1q deposits.

The specificity of the anti-C1q and anti-C4d antibodies was verified by a competition test as follows: for the anti-C1q antibodies, the primary antibodies were incubated with human C1q (Comptech, A099) or C3b (Comptech, A114); for C4d, the primary antibody was preincubated with recombinant human C4d (Abcam, ab 198640) or purified human C3d (Comptech, A112); for C3d, the primary antibody was preincubated with purified human C3d (Comptech, A112) or purified human C1r (Comptech, A102). The incubation was performed for 1 hour at different molar ratios (0:1, 1:1, and 1:2). The staining of the tonsil sections was inhibited after preincubation of the antibody with purified C1q, C4d, or C3d, respectively, but not with purified C3b, C3d, or C1r.

For the C4a/C4d and C1q/C4d double staining, a tyramide system was used. The incubation with AF647 tyramide reagent (1:100 diluted in TBS 1×, H₂O 0.0015%, Life Technologies, B40958) was performed after the secondary horseradish peroxidase (HRP)-coupled antibody and was followed by antibody stripping at 97°C for 10 minutes. This protocol was repeated for the second primary and secondary antibody incubations and AF546 tyramide reagent diluted 1/100 (B40954).

The detection of mRNA expression of C1r and C1s *in situ* in ccRCC tumors was performed by RNAscope technology (ACDbio) using the manufacturer's instructions.

Classification method for C1q, C4 and C3 staining in ccRCC tumors

C1q staining classification. Tumors were scored into three groups according to the percentage of C1q-producing cells within the tumor (at any intensity). This semiquantification was performed by three independent observers as follows: score 0 (weak): cutoff <1% of nonneoplastic cells; 1 (intermediate): 1–30% of nonneoplastic cells; 2 (strong): >30% of nonneoplastic cells. Patients with score 2 staining were found to have a shorter survival than

any other score for both progression-free survival (PFS) and overall survival (OS; $P = 0.0216$ and $P = 0.0165$, respectively). Therefore, all subsequent studies were performed separating tumors into C1q high (score 2) and C1q low (scores 0 to 1) staining. An automated quantification of the immune-reactive area for C1q for the whole slide scans of cohort 3 was performed using HALO Image Analysis Software (Indica Labs). The training of the algorithm to distinguish between infiltrating cells, vessels, and deposits did not result in reliable distinction between the patterns. Therefore, we retained the semiquantification as a method for analysis for this study.

C4/C3 staining classification. Tumors were classified into three staining scores according to the percentage of C4/C3 cytoplasmic staining in tumor cells or C4d/C3d deposits on the membrane of tumor cells (at any intensity). The semiquantification was performed by three independent observers as follows: score 0 (weak): cutoff <1% of nonneoplastic cells; 1 (intermediate): 1–30% of nonneoplastic cells; 2 (strong): >30% of nonneoplastic cells.

The density of C4-producing tumor cells showed a trend toward association with shorter PFS ($P = 0.09$) and a significant association with OS ($P = 0.04$). Because the survival curves overlapped for scores 1 and 2 tumors, these tumors were pooled into a high group.

We found a significant negative impact of C4 activation fragment deposits on PFS ($P = 0.04$) and a trend for the OS ($P = 0.08$) in tumors of patients with staining score 2. Because the survival curves of staining scores 0 and 1 tumors were indistinguishable, these tumors were pooled into a low group.

RNAscope

FFPE human tumor specimens were cut into 3- μ m-thick sections. The detection of mRNA expression of C1r and C1s *in situ* in ccRCC tumors was performed by RNAscope technology using the kit ACDBio universal VS sample Prep Reagents (323220). Negative control probe (ACDbio, 312039), positive control probe (ACDbio, 313909), and probe targeting either human C1s (ACDbio, 508969) or human C1r (ACDbio, 508959) were used.

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Cell lines and culture conditions

The human ccRCC cell lines Caki-1 and A498, as well as control cell lines from colorectal cancer (HCT116 and SW620), were purchased from the ATCC. Caki-1 and HCT116 cells were cultured in McCoy's medium (Gibco) + 10% FCS + 1× penicillin/streptomycin (Gibco). SW620 cells were cultured in Leibovitz medium (Gibco) + 10% FCS + 1× penicillin/streptomycin (Gibco), and A498 cells were cultured in Eagle minimum essential medium (ATCC) + 10% FCS + 1× penicillin/streptomycin (Gibco) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The cells were cultured until approximately 70% confluence and the culture medium was changed to reduced serum medium Opti-MEM (Thermo Fisher Scientific). The supernatant was recovered 48 hours later.

Mouse TC-1, MC38, B16F0 melanoma, LLC lung adenocarcinoma, and MCA205 fibrosarcoma cell lines were tested *in vitro*. The cells were cultured in RPMI supplemented with 5 mmol/L glutamine (Gibco), 10% FCS, 1× penicillin/streptomycin (Gibco), and 50 μmol/L 2-mercaptoethanol (Gibco). No specific authentication of the cell lines was performed. They were routinely tested for *Mycoplasma* and used when negative.

Western blot analysis for complement

After 48 hours of culture in a synthetic medium without serum, the supernatants of the human and mouse cell lines were recovered and concentrated using Amicon Ultracel 3K units (UFC 900324). The samples were prepared with NuPAGE LDS sample buffer (4×; Thermo Fisher Scientific) with or without reducing agent (DTT) and then denatured at 80°C for 10 minutes. Proteins were separated in NuPAGE 10% Bis-Tris gel (Thermo Fisher Scientific). The proteins were transferred onto a nitrocellulose membrane using iBlot (Invitrogen). The membranes were then stained with the SNAP i.d. Protein Detection System (Millipore) using a primary goat anti-human C1s antiserum (Quidel, A302; 1/5000), polyclonal rabbit anti-human C1r (Abcam, ab155060, 1/500), rabbit polyclonal anti-mouse C1r (Abcam ab205546, 1/500), rabbit polyclonal anti-mouse C1s (Abcam ab199418, 1/500), rabbit polyclonal anti-human C4 (Siemens, OSAO, 1/500), and goat polyclonal anti-human C3 (Merck Millipore 204869, 1/5,000). Secondary antibodies were rabbit anti-goat HRP (Santa Cruz Biotechnology H0712, 1/10,000) or a goat anti-rabbit HRP (Santa Cruz Biotechnology J512, 1/5,000). After washes, the membranes were developed with an ECL Reagent (Pierce #32106), and the chemiluminescence was detected with a MyECL Imager (Thermo Fisher Scientific). The purified human proteins C1s (CompTech, A104) or C1r (CompTech, A102) or mouse serum were used as positive controls.

Functional assays for C1 complex formation and activity

C1 complex formation. To test the formation of a C1 complex, an ELISA was used, as described previously (20). A polyclonal rabbit anti-human C1q (Dako, A0136; diluted 1/1,000 in PBS), was coated overnight on 96-well Nunc plates (Nunc MaxiSorp). A 1% BSA solution was then used for blocking for 1 hour at room temperature. The washing steps were performed with TBS Tween with 0.05% CaCl₂ (1 mmol/L). The supernatants of cultured human cell lines, supplemented with increasing doses of human C1q (CompTech, A099, from 0.125 to 2 μg/mL diluted in washing buffer) were added to the plates and incubated for 1 hour at 37°C. Increasing doses of normal human serum were added as positive controls. A goat anti-human C1s antiserum (Quidel, A302; 1/500

diluted in the washing buffer) was used and incubated for 1 hour at 37°C, and a secondary rabbit anti-goat HRP (1/2,000 diluted for human; Santa Cruz Biotechnology, H0712) was then added. The ELISA was revealed with SureBlue TMB Microwell Peroxidase Substrate (KPL), and the reaction was stopped with 2 mol/L sulfuric acid. The optical density at 450 nm was measured by Multiskan Ex (Thermo Fisher Scientific).

Functional activity of C1. To evaluate the functionality of this C1 complex, another ELISA-based functional test was set up as in ref. 21. The 96-well plates were coated with human IgG1 (50 μg/mL) for 1 hour at 37°C. A solution of 1% BSA was then used to block the plate for 1 hour at room temperature. The washing steps were performed with 10 mmol/L HEPES, 75 mmol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl, and 0.05% Tween 20. The supernatants of cultured cell lines and increasing doses of human C1q (from 0.125 to 4 μg/mL, diluted in washing buffer) were added to the plates and incubated for 1 hour at 37°C. In the same plate, increasing doses of human serum (diluted from 1/1,280 to 1/40) were added as a positive control. A solution containing human C4 protein (4 μg/mL; Comptech, A105) and C2 protein (5 μg/mL; Comptech, A112) were then added and incubated for 2 hours and 30 minutes at 37°C. The supernatant from the wells was recovered, and the C2 cleavage was analyzed by Western blotting under reducing conditions using biotinylated antihuman C2 (R&D Systems, BAF1936; diluted 1/400) and then streptavidin HRP (1/3,000; Dako, P0397). The signal was revealed as above. The C4 fragment deposits on the plate were detected using an anti-C4 antibody (Siemens, OSAO; diluted 1/500) and a secondary rabbit anti-goat HRP (Santa Cruz Biotechnology, H0712; diluted 1/2,000).

Interaction of tumor cells with C1q *in vitro*

The interaction of two human ccRCC cell lines (Caki-1 and A498), as well as of the mouse cancer cell line TC-1 with immobilized C1q was studied by IF. SuperFrost Plus slides were divided by Dakopen into four equivalent parts, coated either by BSA (Sigma), human C1q (CompTech, A099), or fibronectin (Sigma, F1141) at 20 μg/mL; 2 × 10⁵ cells/quadrant, suspended in Opti MEM (Gibco, 31985-062) medium were placed in each part. After an overnight incubation at 37°C, the cells on the slides were washed and fixed with 4% paraformaldehyde (PFA) for 30 minutes. After an antigen retrieval at low pH and blocking with protein block (Dako, X0909), goat anti-mouse antibody Na/K ATPase followed by anti-mouse IgG-Cy3 was used. After nuclear staining with DAPI and mounting with ProLong Gold antifade reagent (Thermo Fisher Scientific, P36934), the slides were scanned using AxioScan (Zeiss). The nuclei were counted using Visiopharm software. Alternatively, adhesion on these surfaces was evaluated at 5, 10, and 30 minutes after seeding. Proliferation of the tumor cells in the presence of human C1q, albumin, or buffer was evaluated using staining with CFSE CellTrace (CFSE Cell Proliferation Kit Protocol, Thermo Fisher Scientific).

Macrophage sorting

After tissue dissociation, fresh human tumors were incubated for 1 hour at 4°C with 15 mL of Cell Recovery Solution (Thermo Fisher Scientific). Immune populations were separated using Ficoll-Paque PLUS (GE Healthcare Life Science). Cells were then stained with CD14-APC, CD16-APC-H7, CD3-PE, CD66b-PE,

CD19-PE, CD56-PE, and DAPI for viability (Supplementary Table S2). CD14⁺ cells were sorted using a FACS Cell Sorter BD Aria III with a purity over 95%. These macrophages were recovered in RLT reagent (Qiagen, 79216)- β -mercaptoethanol solution and stored at -80°C .

qRT-PCR

The RNA was extracted from TAMs sorted from the tumors of seven consecutive patients with ccRCC and from mouse and human tumor cell lines or mouse tumors using an RNAeasy Micro Kit (Qiagen, 74004). The quality and quantity of RNA were determined with a 2100 Bioanalyzer (Agilent) using an Agilent RNA 6000 Pico Assay Kit (5067-1513) or Nano Assay Kit (5067-1511). The reverse transcription was performed with 250 ng RNA with the Applied Biosystems High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) for the cell lines and mouse tumors. For the mRNA extracted from TAMs, reverse transcription and preamplification were conducted with the Ovation Pico Kit (Nugen, 3302). The quantitative gene expression was assessed by using custom low-density array plates with a TaqMan 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression levels were determined using threshold cycle (C_t) values normalized to $GAPDH$ (ΔC_t) and expressed with $2^{-\Delta C_t}$. The references of the primers used for human and mouse gene expression are given in Supplementary Tables S3 and S4, respectively. The RNA was also extracted from mouse tumors and the expression of *Vegfc* (Mm00437310_m1) was assessed. Actin served as an endogenous control (Mm00607939).

Mouse models

Study approval. C57BL/6J mice were purchased from Charles River Laboratories. C1q^{-/-} mice, generated and provided by Prof. Marina Botto (Imperial College London, London, UK) were bred in our animal facility as described previously (22, 23). C3^{-/-} and C4^{-/-} mice were from The Jackson Laboratory. Complement-deficient mice were backcrossed in-house for four generations. Male and female 8 to 10 week-old C1q^{-/-}, C4^{-/-}, and C3^{-/-} mice and paired groups of wild-type (WT) mice were used in this study. All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with approvals APAFIS#34\0-2016052518485390v2 and #9853-2017050211531651v5 by the French Ministry of Agriculture.

Experimental procedure. The mouse TC-1 lung epithelial cell line (transformed by human papillomavirus; ref. 24) was used for *in vivo* experiments. The cells were cultured during one week in complete medium. After 2 to 3 passages, cells were recovered at 80% confluence, and 4×10^5 cells were inoculated subcutaneously (s.c.) in the right flank with 200 μL PBS. Tumor size was measured with calipers every 2 to 3 days for 25 days or until reaching the ethical endpoint of tumor size approaching 3,000 mm^3 . Tumors were recovered and were either frozen in liquid nitrogen for IF staining and gene expression analyses or used fresh for flow cytometry analyses.

Flow cytometry on mouse tumors. Intracellular staining for C1q in mouse tumors: freshly recovered mouse tumor tissues were dissociated with enzymatic solution: collagenase I (Thermo Fisher Scientific catalog no. 17100-017, 200 U/mL) and DNase I

(Thermo Fisher Scientific catalog no. 90083, 10 U/mL), and then mechanically dissociated by using gentleMACS (Miltenyi Biotec). The solutions were filtered with 70- and 30- μm nylon membrane filters and washed with PBE (PBS, 0.5% BSA, 2 mmol/L EDTA) to obtain single-cell suspensions. The total number of cells was counted using Kova slides. Two million live cells were distributed in V-shaped 96-well plates and were incubated with Fc Block (anti-CD16/CD32, BD Biosciences) for 20 minutes at 4°C . Between the steps, the cells were washed with PBE. The cells were further incubated with viability marker (Live Dead, Thermo Fisher Scientific) following the manufacturer's protocol and membrane marker antibodies (Supplementary Table S1) diluted in PBE for 30 minutes at 4°C . Then, the cells were washed with PBE and suspended in 4% PFA. For the detection of intracellular C1q, the anti-C1q antibody 7H8 was coupled with Cy5 using an Inova Lightning Link Rapid Cy5 Kit (342-0010) according to the manufacturer's instructions. After membrane staining, the cells were washed with Fix/Perm buffer (Thermo Fisher Scientific, 00-8333-56, 00-5223-56), permeabilized with Fix/Perm solution (Thermo Fisher Scientific, 00-5123-43, 00-5223-56) for 30 minutes at 4°C , and then stained for C1q. Finally, the cells were washed with Fix/Perm buffer. Human and mouse samples were acquired in a FACS Fortessa cytometer with FACSDiva software (BD Biosciences) and data were analyzed with FlowJo 10.0.8 software (Tree Star, Inc.).

Staining on mouse tissue. Freshly isolated mouse tumor tissues were frozen with liquid nitrogen and kept at -80°C . Tissues were cut from frozen blocks with cryostat (Leica) at a 6- μm thickness and fixed by acetone for 8 minutes. Sections were incubated with TBS, 5% BSA for 30 minutes in a humidity chamber at room temperature. Sections were incubated with primary antibody rabbit polyclonal anti-CD31 (Abcam ab124432, 10 $\mu\text{g}/\text{mL}$) or isotype for 45 minutes in TBS, 0.04% Tween20 (TTBS). Between each step, sections were washed two times for 2 minutes in TTBS. Sections were incubated with secondary antibody goat anti-rabbit IgG AF647 (Thermo Fisher Scientific, A-21245; 20 $\mu\text{g}/\text{mL}$) for 45 minutes. Sections were washed with water and incubated for 5 minutes with DAPI, and slides were mounted. Staining of other tissues (spleen, kidney, heart, and liver) was performed for controls. The slides were scanned with Zeiss Axio Scan.

Statistical analyses

The survival analyses were performed with R software version 3.4.2 and the "survival" package. The impact on survival was assessed by using Kaplan–Meier estimates and a log-rank test or by using Cox proportional hazard models, according to what is specified in the text. All survival data were censored at 2,500 days. The association between the distributions of qualitative variables was assessed by Fisher exact test. Relationships between quantitative and qualitative variables were estimated using the Mann–Whitney test. For quantitative variables, the cutoff was chosen according to the distribution curves.

Mouse tumor growth was analyzed using a two-way ANOVA test for the curve and independently each day with a nonparametric Mann–Whitney test. Data from mouse IF quantifications, flow cytometry, and qRT-PCR were analyzed using Mann–Whitney tests. These statistical analyses were performed using GraphPad Prism 6.

Results

Classical complement pathway gene expression in human cancers

By analyzing the TCGA database, we found that the genes encoding for classical complement pathway proteins were heterogeneously expressed in human cancers (Supplementary Fig. S2). ccRCC showed overexpression of all tested classical pathway genes, supporting a working hypothesis that C1q and the classical pathway play a major role in this cancer.

The density of C1q⁺ cells is associated with poor prognosis in advanced ccRCC

C1q expression and its correlation with clinical outcome was analyzed in primary tumors from a retrospective cohort of 106 patients with stages I–IV ccRCC (Cohort 1, Table 1). We semi-quantified the density of intratumoral C1q-producing cells (Fig. 1A) as low and high, using a specific antibody (Supplementary Fig. S1A). Compared with a low density of C1q-producing cells, a high density of intratumoral C1q-producing cells had a significant negative impact on PFS ($P = 0.008$) and OS ($P = 0.0016$; Fig. 1B). When patients were stratified into early (stages I–II, Fig. 1C) and advanced (stages III–IV, Fig. 1D) cancers, the negative clinical impact of a high density of C1q⁺ cells was evident only in patients with advanced cancers (PFS: $P = 0.004$; OS: $P = 0.002$; Fig. 1D).

This finding was validated using two independent cohorts. Cohort 2 (Table 1) included 154 patients: 68 with early (stages I–II) and 86 with advanced (stages III–IV) ccRCC. In this cohort, we had access only to PFS data and again showed the negative clinical impact of a high density of C1q⁺ cells in advanced stage cancers (PFS: $P = 0.0109$) but not in early-stage cancers (PFS: $P = 0.527$; Fig. 1E and F). In cohort 3, composed of 43 stage IV metastatic patients with ccRCC (Table 1), we confirmed the shorter PFS ($P = 0.00276$) and OS ($P = 0.0126$; Fig. 1G) of patients having a high density of C1q⁺ cells in their primary tumors.

TAMs are the most abundant cell type producing C1q in ccRCC

The C1q⁺ cells were characterized by double labeling using IF (Fig. 2A–D). Cytoplasmic C1q staining was detected in infiltrating cells. We observed that some CD31⁺ vascular endothelial cells were C1q-positive (Fig. 2A), in agreement with previous data (25), whereas the podoplanin-positive lymphatic endothelium and the SMA-positive fibroblasts were negative. Macrophages were the major cell type producing C1q in ccRCC tumors (Fig. 2B). The majority of C1q⁺ cells expressed both CD68 and CD163 (Fig. 2B and C). Tumor cells stained negative for cytoplasmic C1q, but membranous deposits on their surface were detected in a fraction of the tumors (Fig. 2D). The density of CD163⁺ macrophages was higher in the C1q-high tumors (Fig. 2E, $P = 3.4 \times 10^{-5}$), and quantification of the colocalization of the staining revealed that about 80% of the C1q⁺-infiltrating cells were CD68⁺CD163⁺ macrophages.

Therefore, we further investigated the macrophage orientation and TME characteristics in ccRCC tumors. C1q staining showed variable intensity (by IF) among TAMs in ccRCC. A study reports that a subgroup of TAMs, specifically, CD14⁺HLA-DR⁺CD204⁺CD38⁺CD206⁻ cells named M5, associates with exhausted T cells in ccRCC tumors (6). We reanalyzed the RNA-Seq data published by Chevrier and colleagues (6) and

found significantly higher expression of *C1QA* ($P = 3.2 \times 10^{-4}$), *C1QB* ($P = 1.6 \times 10^{-4}$), and *C1QC* ($P = 1.6 \times 10^{-4}$) than in control TAMs (Fig. 2F). M5 TAMs expressed significantly higher PD-L2 (*PDCD1LG2*, $P = 1.6 \times 10^{-4}$), as well as the complement receptor for C1q *LAI1* ($P = 6.5 \times 10^{-4}$; Fig. 2F). We further analyzed the expression of several of these genes in CD14⁺ macrophages sorted from 7 fresh ccRCC tumors (Table 1). *C1QA* showed a significant correlation with the mRNA expression of PD-L2 (*PDCD1LG2*; $R = 0.913$, $P = 0.004$) and a trend toward a correlation with the C1q receptor *LAI1* ($R = 0.75$, $P = 0.054$; Fig. 2G).

C1q expression is associated with immune checkpoint expression in ccRCC

Tumors from 102 patients from cohort 1 were stained for PD-1 and LAG3. A positive correlation was found between C1q⁺ cell density and PD-1 ($P = 0.012$; Fig. 2H), as well as LAG3 ($P = 0.0008$; Fig. 2I). We also searched for a potential association between C1q expression and a T-cell signature, evaluated by *CD3*, *CD4*, and *CD8* signatures expression in public databases (TCGA), without finding a significant correlation. However, C1q gene expression correlated with that of immune exhaustion markers in ccRCC tumors in publicly available transcriptomic data from the TCGA database ($n = 537$). We found a correlation between *C1QA* gene expression and PD-L2 (*CD273* or *PDCD1LG2*, $P = 3.1 \times 10^{-56}$), as well as a correlation with PD-L1 (*CD274*, $P = 0.0003$; Fig. 2J). A correlation was also observed between *C1QA* and the immune checkpoint molecules PD-1 (*PDCD1*, $P = 1.5 \times 10^{-70}$), LAG3 ($P = 2 \times 10^{-70}$), TIM-3 (*HAVCR2*, $P = 6.5 \times 10^{-23}$), and *CTLA4* ($P = 1.3 \times 10^{-39}$; Fig. 2K).

The classical complement pathway is activated *in situ* in ccRCC

The classical pathway can be activated by IgG-containing immune complexes. IgG staining by IF revealed IgG deposits on tumor cells, which colocalized with C1q deposits in about 30% of the cases (Fig. 3A). In the areas rich in deposits, up to 90% of the C1q deposits colocalized with IgG. The C1q deposits on tumor cells partially colocalized with the C4d staining (about half, Fig. 3B), indicating activation of the classical pathway. Membranous C1q staining outside IgG deposits was scarce, but could be related to a direct interaction of C1q with the tumor cells or with other C1q ligands. Indeed, two ccRCC cell lines, A498 and Caki-1, interacted with a C1q-coated surface at a similar level as to fibronectin (FN, positive control) and contrary to an irrelevant protein (albumin) after 10 minutes of incubation (Supplementary Fig. S3A), and the cells adhered better on C1q-coated or fibronectin-coated wells than on albumin-coated wells at 12 hours (Supplementary Fig. S3B). The higher cell density was not due to an increased proliferation rate, as measured by carboxyfluorescein succinimidyl ester method, but to better cell adherence.

Tumor cells stained positive for mRNA encoding the remaining components of the C1 complex, namely *C1R* and *C1S*, as revealed by an RNAscope assay (Fig. 3C and D). This was substantiated by expression of *C1R* and *C1S* mRNA (Supplementary Fig. S4A and S4B) and protein (Fig. 3E and F) in the human ccRCC cell lines. C4 was detected in ccRCC cell lines as measured by mRNA expression (Supplementary Fig. S4C) and at the protein level by Western blot analysis (Fig. 3G).

The native C4 protein was produced *in situ* by the tumor cells, as visualized by the colocalization of the cytoplasmic staining of cytokeratin with the staining with an anti-C4 antibody

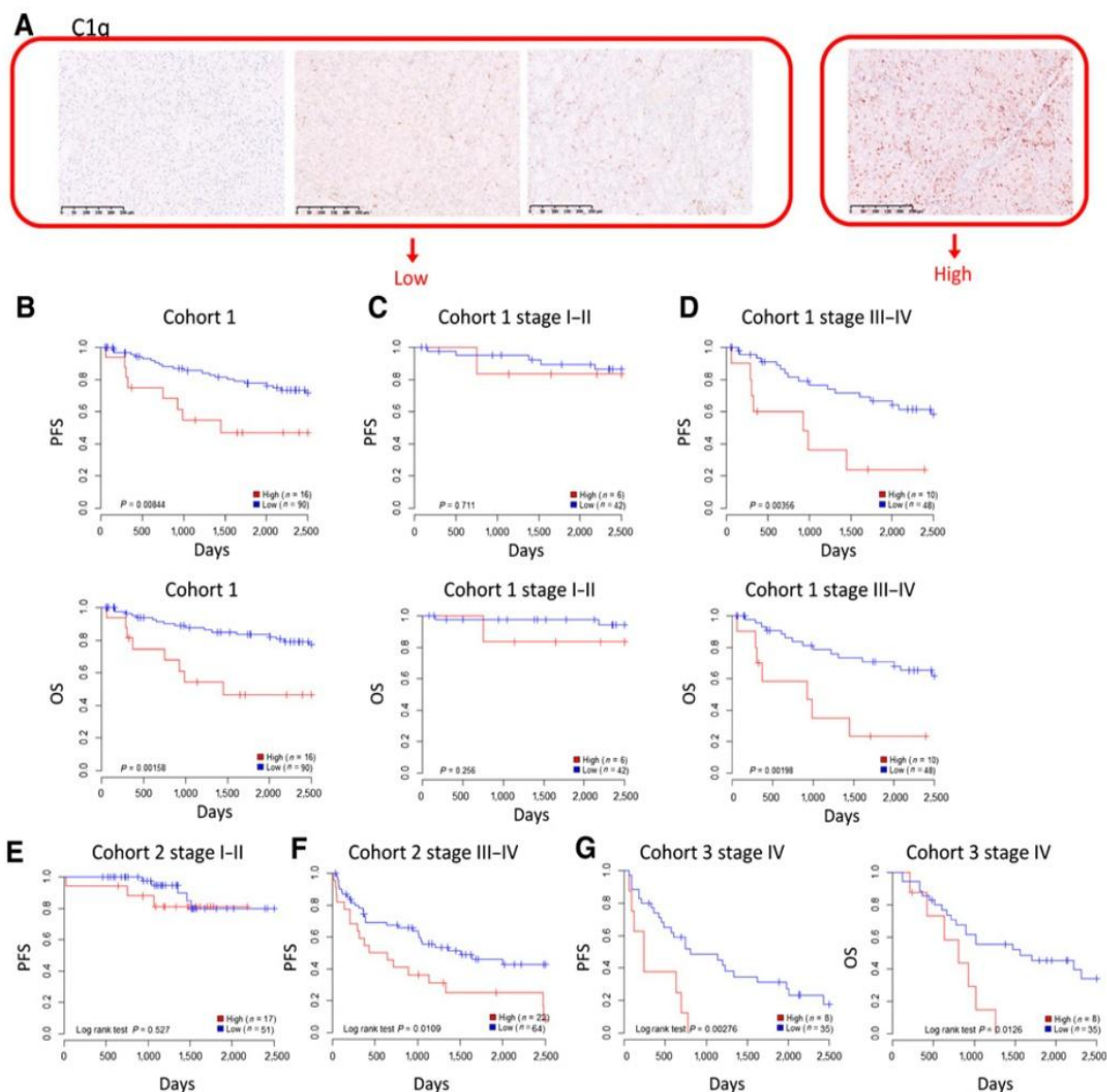


Figure 1.

The density of C1q⁺ cells is associated with poor prognosis in advanced ccRCC. **A**, Tumor scores for C1q staining as revealed by IHC on paraffin-embedded tumor sections (200 \times). Low – less than 30% of nonneoplastic cells; High – over 30% of nonneoplastic cells. **B–D**, Kaplan–Meier curves of PFS and OS according to the C1q staining for Cohort 1 ($n = 106$). **B**, PFS and OS for the total Cohort 1. **C**, Prognostic value of C1q⁺ cells. PFS and OS according to the presence of high or low densities of C1q⁺ cells in Cohort 1 in localized stages I–II. **D**, PFS and OS according to the presence of high or low densities of C1q⁺ cells in Cohort 1 in advanced stages III–IV. Prognostic value of C1q⁺ cells on PFS of patients from Cohort 2 ($n = 154$) with localized stages I–II (**E**) and advanced stages III–IV (**F**). **G**, PFS and OS of Cohort 3 ($n = 43$) patients with high and low C1q⁺ cell densities in metastatic stage IV. Number of patients per curve indicated on figure. Log-rank test was used and $P \leq 0.05$ was significant.

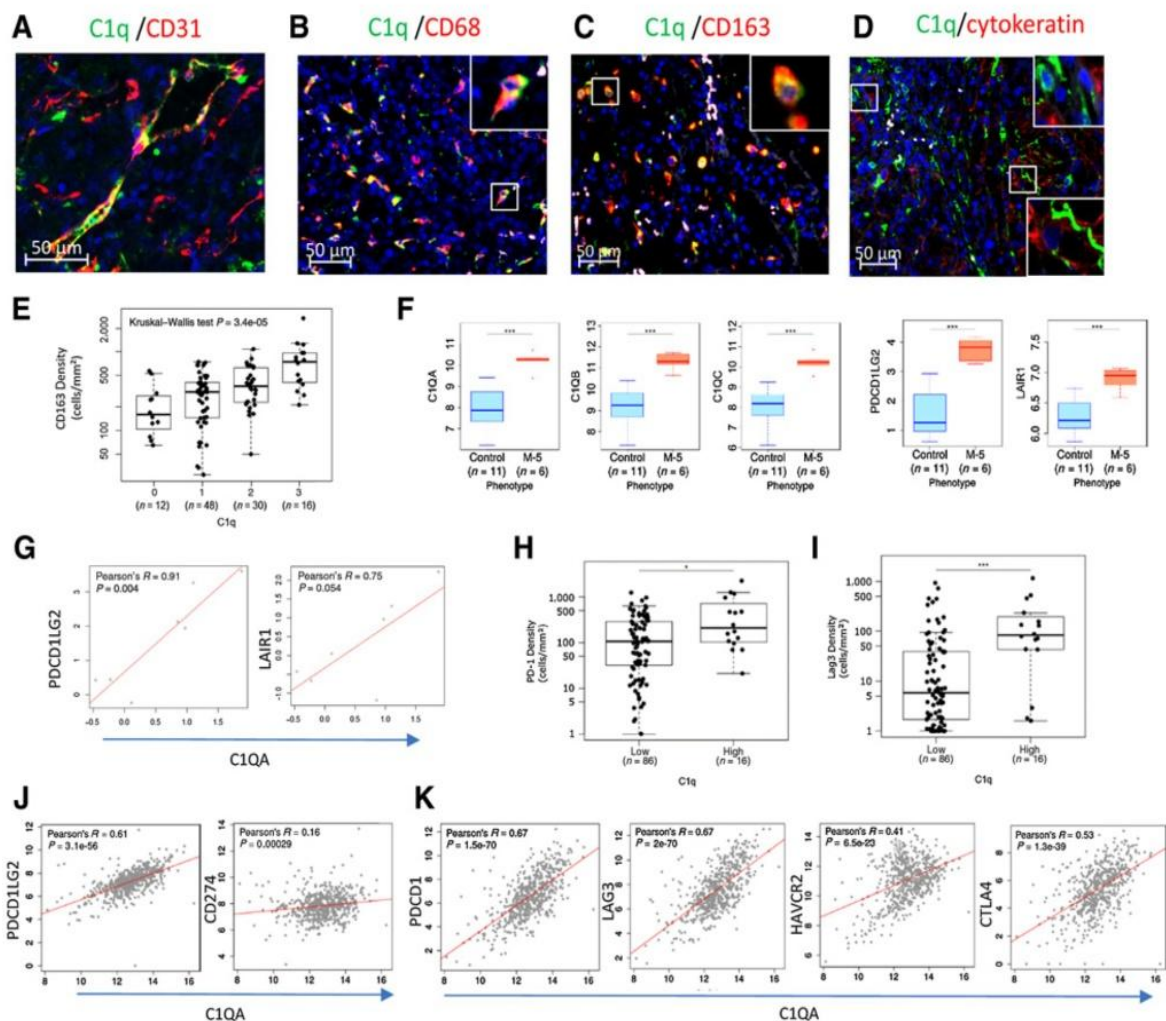
recognizing an epitope in the C4a region of the intact molecule (Fig. 3H; Supplementary Fig. S4D). Deposits of C4 activation fragments were detected on the tumor cell surface, as evidenced by using an antibody preferentially detecting C4d (Fig. 3G), colocalizing with cytokeratin (Supplementary Fig. S4E). The C4d⁺ deposits were localized at the surface of tumor cells that could also produce C4 (Fig. 3H). Similar results were obtained for C3 (Supplementary Fig. S4F–S4H). It was produced by the ccRCC tumor cell lines at mRNA and protein level (Supplementary Fig.

S4F and S4G), and C3d⁺ deposits were detected on the surface of tumor cells of human ccRCC (Supplementary Fig. S4H).

The classical complement pathway is activated in an *in vitro* model of ccRCC

We further investigated the role of tumor cells in the formation of the C1 complex and in the activation of the classical pathway using cancer cell lines. The two ccRCC cell lines Caki-1 and A498 expressed mRNA for *C1R*, *C1S*, *C4*, and *C3* and produced their

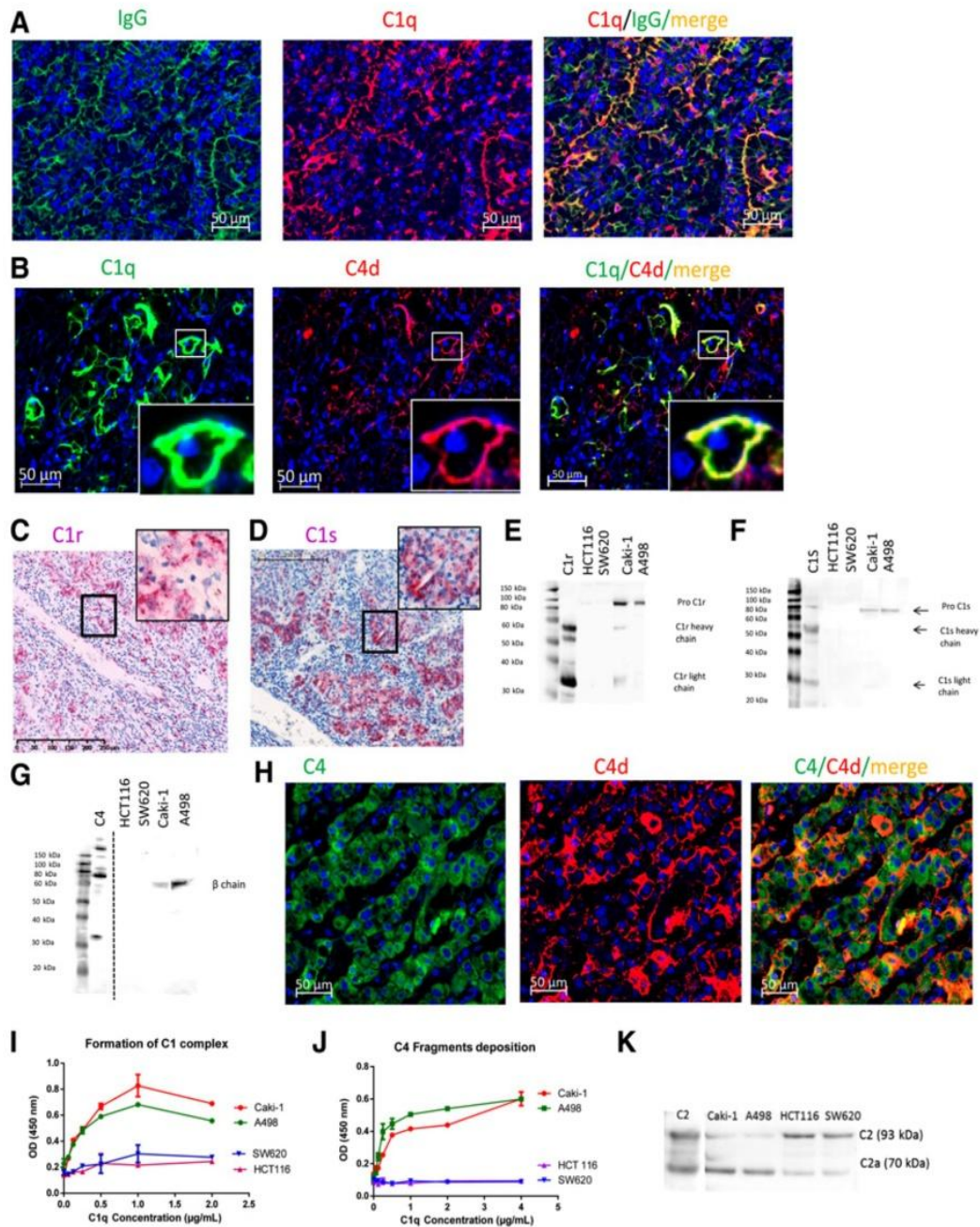
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**Figure 2.**

C1q expression is associated with a subtype of TAMs and T-cell exhaustion. **A–D**, Identification of C1q⁺ cells in ccRCC. ccRCC sections were double-stained for IF: C1q (green) and CD31 (endothelial cell marker, red; **A**), CD68 (macrophage marker, red; **B**), CD163 (M2 macrophage marker, red; **C**), and cytokeratin AE1/AE3 (tumoral cell marker, red; **D**). The double-positive cells appear in yellow. **D**, Double staining (top right insert) show representative intracellular C1q staining in tumor-infiltrating cells, and the lower left insert shows C1q deposits around cytokeratin⁺ tumor cells. **E**, Densities of CD163⁺ cells in the C1q-low (classified as 0, 1, and 2) and C1q-high (classified as 3) groups, determined by IHC in Cohort 1. Box plots represent median (wide bar) and interquartile range (IQR). Kruskal–Wallis test was used and $P \leq 0.05$ was significant. **F**, Expression of *CIQA*, *CIQB*, and *CIQC* mRNA in sorted M5 macrophages (CD14⁺HLA-DR⁺CD204⁺CD38[−]CD206[−]) compared with control macrophages (CD14⁺HLA-DR⁺CD204[−]CD38[−]CD206[−]) from ccRCC tumors according to the transcriptomic data (RNAseq) in Chevrier and colleagues (6). Gene expression of immune checkpoint PD-L2 (*PDCD1LG2*) and C1q receptor *LAIR1* in M5 versus control macrophages in the same dataset is also shown. Box plots represent median (wide bar) and IQR. Kruskal–Wallis test was used and $P \leq 0.05$ was significant. **G**, Correlation between the gene expression of *CIQA* and PD-L2 (*PDCD1LG2*) and *LAIR1* in TAMs purified from seven ccRCC fresh tumors (CD14⁺ sorting) Pearson *R* test is used and $P \leq 0.05$ was significant. Densities of PD-1⁺ (**H**) and Lag3⁺ (**I**) cells in the C1q^{low} and C1q^{high} groups, determined by IHC in Cohort 1. Data were analyzed in the invasive margin and tumor core. Box plots represent median (wide bar) and IQR. Kruskal–Wallis test was used and $P \leq 0.05$ was significant. **J**, Correlation between mRNA expression of *CIQA* and *PDCD1LG2* (CD274) in the TCGA cohort ($n = 537$). Pearson *R* test was used and $P \leq 0.05$ was significant. **K**, Correlation between mRNA expression levels of *CIQA* and immune checkpoint molecules (*PDCD1*, *LAG3*, *HAVCR2*, *CTLA4*) in the same TCGA cohort. Pearson *R* test was used and $P \leq 0.05$ was significant.

encoded proteins, contrary to the two colon cancer cell lines (HCT116 and SW620) used as negative controls (Fig. 3E and G; Supplementary Fig. S4A–S4C, S4F, and S4G). None of these cell lines expressed detectable C1q mRNA and protein. Addition of C1r- and C1s-containing supernatants of Caki-1 and A498 to purified human C1q allowed the formation of the C1 complex (Fig. 3I), as revealed by ELISA. The first substrate of the activated

C1s is C4, followed by C2. The low concentration of endogenous C4 in the supernatants precluded reliable detection of its cleavage by the C1 complex in this setting. The serine protease activity of C1s is activated when the C1 complex is assembled. To test whether the C1 complex was functionally active, exogenous purified human C1q (without C1r and C1s), C4, and C2 were added to the cancer cell line supernatants and incubated with

**Figure 3.**

The classical complement pathway is activated in ccRCC tumors. **A**, IgG deposits present in ccRCC on tumor cells. Double-staining for IF: C1q (green), IgG (red), and merged imaging (yellow). **B**, C1q and C4d deposits in ccRCC tumors of cohort 1. Double-staining with an anti-C1q (green) and an anti-C4d (red). The double-positive tumor cells appear in yellow. Detection of cells positive for *C1R* (**C**) and *C1S* (**D**) mRNA by an *in situ* hybridization RNAscope assay on paraffin-embedded ccRCC tumor sections. Detection of secreted C1r (**E**) and C1s (**F**) in the culture supernatant of ccRCC cell lines (A498 and Caki-1) compared with control cell lines (colorectal cancer cell lines HCT116 and SW620) by Western blot analysis. Human plasma-purified activated C1r and C1s were used as controls; representative image of three experiments. **G**, Detection of secreted C4 in the culture supernatant of Caki-1 and A498 compared control cell lines (colorectal cancer cell lines HCT116 and SW620) by Western blot analysis. **H**, Tumor cell C4 and C4d deposits in ccRCC tumors of cohort 1. Double-staining with an anti-C4a (green) and an antibody preferentially recognizing C4d and activated fragments of C4 in red. The double-positive cells appear in yellow. **I**, Formation of the C1 complex in supernatants of ccRCC (A498 and Caki-1) and control (SW620 and HCT116) cell lines after the addition of purified human C1q, revealed by ELISA (mean \pm SD; experiments performed in triplicate; representative results of three independent experiments). **J** and **K**, Evaluation of the activity of the C1 complex formed (as in **I**); (mean \pm SD; samples tested in triplicate; representative results of two independent experiments). **J**, The preformed C1 complex (C1q⁺ cell supernatant) was allowed to interact with the IgG-coated surface. Purified C4 and C2 were added to the wells, and C4 activation fragment deposition was detected by ELISA. **K**, C2 cleavage by the C1q complex. Supernatants from the experiment in **J** were recovered and resolved on gels to detect the C2 fragment generation.

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surface-immobilized IgG, as a model of immune complexes. C4 activation fragment deposition (Fig. 3I) and cleavage of C2 (Fig. 3K) were detected in the presence of the ccRCC cell line supernatants, contrary to the control supernatants, demonstrating that the formed C1 complex was functionally active. The ccRCC cell lines had high expression of membrane complement regulators, such as CD46, CD55, and CD59 (Supplementary Fig. S5A–S5C), as well as soluble ones, like Factor H and Factor I (Supplementary Fig. S5D and S5E), which can protect them from the formation of cytotoxic membrane attack complex C5b-9.

The density of C4⁺ and C3⁺ cells and C4d⁺ deposits correlates with poor prognosis

We semiquantified the density of C4- (Fig. 4A) and C3-producing (Supplementary Fig. S6A) tumor cells in cohort 1 (106 patients, Table 1). Patients with tumors having a high density of C4-producing tumor cells had significantly decreased PFS (Fig. 4B, left, $P = 0.02$) and OS (Fig. 4B, right; $P = 0.03$). Similarly, the density of C3-producing tumor cells (Supplementary Fig. S6A and S6B) showed a significant association with shorter PFS (Supplementary Fig. S6B, left; $P = 0.035$) and a trend toward association with shorter OS (Supplementary Fig. S6B, right; $P = 0.07$).

Comparing low and high staining scores for C4 activation fragment deposits (Fig. 4C) revealed a significant association with poor prognosis for the high group for both PFS (Fig. 4D, left; $P = 0.013$) and OS (Fig. 4D, right; $P = 0.007$). Combining the densities of C4-producing cells and C4 deposits yielded a deleterious prognosis for both PFS and OS ($P = 0.018$ and $P = 0.0036$, respectively) in the group of patients with high C4-producing cells and high deposits (Fig. 4E). The C3 activation fragment deposits did not correlate with survival (Supplementary Fig. S6C and S6D).

Correlation between local production and complement deposits in patients with ccRCC

C1q deposits correlated with the deposits of C4 activation fragments in the tumors from Cohort 1 (Supplementary Fig. S7A, $P = 0.031$). Cytoplasmic staining and deposits of C3 (Supplementary Fig. S7B and S7C) correlated with the C1q and C4 activation fragment deposition ($P = 0.002$ and $P = 0.001$, respectively). The local production of C4 and C3, revealed by the cytoplasmic staining in the tumor cells, was correlated with the local deposits (Supplementary Fig. S7D and S7E; $P = 0.033$ for C4 and $P = 0.018$ for C3).

Complement is an independent prognostic factor in ccRCC

Finally, univariate Cox proportional hazards models were fitted for clinicopathologic parameters (sex, age, stage, Fuhrman grade, and presence of a sarcomatoid component), as well as complement-related variables for both PFS and OS (Table 2). All variables significantly associated with prognosis were then integrated into multivariate models integrating either all complement-associated variables or C1q. The prognostic impact of C1q on PFS and OS was found to be independent from clinical parameters (Fuhrman grade, TNM stage, and sarcomatoid component; $P = 0.014$ and $P = 0.007$, respectively). In contrast, C1q was not an independent marker when the remaining complement-related parameters were incorporated, probably because of the correlation of complement-associated variables.

Ablation of C1q, C4, and C3 in mice is associated with decreased tumor growth

To evaluate the impact of C1q and the classical complement pathway activation *in vivo*, we analyzed tumor models in C1q^{-/-} mice on the C57BL/6 background. We searched for syngeneic tumor models, in which the tumor cells produce C1r, C1s, C3, and C4 and in which C1q could be present in the TME. In the absence of RCC models growing in C57BL/6 mice, we screened five tumor cell lines and found that they expressed detectable *C1r* and *C1s* at the mRNA level (Fig. 5A) but not *C1q* or *Mbl2* (MBL), similarly to the human ccRCC cell lines. These cell lines also had a heterogeneous expression of C4, C3, and C2. We selected the TC-1 cell line because it expressed all the genes of interest and represented a model where complement activation contributes to tumor growth (11).

Intracellular staining for C1q from harvested tumors by flow cytometry showed that a minority of CD45⁺ cells were C1q⁺ (presumably endothelial cells; Fig. 5B, left). Positivity was detected in dendritic cells (DC; CD45⁺CD3⁻CD11b⁺CD11c⁺), but they represented only approximately 5% of the CD45⁺ cells (Fig. 5B, middle). The major C1q-expressing population in the TC-1 tumors were the macrophages (CD45⁺CD3⁻CD11b⁺CD11c^{low}Ly6C^{low}Ly6G⁻; Fig. 5B, right) representing approximately 60% of the CD45⁺ cells at an early timepoint (day 10).

To establish whether activation of the early steps of the complement cascade could be involved in tumor progression, we grafted TC-1 cells into C3^{-/-}, C4^{-/-}, and C1q^{-/-} mice. The C3^{-/-} mice were nearly completely protected from tumor growth in these experimental settings, (Fig. 5C) and a significant reduction of the tumor size in C4^{-/-} mice was seen (Fig. 5D) for the late time points, in agreement with previous observations (11). After day 15 in C1q^{-/-} mice, TC-1 tumors were significantly smaller than those grafted into WT mice (Fig. 5E), demonstrating the implication of the early steps of complement activation in tumor progression.

Impact of C1q on neoangiogenesis

C1q is reported to impact neoangiogenesis in mouse tumor models (26). Indeed, we detected a difference in the morphology of the vasculature between tumors growing in C1q^{-/-} mice and those growing in WT mice in the TC-1 model (Supplementary Fig. S8A). Tumors growing in C1q^{-/-} mice exhibited shorter vessels with disrupted organization (data shown for day 17). In contrast, the staining of tumors from C4^{-/-} mice did not show a difference compared with tumors in WT mice (Supplementary Fig. S8B). In human ccRCC, no correlation was observed between *C1QA* gene expression and the endothelial cell signature, defined by the MCP-counter approach (18), in the TCGA cohort and in our cohort 3 (Supplementary Fig. S8C). The presence of C1q-positive staining in vessels did not affect survival. Nevertheless, among the neoangiogenesis-related genes tested, *VEGFC* showed a positive correlation with *C1QA* gene in both cohorts (TCGA: $r = 0.412$, $P = 7.10^{-24}$, Cohort 3: $r = 0.141$, $P = 0.00075$; Supplementary Fig. S8D). The *Vegfc* gene expression was downregulated in the tumors of C1q^{-/-} mice compared with tumors from WT mice (Supplementary Fig. S8E).

Discussion

Here, we described the protumoral properties of a population of TAMs expressing C1q in ccRCC. TAM-derived C1q is hijacked by the cancer cells, which produced C1r, C1s, C4, and C3 to

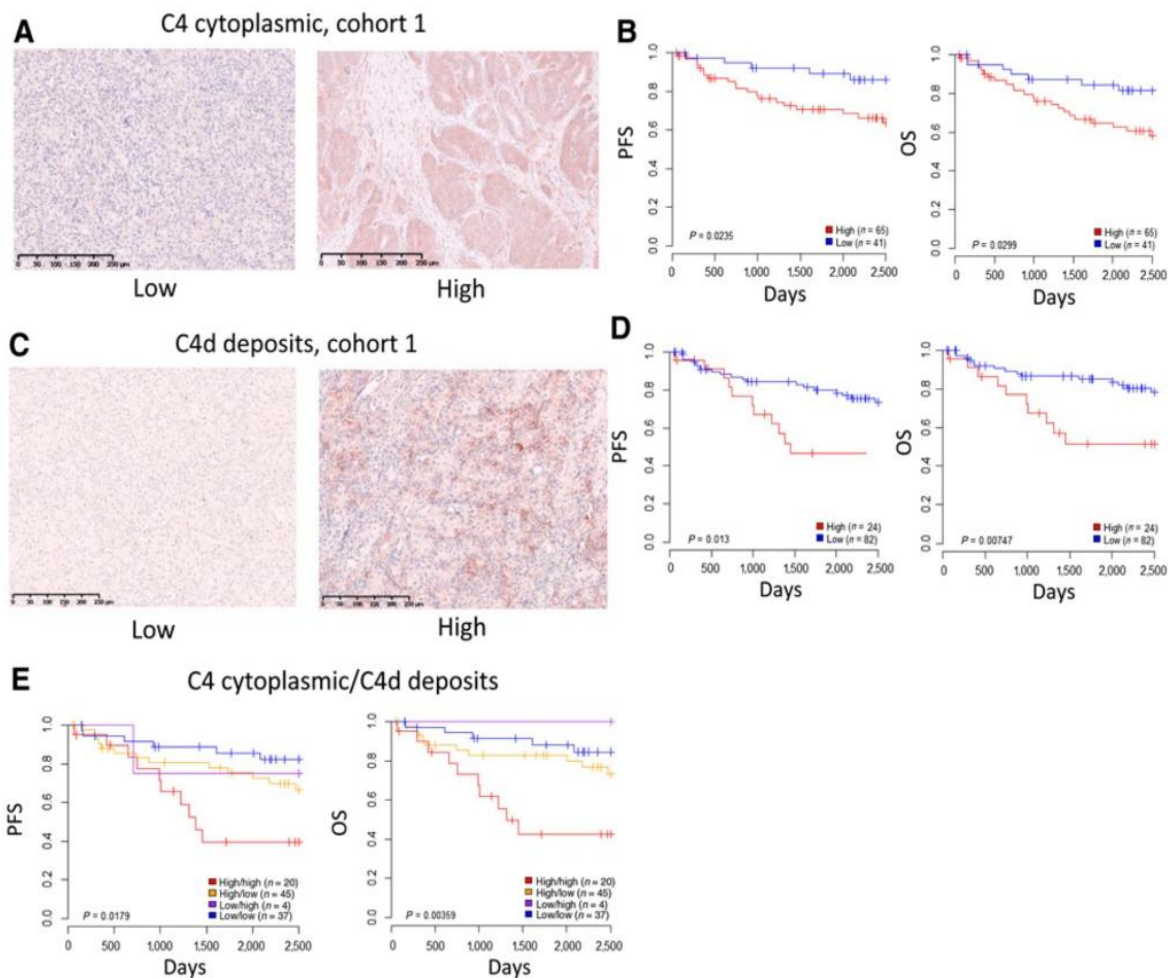


Figure 4.

The density of C4⁺ cells and C4 activation fragment deposits are associated with poor prognosis in ccRCC. **A**, Tumor scores for C4 cytoplasmic staining of tumor cells (low: below 30% of the tumor cells, high: over 30% positive tumor cells) revealed by IHC on paraffin-embedded tumor sections for Cohort 1. **B**, Kaplan-Meier curves of PFS and OS according to C4 cytoplasmic staining on tumor cells for cohort 1. Log-rank test was used and $P \leq 0.05$ was significant. **C**, Tumor classification for C4d deposits on tumor cells (0: <1%, 1: 1–30%, 2: >30% positive cells) as revealed by IHC on paraffin-embedded tumor sections. Tumors were classified as low or high for C4d deposits. **D**, Kaplan-Meier curves of PFS and OS according to C4d deposits on tumor cells for Cohort 1. Log-rank test was used and $P \leq 0.05$ was significant. **E**, Kaplan-Meier curves according to combined intensities of cytoplasmic C4 staining and C4d deposits for Cohort 1. Log-rank test was used and $P \leq 0.05$ was significant. Number of patients per curve indicated on figure.

initiate the classical pathway of the complement cascade on intratumoral IgG immune complexes. Inflammation and T-cell exhaustion, promoted by C1q-expressing TAMs and complement activation products, fueled tumor progression. We identified C1q-expressing TAMs and C4d⁺ deposits at high densities on tumor cells as markers for deleterious prognosis in ccRCC.

C1q is a multifunctional molecule, activating the classical complement pathway and acting outside the cascade as a modulator of the phenotype of immune cells, as a mediator of immune tolerance in apoptotic cell clearance, as an angiogenic factor, and/or as a modulator of cell proliferation (27, 28). C1q can be produced by the M2 macrophages, and it favors M2 polarization *in vitro*, independently of its actions within the cascade (29, 30). C1q also inhibits CD8⁺ T-cell activation, proliferation, and cytotoxic functions under suboptimal stimulation *in vitro* (31), a situation that may occur in the TME.

We found that in ccRCC, C1q is produced mainly by the TAMs and that the high density of C1q-producing cells is a robust marker for unfavorable prognosis in advanced stages of ccRCC (III and IV) in three independent cohorts. To find out the mechanism behind this association, we studied the main functions of C1q, namely its capacity to activate complement, to promote neoangiogenesis, and to modulate the phenotype of T cells.

A major factor affecting tumor growth is the phenotype of TAMs and tumor-infiltrating T cells. M2 macrophages are considered as having a tumor-promoting phenotype in renal cancer (3, 32). We found that C1q is produced mainly by a subset of TAMs in ccRCC, which belong to the large class of the M2 (CD163⁺) macrophages. Reanalyzing the transcriptomic profile of reported M5 macrophages (6), we noticed high expression of C1q-related genes, suggesting that this subtype could be the main source of C1q in ccRCC. These TAMs also had higher expression C1q receptors and

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Table 2. Univariate and multivariate analysis of PFS and OS in patients with ccRCC, Cohort 1

Progression-free survival	Univariate		Multivariate		Multivariate C1q vs. clinical data	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Sex, Male vs. Female	1.000 (0.45-2.25)	0.994				
Age	1.030 (0.99-1.06)	0.115				
Stage.UICC.1997 III/IV vs. I/II	4.31 (0.23-1.76)	0.00141	2.902 (1.14-7.42)	0.261	2.838 (1.11-7.25)	0.0292
Fuhrman Grade 3/4 vs. 1/2	7.11 (1.70-29.89)	0.00742	4.094 (0.92-18.28)	0.649	4.860 (1.12-21.07)	0.0346
Sarcomatoid	3.95 (1.73-9.00)	0.00109	1.791 (0.74-4.33)	0.195	2.075 (0.88-4.88)	0.0945
C1q High vs. Low	2.855 (1.26-6.46)	0.0118	2.122 (0.84-5.34)	0.11	2.861 (1.23-6.63)	0.0142
C4 Production High vs. Low	2.477 (1.06-5.78)	0.0359	1.230 (0.50-3.02)	0.652		
C4d Deposit High vs. Low	2.5 (1.18-5.28)	0.0164	1.480 (0.64-3.40)	0.356		
C3 Production High vs. Low	2.243 (1.03-4.90)	0.0428	1.356 (0.59-3.13)	0.476		
C3d Deposit High vs. Low	0.867 (0.35-2.13)	0.758				

OS	Univariate		Multivariate		Multivariate C1q vs. clinical data	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Sex, Male vs. Female	1.156 (0.46-2.89)	0.757				
Age	1.023 (0.99-1.06)	0.193				
Stage.UICC.1997 III/IV vs. I/II	7.908 (2.36-26.46)	0.000791	5.024 (1.45-17.45)	0.0111	5.034 (1.45-17.48)	0.0109
Fuhrman grade 3/4 vs. 1/2	12.12 (1.64-89.69)	0.0146	6.805 (0.87-53.30)	0.068	7.345 (0.96-56.12)	0.0546
Sarcomatoid	4.042 (1.66-9.84)	0.00208	1.579 (0.32-4.03)	0.339	1.870 (0.75-4.65)	0.1779
C1q High vs. Low	3.605 (1.54-8.43)	0.0031	2.791 (1.04-7.49)	0.041	3.333 (1.39-8.03)	0.0072
C4 Production High vs. Low	2.947 (1.10-7.86)	0.0309	1.373 (0.48-3.92)	0.554		
C4d Deposit High vs. Low	2.859 (1.28-6.39)	0.0106	1.544 (0.63-3.80)	0.345		
C3 Production High vs. Low	1.706 (0.75-3.86)	0.2	0.876 (0.35-2.16)	0.774		
C3d Deposit High vs. Low	0.879 (0.33-2.34)	0.796				

NOTE: The *P*-values reaching statistical significance are in bold.

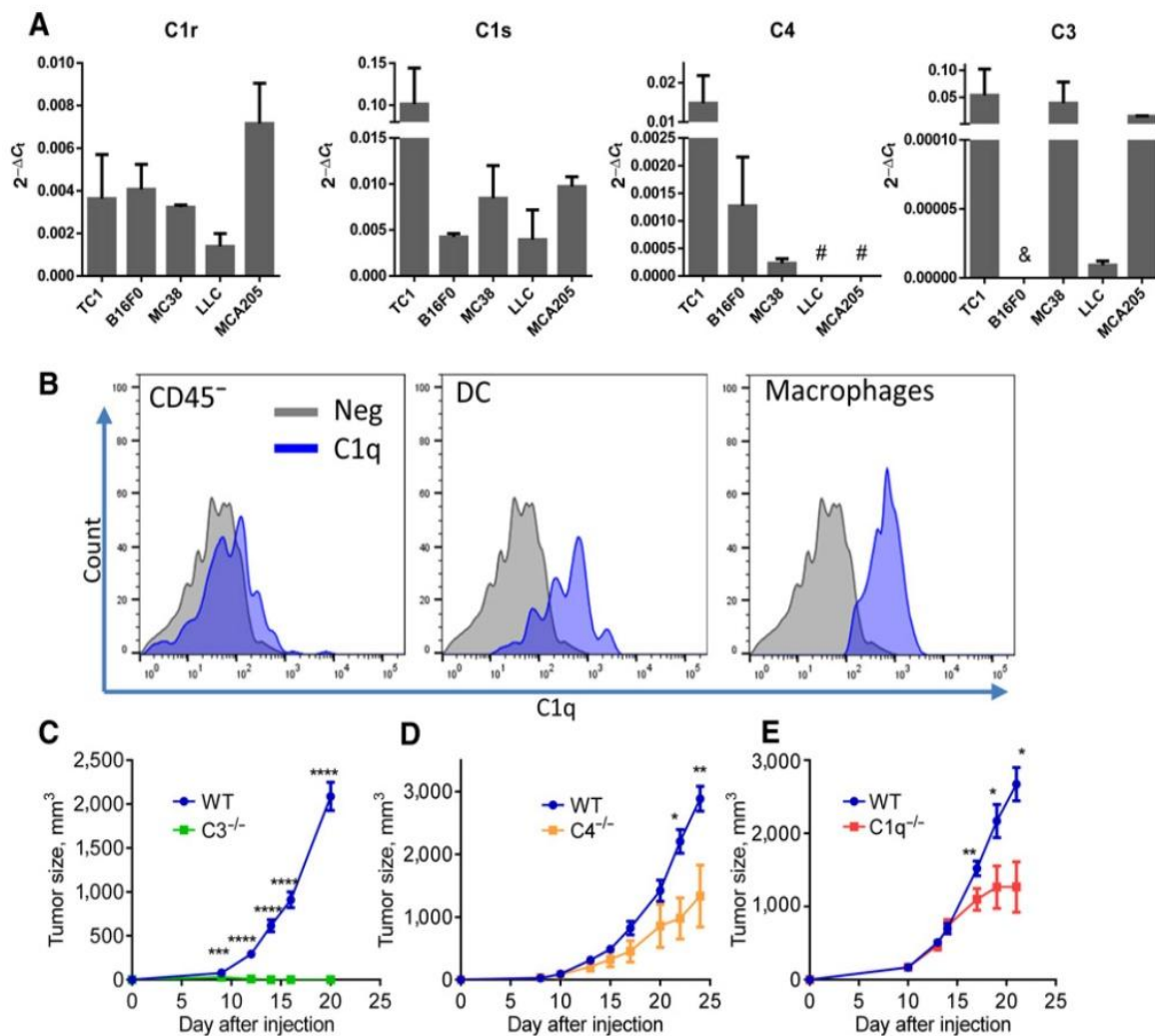
C3aR, making them responsive to C1q and C3a. TAMs also overexpressed PD-L2. It is tempting to speculate that M5 macrophages exert their immunosuppressive activity via the action of C1q. Indeed, it was shown that C1q, in the context of phagocytosis of dying cells, induces a tolerogenic/immunosuppressed phenotype in macrophages *in vitro*, which is associated with the upregulation of PD-L1 and PD-L2, as well as reduced proliferation of T cells (33). C1q also exerts direct effects on T cells by inhibiting their proliferation (34) and by modulation of the mitochondrial metabolism of CD8⁺ T cells, restraining their activation (31). Herein, ccRCC tumors with the highest C1q expression were enriched in PD-1⁺ and LAG3⁺ cells, suggesting immune suppression/T-cell exhaustion. This was confirmed at the gene expression level in ccRCC tumors from the TCGA database. Altogether, these results point toward an (autocrine) mechanism by which a subtype of TAMs produce C1q, which contributes to PD-L1 and PD-L2 expression and subsequent T-cell exhaustion. These data demonstrated the importance of C1q for the phenotype of TAMs and their interaction with the T-cell subsets. The role of complement anaphylatoxins in this process requires further studies.

The contribution of complement to cancer progression is a complex phenomenon. Data support the protumoral effects of C3a/C3aR and C5a/C5aR axes in experimental models and patients (13), but little is known about the complement activation pathways and their triggers (35). The most detailed characterization of the protumoral effect of C5a/C5aR axis was done using the TC-1 mouse tumor model, but the initiation mechanisms were not described (11). We found that the TC-1 tumor cells express a similar set of classical pathway genes as the ccRCC tumor cells and that the TAMs from this model produced C1q. The TC-1 tumors had a slower progression in C1q^{-/-}, C4^{-/-}, and C3^{-/-} mice, demonstrating the protumoral properties of C1q and the classical pathway *in vivo*. The protective effect of the C3 deficiency was more pronounced compared with C1q^{-/-} and C4^{-/-}. Bonavita and colleagues showed that C3^{-/-} mice develop smaller tumors in a carcinogen-induced sarcoma model, providing a genetic evidence

for the protective role of the C3 deficiency in carcinogenesis (36). The intratumoral activation of the alternative pathway could play a role, as well as the intracellular, noncanonical functions of C3. Noncanonical intracellular tumor cell-derived C3 activation also suppresses antitumor immunity (37).

In mice, C1q has a critical role in promoting neoangiogenesis (25, 26, 38, 39). It is important to note that ccRCC is a very highly vascularized tumor, due to the dominant effect of the VHL mutation, (40) and therefore, the role of C1q on the angiogenesis may not be visible in this tumor type. Renal endothelium shows unique features, lacking in other vascular beds (41). However, despite the lack of correlation between the C1q genes and the endothelial cell signature, a link with the expression of *VEGFC* both in patients and in the mouse model was seen. The intratumoral vascular network was disorganized in the tumors from C1q^{-/-} mice. This pattern was not observed in C4^{-/-}, suggesting a noncanonical function of C1q outside of the complement cascade, likely related to *VEGFC*.

ccRCC is particular in its capacity to express components of the classical complement pathway. We found a correlation between the local production and deposition of complement in this cancer. The classical complement pathway requires a trigger. Dying cells or pentraxins could serve as C1q-binding targets, but the most common ones are IgG- and IgM-containing immune complexes (41-44). We detected the presence of IgG deposits on tumor cells in ccRCC. IgG colocalized with C1q deposits and C1q deposits with C4d, showing that all elements needed for classical pathway activation coincided spatially and temporarily in ccRCC. C1q bound directly to the tumor cells of ccRCC *in situ* and *in vitro*, in agreement with observations for other cancer cell lines (26, 39, 45). This suggests a possible activation of complement, independently of immune complexes. Therefore, classical pathway initiators are present in ccRCC. Tumor cells and cell lines produced C1r and C1s that could assemble with C1q, enabling the formation of a functional C1 complex and complement activation in ccRCC tumors. Tumor cells were the local source of C4 and C3.

**Figure 5.**

C1q and the classical pathway are implicated in tumor progression in a mouse model of tumor progression. **A**, Expression of complement genes by murine cancer cell lines TC-1, B16F0, MC38, LLC, and MCA205 (mean \pm SD, $n = 4$ independent experiments). Data for C1r, C1s, C4, and C3 shown. C1qA, C1qB, C1qC, and Mbl2 genes showed no expression. **B**, Flow cytometry analyses of the C1q-producing cells in the tumors after injection of the selected TC-1 cells. Intracellular staining for C1q in CD45⁺ cells (left), DCs (middle), and TAMs (right; day 10). **C-E**, Tumor growth after subcutaneous injection of TC-1 tumor cells into WT and complement-deficient mice. At each timepoint the groups were compared (Mann-Whitney, *, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.001$, ****, $P \leq 0.0001$). **C**, WT versus C3^{-/-} mice (mean \pm SEM; $n = 10$ WT and $n = 10$ C3^{-/-} mice/group; representative experiment out of two performed). **D**, WT versus C4^{-/-} mice (mean \pm SEM; $n = 10$ WT and $n = 9$ C4^{-/-} mice/group; one out of two experiments performed). **E**, WT versus C1q^{-/-} mice (mean \pm SEM; $n = 20$ WT and $n = 18$ C1q^{-/-} mice/group; representative experiment, six other experiments performed with 5–10 mice/group).

C3 and C4 were cleaved, and their activation fragments were deposited on tumor cell membranes, reflecting complement activation. Indeed, our results suggest that the deleterious effect of C4 on clinical outcome was due to its activation and deposition, rather than only its production, similar to observations in lung cancer (46, 47). The local production of C3 was also associated with poor outcome, but the C3 activation fragment deposits did not show prognostic value. We hypothesize that this finding could be due to the large variety of C3 activation fragments that may be present on the cell surface (C3b, iC3b, and C3d), each having opposing functions both on the complement cascade and on immune cells (10). Taken together, our results in ccRCC and in the mouse model demonstrated that classical complement path-

way activation occurs in cancer and has a protumoral effect. Our data fit with the findings that positive C5a/C5aR staining is associated with a poor prognosis in ccRCC (48, 49), and genetic partial C4 deficiency is related to prolonged survival (50), hinting that complement activation could promote tumor growth. Our data indicate that the local production of complement components is mandatory for the efficient, cancer-promoting activation of the complement cascade. However, this complement activation does not end up in cell killing, most likely due to the strong expression of complement regulators.

In conclusion, classical complement pathway activation occurs in human ccRCC through the orchestrated production of C1q by TAMs and other complement components by cancer

cells. This unique cooperative activation process fuels inflammation and has a deleterious impact on patients' prognosis. These results open the gateway for designing novel therapeutic strategies in ccRCC.

Disclosure of Potential Conflicts of Interest

L.T. Roumenina reports receiving a commercial research grant from the Pierre Fabre Research Institute. E. Chetaille is the head of the Oncology Innovation Unit at Pierre Fabre. W.H. Fridman reports receiving a commercial research grant from Pierre Fabre Medicaments and is a consultant/advisory board member for Pierre Fabre Medicaments. No potential conflicts of interest were disclosed by other authors.

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Acknowledgments

We thank Veronique Fremaux-Bacchi (CRC) for the stimulating discussions as well as Alexia Tavares, Jennifer Tardiveau, Carine Torset, Sarah Bourass, Ivo Natario, Benedicte Buttard, Margot Revel, and Natalie Jupiter at Cordeliers Research Center (CRC) for their technical assistance. The slides stained for IF were scanned and analyzed at the Centre d'Histologie, d'Imagerie et de Cytométrie (CHIC), Centre de Recherche des Cordeliers UMRS1138 (Paris, France). CHIC is a member of the Sorbonne University Flow Cytometry Network (RECYF). We are grateful for the excellent technical assistance of the CHIC, CEF, and CGB crew of Centre de Recherche des Cordeliers for their support with the imaging and animal experimentation. We thank Isabelle Sauret and Simon Lefranc from Centre de Ressources Biologiques and the Pathology Department at the IMM for their help in the sample storage and collection. This work was supported by a grant from Pierre Fabre Research Institute (to W.H. Fridman and L.T. Roumenina), grants from Association pour la Recherche sur le Cancer (ARC) and Cancer Research for Personalized Medicine (CARPEM; to L.T. Roumenina); La Ligue contre le cancer (RS19/75-111 to L.T. Roumenina); Institut du Cancer (INCa) HTE Plan Cancer (C1608DS to C. Sautes-Fridman), PRITK G26 NIVOREN and BioniKK (to C. Sautes-Fridman) programs. This work was also supported by INSERM, University Paris Descartes, Sorbonne University, CARPEM T8, and the Labex Immuno-Oncology Excellence Program. M.V. Daugan received a PhD fellowship from ARC. F. Petitprez and Y.A. Vano were supported by CARPEM doctorate fellowships.

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Received December 14, 2018; revised March 1, 2019; accepted May 30, 2019; published first June 4, 2019.

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Cancer Immunology Research

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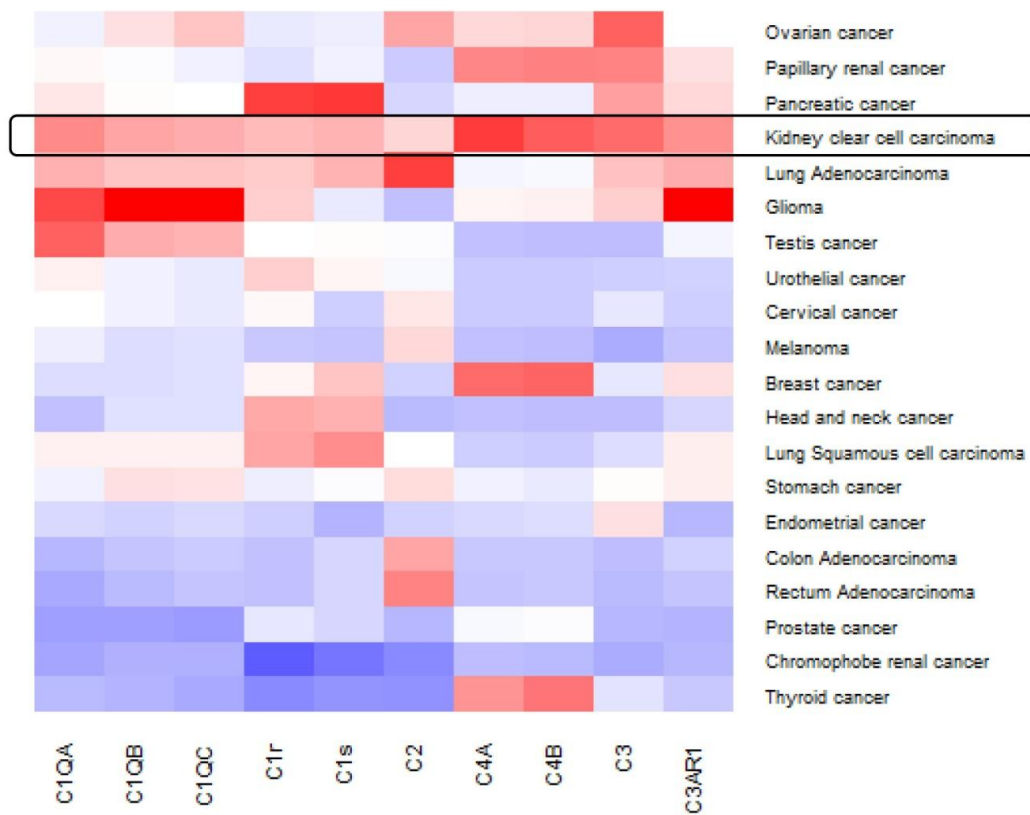
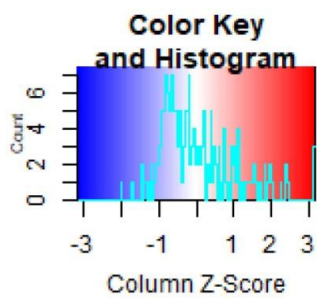
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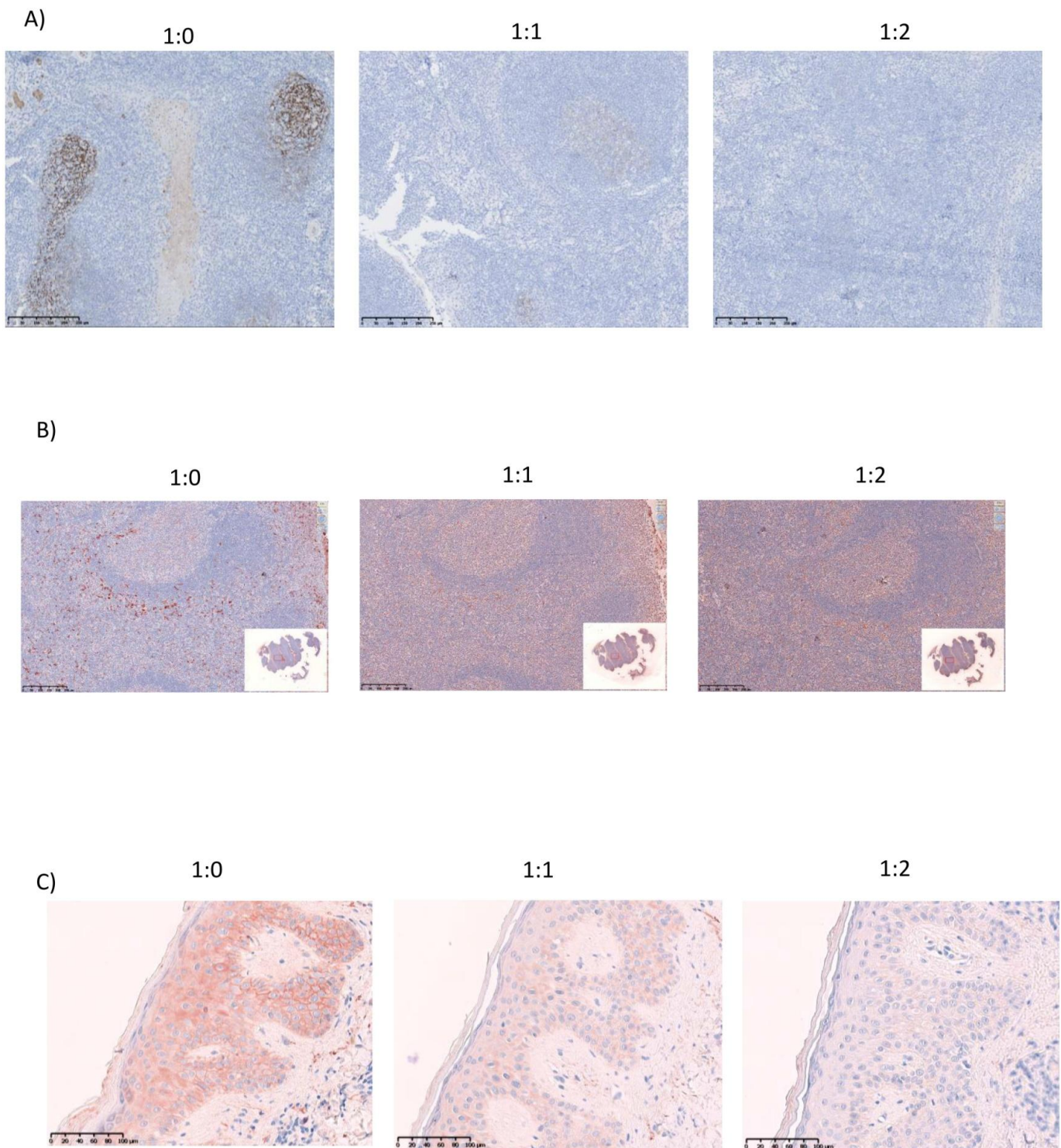
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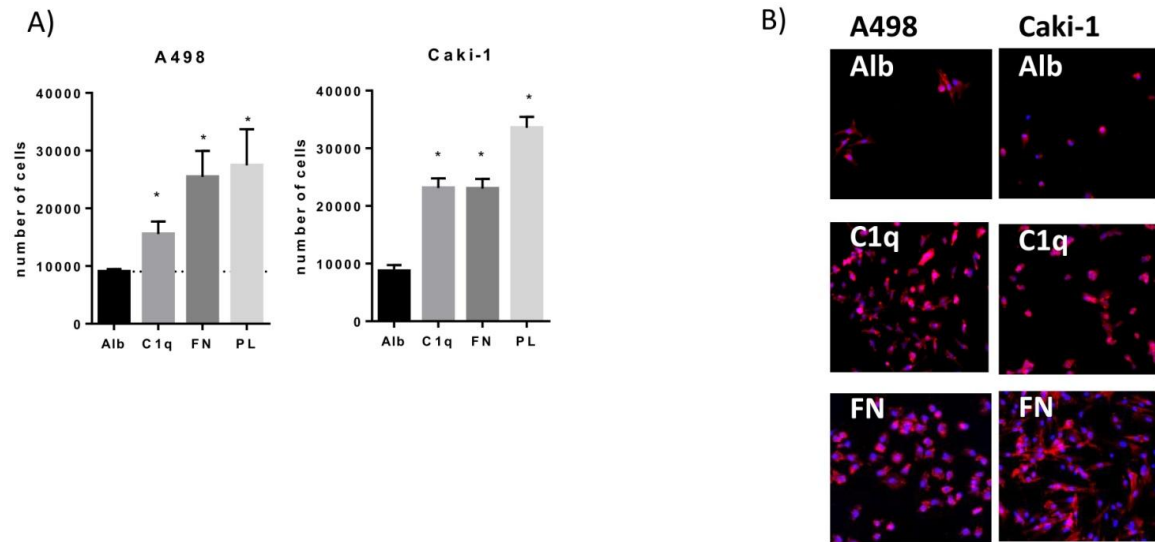
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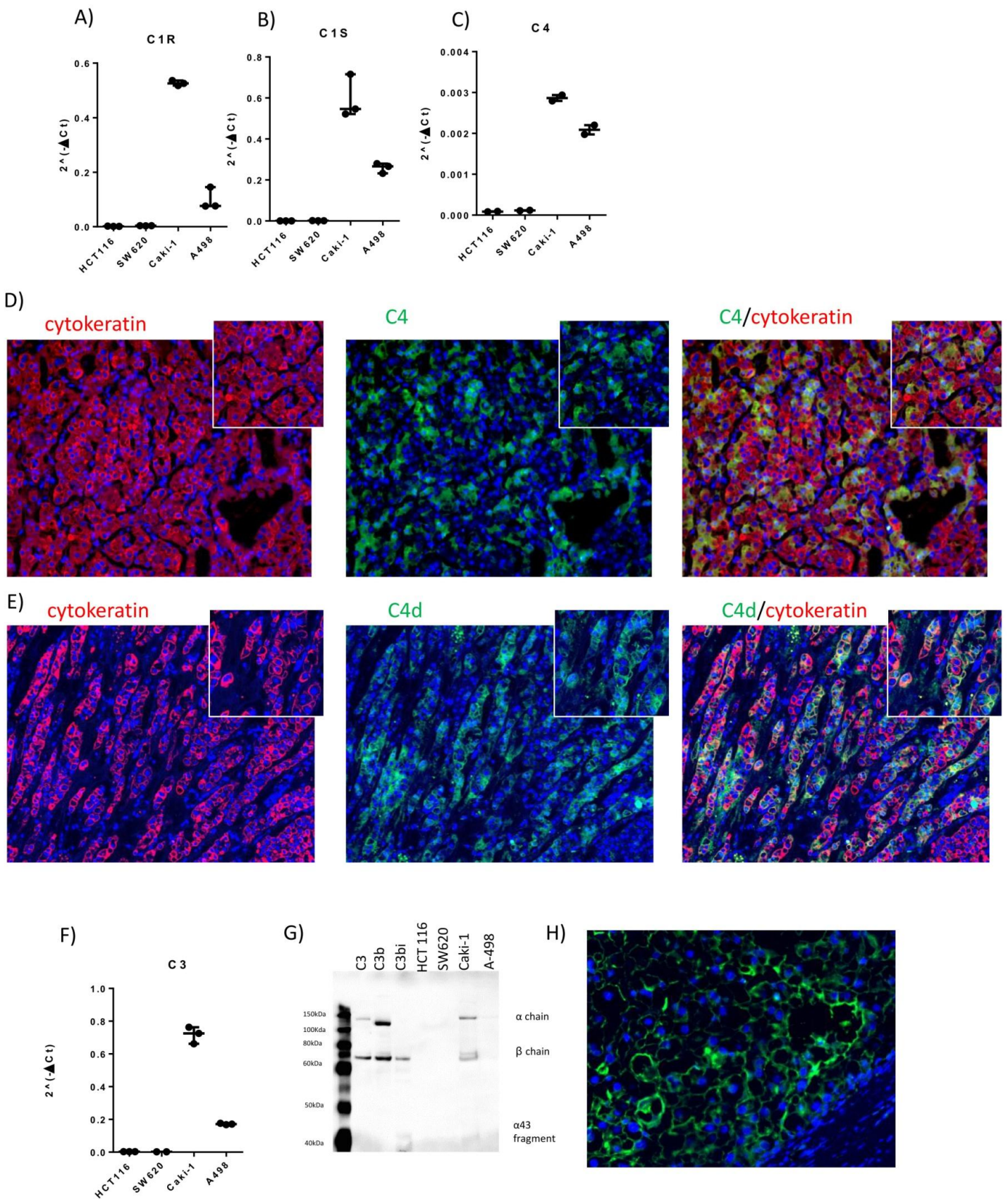
Supplementary Figure 1. Relative gene expression for the components of the classical complement pathway in 21 different cancer types. Analyses using the Protein Atlas database.



Supplementary Figure S2 Specificity of the staining for complement components and activation fragments as shown by immunohistochemistry. A) C1q staining on macrophages of tonsil, serving as a positive control tissue. Inhibition of the staining by pre-incubation of the anti-C1q antibody with purified C1q at different ratios (0:1, 1:1, 1:2) on FFPE sections of tonsil. B) C4d deposits on germinal center of tonsil, serving as a positive control tissue. Inhibition of the staining by pre-incubation of the anti-C4d antibody with purified C4d at different ratios (0:1, 1:1, 1:2). C) C3d staining on FFPE sections of skin of a patient with pemphigus vulgaris, serving as a positive control tissue. Inhibition of the staining by pre-incubation of the anti-C3d antibody with purified C3d at different ratios (0:1, 1:1, 1:2)

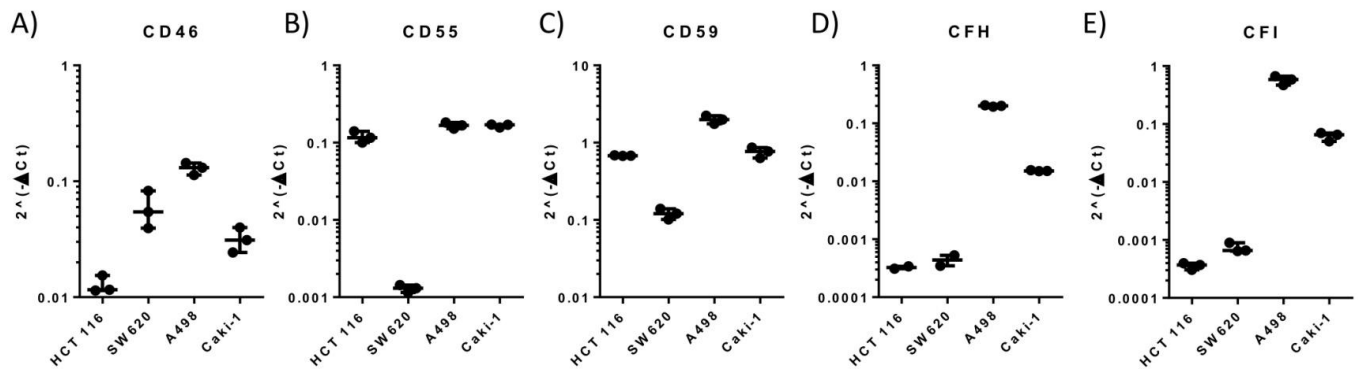


Supplementary Figure S3. Characterization of the interaction C1q with human ccRCC cell lines. A) Interaction of human ccRCC cancer cell lines A498 and Caki-1 with C1q and fibronectin (FN). Quantification of the number of cells, adhering to albumin (Alb), C1q, fibronectin (FN) or poly L-lysine (PL)-coated plates after 10 minutes of incubation. B) Visualization of A498 and Caki-1 cells after overnight culture on albumin, C1q or fibronectin-coated slides. Cell membranes were stained with Na/K ATPase (a membrane marker, red) and nuclei with DAPI (blue).

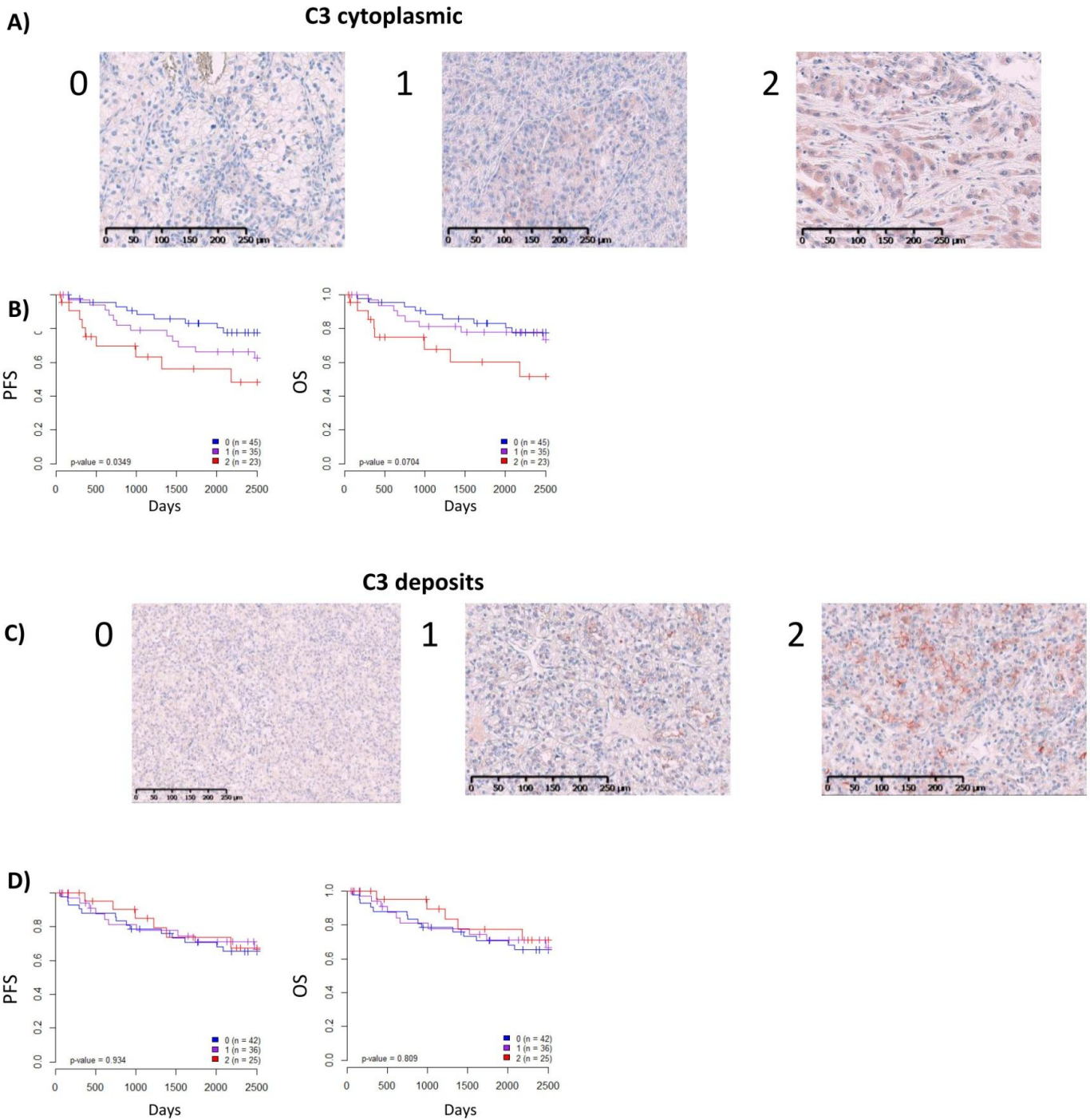


Supplementary Figure S4. Characterization of the production of complement by human ccRCC tumor cells and cell lines. Deposits of C4 and C3 on ccRCC tumors

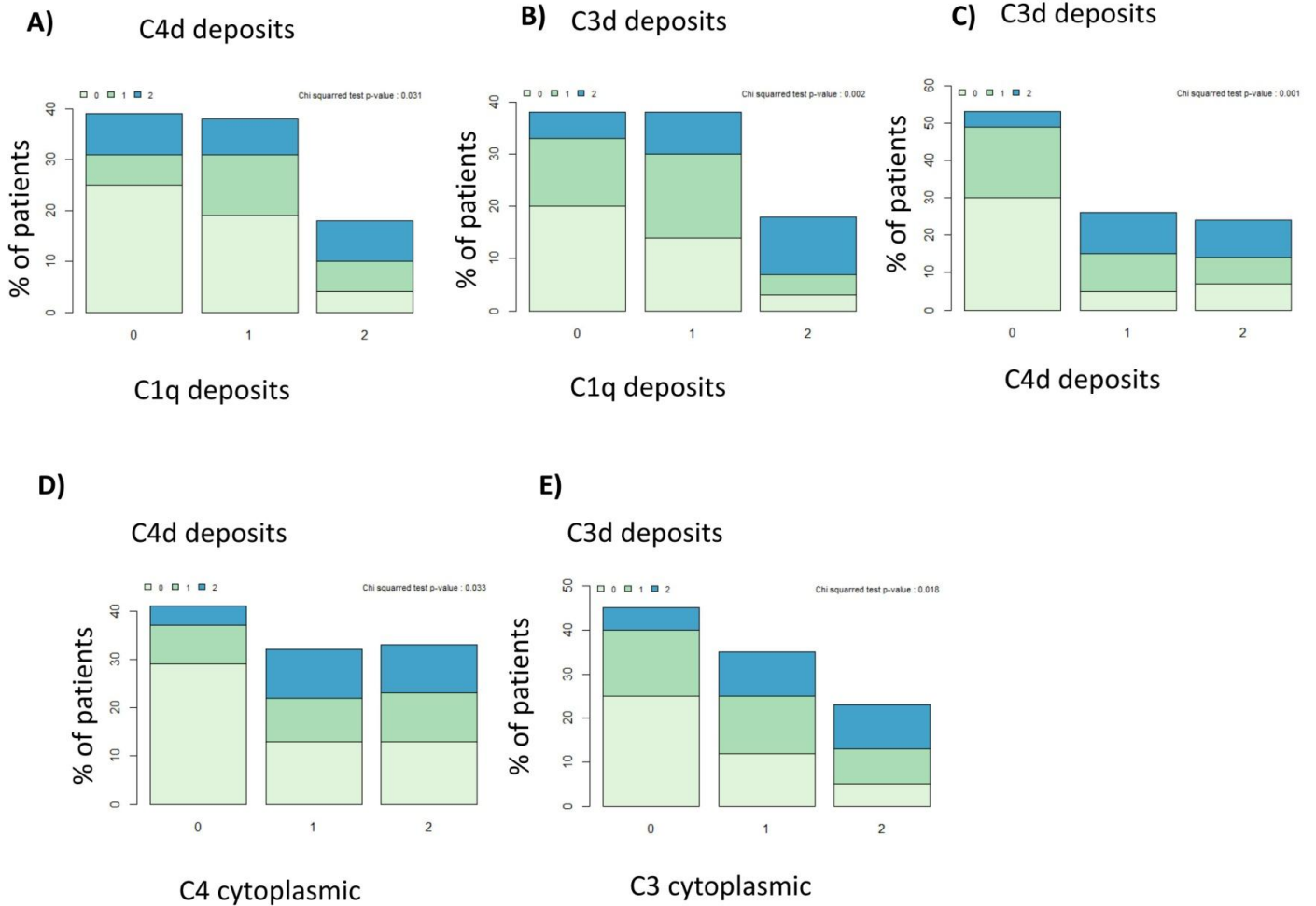
Supplementary Figure S4. Characterization of the production of complement by tumor cell lines. Expression of the genes encoding C1r (A), C1s (B), C4 (C) in human ccRCC tumor cell lines (Caki-1 and A498) and control colorectal cancer cell lines (HCT116, SW620) by RTqPCR. Mean±SD. D) Double staining of ccRCC tumor section with cytokeratin (red) and with an anti-C4a antibody (green). E) Double staining of ccRCC tumor section with cytokeratin (red) and with an anti-C4d antibody (green). F) Expression of the gene encoding C3 in human ccRCC tumor cell lines (Caki-1 and A498) and control colorectal cancer cell lines (HCT116, SW620) by RTqPCR. Mean±SD. G) Detection of secreted C3 in the culture supernatant of ccRCC cell lines (Caki-1 and A498) compared to control cell lines (colorectal cancer cell lines HCT116 and SW620) by Western Blot. Human plasma-purified C3, C3b and iC3b were used as controls for the western blots. H) C3d (green) staining on paraffin-embedded ccRCC sections by immunofluorescence reveal presence of C3 activation fragments deposits.



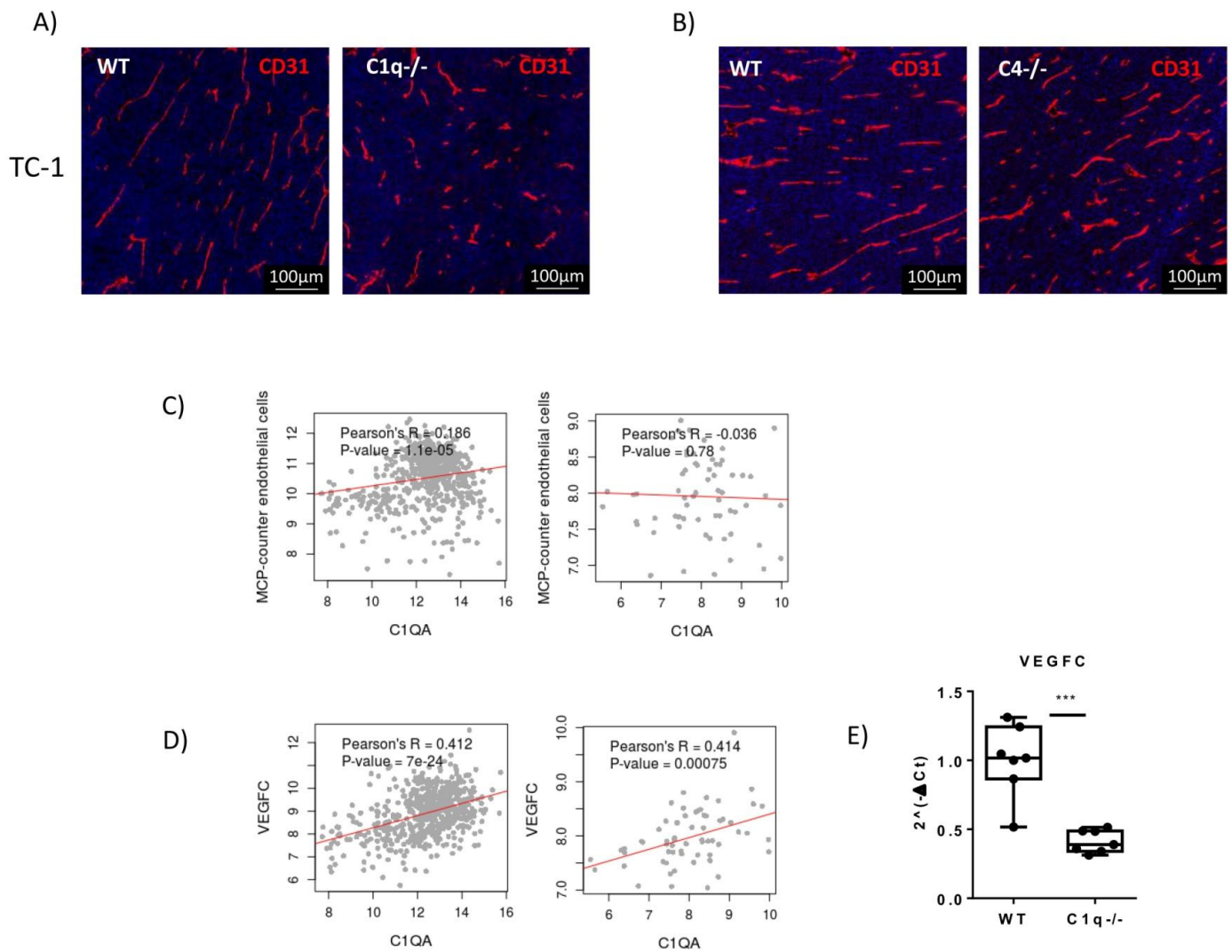
Supplementary Figure S5. Characterization of the expression of complement regulators by human ccRCC cell lines. Gene expression of CD46 (A), CD55 (B), CD59 (C), CFH (D) and CFI (E) in human ccRCC tumor cell lines (Caki-1 and A498) and control colorectal cancer cell lines (HCT116, SW620) by RTqPCR.



Supplementary Figure S6. C3 production and deposition in ccRCC tumors. A) Tumor stratification for C3 cytoplasmic staining of tumor cells (0/<1%, 1 /1-30%, 2/>30% positive cells) as revealed by IHC on paraffin-embedded tumor sections. B) Kaplan-Meier curves of PFS and OS according to C3 cytoplasmic staining on tumor cells for cohort 1. C) Tumor classification for C3d deposits on tumor cells (0/<1%, 1 /1-30%, 2/>30% positive cells) as revealed by IHC on paraffin-embedded tumor sections. D) Kaplan-Meier curves of PFS and OS according to C3d deposits on tumor cells for cohort 1.



Supplementary Figure S7: Correlations between complement components production and deposition in ccRCC. A) C4d deposits vs. C1q deposits. B) C3d deposits vs. C1q deposits. C) C3d deposits vs. C4d deposits. D) C4d deposits vs. C4 cytoplasmic staining. E) C3d deposits vs. C3 cytoplasmic staining. Data for cohort 1. The staining for complement is stratified as described in the Methods section as follows (0/<1%, 1 /1-30%, 2/>30% positive cells).



Supplementary Figure S8. C1q deficiency perturbs the neovascularization in mouse TC-1 tumors and the VEGFC expression in ccRCC and TC-1 mouse tumors. Staining for CD31 (red) on frozen sections of TC-1 tumors at day 17 from A) WT and C1q^{-/-} mice or B) WT and C4^{-/-} mice. Nuclei are stained with DAPI (blue). C) Lack of correlation between the endothelial MCP counter signature and C1QA gene in the TCGA cohort and our cohort 3, revealed by analysis of transcriptomics data. D) Positive correlation between VEGFC and C1QA in the same cohorts. E) Expression of VEGFC gene in TC-1 tumors from WT and C1q^{-/-} mice, measured by a RTqPCR (mean±SD, n=7 WT and 7 C1q^{-/-} mice per group).

Supplementary Table 1. Antibodies and experimental conditions for immunohistochemistry/immunofluorescence in human tissues

Antibody	References	Species	Clone	Supplier	Concentration (ug/mL)	Antigen Retrieval	Secondary Antibody
Immunohistochemistry							
C1q	A0136	Rabbit IgG	Polyclonal	Dako	7,90	pH High	Anti-rabbit EnVision FLEX/HRP labeled polymer (Dako, k4003)
CD163	Mob460	Mouse IgG1	10D6	Diagnostic biosystem	1,00	pH Low	Anti-mouse EnVision FLEX/HRP labeled polymer (Dako, k4001)
C4d	DB107	Rabbit IgG	A24-T	DB-biotech	4,00	pH High	Anti-rabbit EnVision FLEX/HRP labeled polymer (Dako, k4003)
C3d	A0063	Rabbit IgG	Polyclonal	Dako	6,2	pH Low	Anti-rabbit EnVision FLEX/HRP labeled polymer (Dako, k4003)
PD-1		Mouse IgG2a	EH33	CoStim (1)	1,26	pH Low	Anti-mouse EnVision FLEX/HRP labeled polymer (Dako, k4001)
LAG3		Mouse IgG1	17B4	CoStim (1)	0,50	pH High	Anti-mouse EnVision FLEX/HRP labeled polymer (Dako, k4001)
MBL	ab23457	Mouse IgG1	2B6	Abcam	4,00	pH High	Anti-mouse EnVision FLEX/HRP labeled polymer (Dako, k4001)
Immunofluorescence							
C1q	A0136	Rabbit IgG	Polyclonal	Dako	7,90	pH High	Goat Anti-Rabbit IgG Cy5 (H+L), (JIR 111-175-144)
CD163	Mob460	Mouse IgG1	10D6	Diagnostic biosystem	1,00	pH Low	Goat anti mouse Cy3 IgG1 (JIR 115-165-205)
CD31	NCL-CD31-1A10	Mouse IgG1	1A10	Leica	1,14	pH High	Goat anti mouse Cy3 IgG1 (JIR 115-165-205)
Cytokeratin	M3515	Mouse IgG1	AE1/AE3	Dako	3,56	pH High	Goat anti mouse Cy3 IgG1 (JIR 115-165-205)
α SMA	M0851	Mouse IgG2a	1A4	Dako	0,71	pH High	Goat anti mouse Cy3 pan IgG (Biolegend 405309)
Podoplanin	M3619	Mouse IgG1k	D2-40	Dako	0,12	pH High	Goat anti mouse Cy3 IgG1 (JIR 115-165-205)
CD68	M0814	Mouse IgG1k	KP1	Dako	0.2	pH Low	Goat anti mouse Cy3 IgG1 (JIR 115-165-205)
Na/K	SC-21712	Mouse IgG1	C464	SantaCruz biotechnologies	4,00	pH Low	
IgG	A0423	Rabbit IgG	Polyclonal	Dako	9,20	Proteinase K	Goat Anti-Rabbit IgG Cy5 (H+L), (JIR 111-175-144)
C4a	A206	Rabbit serum	Polyclonal	Comptech	20,00	pH High	Anti-rabbit EnVision FLEX/HRP labeled polymer (Dako, k4003) + AF546 tyramide
C4d	DB107	Rabbit IgG	A24-T	DB-biotech	4,00	pH High	Anti-rabbit EnVision FLEX/HRP labeled polymer (Dako, k4003) + AF647 tyramide

Isotype controls					
Mouse IgG1	X0931			Dako	As a function of the corresponding primary antibody
Mouse IgG2a	X0943			Dako	As a function of the corresponding primary antibody
Rabbit IgG	011-000-003			Jackson Immunology	As a function of the corresponding primary antibody
Normal rabbit serum	31883			Invitrogen	As a function of the corresponding primary antibody

Supplementary Table 2. Antibodies for flow cytometry of human TAM

Antibody	Clone	Source	Fluorochrome	Concentration (μ L/million)
Macrophage sorting				
CD14	Mfp9	BD	APC	6
CD16	3G8	BD	APC-H7	8
CD3	UCHT1	BD	PE	6
CD19	HIB19	BD	PE	4
CD56	B159	BD	PE	4

Supplementary Table 3.

Primers for evaluation of the expression level of human genes by a custom low-density array

Gene	References
GAPDH	Hs02786624_g1
C5AR1	Hs00704891_s1
LAIR1	Hs00253790_m1
CD274	Hs00204257_m1
C3AR1	Hs00269693_s1
C1QC	Hs00757779_m1

PDCD1LG2	Hs00228839_m1
C1QA	Hs00706358_s1
C1R	Hs00354278_m1
C1S	Hs00156159_m1
C4	Hs00246758_m1
FH	Hs00962373_m1
FI	Hs00989715_m1
CD46	Hs00611257_m1
CD55	Hs00892618_m1
CD59	Hs00174141_m1

Supplementary Table 4.

Primers for mRNA expression analyses of murine complement genes.

Gene	Reference
C1qa	Mm00432142_m1
C1qb	Mm01179619_m1
C1qc	Mm00776126_m1
C1rl	Mm00619332_m1
C1s1	Mm00663210_mH
C2	Mm00442726_m1
C4b	Mm00437893_g1
Serping1	Mm00437835_m1
Mbl2	Mm00487623_m1
C3ar1	Mm02620006_s1
C5ar1	Mm00500292_s1
C5ar2	Mm01267981_s1
Gapdh	Mm99999915_g1
18S	Hs99999901_s1
Actb	Mm00607939_s1

MANUSCRIPT 4: Marie V. Daugan, Margot Revel, Romane Thouenon, Tania Rybkine, Carine Torset, Nicolas S. Merle, Rémi Noé, Virginie Verkarre, Stephane Marie Oudard, Arnaud Mejean, Pierre Validire, Xavier Cathelineau, Raphael Sanchez-Salas, Mathew Pickering, Marie-Agnès Dragon-Durey, Isabelle Cremer, Audrey Lupo, Marco Alifano, Catherine Sautés-Fridman, Diane Damotte, Wolf H. Fridman, Lubka T. Roumenina, " Implication of intracellular FH as a novel modulator of tumor cell biology" – *In preparation*

Summary and novelty: Evidences from the literature suggested that complement factor H (FH) can support tumor growth. This pro-tumoral effect was mainly attributed to the role of FH as complement regulator of the alternative pathway, limiting C3b opsonization and MAC formation. In this manuscript, we show that in clear cell renal cell carcinoma (ccRCC) patients, FH is localized at the surface and within the tumor cells. Interestingly, FH at the membrane play its role as complement regulator, but had no impact in vitro on tumor cells as well as in patients, on survival. However, we described for the first time that intracellular FH localized in the vesicles of tumor cells strongly modulate tumor cell phenotype in ccRCC and lung adenocarcinoma by acting on proliferation, cell cycle, cell morphology, adhesion and migration. These effects were tumor cells-specific, since no modifications occurred in normal FH-expressing cells. The discovery of these unexpected intracellular functions redefines the role of FH in tumor progression and its use as a prognosis biomarker or potential therapeutic target.

Implication of intracellular FH as a novel modulator of tumor cell biology

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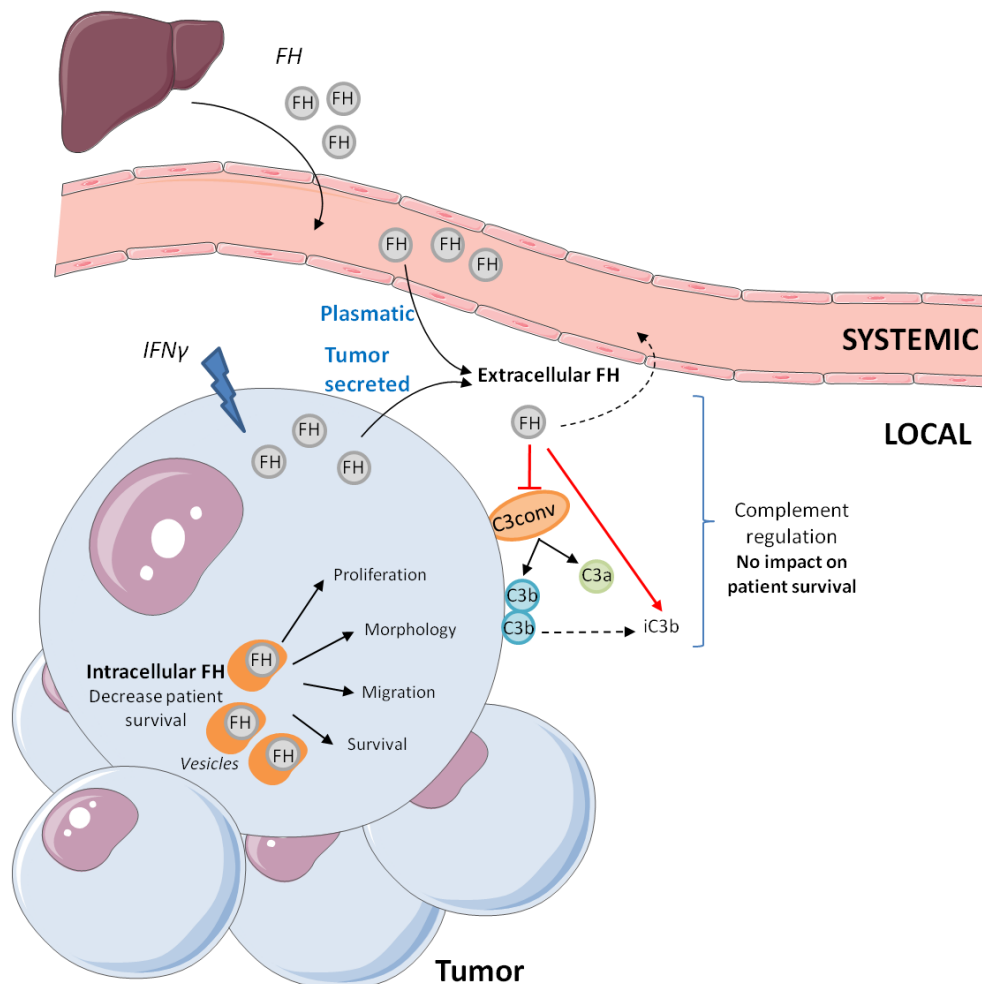
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Summary

Factor H (FH) is an essential plasmatic regulator of the innate immune complement system. We discovered that in clear cell renal cell carcinoma (ccRCC), FH is present in two distinct compartments; extracellular (ext-FH) and intracellular (int-FH). The ext-FH, localized at tumor cell surface, comes from local production by tumor cells and recruitment from circulation. Ext-FH is implicated in the regulation of complement activation but had no impact on tumor cell phenotype or patient survival. In contrast, int-FH, exerts specific pro-tumoral functions, altering tumor cells phenotype and promoting a negative impact on ccRCC patient clinical outcome. Moreover, the pro-tumoral effects of int-FH are not restricted to ccRCC but are shared with other cancers like lung adenocarcinoma.

Keyword: complement system, Factor H, intracellular complement, complosome, non-canonical functions of complement, clear cell renal cell carcinoma, lung adenocarcinoma



Significance

Complement FH is expressed by a multitude of cancers and is part of the tumor microenvironment (ext-FH) or located within cancer cells (int-FH). Ext-FH controls complement attack on tumor cells. Nevertheless, ext-FH has no impact on ccRCC patient survival or on the phenotype of renal and lung cancer cells *in vitro*. We found that in ccRCC and lung adenocarcinoma, FH exerts pro-tumoral action through an intracellular, non-canonical mechanism, acting on proliferation, cell cycle, cell morphology, adhesion and migration. These effects were tumor cells-specific, since no modifications occurred in normal FH-expressing cells. The discovery of these unexpected intracellular functions redefines the role of FH in tumor progression and its use as a prognosis biomarker or potential therapeutic target.

Introduction

The complement cascade is a part of the innate immunity¹. It kills pathogens and helps to maintain host homeostasis². However, its actions within cancers are context-dependent³. Tumors are a complement rich environment where numerous cell types contribute to the local production of complement component such as immune cells⁴, endothelial cells^{5,6}, fibroblasts⁷ or malignant cells themselves^{3,6}. It is well described that to turn complement activation in their favor, tumor cells develop mechanisms to escape from the canonical functions of the complement cascade. Due to overexpression of complement regulators, such as FH, they block the opsonization with C3b and the lytic membrane attack complex (MAC) formation⁸⁻¹⁰, while still benefiting from the chronic inflammation due to generation of anaphylatoxins¹¹⁻¹⁵. It is now clear that cancer cells produce complement proteins, which are not all secreted, but partially remain within the cell¹⁶. The role of these intracellular complement proteins in cancer is unknown, but in T-cells, C3 and C5 have intracellular functions outside of the complement cascade, termed thus non-canonical^{17,18}. Intracellular staining of FH is reported for ovarian cancer cells¹⁹, glioma cells^{20,21}, cutaneous squamous cell carcinoma (cSCC)²² and lung cancer cells⁹, but the function and relevance for tumor progression of this FH are poorly understood. FH is produced mainly by the liver (up to 80%²³), but endothelial cells, fibroblasts, renal proximal tubular epithelial cells, etc contribute to the local production of FH. However, the role of the intracellular FH within these natural FH-producing cells is unknown.

FH is the master regulator of the central enzyme of the complement alternative pathway (AP): the C3 convertase. It blocks its formation, enhances its dissociation and exerts a cofactor activity for FI, an enzyme cleaving C3b to inactive fragment iC3b¹. The presence of FH in a tumor can come from the circulation or the *in situ* production by tumor cells. Within the

tumor microenvironment (TME), FH could have an anti-tumoral effect by regulating the generation of the anaphylatoxins, which promoted an immunosuppressive environment as in a mouse model of sarcoma¹⁵. If the tumor cells act as a strong complement-activating surface, FH would allow them to escape from complement attack. Indeed, in a mouse model of lung cancer, the injection of tumor cells deficient for FH resulted in a decreased tumor growth, associated with an enhanced complement cascade activation and leading to tumor cell death²⁴. Injection of a blocking FH antibody also reduced lung cancer growth in mice²⁵. However, recent studies showed that FH can have an intrinsic role, independent from the complement cascade²⁶. In liver cancer, FH is required for the maintenance of stemness²⁷. Moreover, in cSCC, silencing of FH production in tumor cells decreased the proliferation, migration and altered cell signaling²².

The kidney is one of the major extrahepatic sources of FH and is an organ particularly susceptible to complement attack^{23,28}. Here we show that FH is overexpressed by the tumor cells in a subset of patients with clear cell renal cell carcinoma (ccRCC) but also with lung adenocarcinoma and is associated with poor prognosis. The pro-tumoral effect of FH results from an unexpected tumor cells-specific intracellular mode of action, promoting their proliferation, survival and migration, independently of the complement activation.

Material and methods

Bioinformatic analysis

The Kaplan-Meier curves for *CFH* in KIRC (n= 516) are generated with GEPIA by using disease free survival and overall survival data with a median cutoff. The boxplot is generated by GEPIA tool and represents the expression of *CFH* using $\log_2(1+TPM)$ in KIRC (ccRCC) tumors vs normal kidney from TCGA normal and GTEx normal database (n=100). Correlation between expression of *CFH* and *CFHL-1* (\log_2TPM) isoform in KIRC and the Kaplan-meier for *CFHL-1* in KIRC are assessed by GEPIA. 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. The heatmap is generated using R software 3.4.2 with heatmap.2 package with the clustering method “complete” and represents the \log_2 Hazard ratio in each cancer type with a scale centered in 0. The surrounded boxes represent a log-rank p-value <0.05.

Patients

The patients included in this study have signed an informed consent form prior to inclusion. The research is approved (N° CEPAR 2014-001) by the medical ethics board and conducted according to the recommendations in the Helsinki declaration. Primary ccRCC tumor specimens were collected from two retrospective studies (Supplementary Table 1). The inclusion criteria are; histology type ccRCC, all TNM stages, tissue with good quality for analysis and presence of the clinical data. Cohort 1 is composed of 133 ccRCC patients operated at Institut Mutualiste Montsouris (Paris, France) between 2002 and 2010. Cohort 2 is composed of 91 ccRCC patients that undergone nephrectomy at Necker-Enfants Malades Hospital (Paris, France) between 1999 and 2003²⁹. Histopathologic features such as Fuhrman grade, TNM stages and size and histologic subtypes are available for the majority of the patients.

For NSCLC, one retrospective cohort is used in this study (Cohort 3)³⁰. Cohort 3 includes 212 untreated patients seen between 2001 and 2005 at the Department of Thoracic Surgery of Hôtel-Dieu Hospital (Paris, France). The inclusion criteria are histology type squamous cell carcinoma (n= 100) or lung adenocarcinoma (n=112), all TNM stages, tissue with good quality for analysis and presence of the clinical data. Histopathologic features such as histologic subtypes, ADK grade, TNM stages are available for the majority of the patients.

Plasma samples of 69 patients with ccRCC were collected prospectively at time of surgery at Institut Mutualiste Montsouris (Paris, France) since 2014 until now. Thirty-one plasma samples from healthy donors were used as a control for FH quantification.

Immunofluorescence(IF)/immunohistochemistry(IHC)

Formalin-fixed paraffin-embedded (FFPE) human tumors specimens of RCC or NSCLC were cut into 3µm thick sections and stained for FH, C3d, Cytokeratin, αSMA, CD31, CD163, CD20, CD3, N-cadherin, E-cadherin, vimentin, lamp-1, calnexin or tom20 (Supplementary table 2). Human liver sections were used as positive control for FH. *Pemphigus vulgaris* (geneticist) is used as positive control for C3d. Tonsil (Geneticist) is used as positive control for cytokeratin, αSMA, CD31, CD163, CD20, CD3. Tissues were deparaffinized and rehydrated by successive baths of Clearene and ethanol gradient (100%, 90%, 70%, 50%). The antigen retrieval was performed with PT-link (Dako) using the EnVision FLEX Retrieval Solutions (Dako) with pH High or Low depending of the antibody. The endogenous peroxidase are then block by using 3% H₂O₂ (Gifrer) solution and follow by a blocking of non-specific staining with protein block (Dako).

The conditions for each staining are summarized in Supplementary Table 2. For IHC, FH primary antibody is used at 11.3µg/mL. The staining was revealed by 3-amino-9-ethylcarbazole substrate (Vector Laboratories). The slides are then counterstained with hematoxylin (dako), mounted with glycergel (Dako) and scanned with Nanozommer (Hamamastu). For double staining by IF, a tyramid system is used in order to avoid species compatibility problems and increase the fluorescence signal. This system needs a supplementary step after the secondary HRP antibody that consists of incubation with AF647 tyramid reagent (diluted 1:100 in TBS 1X, H2O2 0.0015%, Life technologies). The entire staining protocol is then repeated for the second primary antibody but this time with AF546 tyramid reagent (Life technologies). The nucleus is stained with DAPI. The slides are then mounted with Prolong Glass antifade reagent and scanned with AxioScan (Zeiss). The colocalization between FH and CK is analyzed by using HALO Image Analysis software (Indica Labs).

Staining specificity. The specificity of FH staining is assessed by a competition test on liver sections (Supplementary Figure 1). In this test, anti-FH antibody clone Ox24 and FH purified (Comptech) protein are pre-incubated at different molecular ratios during 1 hour at 37°C before putting it on liver tissue sections.

Patient stratification. For patient stratification, the tumors are classified into 2 groups High or Low by a semi-quantification method in order to distinguish the different types of FH staining; intracellular (int-FH) or extracellular (ext-FH). For FH int-FH or ext-FH staining, the high group corresponds to a number of FH positive cells > 30% and the low group < 30%. Three independent observers performed the analysis in order to avoid any bias. Automated quantification was unfeasible, since it was not possible to train the algorithm to distinguish reliably intracellular staining from membrane deposits.

Mouse tumor staining. Freshly isolated mouse tumor tissues were frozen with liquid nitrogen and cut into 6µm section with cryostat (Leica). Tissues were then fixer with cold acetone for 8 minutes and blocked with TBS BSA 5% for 30 minutes. To stain the vessels, a rabbit polyclonal anti-CD31 (abcam, ab124432) or a goat antiserum polyclonal anti-FH (Quidel, A312) as primary antibodies and a goat anti rabbit coupled with AF647 (Thermofisher scientific, A-21245) or donkey anti-goat coupled with AF647 (Thermofisher scientific, A-21447) s secondary antibodies. The nuclei were stained with DAPI and the slides were mounted with Prolong glass. The entire tissues were scanned with AxioScan (Zeiss) and analyzed with HALO Image Analysis software (Indica Labs).

Cell lines

Human ccRCC cell lines (Caki-1 and A498), human lung adenocarcinoma cell line (A549), human umbilical vein endothelial cells (HUVEC) and renal proximal tubule cells (RPTEC) were used in this study. Cells were cultured with the appropriate medium in humidified atmosphere of 5% CO₂ at 37°C until 90% confluence. Caki-1 was cultured in McCoy's medium (Gibco) +10% FCS +1X penicillin/streptomycin (Gibco). A498 was cultured in Eagle minimum essential medium (ATCC) +10% FCS +1X penicillin/streptomycin (Gibco). A549 was cultured in Dulbecco's modified eagle medium nutrient mixture F12 (Gibco) +10% FCS +1X penicillin/streptomycin (Gibco) +1X L-glutamine (Gibco) +1X HEPES (Gibco) +1X Non-essential amino-acids solution (Gibco). HUVEC was cultured in M199 (Gibco) +20% FCS +1X penicillin/streptomycin (Gibco) +20% EG2-MV (Lonza). RPTEC is cultured in RGEM medium (Lonza). All cell lines are routinely tested for *mycoplasma*.

Mouse lung cancer TC-1 cell lines was cultured in RPMI supplemented with 10% FCS, 1X penicillin/streptomycin (Gibco), 5mmol/L glutamine (Gibco) and 50µmol/L of 2-mercaptoethanol.

Silencing of *CFH*

On day before transfection, the cells were plated in a 6-well plate at 50% confluence in their specific medium without antibiotics. The cells were then transfected with a pre-prepared mixture of two siRNAs against *CFH/CFHL-1* at the concentration of 50nM (Qiagen Hs_CFH_3_Flexitube siRNA SI00003983 and Hs_CFH_4_Flexitube siRNA SI00003990) or siRNA control 50nM (Qiagen AllStars negative control siRNA, SI03650318), 5µL of lipofectamine RNAiMAX Transfection reagent (Thermofisher, 13778030) in an OptiMEM medium (Gibco). After 24h, the transfection is stopped by changing the OptiMEM medium for appropriate medium without antibiotics. The cells were ready to use for the functional experiments 72h post-transfection. Photos under microscope were taken 72h post transfection to attest the difference in cell number and morphology. The efficiency of the silencing was attested by qRT-PCR and ELISA for cell lysates and supernatants.

Protein extraction

Cell lines are lysated by RIPA buffer to obtain a total cell lysate. A specific cell fractionation kit (Abcam, ab109719) was also used to allow preparation of organelle, cytoplasmic and nuclear fractions from cultured cells according to manufacturer's instructions.

ELISA

FH or C3 concentrations in cell supernatants and cell lysates were measured by a sandwich ELISA method. FH concentration in the supernatant was measured under non stimulated conditions or after treatment with different cytokines (IFN γ , TNF α and TGF β) at 10 or 100ng/mL for 72 hours. Briefly, a polyclonal antibody against FH was purified from goat anti-serum using protein G and left native or was biotinylated to generate a home-made ELISA kit³¹. The native anti-FH or anti-C3c (Calbiochem, 201869) was coated on a Nunc Maxisorb ELISA 96-well plate overnight 4°C. After washing with PBS Tween 0.1% and blocking the free reactive sites with PBS BSA 1% during 1 hour at room temperature (RT), the supernatant and cell lysate were added to the plate and incubated during one hour at RT. After washing, plate was incubated with an in-house biotinylated antibody anti-FH or anti-C3 (purified from antiserum from Calbiochem) for 1 hour at RT. After additional washings, streptavidine coupled with horseradish peroxydase (HRP) (Dako, P039701-2) was added for 1 hour at RT. FH concentration was revealed by SureBlue TMB microwell peroxidase substrate (KPL) and the reaction was stopped by sulfuric acid 2mol/L. Multiskan Ex (Thermofisher Scientific) was used to read the optical density at 450nm. Results are expressed in $\mu\text{g/mL}$ according to the standard curve, made using commercial purified FH and C3 (Comptech).

Western blot

The supernatants of cells cultured without serum for 48h were recovered and concentrated using Amicon Ultracel 3K units (UFC, 900324). NuPAGE LDS sample buffer (4X, Thermofisher Scientific) with or without reducing reagent DTT is added to the concentrated supernatants and then denatured at 80°C for 10 minutes. A NuPAGE 10% Bis-Tris gel (Thermofisher Scientific) is used to separate protein before transfer onto a nitrocellulose membrane. After 1 hour of blocking using TBS BSA 5%, the membrane is incubated overnight at 4°C under a rocking chair platform with the primary antibodies; goat polyclonal antiserum human FH (Quidel, A312), goat polyclonal antiserum human FI (Quidel, A313), anti-human C3 (Calbiochem,) diluted 1:1000 in PBS BSA 5%. Purified C3 (Calbiochem, 204885), purified C3b (Calbiochem, 204860) or purified iC3b (Calbiochem, 204863) was used as control. Secondary antibodies were rabbit anti goat HRP 1:1000 (Covalab, lab0048) or goat anti-mouse HRP 1:1000 (Covalab, lab0365) or goat anti-rabbit HRP 1:1000 (Covalab, lab0363). After washing with TBS Tween 0.1%, the membranes were developed with ECL reagent (Pierce, 32106) and the chemiluminescence was read with iBright CL100 (Thermofisher).

qRT-PCR

The cell lines RNA was extracted using Maxwell cell 16LEV simplyRNA purification kit (Promega, AS1270). The quality and quantity of RNA were determined by 2100 Bioanalyzer (Agilent) using an Agilent RNA 6000 Nano Assay kit (5067-1511). Applied Biosystem High-capacity cDNA reverse transcription kit (Applied Biosystem, 4368814) was used for reverse transcription. The gene expression is determined by Taqman using primers of human *CFH* (Thermofisher, Hs0092373_m1), mouse *Vegfa* (Thermofisher, Mm00437306_m1), mouse *Vegfb* (Thermofisher, Mm00442102_m1) mouse *Vegfc* (Thermofisher, Mm00437310_m1), mouse *Pecam-1* (Thermofisher, Mm01242576_m1), mouse *Flt4* (Thermofisher, Mm01292604_m1) mouse *Kdr* (Thermofisher, Mm01222421_m1), mouse *Prokr1* (Thermofisher, Mm00517546_m1) mouse *Vwf* (Thermofisher, Mm00550376_m1).

Analysis of differentially expressed genes

The RNA of 3 independent biological replicate for each conditions (siFH vs siC) are used for the RNAseq study. The preparation of libraries with NEBNext Ultra II Directional RNA Library Prep Kit follows the successive steps; purification of PolyA containing RNA molecules using poly-T oligo attached magnetic beads from 1 μ g total RNA, fragmentation using divalent cations under elevated temperature to obtain approximately 300bp pieces, double strand cDNA synthesis adapters ligation and cDNA library amplification by PCR for sequencing. Sequencing is then carried out on paired-end 75b of Illumina HiSeq 4000 instrument. The quality of FASTQ files are verified by using FASTQC and then aligned to a reference genome using STAR tool. Once the data are mapped on the genome, HTSeqCount is used to count and assign reads to a given exon and generate a count table. Before proceeding to differential analysis, non-expressed genes with a count equal to 0 are removed from the analysis. The differential expression analysis between siFH and siC is then performed by using R software and DESeq2 package³². DESeq2 uses the tables of counts to determine differentially expressed genes between siFH and siC cells. Results were considered statistically significant at an adjusted $p < 0.05$. The top 50 most significant genes were sorted by adjusted p-value. This was plotted in volcano plots and heatmap using heatmap.2 and EnhancedVolcano packages in R. Gene ontology enrichment analysis was performed with WEV-based Gene Set Analysis Toolkit (WebGestalt) by using the GO biological process category.

Flow cytometry

C3 deposits. The cells are detached by trypsin and washed two times with PBS. Human normal serum diluted to 33% in PBS is then added to the cells for 30min at 37°C with or without anti-FH blocking antibody (hybridoma, Ox-24). Staining was performed with mouse monoclonal anti-C3c (Quidel, A205) or isotype control IgG1 follow by a secondary goat anti mouse AF700 (Invitrogen, A-21036). C3 deposits were quantified by flow cytometry (BD Fortessa) and further analyzed by FlowJo X.

Proliferation and mortality. Cell are detached by trypsin and washed two times with PBS before adding CFSE reagent 1:1000 (Invitrogen, C34554) for 20 minutes at 37°C. Complete culture medium was added to stop CFSE staining and incubated 5minutes at 37°C. After two washing steps, the cells were seeded on a 6-well plate and cultured for 72h in presence of complete medium. Supernatants containing dead cells and adherent cells were recovered and stained for DAPI before analysis by flow cytometry (BD Fortessa).

Cell cycle. Cell are detached by trypsin and washed two times with PBS before fixation in cold ethanol 70% and storage for 24h in -20°C. After two washes in PBS, 3µM of propidium iodide (PI, Invitrogen, P1304MP) was added in presence of RNase for 15minutes at room temperature. PI nuclear staining was then quantified by flow cytometry (BD Fortessa) and further analyzed by FlowJo X with the cell cycle tool.

FH staining. For intracellular FH staining, after blocking with BSA 5%, cells were stained with DAPI 1:1000 for 10min at RT. The cells were then permeabilized with BD cytofix/cytoperm kit (BD, 554714). Anti-FH antibody 1:100 (Quidel, A224) or isotype control were added to cells for 30min at RT. After two washing steps, cells were incubated with goat anti mouse AF488 (Thermofisher, A28175) diluted 1:100 in PBE buffer. After two supplementary washes, intracellular FH on A498 cell was quantified by flow cytometry (BD Fortessa) and further analyzed by FlowJo X.

For evaluation of complement deposition, normal human serum diluted to 33% in PBS is added to the cells for 30min at 37°C with or without anti-FH blocking antibody (hybridoma, Ox-24). Staining was performed with mouse monoclonal anti-C3c (Quidel, A205) or isotype control IgG1 follow by a secondary goat anti mouse AF700 (Invitrogen, A-21036). C3 deposits were quantified by flow cytometry (BD Fortessa) and further analyzed by FlowJo X.

Immunocytofluorescence

Cells were seeded on a round cover glass in the bottom on a 24-well plate in presence of their specific medium. After one day of adherence, cells were fixed with PFA 4% for 30min at RT. Cells are stained for their actin cytoskeleton with Phalloidin-IFluor 488 reagent (Abcam, ab176753) and the nucleus is stained with DAPI. The cover glass was recovered and mounted on a microscope slide with Prolong Glass antifade reagent and scanned with AxioScan (Zeiss).

Migration

A scratch test is used to study migration capacity of the cells. When 90% cell confluence was reached, a wound on the cell monolayer was realized with a tip. Photos of the scratch with a microscope were taken just after the scratch and 12hours later. The scratch area was determined with ImageJ using the macro Wound healing tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool) in order to calculate the percentage of recovery.

Mouse model

Animals - Animal experimentation was approved by Charles Darwin ethical committee (Paris, France) and by the French Ministry of Agriculture (Paris, France) number APAFIS#201904111223718. All procedures were conducted in accordance with their recommendations for care and use of laboratory animals. Male and female wild type C57Bl/6 mice (WT) were purchased from Charles River Laboratories (L'Arbresle, France). The hepatocyte-CFH-/- C57BL/6 mice (hepatoFH-/-) were previously described by Vernon et al²³. HepatoFH-/- mice have been generated by crossing alb-Cre (Jackson Laboratories, 003574) with FH-loxP mice (CFHloxP/loxP) (a gift from M.C Pickering (Imperial College London, London, United Kingdom)).

Experimental procedure - The mouse TC-1 tumor cell line corresponds to lung epithelial cells transformed by HPV. Cells were recovered at 80% confluence and 400 000 cells were inoculated s.c. in the right flank with 200 µl PBS. Tumor size was measured with calipers every 2-3 days for 25 days or until reaching the ethical endpoint of tumor size of 3000 mm³. Tumors were snap-frozen in liquid nitrogen and kept in -80°C for further analyses. Two independent experiments were performed with 8-10 weeks old 5 WT and 5 hepatoFH-/- (experiment 1) and with 10 WT and 5 hepatoFH-/- (experiment 2).

Statistical analysis

The Kaplan-Meier curves were realized with R software 3.4.2 and the package “survival”. The survival data were censored at 2500 days and the impact on survival was assessed by a log-rank test. Correlation between the distributions of two semi-quantitative variables was assessed by a Chi2 test and correlation between one semi-quantitative variable and one quantitative variable by a Kruskal-Wallis test. A paired T-test was used for analysis of FH concentration in cell supernatant or lysate determined by ELISA. The difference between siFH and siC cells for proliferation, cell cycle and survival was assessed by a paired T-test and unpaired T test is used for cell migration. Mouse tumor growth was analyzed by a two-way ANOVA test. Data from mouse IF quantification were analyzed by Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

FH is locally produced by tumor and stromal cells and upregulated by IFN γ

To identify the FH producing cells in the context of ccRCC tumors, double staining using IF were performed (Figure 1A-F). The colocalization between FH and cytokeratin revealed that tumor cells are the main local source of FH with ~85% of the FH staining localized in tumor cells after automatic quantification (Figure 1A). To a less extent, stromal cells and especially fibroblasts, endothelial cells and a fraction of the macrophages were also able to produce FH locally as attested by the colocalization between FH and α SMA, CD31 or some CD163+ cells (Figure 1B-D), whereas CD3+ T cells and CD20+ B cells were negative (Figure 1E-F). Plasmatic concentration of FH was not different between ccRCC patients and healthy donors (Figure 1G), suggesting that FH production by the tumor itself contributes little if any to the plasmatic pool. As an *in vitro* model, human ccRCC cell lines A498 and Caki-1 were chosen for their expression of FH at gene and protein level (Supplementary Figure 2A-F). Moreover, in both cell lines FH is present both intracellularly in the lysate and is secreted in the supernatant (Figure 1H, Supplementary Figure 2). IFN γ increases the FH production by healthy cells, as hepatocytes, endothelial cells or fibroblasts³³. Similarly, the production of FH by A498 is upregulated when cells are treated with IFN γ with an 8-fold increased expression after treatment with IFN γ 100ng/mL (Figure 1I) but not by TNF α or TGF β .

FH modifies the phenotype of ccRCC tumor cells

We next examined the role of FH on key characteristics of tumor cells such as proliferation, survival, morphology and migration capacity. After silencing the secretion of FH is reduced by 90-96% in ccRCC cell lines and 85-90% in the tumor cell lysate (Supplementary Figure 2A-B and D-E). The silencing of FH by a siRNA revealed a high impact on ccRCC tumor cell phenotype (Figure 2A and Supplementary figure 3A). To identify the biological processes impacted by FH silencing, RNAseq was performed on 3 biological replicates for each condition for the two ccRCC cell lines A498 and Caki-1 (siFH cells or siC cells). We used $p < 0.05$ and \log_2 fold change > 1 to select the differentially expressed genes. With these criteria, in A498 cells, 235 were differentially regulated after FH silencing (Figure 2B). Among these genes, 139 were upregulated and 96 were downregulated. For Caki-1 cells, 251 genes were downregulated and 192 were upregulated after FH silencing (Supplementary Figure 3B). The 50 most differentially expressed genes based on adjusted p-value (p_{adj}) for ccRCC cell lines siFH vs. siC were represented in the heatmap (Figure 2C and Supplementary figure 3C). The functional classification analyses of up and down-regulated genes using gene ontology “Biological Pathway” category reveals that these genes were implicated in different biological

processes (Figure 2D and Supplementary 3D). First, we observed an enrichment of genes implicated in cell proliferation in siFH cells and in parallel a downregulation of genes implicated in G1/S transition of mitotic cycle. We further confirmed these pathways were considerably altered in FH silenced cells with a significantly reduced cell proliferation capacity of ccRCC cells (Figure 2E-F and Supplementary figure 3E-F) and blocked the cells in the G0-G1 phase (Figure 2G and Supplementary figure 3G). The cell viability is also affected with an increase in the number of dead cells when tumor cells were deficient for FH (Figure 2H and Supplementary figure 3H). Moreover, some modifications of genes involved in cell adhesion, extracellular matrix organization and cell morphogenesis were also detected with RNASeq analysis. Importantly, these transcriptional changes had functional repercussions with a strong modification of the actin cytoskeleton. Indeed, FH silenced cells were more rounds with less actin filament and stress fibers (Figure 2I and Supplementary figure 3I). Finally, there were also some differentially expressed genes that were involved in cell location and cellular movement. *In vitro*, we confirmed that migration capacity was also decreased with only 5% of scratch recovery at 12h for A498 siFH in comparison with 40% of scratch area recovery for A498 siC (Figure 2J-K). Interestingly, the study of the signaling pathway revealed a higher p53 expression in A498 siFH which is phosphorylated in S46 and S15 position (Figure 2L). The observed effects of FH silencing on tumor cell phenotype could be due to autocrine effect upon secretion in the supernatant or due to intracellular impact.

Extracellular FH comes from local production by tumor cells and from plasma

First we sought to explore the possibility that FH acts extracellularly. By immunohistochemistry on ccRCC sections, we found FH deposits (ext-FH) on the membrane of tumor cells (Figure 3A). This staining is very heterogeneous between patients with some tumors with high level of FH deposits and others with low or no ext-FH positive staining. This ext-FH could come from two main sources: a local production by tumor cells and a plasmatic origin. The existence of a positive correlation between FH staining (reflecting FH production) and the presence of FH deposits at tumor cells surface suggest a local origin (Figure 3B). Nevertheless, this local production only partially explains the ext-FH since some tumors presented ext-FH without local production by tumor cells. In a mouse model with an absence of a hepatic production of FH, the presence of FH within the plasma is largely decreased. Only 20% of FH, produced by extrahepatic sources, remained in the circulation²³ (Supplementary figure 4A). In order to study the impact of plasmatic FH in tumor growth, we injected subcutaneously TC-1 cell lines that expressed very low level of FH (Supplementary figure 4B). When, TC-1 cells were inoculated in hepato^{-/-} mice, the level of FH inside the tumor is largely decreased (Figure 3C-D) meaning that plasmatic FH is an important source of FH for the tumor. However, the tumor growth was not modified in FH hepato^{-/-} mice

compared to WT mice (Figure 3E). FH was suggested to inhibit neoangiogenesis³⁴. Nevertheless, no modification in vascular density or architecture (Supplementary figure 4C) as well as angiogenic genes expression was observed in tumors from hepato FH $-/-$ compared to WT mice (Supplementary figure 4D).

Ext-FH is responsible of complement regulation

We next focused on the FH deposits at the surface of tumor cells. Indeed, previous studies indicated that tumor cells produce FH, which can be used as a potential mechanism to escape from complement attack²⁴. Interestingly, ext-FH colocalized and correlated with the presence of C3d-deposits at the surface of tumor cells, suggesting that FH plays its role as complement regulator (Figure 4A-B). When A498 or Caki-1 cells were silenced for FH and exposed to serum, an increased amount of C3 fragments deposits was observed, when compared to siC cells (Figure 4C and Supplementary Figure 5A). Moreover, in the supernatant of A498 and Caki-1 siFH cells, the α 43 band corresponding to the inactive fragment of C3b and C3(H₂O) (iC3b, iC3(H₂O)), was largely reduced due to a loss of factor I (FI) cofactor activity (Figure 4D and Supplementary Figure 5B). Indeed, factor I was secreted in the supernatants of ccRCC tumor cells (Supplementary Figure 5C). Of note, the presence of C3b fragment was only detected on Caki-1 supernatant. This indicated that the silencing of FH results in a deregulation of the complement system and an increased level of C3 activation. In order to decipher if this over-activation of complement can impact tumor cell phenotype, we treated A498 cells with a FH blocking antibody in excess comparing to the amount of FH secreted, Ox-24, at 20 μ g/mL. The level of C3 deposits increased when cells are treated with this blocking antibody, reflecting its capacity to prevent FH binding to C3b and its regulatory function (Figure 4E). However, the tumor cells phenotype was not modified, contrary to the condition when cells are silenced for FH (Figure 4F) suggesting that complement regulatory functions had no impact in ccRCC.

Extracellular FH does not reverse the tumor cell phenotype after FH silencing

A498 cells silenced for FH displayed reduced proliferation capacity. The addition of 2 μ g/mL of exogenous FH to A498-siC cells did not modify their ability to proliferate. Furthermore, the addition of exogenous FH to A498-siFH did not restore their basal proliferation level suggesting that intracellular but not plasma-derived/extracellular FH is essential for tumor cell phenotype (Figure 5A). Moreover, ccRCC cell lines stained for FH by flow cytometry only after a permeabilization step (Figure 5B and Supplementary figure 6A).

FH positivity is associated with mesenchymal phenotype in ccRCC tumors

In situ on ccRCC sections, FH colocalized with tumor cells and especially those with low level of the epithelial marker cytokeratin (CK) (Figure 5C). An increased intensity of CK staining was associated with a decreased positivity for FH: cells with low intensity for CK represented 65% of FH+ cells whereas tumor cells with medium and high intensity of CK represented only 15% and 5% for the FH+ cells respectively (Figure 5D). In addition, in ccRCC, FH expressing cells also expressed mesenchymal markers N-cadherin and vimentin (Figure 5E-F).

FH is localized in intracellular vesicles

FH presented punctiform staining in ccRCC tumors. By using successive detergent and centrifugations steps, we were able to fractionate the different cell compartments from ccRCC as well as from lung ADK cell lines. In the tumor cell lines, the majority of FH protein was detected in a compartment, containing organelle fraction (mitochondria/lysosomes/endoplasmic reticulum/vesicles) and no FH was found in the cytosolic or nuclear compartment (Figure 5G and Supplementary figure 6B). Visually FH staining was localized in a vesicular pattern but did not colocalized with endoplasmic reticulum (FH/calnexin), lysosome (FH/Lamp1) or mitochondria (FH/Tom20) (Figure 5H). In contrast, C3 - the main plasmatic partner of FH, was localized principally in the cytosolic and nuclei compartment of tumor cells for all tested cell lines (Supplementary figure 7A-B).

Intracellular FH but not extracellular FH is associated with a negative impact on ccRCC patient survival

In TCGA KIRC cohort, there was no significant difference in CFH expression between tumors and normal kidney tissue (Supplementary 8A). Nevertheless, a high expression of *CFH*, above the median, was associated with a decrease disease free survival (DFS) and overall survival (OS) (Figure Supplementary 8B-C) for ccRCC patients (TCGA KIRC cohort). In order to have access to the specific impact of Ext-FH and Int-FH we stained 2 cohorts of ccRCC patients by immunohistochemistry. Extracellular FH staining localized at the surface of tumor cells was very heterogeneous between patients and inside the same tumor with positive and negative areas. This variability allowed to classify the patients into two groups according to the number of FH deposits (Figure 6A). In ccRCC cohort 1, the presence of such deposits did not have any impact on DFS ($p=0.14$) (Figure 6B). This absence of prognostic value was confirmed in the second cohort, with no effect on PFS ($p=0.226$) and OS ($p=0.627$) (Figure 6C-D). The same staining heterogeneity was observed for intracellular

FH and two groups of patients were constituted depending on the number of FH producing cells (Figure 6E). Compared to low density of int-FH-positive cells, a high density of int-FH-positive cells had a negative impact on the DFS of the cohort 1 ($p=0.00432$) (Figure 6F). This finding was validated in another independent cohort of ccRCC patients (PFS: $p=0.0274$, OS: $p=0.0727$) (Figure 6G-H). The presence of FH within the tumor cells correlated with tumor size and Fuhrman grade and also with the other complement staining, especially for intracellular C4 and C3. On the other hand, no association between int-FH and the immune infiltrate were found (Supplementary table 4).

The alternative splice variant FHL1 did not affect ccRCC progression

FH presents an alternative splice variant called FHL1, sharing the first seven domains of FH. The A498 and Caki cell lines expressed a lower amount of FHL-1 detectable by WB, without any evidence for FHR1, FHR2 and FHR5. After the treatment with siRNA against *CFH*, an extinction of FHL-1 was also observed (Supplementary figure 2C and 2F). A *CFH*-specific siRNA affecting exclusively FH expression and not FHL-1 was tested but silencing was not efficient, which precluded its use for this study and all experiments were performed with concomitant downregulation of FH and to a less extend, FHL1. Nevertheless, a stable extinction using shRNA, targeting specifically *CFH*, was developed in A498 and Caki-1 cell lines, showing modification of cell morphology and survival (data not shown), pointing to a specific effect of FH.

Moreover, the splicing form of CFH called CFHL-1, was also expressed in ccRCC and represents around 20% of total CFH expression. The production of FH in ccRCC tumors was confirmed by a staining with an antibody, which does not recognize FHL1 (clone L20, binding to the C-terminal part, not shown). The expression of CFHL-1 was correlated with CFH (Supplementary figure 8D). However, CFHL-1 had no impact on DFS and OS (Supplementary figure 8E-F). These results suggest that the main actor on tumor cell phenotype is FH, although a contribution of FHL1 cannot be excluded.

The biological effect of FH is specific for tumor cells

Since ccRCC derived from proximal tubules, we tested whether the results for the cancer cells could be extrapolated to this cell type. First, we clearly see that proximal tubules can produce FH in the peri-tumoral tissue (Figure 7A). In primary human renal proximal tubule epithelial cells (RPTEC), we detect FH in the lysate and the supernatant (Figure 7B). Within these cells, FH was localized in the same fractions as for the tumor counterpart (Figure 7C) but contrary to tumor cells the majority of FH staining colocalized with lysosomes (FH/lamp1) and not

with endoplasmic reticulum (FH/calnexin) or mitochondria (FH/tom20) (Figure 7D). Interestingly, despite the efficient FH silencing (validated by ELISA and Western blot on cell lysate on supernatant, Supplementary figure 9A-B), no modification of the transcriptomic program of the cells were observed with only 11 differentially expressed genes including CFH between RPTEC siFH and siC (Figure 7E) against 228 for A498. Moreover, only 2 changes were commonly differentially expressed in A498 and RPTEC; CFH and NCKAP5. Moreover, from a functional point of view, there were no effect on cell phenotype in terms of viability, number of cells and morphology (Figure 7F). The actin cytoskeleton of the cells remains the same whether FH is silenced or not (Figure 7G) and the migration capacity did not change (Figure 7H). Thus, the effect of FH seems to be specific of tumor cells. Within the tumor, endothelial cells can also produce FH (Supplementary figure 10A). In vitro, endothelial cells (HUVEC) also produce and secrete FH (Supplementary figure 10B). After efficient silencing (Supplementary figure 10C-D), again, no alterations of phenotype was found in terms of cell number, morphology, actin cytoskeleton and migration capacity (Supplementary figure 10F-G).

Pro-tumoral effect of FH in lung adenocarcinoma.

In non-small cell lung cancer (NSCLC), we also detected int-FH. The pattern of FH staining was the same as for ccRCC. A high production of FH was found by tumor cells expressing CK (Figure 8A) and especially by mesenchymal tumor cells that expressed high level of N-cadherin and low level of E-cadherin (Figure 8B-C). Moreover, stromal cells such as α SMA-positive fibroblasts and CD31-positive endothelial cells can also contribute to the local production of FH (Figure 8E-F). On the other hand, CD163+ macrophages (Figure 8D), CD20+ B cells (Figure 8G) and CD3+ T cells (Figure 8H) did not produce FH locally. However, very rare FH deposits were found in NSCLC patients (data not shown). In order to know if the pro-tumoral effect of int-FH could be relevant in NSCLC, the lung adenocarcinoma (ADK) cell line, A549 was selected to perform the *in vitro* experiments. A549 produce and secrete equivalent amounts of FH to ccRCC cell lines (Figure 8I). Moreover, intracellularly, FH is localized mainly on the same compartment as in ccRCC (Figure 8J) that was distinct from the cellular compartment for C3 (Supplementary figure 11F). After efficient silencing (Supplementary figure 11A-C), an increased complement activation was detected (Supplementary figure 11D-E). The silencing of FH modified strongly the cell phenotype (Figure 8K) with difference in morphology and proliferation capacity (Figure 8L). On the contrary, in a squamous cell lung carcinoma (SSC) cell line (SK-MES) no effects of FH silencing was detected (Supplementary figure 12). NSCLC patients with either lung ADK or SCC from cohort 3 were classified into two groups according to the number of int-FH positive cells (Figure 8M). Contrary to lung ADK where of int-FH-positive cells was

associated with a negative impact on OS ($p=0.0156$) (Figure 8N), in lung SCC, int-FH had no impact on OS ($p=0.798$) (Figure 8O). The negative impact on lung ADK was independent from clinical or histological features and did not correlate with the immune infiltrate (Supplementary table 3). These findings suggested that the pro-tumoral effect of FH is context-dependent but can be extended to other cancer types. Indeed, the study of prognostic value of CFH gene expression in 28 cancer types from the TCGA database revealed that in some cancer CFH gene expression was protective like in melanoma (SKCM) or aggressive in low grade glioma (LGG) or stomach cancer (STAD) (Supplementary Figure 13).

Discussion

Complement components form a plasmatic innate immune cascade, but could serve also as multitasking proteins, having functions outside of this system. Here we show that the master regulator of the alternative complement pathway FH is locally expressed by multiple types of human tumors. Although FH exerts its role as a complement regulator in the TME of ccRCC and lung ADK, we could not find a functional or prognostic impact of this ext-FH. Here we provide a paradigm shift for the impact of FH in cancer progression, showing a previously unrecognized, likely tumor cells-specific, intracellular function of FH outside the complement cascade. Int-FH served as a driver of proliferation and migration of ccRCC and lung ADK cells but not of normal cells or lung SCC. The presence of int-FH staining on tumor cells conferred poor prognosis in ccRCC and lung ADK.

In ccRCC, we described that tumor cells and to a lesser extent endothelial cells, fibroblast and macrophages participate to the local production of FH. Intratumoral FH synthesis is not responsible for an enrichment of the systemic compartment but rather accumulates locally. We distinguished two locations of FH within the ccRCC tumors: intracellular and extracellular. We found that ext-FH exerted canonical functions as complement regulator, binding to C3 activation fragments in tissues, protecting the tumor cells *in vitro* from complement deposition and transforming cell-derived C3(H₂O)/C3b into iC3(H₂O)/iC3b. This act of regulation happens likely in the extracellular space, since within the cell FH and C3 are not localized in the same compartment. The C3 cleavage is assured by FI, which is also secreted by the ccRCC cells. A fraction of the ext-FH within the tumor could come from the circulation, as evident from the mouse model of hepatic FH deficiency. Despite its clear cut regulatory functions, the ext-FH had no impact on patient survival. Moreover, in ccRCC patients the presence of deposits of C3b/iC3b/C3d were not associated with survival, despite the clear prognostic impact of the classical pathway activation⁶. Outside cancer, FH can also act as an immune regulator by modulating the recruitment and functionality of various immune cell types, such as B cells, monocytes, neutrophils or dendritic cells³⁹⁻⁴⁴. However, in our cohorts, no associations between int-FH or ext-FH and immune infiltrate have been observed. These observations forced the conclusion that the function of FH as a complement regulator is not a major driver of tumor progression, at least in ccRCC.

In contrast to ext-FH, the int-FH showed a major impact on patient survival both at gene expression and protein staining level. This association with poor prognosis is not a mere biomarker but reflects a novel function of FH, acting outside of the complement cascade. Indeed, the silencing of FH in ccRCC cell lines induced a strong modulation of tumor cell phenotype, including proliferation, cell cycle, morphology, viability and migration. This can be explained by the profound modification of the transcriptional program of the siFH tumor

cells lines, marked by alteration of the expression of genes, governing the biological processes of adhesion and locomotion. Moreover, within the tumors int-FH-positive cells were frequently also positive for mesenchymal markers, suggesting a more motile phenotype. FH is localized within intracellular vesicles corresponding to the organelle subcellular fraction and not in the nucleus. Therefore, it is less likely that it serves directly as a transcription factor or regulator of genes expression. It rather participates in a complex network of biomolecular interactions, which has to be further elucidated.

The reduced proliferation and mortality of siFH cells could be explained by cell arrest at G0/G1. At molecular level, these biological functions could be a consequence of the siFH-mediated up-regulation at protein level and phosphorylation of p53, which is a powerful tumor suppressor. P53 is well known to arrest the cells in G0/G1, to reduce survival and control the proliferation⁴⁵. How the down-regulation of FH results in up-regulation of p53 requires further investigations. The *CFH* gene encodes for two alternative splice forms: FH and FHL-1. In our study FH silencing also affected the expression FHL-1, as previously observed in lung cancer and cSCC. We cannot completely rule out the hypothesis that FHL-1 play a role in the pro-tumoral effect that we observed. Nevertheless, we found much higher expression of *CFH* within the tumor, an absence of effect of *CFHL-1* on ccRCC patient survival and the use of a shRNA against the C-terminal part of FH, that does not affect FHL-1, induced the same phenotypic modifications (data not shown). These data supported the conclusion that FH is the main actor responsible for the biological functions, altered after *CFH/CFHL-1* silencing and that it is FH and not FHL-1 that drives tumor progression in ccRCC. Moreover, the absence of FH effects in normal cells suggested an oncogenic role and raised questions about its mechanism of action in tumor vs. normal cells. *In situ*, FH was found intracellularly in vesicles in both tumor and peritumoral tissues. While FH was mainly localized in lysosomes in normal cells, it was not the case in cancer cells. This difference of cellular compartment could impact FH action on its microenvironment and interactome. They may explain why FH silencing affects tumor but not healthy cells. *Experiments by mass spectrometry are currently on going to determine the intracellular partner of FH in tumor lysate.*

The pro-tumoral effects of int-FH are not exclusive of ccRCC. We replicated our findings in lung adenocarcinoma. However, FH can be pro- or anti-tumoral according to tumor types. In hepatocellular carcinoma, a high expression of *CFH* is associated with a lower risk of recurrence⁴⁶. Pan-cancer transcriptomic analysis confirmed this duality of FH effects with either a protective or an aggressive effect depending on cancer types. Moreover, besides the cancer types, our data indicates that the histologic subtype is also important to take into account. Indeed, while FH exert pro-tumoral effect on lung adenocarcinoma, no effects of FH were detected in the case of squamous cell lung cancer.

Further studies are needed to understand the heterogeneity of FH effect between cancer types /subtypes. Indeed, FH functionality can be modulated by different parameters that modify its effects on cancer. In lung cancer, the FH variant Y402H is overrepresented among patients compared to control and associated with increased risk of lung cancer among smokers^{47,48}. Moreover, the glycosylation level of FH is modulated in the context of cancer. In ovarian cancer, the sialylation level of FH in sera is lower in advanced stage patients than in healthy donors⁴⁹. In hepatocellular carcinoma, an enhanced fucosylation of FH is an indicator of premalignant liver disease⁵⁰. The reduced form of FH is also a potential parameter that can affect its functions in cancer progression. In lung cancer, FH is present under a reduced form^{25,51} that display a decrease C3b binding capacity and a loss of FI cofactor activity³⁶. Finally, the intracellular partners of FH are not yet identified and can be a potential explanation for this discrepancy.

Based on these data we hypothesize that FH could be not only a potential biomarker but also a therapeutic target for personalized medicine at least for a fraction of ccRCC and lung ADK patients. Interestingly, in a lung cancer model, targeting the reduced FH by a specific antibody delayed tumor growth in mice²⁵. In our study, modulating the extracellular/systemic FH levels *in vitro* and in mice failed to suppress tumor growth. This could be explained by the lack of production of reduced FH by the tested cell lines. Moreover, in our model any antibody targeting C3b/FH interaction may not be efficient, since the int-FH effects are likely independent of C3. Therefore, for such cases where the int-FH produced by tumor cells is pathogenic, FH targeted therapy which can enter to tumor cells has to be developed.

A particular care should be taken in such an approach to avoid potential side effects, such as renal thrombotic microangiopathy (atypical hemolytic uremic syndrome) or C3 glomerulopathy, which are characteristic for patients with FH deficiency, mutations or auto-antibodies²⁸. In addition, deficiency of FH in mice leads not only to renal injury but also to altered splenic B cell development and autoimmunity via C3 over-activation. Interestingly, the biological functions of int-FH are complement-independent and seem to be restricted to tumor cells. Indeed, renal proximal tubule cells, the cells from which ccRCC is derived, are not affected by FH silencing. Moreover, the downregulation of FH expression in endothelial cells does not alter their phenotype. Finally, the hepatic FH deficient mice do not show any apparent liver abnormality. The FH genetic abnormalities in patients are a predisposing factor renal injury but the disease penetrance is incomplete, requiring a primary and a secondary hit for disease manifestation^{28,52,53}. This relative specificity of int-FH to function in tumor cells is favorable argument to consider for the potential development of an int-FH targeting inhibitor for cancer. Nevertheless, particular effort should be made to target the therapeutic molecule to the right type of cells to avoid potential renal side effects.

Acknowledgements

This work was supported by a grant from the Cancer Research for Personalized Medicine (CARPEM) to LTR. This work was also supported by INSERM, University of Paris, Sorbonne University, and the Labex Immuno-Oncology Excellence Program. MVD received a PhD fellowship from ARC.

The authors declare no conflict of interest

Legend

Figure 1: Tumor cells are the main producer of FH in ccRCC tumor

A-F Identification of FH⁺ cells in ccRCC by IF double staining. **A)** FH (green) and CK (epithelial tumor cell marker, red). **B)** FH (green) and α SMA (fibroblast marker, red). **C)** FH (green) and CD31 (endothelial cell marker, red). **D)** FH (green) and CD163 (macrophages marker, red). **E)** FH (green) and CD20 (B cell marker, red). **F)** FH (green) and CD3 (T cell marker, red). **G)** Optical density of FH in the plasma of ccRCC patients from cohort 4 (n=69) or healthy donors (n=31) determined by ELISA. **H)** FH concentration in the lysate and the supernatant of A498 cell lines determined by ELISA. **I)** Fold change of FH concentration in the supernatant of A498 cell lines determined by ELISA after 72h of stimulation with cytokines (TNF α , IFN γ and TGF β) at 10ng/mL or 100ng/mL.

Figure 2: FH modifies ccRCC A498 tumor cell phenotype

A) Images (x100) of A498 cells 72h post-transfection with siFH or siC. **B)** Volcanoplot depicting the results of differential expression obtained with DESeq2 analysis between A498 siFH (n=3) and A498 siC (n=3). The volcano plot was generated with R software and EnhancedVolcano package. Red points represented genes with a log₂ fold change >1 and P_{adj}< 0.05. **C)** Heatmap of log₂ counts for top 50 differentially expressed genes after silencing of with siFH compared with siC in A498. The most differentially expressed genes are selected on adjusted p-value. Expression across each gene have been row scaled with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. Lighter shades and white represent genes with intermediate expression levels. **D)** Gene ontology analysis of functional annotations (biological process) up- or downregulated by FH silencing vs. A498 siC **E)** Histogram representing CFSE intensity on A498 siFH or A498 siC after 72h of proliferation (green = unstained A498 cells,

orange = A498 taken at the time of CFSE staining T0, red = A498 siC after 72h of proliferation, blue = A498 siFH after 72h of proliferation). **F)** Fold change of CFSE geometric means in A498 siFH vs. A498 siC on 4 independent experiments. **G)** Percentage of cells in each cell cycle phase (G0-G1/S/G2-M) determined by flow cytometry analysis of PI staining on A498 siFH or siC cells on four independent experiments. **H)** Percentage of DAPI positive dead cells determined by flow cytometry after 72h of culture in A498 siFH or A498 siC in five independent experiments. **I)** Immunofluorescence staining of phalloidin (red) in A498 siFH (right) or siC (left). **J)** Analysis of migration capacity of A498 siFH cells and A498 siC cells. A scratch on A498 monolayer was realized at T0 and photo of the scratch area were taken. After 12h, a photo of the same area was taken to compare the migration capacity. **K)** ImageJ Wound healing macro was used to determine the area of the scratch at each time point. The percentage of closing of the scratch area was calculated with the following calculation; $100 - (\text{scratch area T0} / \text{scratch area t+12h})$. **L)** Western blot analysis on A498 siFH or siC lysate for p53, p53 S15 and p53 S46.

Figure 3: Extracellular FH comes from the circulation and the local production by tumor cells.

A) FH staining on ccRCC paraffin sections representing FH deposits. **B)** Histogram representing the correlation between int-FH staining within tumor cells (FH IC) and ext-FH from cohort 2. The association was calculated by using a Chi2 test. **C)** FH immunofluorescence staining (green) on frozen sections of TC-1 tumors from WT or FH hepato-/- mice at day 24. Nuclei were stained with DAPI. **D)** The quantification of FH staining surface was determined by HALO software for 4 WT mice and 4 hepato -/- mice. The boxplot represents the ratio between FH staining area and total tissue area for each mouse. **E)** Tumor growth after subcutaneous injection of TC-1 tumor cells into WT (n=9) or FH hepato -/- mice (n=5) on two independent experiments.

Figure 4: Extracellular FH acts as complement regulator

A) Double staining by IF of FH (green) and C3d (red) on paraffin-embedded ccRCC sections. **B)** Histogram representing the correlation between FH deposits (ext-FH) on tumor cell and C3d deposits (C3d D) from cohort 2. The association was calculated by using a Chi2 test. **C)** C3 deposits on A498 cell surface after incubation with NHS determined by flow cytometry (red: isotype control, blue: A498 siC, orange: A498 siFH). **D)** Western blot for C3 and its fragments in reducing conditions in the supernatant of A498 siFH and A498 siC. **E)** C3 deposits on A498 cell surface after incubation with NHS in presence or not of anti-FH blocking antibody (Ox-24) 20µg/mL determined by flow cytometry (red: isotype control,

green: A498 treated with ox-24, orange: A498 ST). **F)** Images (x100) of A498 cells 72h post-transfection with siFH or siC or after treatment with Ox-24.

Figure 5: Intracellular FH produced by specific vesicles within tumor cells

A) Histogram representing CFSE intensity on A498 siFH or A498 siC with or without purified FH 2 μ g/mL after 72h of proliferation (purple = unstained A498 cells, blue = A498 taken at the time of CFSE staining T0, darkgreen = A498 siC + purified FH, lightgreen = A498 siC + purified FH, red= a498 siFH, orange = A498 siFH + purified FH). **B)** Histogram representing intracellular FH staining on A498 cells (with permeabilization step) (red= isotype, blue = FH staining). **C)** Double staining by IF of FH (green) and CK (red) on paraffin-embedded ccRCC sections. Merged (left), FH (middle), CK (right). **D)** Quantification by HALO software of the percentage of FH positive cells according to the intensity of CK staining (low, medium and high). **E)** Double staining by IF of FH (green) and Ncad (red) on paraffin-embedded ccRCC sections. **F)** Double staining by IF of FH (green) and Vimentin (red) on paraffin-embedded ccRCC sections. **G)** FH quantification by ELISA on the subcellular fraction of A498 cells (cytoplasmic, organelle and nucleus). **H)** Double staining by IF of FH (green) and calnexin/lamp1/tom20 (red) on paraffin-embedded ccRCC sections.

Figure 6: Intracellular FH produced by tumor cells but not extracellular FH is associated with poor prognosis

A) Presence of FH deposits (ext-FH) at the surface of tumor cells by IHC on paraffin-embedded ccRCC sections. The tumors were classified into 2 groups High or Low. High – over 30% of FH+ cells and Low – less than 30% of FH+ cells. **B)** Kaplan-Meier curve of DFS according to the High/Low classification for FH deposits (ext-FH) on ccRCC cohort 1. Log-rank test was used and $p < 0.05$ was considered as significant. **C-D)** Kaplan-Meier curves of PFS and OS according to the High/Low classification for FH deposits (ext-FH) on ccRCC cohort 2. Log-rank test was used and $p < 0.05$ was considered as significant. Log-rank test was used and $p < 0.05$ was considered as significant. **E)** Presence of FH within the cytoplasm of tumor cells by IHC on paraffin-embedded ccRCC sections. The tumors were classified into 2 groups High or Low. High – over 30% of FH+ cells and Low – less than 30% of FH+ cells. **F)** Kaplan-Meier curve of DFS according to the High/Low classification for int-FH on ccRCC cohort 1. Log-rank test was used and $p < 0.05$ was considered as significant. **G-H)** Kaplan-Meier curves of PFS and OS according to the High/Low classification for int-FH on ccRCC cohort 2. Log-rank test was used and $p < 0.05$ was considered as significant. Log-rank test was used and $p < 0.05$ was considered as significant.

Figure 7: No effect of FH produced by renal proximal tubule cells (RPTEC)

A) FH staining on tubular cells in peritumoral tissue by IHC. **B)** FH concentration in the lysate and the supernatant of RPTEC determined by ELISA. **C)** FH quantification by ELISA on the subcellular fraction of RPTEC (cytoplasmic, organelle and nucleus). **D)** Double staining by IF of FH (green) and calnexin/lamp1/tom20 (red) on paraffin-embedded ccRCC sections in the peritumoral part. **E)** Heatmap of log₂ counts for differentially expressed genes after silencing of with siFH compared with siC in RPTEC. Expression across each gene have been row scaled with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. Lighter shades and white represent genes with intermediate expression levels. **F)** Images (x100) of RPTEC cells 72h post-transfection with siFH or siC. **G)** Immunofluorescence staining of phalloïdin (red) in RPTEC siFH (right) or siC (left). **H)** Analysis of migration capacity of RPTEC siFH cells and RPTEC siC cells. A scratch on RPTEC monolayer was realized at T0 and photo of the scratch area were taken. After 12h, a photo of the same area was taken to compare the migration capacity. ImageJ Wound healing macro was used to determine the area of the scratch at each time point. The percentage of closing of the scratch area was calculated with the following calculation; 100-(scratch area T0/scratch area t+12h).

Figure 8: FH exerts pro-tumoral effect in lung adenocarcinoma

A-H) Identification of FH⁺ cells in ccRCC by IF double staining. **A)** FH (green) and CK (epithelial tumor cell marker, red). **B)** FH (green) and E-cadherin (epithelial cell marker, red). **C)** FH (green) and N-cadherin (mesenchymal cell marker, red). **D)** FH (green) and CD163 (macrophages marker, red). **E)** FH (green) and α SMA (fibroblast marker, red). **F)** FH (green) and CD31 (endothelial cell marker, red). **G)** FH (green) and CD20 (B cell marker, red). **H)** FH (green) and CD3 (T cell marker, red). **I)** FH concentration in the lysate and the supernatant of A549 determined by ELISA. **J)** FH quantification by ELISA on the subcellular fraction of A549 (cytoplasmic, organelle and nucleus). **K)** Images (x100) of A549 cells 72h post-transfection with siFH or siC. **L)** Histogram representing CFSE intensity on A549 siFH or A549siC after 72h of proliferation (green = unstained A549 cells, orange = A549 taken at the time of CFSE staining T0, red = A549 siC after 72h of proliferation, blue = A549 siFH after 72h of proliferation). **M)** Presence of FH within the cytoplasm of tumor cells by IHC on paraffin-embedded NSCLC sections. The tumors were classified into 2 groups High or Low. High – over 30% of FH⁺ cells and Low – less than 30% of FH⁺ cells. **N-O)** Kaplan-Meier curves on OS according to the High/Low classification for int-FH on NSCLC cohort 3. **N)** Lung adenocarcinoma. **O)** Lung squamous cell carcinoma. Log-rank test was used and p<0.05

was considered as significant. Log-rank test was used and $p < 0.05$ was considered as significant.

Supplementary

Supplementary table 1: Cohort description

Supplementary table 2: List of antibodies for immunofluorescence

Supplementary table 3: Correlation between FH staining High low and clinical, histological features and immune infiltrate

Supplementary figure 1: Staining specificity of FH.

Paraffin-embedded liver sections was stained with an anti-FH antibody, clone Ox-24 or isotype control. The inhibition of FH staining by pre-incubation of anti-FH antibody with 10 molar equivalent of purified FH (Comptech, A137) reflects the specificity.

Supplementary figure 2: Efficiency of FH silencing

A) FH concentration in the supernatant of A498 cells (siFH, siC or WT) determined by ELISA. **B)** FH concentration in the lysate of A498 cells (siFH, siC) determined by ELISA. **C)** Western blot of FH in the supernatant of A498 cells (siFH, siC) under non reducing conditions. **D)** FH concentration in the supernatant of Caki-1 cells (siFH, siC or WT) determined by ELISA. **E)** FH concentration in the lysate of Caki-1 cells (siFH, siC) determined by ELISA. **F)** Western blot of FH in the supernatant of Caki-1 cells (siFH, siC) under non reducing conditions.

Supplementary Figure 3: FH modifies ccRCC Caki-1 tumor cell phenotype

A) Images (x100) of Caki-1 cells 72h post-transfection with siFH or siC. **B)** Volcanoplot depicting the results of differential expression obtained with DESeq2 analysis between Caki-1 siFH (n=3) and Caki-1 siC (n=3). The volcano plot was generated with R software and EnhancedVolcano package. Red points represented genes with a \log_2 fold change > 1 and $P_{adj} < 0.05$. **C)** Heatmap of \log_2 counts for top 50 differentially expressed genes after silencing of with siFH compared with siC in Caki-1. The most differentially expressed genes are selected on adjusted p-value. Expression across each gene have been row scaled with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. Lighter shades and white represent genes with intermediate expression levels. **D)** Gene ontology analysis of functional annotations (biological process)

up- or downregulated by FH silencing vs. Caki-1 siC. **E)** Histogram representing CFSE intensity on Caki-1 siFH or Caki-1 siC after 72h of proliferation (green = unstained Caki-1 cells, orange = Caki-1 taken at the time of CFSE staining T0, red = Caki-1 siC after 72h of proliferation, blue = Caki-1 siFH after 72h of proliferation). **F)** Fold change of CFSE geometric means in Caki-1 siFH vs. Caki-1 siC on 6 independent experiments. **G)** Percentage of cells in each cell cycle phase (G0-G1/S/G2-M) determined by flow cytometry analysis of PI staining on Caki-1 siFH or siC cells on three independent experiments. **H)** Percentage of DAPI positive dead cells determined by flow cytometry after 72h of culture in Caki-1 siFH or Caki-1 siC in eight independent experiments. **I)** Immunofluorescence staining of phalloïdin (red) in Caki-1 siFH (right) or siC (left).

Supplementary Figure 4: Reduction of plasmatic FH in hepato -/- mice doesn't alter angiogenesis

A) Western blot for FH on plasma from WT (n=3) or hepato -/- mice (n=4). **B)** Western blot for FH on TC-1 supernatant. Mice plasma is used as positive control. **C)** Staining for CD31 (green) on frozen sections of TC-1 tumors at day 24 from WT mice (left) or hepato -/- mice (right). **D)** Expression of angiogenic genes in Tcv-1 tumors from WT (n=4) or FH hepato -/- mice (n=4). Measured by RTqPCR and represented by using $2^{-\Delta Ct}$.

Supplementary Figure 5: Increase complement activation after FH silencing

A) C3 deposits on Caki-1 cell surface after incubation with NHS determined by flow cytometry (green: isotype control, red: Caki-1 siC, blue: Caki-1 siFH). **B)** Western blot for C3 and its fragments in reducing conditions in the supernatant of Caki-1 siFH and Caki-1 siC. **C)** Western blot for FI in reducing conditions in the supernatant of Caki-1 and A498.

Supplementary Figure 6: Intracellular FH is localized on the organelle fraction of Caki-1 cells

A) Histogram representing intracellular FH staining on Caki-1 cells (with permeabilization step) (red= isotype, blue = FH staining). **B)** FH quantification by ELISA on the subcellular fraction of Caki-1 cells (cytoplasmic, organelle and nucleus).

Supplementary Figure 7: Intracellular C3 is mainly localized on the cytoplasmic and nuclei fractions of A498 and Caki-1

C3 dosage by ELISA on the subcellular fractions (cytoplasmic, organelle and nucleus) of A498 (**A**) and Caki-1 (**B**).

Supplementary figure 8: Prognostic value of CFH and CFHL-1 gene expression in TCGA KIRC cohort

A) CFH expression (log₂TPM) in patients with ccRCC (TCGA KIRC) compared to normal kidney tissues from TCGA normal and GTEx database. B-C) Kaplan-meier curves of DFS and OS according to CFH gene expression level using median cut-off on KIRC TCGA cohort. Log-rank test was used and $p < 0.05$ was considered as significant. D) Correlation between gene expression (TPM) of CFH and CFHL-1 determined by calculating Pearson correlation coefficient. Generated with GEPIA <http://gepia.cancer-pku.cn/detail.php> E-F) Kaplan-meier curves of DFS and OS according to CFHL-1 gene expression level using median cut-off on KIRC TCGA cohort. Log-rank test was used and $p < 0.05$ was considered as significant.

Supplementary Figure 9: Efficiency of FH silencing on RPTEC

A) FH concentration in the supernatant of RPTEC cells (siFH, siC) determined by ELISA. B) FH concentration in the lysate of RPTEC cells (siFH, siC) determined by ELISA.

Supplementary Figure 10: No effect of FH produced by HUVEC on their phenotype

A) FH staining on endothelial cells inside ccRCC tumors. B) FH concentration in the lysate and the supernatant of HUVEC determined by ELISA. C) FH concentration in the supernatant of HUVEC cells (siFH, siC) determined by ELISA. D) FH concentration in the lysate of HUVEC cells (siFH, siC) determined by ELISA. E) Western blot of FH in the supernatant of HUVEC cells (siFH, siC) under non reducing conditions. F) Immunofluorescence staining of phalloïdin (red) in HUVEC siFH (right) or siC (left). G) Analysis of migration capacity of HUVEC siFH cells and RPTEC siC cells. A scratch on RPTEC monolayer was realized at T₀ and photo of the scratch area were taken. After 12h, a photo of the same area was taken to compare the migration capacity. ImageJ Wound healing macro was used to determine the area of the scratch at each time point. The percentage of closing of the scratch area was calculated with the following calculation; $100 - (\text{scratch area T}_0 / \text{scratch area t+12h})$.

Supplementary Figure 11: Increase complement activation after FH silencing on A549

A) FH concentration in the supernatant of A549 cells (siFH, siC) determined by ELISA. B) FH concentration in the lysate of A549 cells (siFH, siC) determined by ELISA. C) Western blot of FH in the supernatant of A549 cells (siFH, siC) under non reducing conditions. D) C3 deposits on A549 cell surface after incubation with NHS determined by flow cytometry (green: isotype control, red: A549 siC, blue: A549 siFH). E) Western blot for C3 and its

fragments in reducing conditions in the supernatant of A549 siFH and A549 siC. **F)** C3 dosage by ELISA on the subcellular fractions (cytoplasmic, organelles and nucleus) of A549

Supplementary Figure 12: No effects of FH silencing on SK-MES cells

A) FH concentration in the supernatant of SK-MES cells (siFH, siC) determined by ELISA. **B)** FH concentration in the lysate of SK-MES cells (siFH, siC) determined by ELISA. **C)** Images (x100) of SK-MES cells 72h post-transfection with siFH or siC and stained with crystal violet. **D)** Histogram representing CFSE intensity on SK-MES siFH or SK-MES siC after 72h of proliferation (green = unstained SK-MES cells, orange = SK-MES taken at the time of CFSE staining T0, red = SK-MES siC after 72h of proliferation, blue = SK-MES siFH after 72h of proliferation). **E)** Fold change of CFSE geometric means in SK-MES siFH vs. SK-MES siC on 2 independent experiments. **F)** Percentage of cells in each cell cycle phase (G0-G1/S/G2-M) determined by flow cytometry analysis of PI staining on SK-MES siFH or siC cells on three independent experiments. **G)** Percentage of DAPI positive dead cells determined by flow cytometry after 72h of culture in SK-MES siFH or SK-MES siC in four independent experiments. **H)** Immunofluorescence staining of phalloïdin (red) in SK-MES siFH (right) or siC (left). **I)** C3 deposits on SK-MES cell surface after incubation with NHS determined by flow cytometry (green: isotype control, red: SK-MES siC, blue: SK-MES siFH).

Supplementary Figure 13: Pan cancer prognostic impact of FH gene expression

Survival map representing *CFH* hazard ratio in 29 cancer types from the TCGA PanCancer atlas. The survival analysis was performed by using Gene Expression Profiling Interactive Analysis (GEPIA) tool⁵⁴. Overall survival (OS) analysis based on gene expression with a median cut-off was used to calculate hazards ratio based on Cox PH Model and Log-rank p-value. The heatmap representing the log₂ HR with a scale centered in 0 was generated by using the R package “pheatmap” and cancer types were sorted in ascending order. The surrounded boxes correspond to a log-rank p-value <0.05. . 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.

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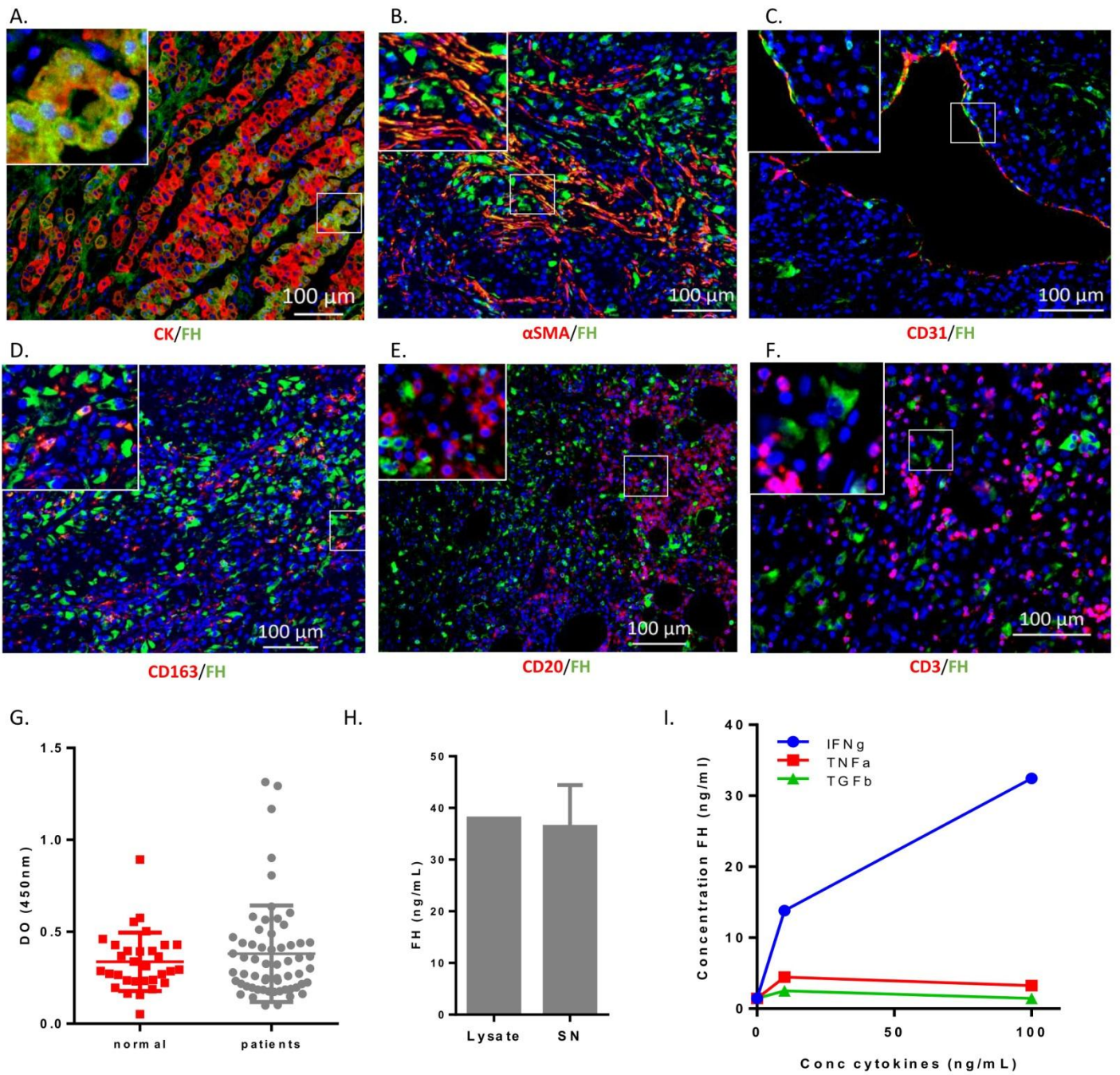


Figure 1: Tumor cells are the main producer of FH in ccRCC tumor

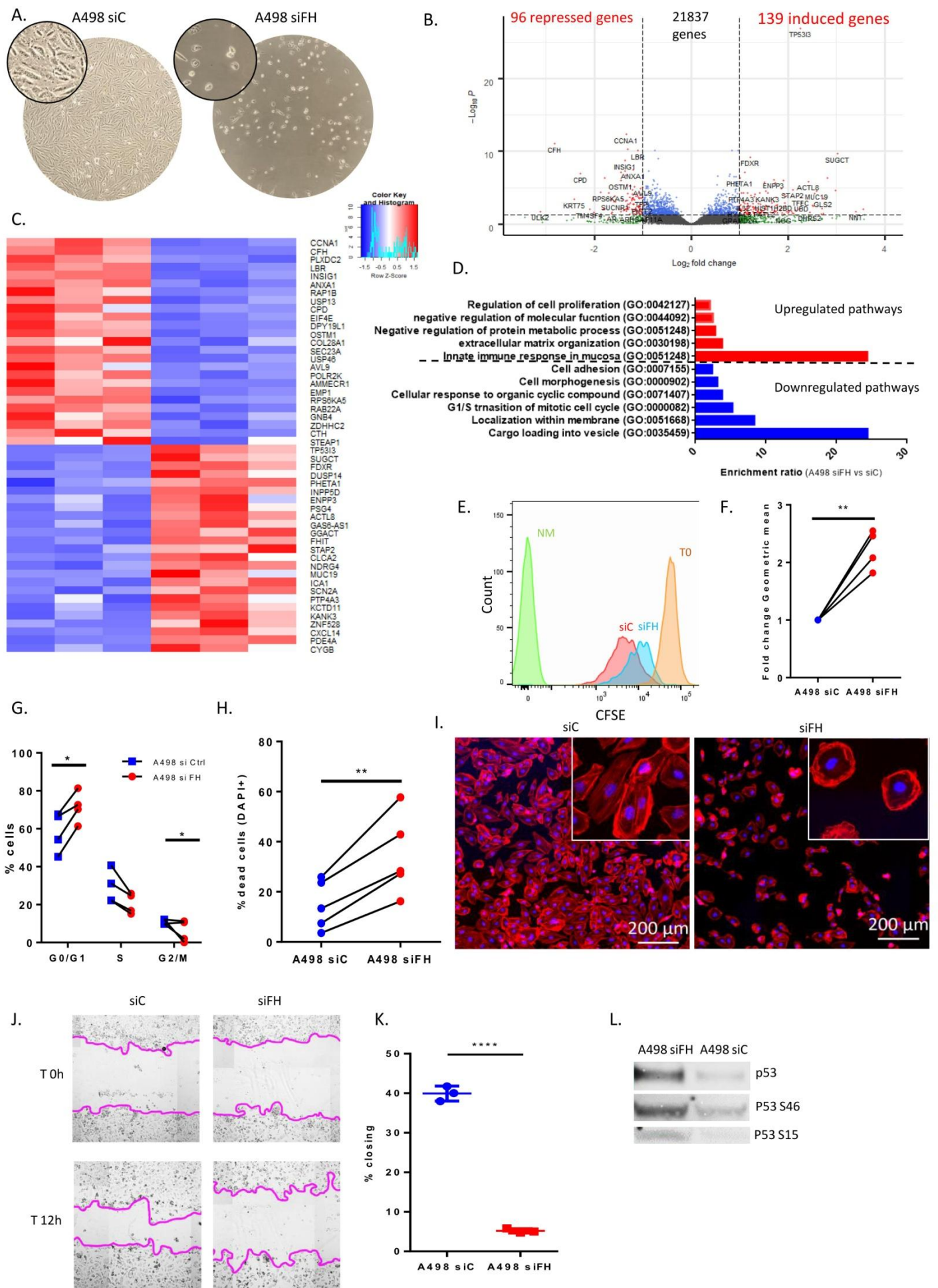


Figure 2: FH modifies tumor cell phenotype

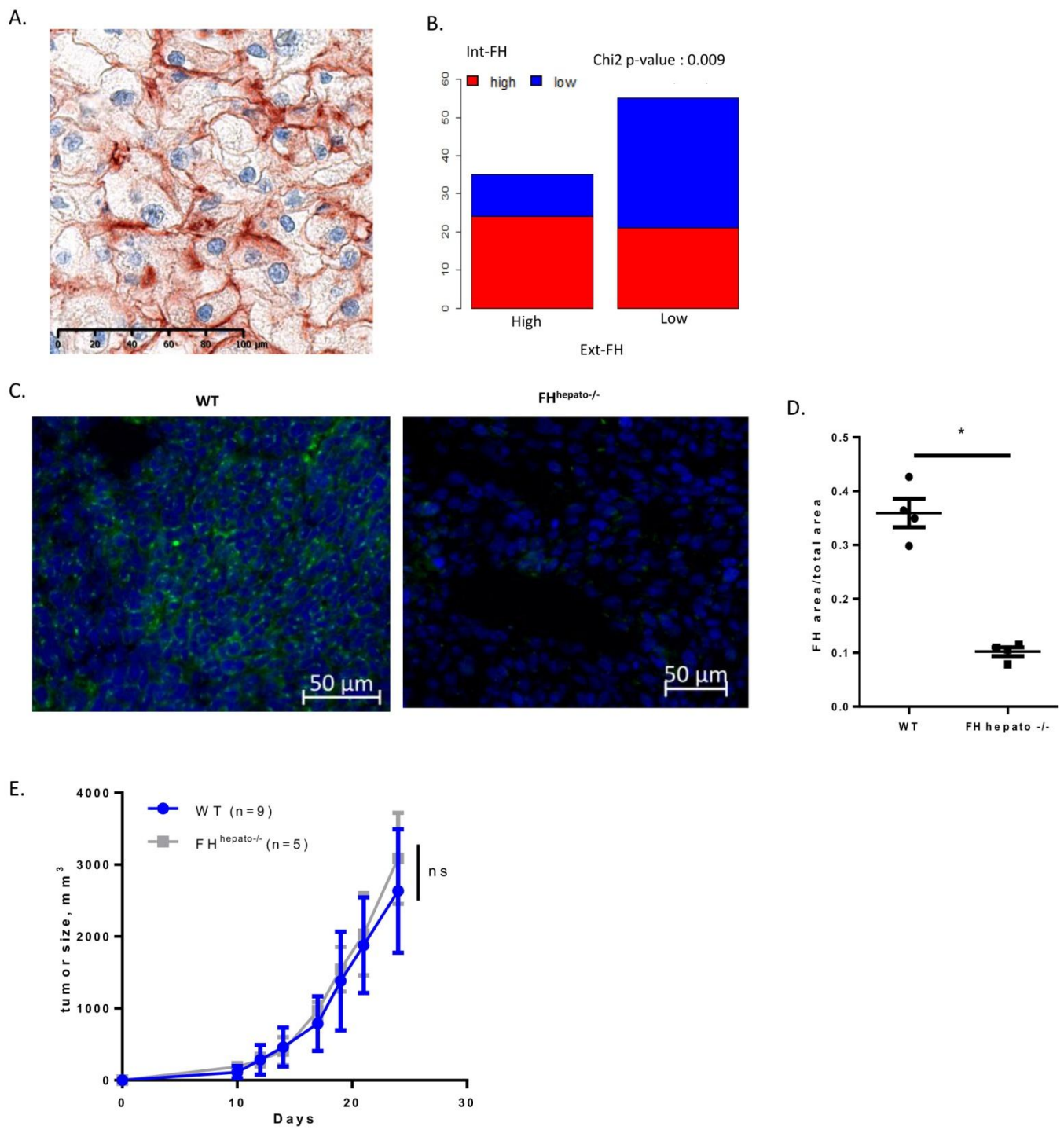


Figure 3: Extracellular FH comes from the circulation and the local production by tumor cells.

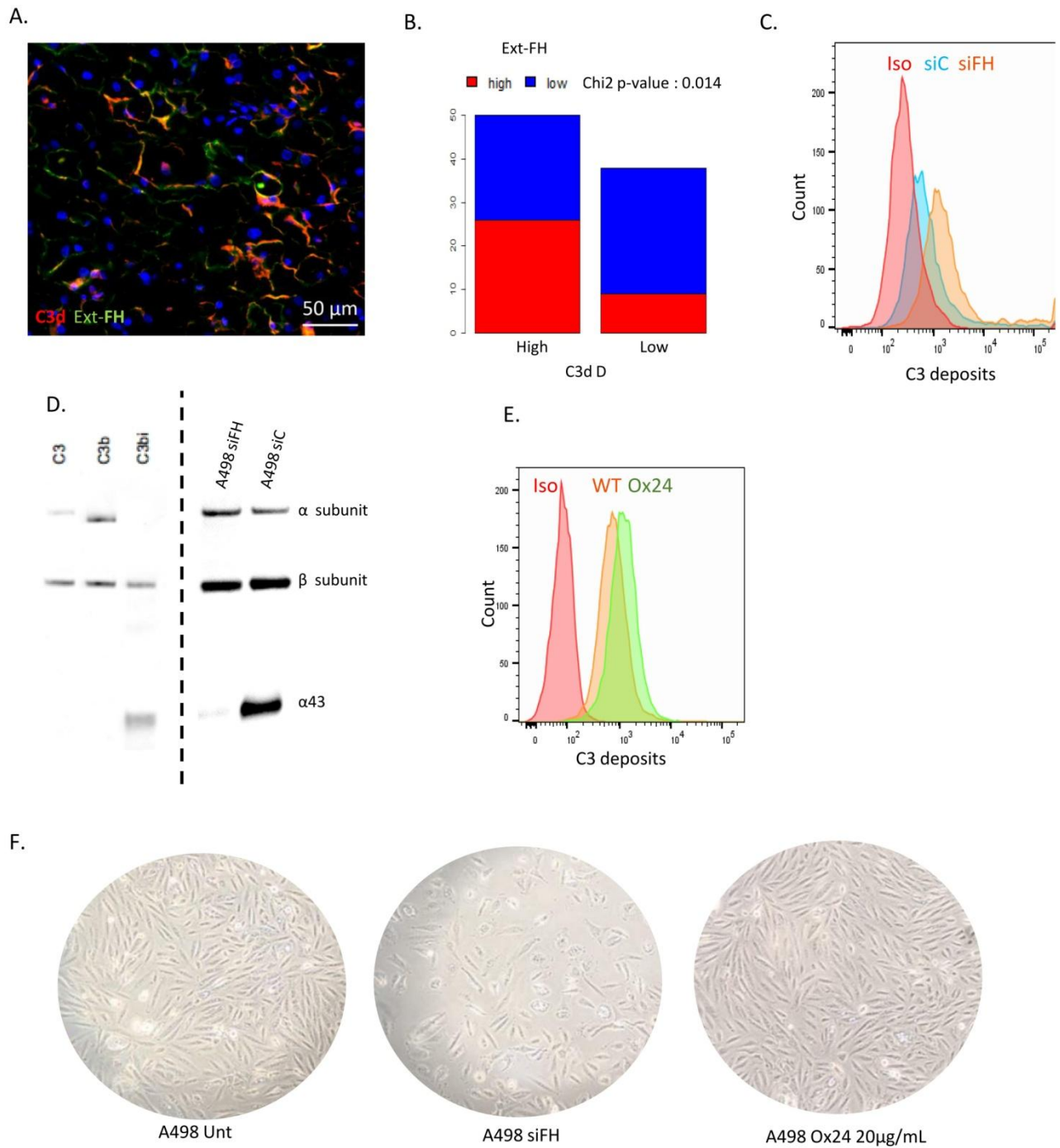


Figure 4: Extracellular FH acts as complement regulator

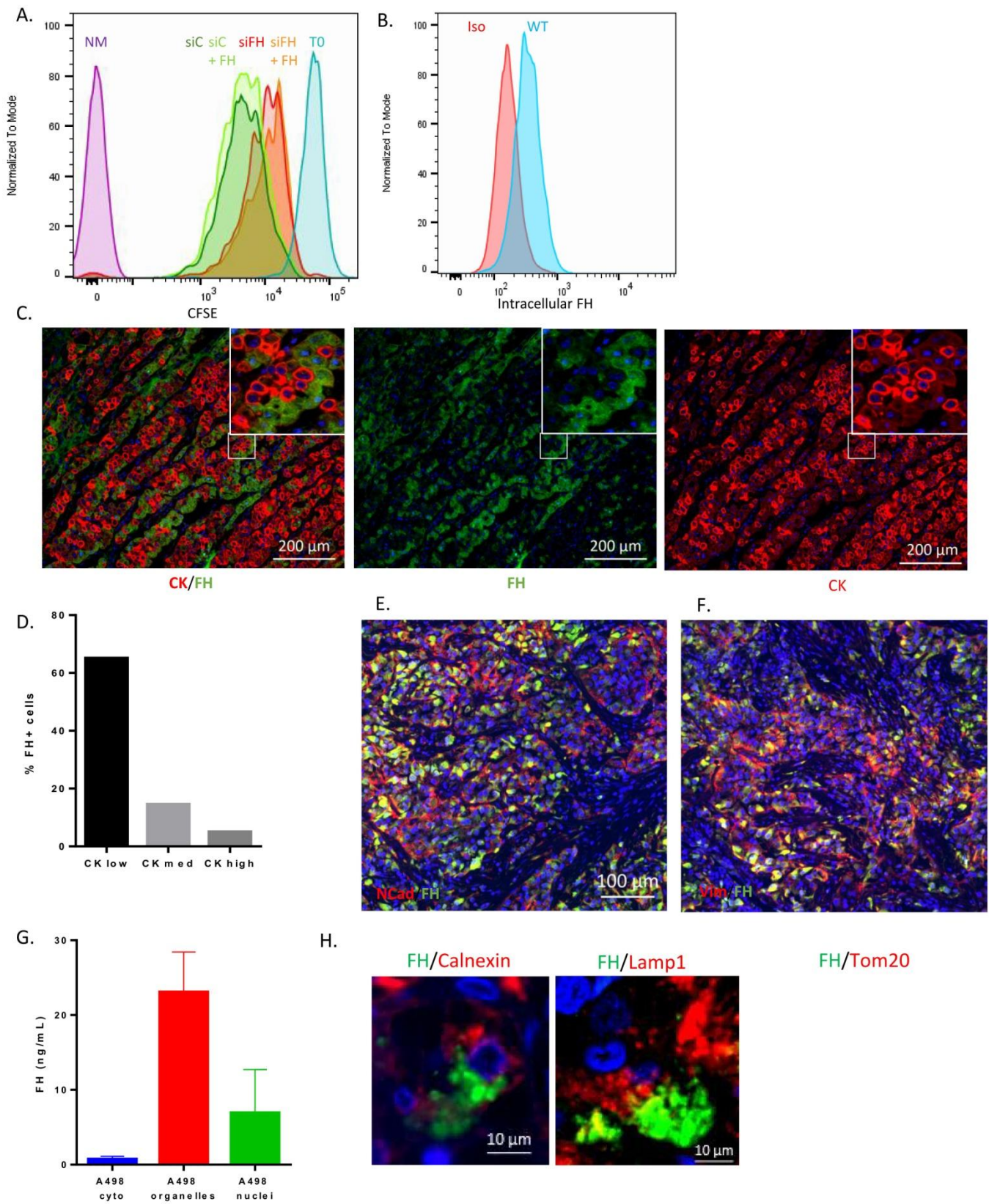


Figure 5: Intracellular FH is produced by specific vesicles within tumor cells

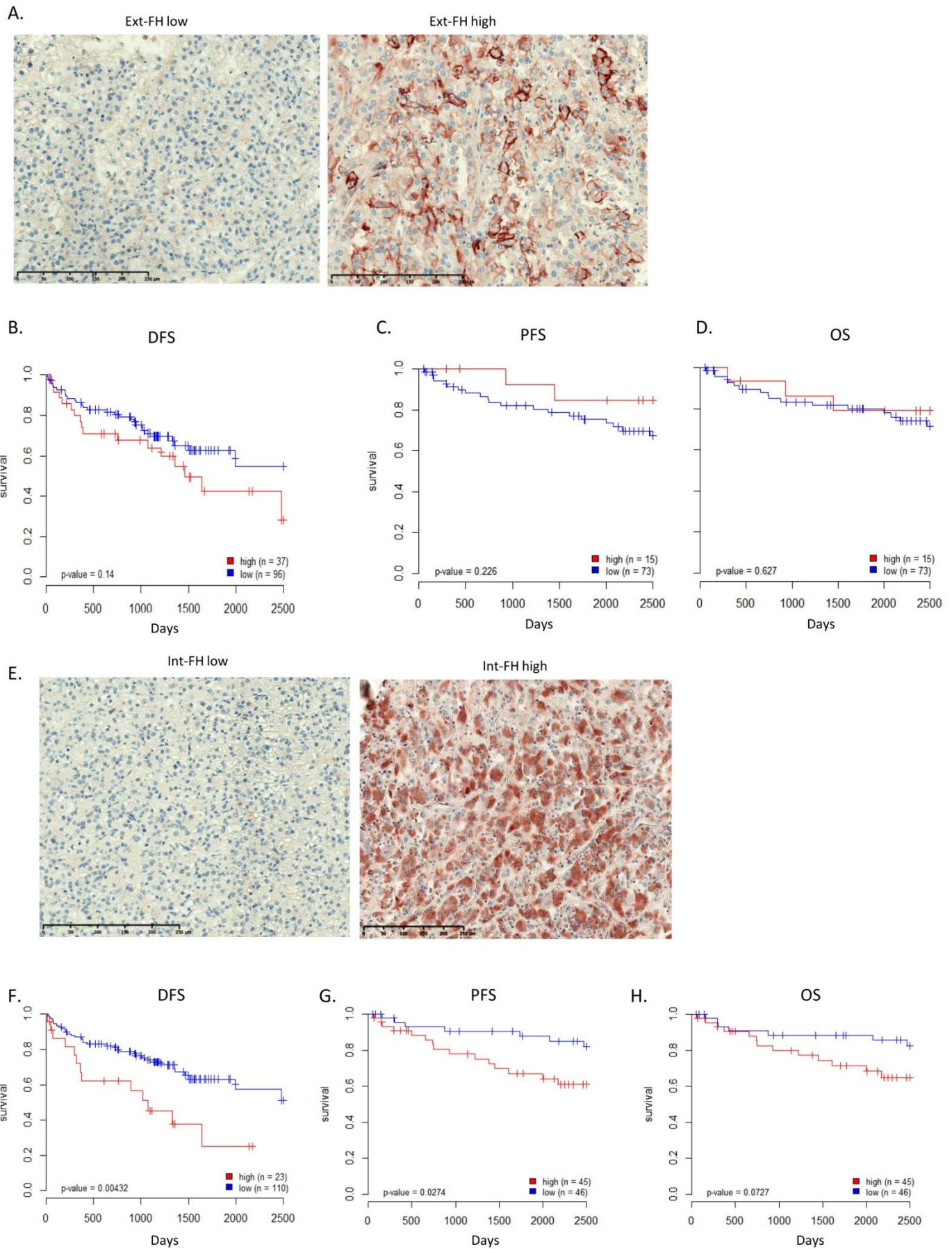


Figure 6: Intracellular FH produced by tumor cells but not extracellular FH is associated with poor prognosis

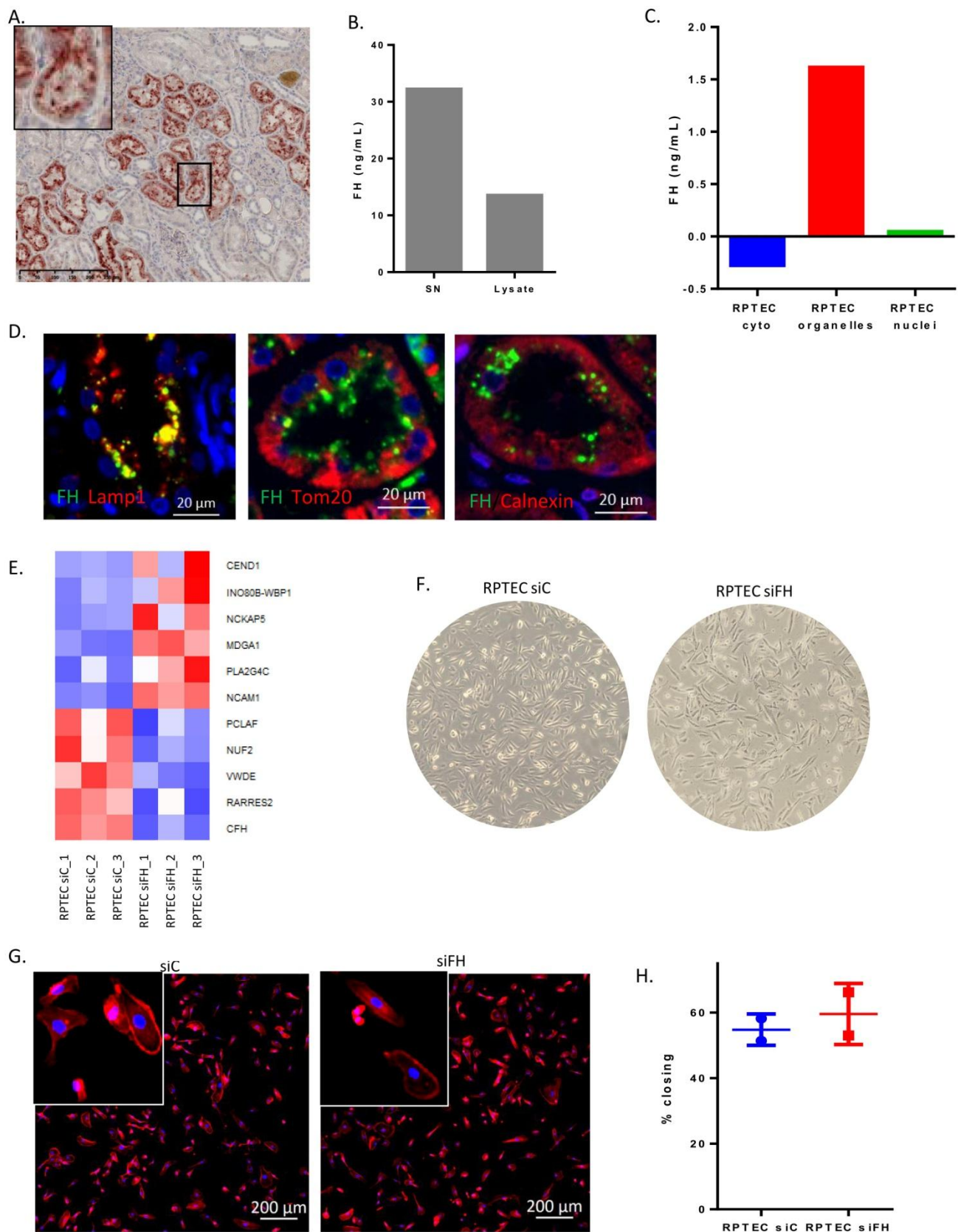


Figure 7: No effect of FH produced by renal proximal tubule cells (RPTEC)

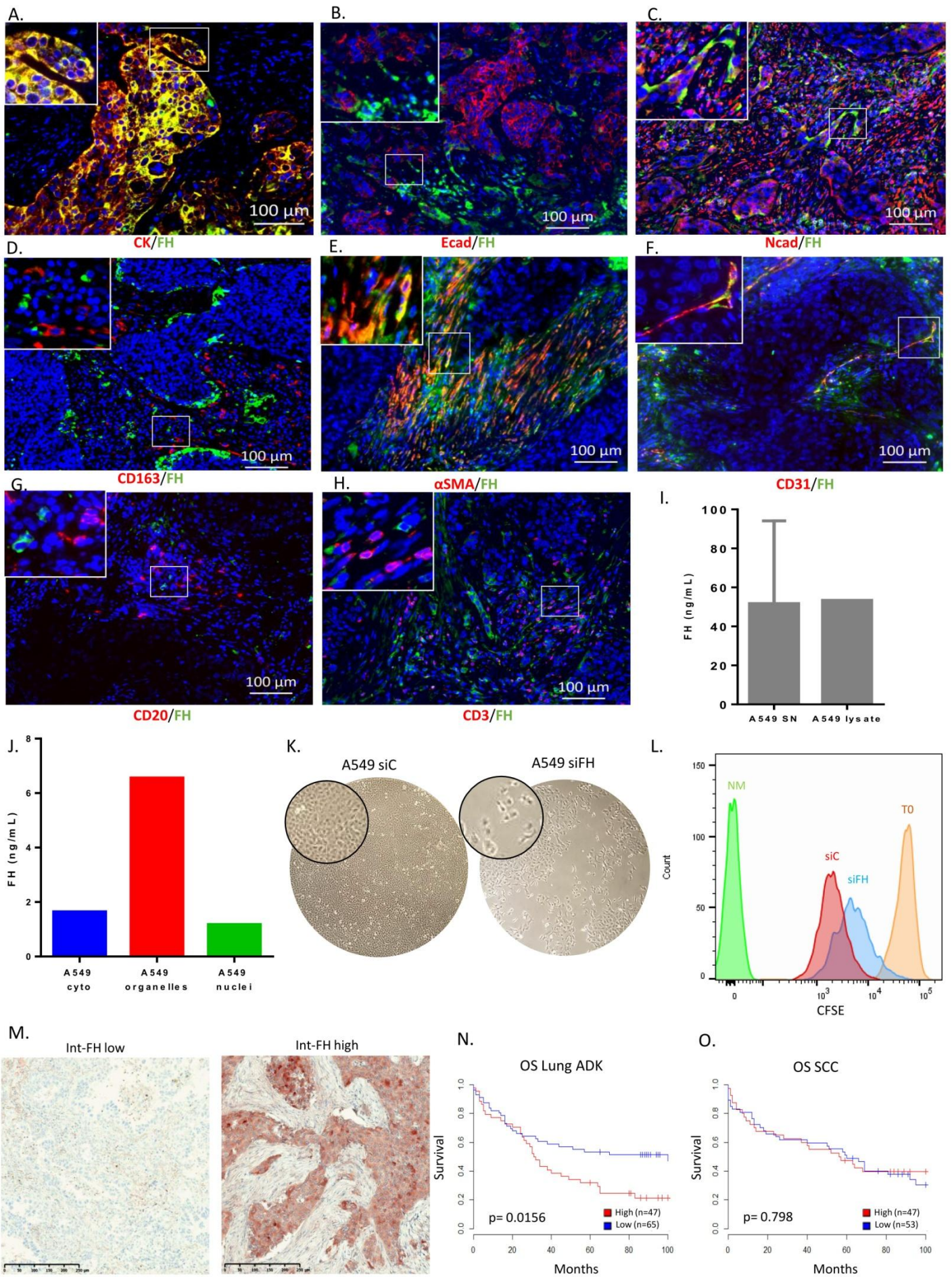


Figure 8: FH exerts pro-tumoral effect in lung adenocarcinoma

Supplementary

	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Histologic type	ccRCC	ccRCC	ADK(112), SCC (100)	ccRCC
Type	Retrospective	Retrospective	Retrospective	Prospective
Number of patients	133	91	212	61
Age (median)	64	63	61,5	67
Sex (F/M)	43/90	23/68	39/173	20/35/6
Fuhrman grade (1/2/3/4)	1/32/85/15	5/20/54/11/1	NA	1/36/13/5/6
Tumor stade (1/2/3/4)	54/6/70/3	40/5/34/12	79/59/68/5/1	39/5/9/2/6

Supplementary table 1 : Cohort description

Antigen	Clone	Species	Ref	Antigen retrieval pH	Dilution	Secondary Ab	Tyramide
FH	Ox-24	Mouse	Hybridoma		61:500	HRP labelled polymer anti-mouse, Dako K4001	AF647
C3d	Polyclonal	Rabbit	Dako, A0063		61:1000	HRP labelled polymer anti-rabbit, Dako K4002	AF546
Cytokeratin	AE1/AE3	Mouse	Dako, M3515		61:200	HRP labelled polymer anti-mouse, Dako K4001	AF546
α SMA	1A4	Mouse	Dako, M0851		61:400	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD31	1A10	Mouse	Leica, PA0250		91:400	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD163	10D6	Mouse	Dako, Mob460		91:200	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD20	L26	Mouse	Dako, M0755		61:1000	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD3	2GV6	Rabbit	Ventana, 790-4341		91:200	HRP labelled polymer anti-rabbit, Dako K4002	AF546
N-cadherin	EPR1791-4	Rabbit	Abcam, ab76011		61:200	HRP labelled polymer anti-rabbit, Dako K4002	AF546
E-cadherin	NCH-38	Mouse	Dako, M3612		91:200	HRP labelled polymer anti-mouse, Dako K4001	AF546
Vimentin	V9	Mouse	Dako, M0725		61:500	HRP labelled polymer anti-mouse, Dako K4001	AF546
Lamp-1	D4O1S	Mouse	Cell signaling, 15665		91:100	HRP labelled polymer anti-mouse, Dako K4001	AF546
Calnexin	C5C9	Rabbit	Cell signaling, 2679		61:100	HRP labelled polymer anti-rabbit, Dako K4002	AF546
Tom20	F-10	Mouse	sc-17764		91:100	HRP labelled polymer anti-mouse, Dako K4001	AF546

Supplementary table 2 : List of antibodies for immunofluorescence

	Ref	Dilution
p53	Sc-126	1:200
p53 pS15	R&D, AF1043	1:200
p53 pS46	R&D, MAB1489	1:200

Supplementary table 3 : List of antibodies for western blot

Cohort 1

Correlation (test Chi2)

	FH cyto HiLo
Sex	0,602
pT	0,03
N	0,924
Stage UICC	0,078
Grade Fuhrman	0,229
C4 cyto	0
C4 depot	0,066
Ext-FH	0,002
CD8 IM	0,22
CD8 TC	0,85
CD8 IMTC	0,91

Cohort 2

Correlation (test Chi2)

	FH cyto HiLo
Sex	0,589
pT	0,651
N	0,602
MET	0,374
Stage UICC	0,565
Grade Fuhrman	0,034
PDL1	0,906
PDL2	0,245
C4 cyto	0,019
C4 depot	0,373
C3 cyto	0
C3 depot	0,014
Ext-FH	0,019

Correlation (test Kruskal-Wallis)

	FH cyto HiLo
DC	1
CD8	0,12
PD1	0,07
LAG3	0,16
CD163	0,92

Cohort 3

Correlation (test Chi2)

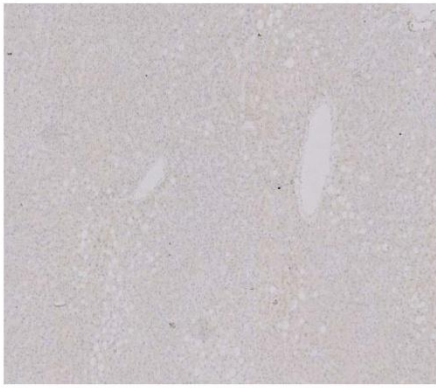
	FH cyto HiLo
Sex	0,271
Post chimio	1
Histological type	0,758
ADK Grade	0,562
TTF1	0,665
TNM Stage	0,189
Pleura	0,111
Emboli	0,673
COPD status	0,684
Smoking status	0,621

Correlation (test Kruskal-Wallis)

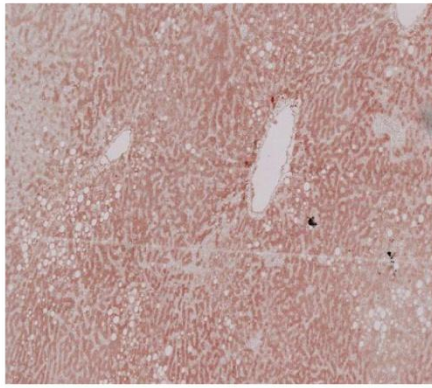
	FH cyto HiLo
NK	0,21
DC lamp	0,15
CD8	0,46
B cells	0,32
CD68	0,61
CD66b	0,31
CRT	0,33

Supplementary table 4: Correlation between FH staining High low and clinical, histopathologic features and immune infiltrate

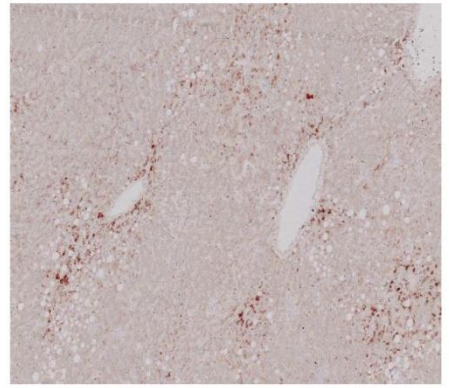
Isotype



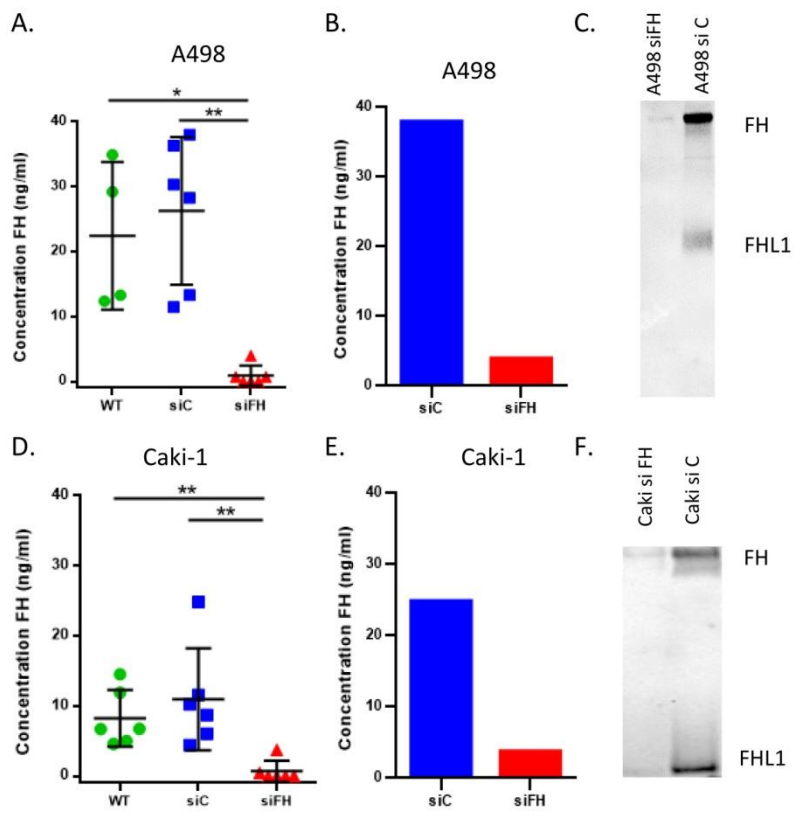
Ox24



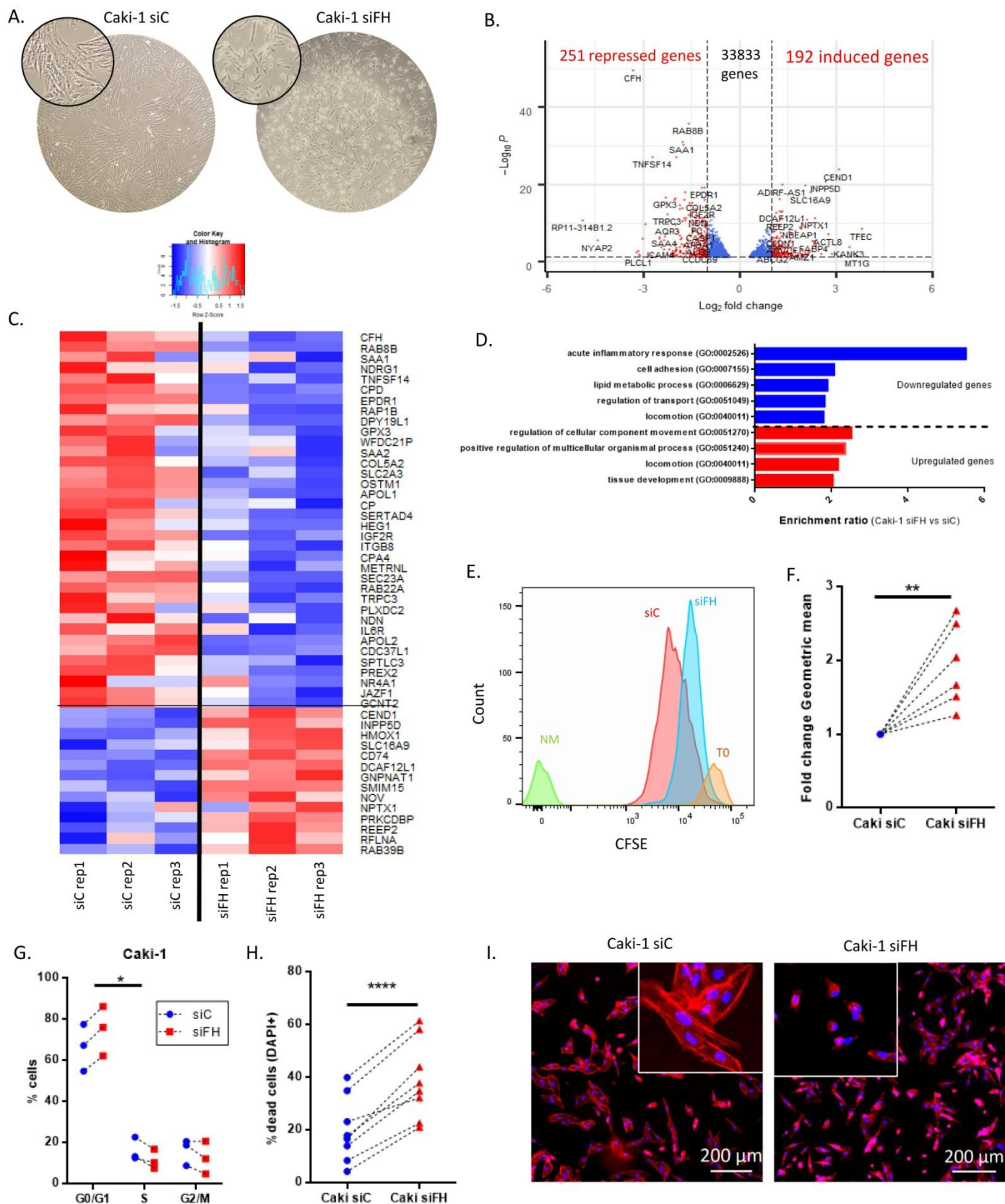
Ox24 + purified FH 1:10



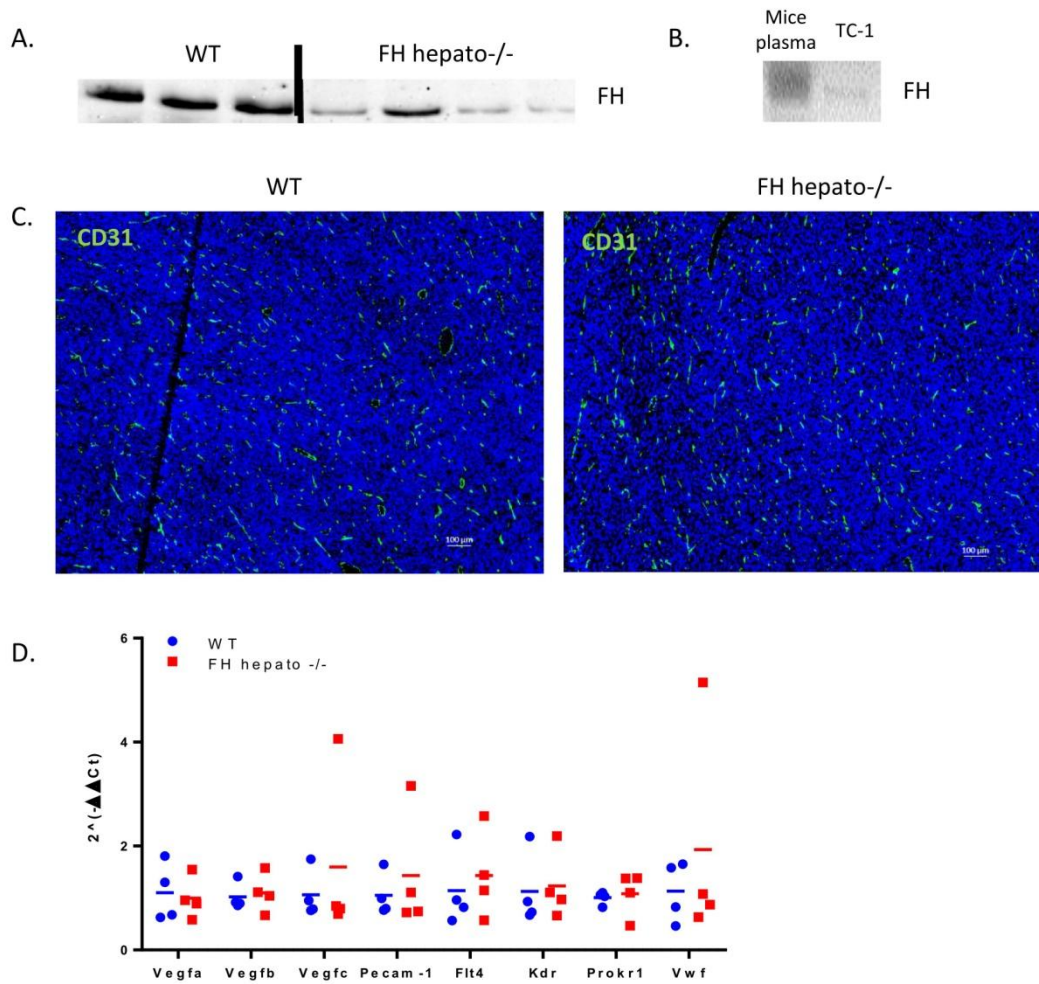
Supplementary figure 1 : Staining specificity of FH.



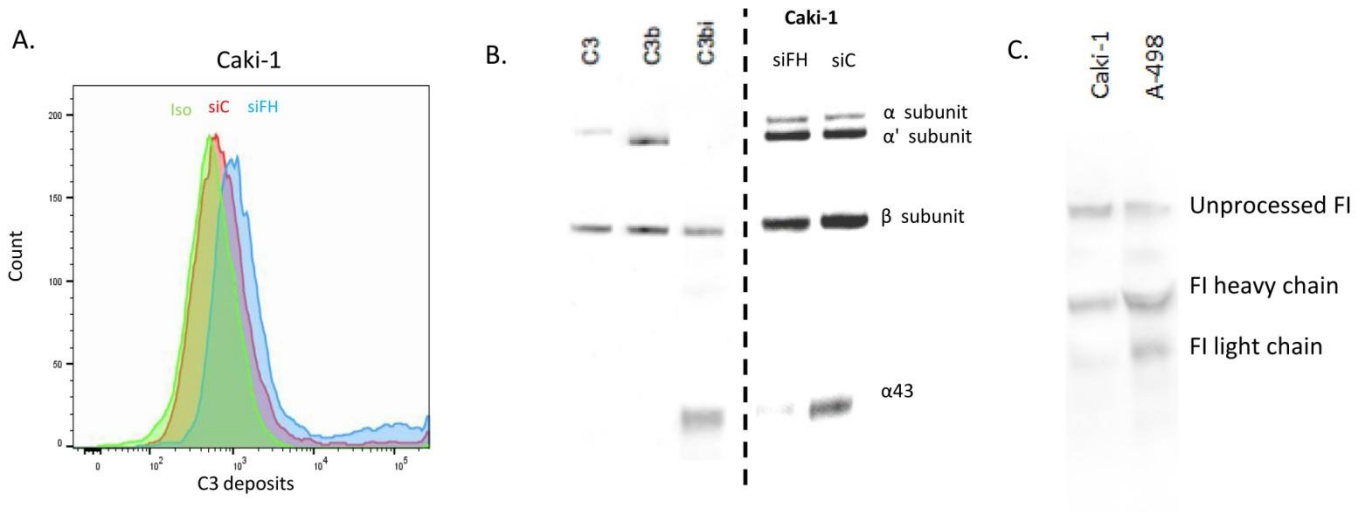
Supplementary figure 2: Efficiency of FH silencing on ccRCC cell lines



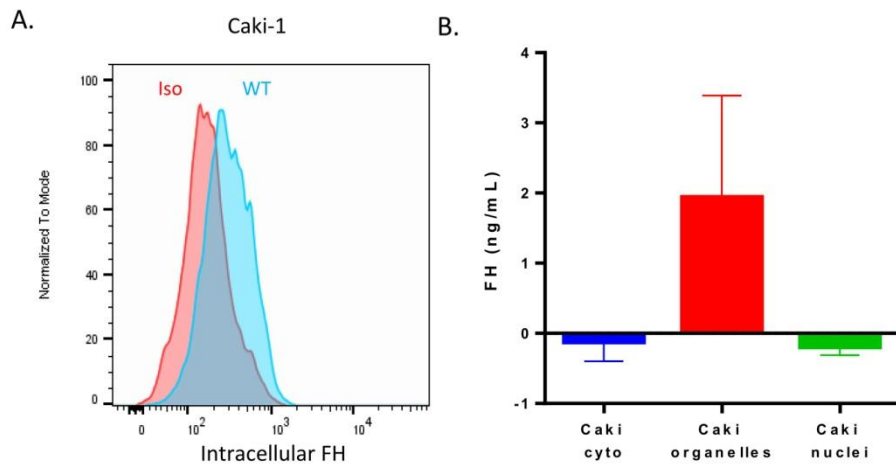
Supplementary Figure 3: FH modifies ccRCC Caki-1 tumor cell phenotype



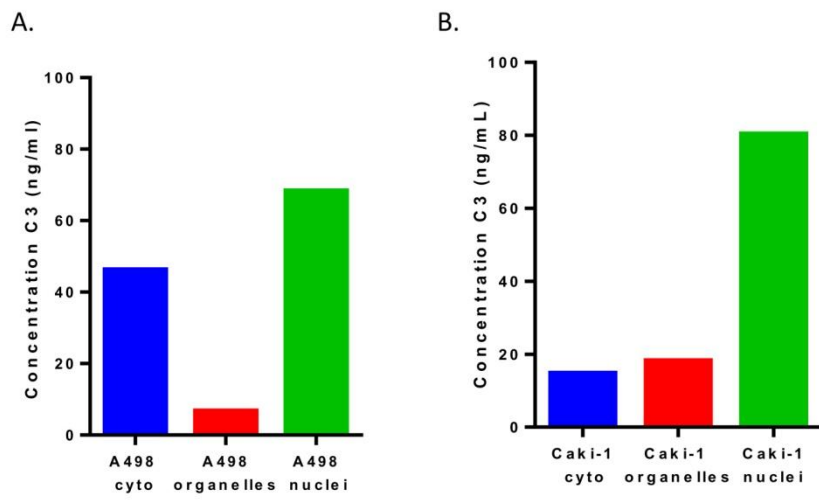
Supplementary Figure 4: Reduction of plasmatic FH in hepato^{-/-} mice doesn't alter angiogenesis



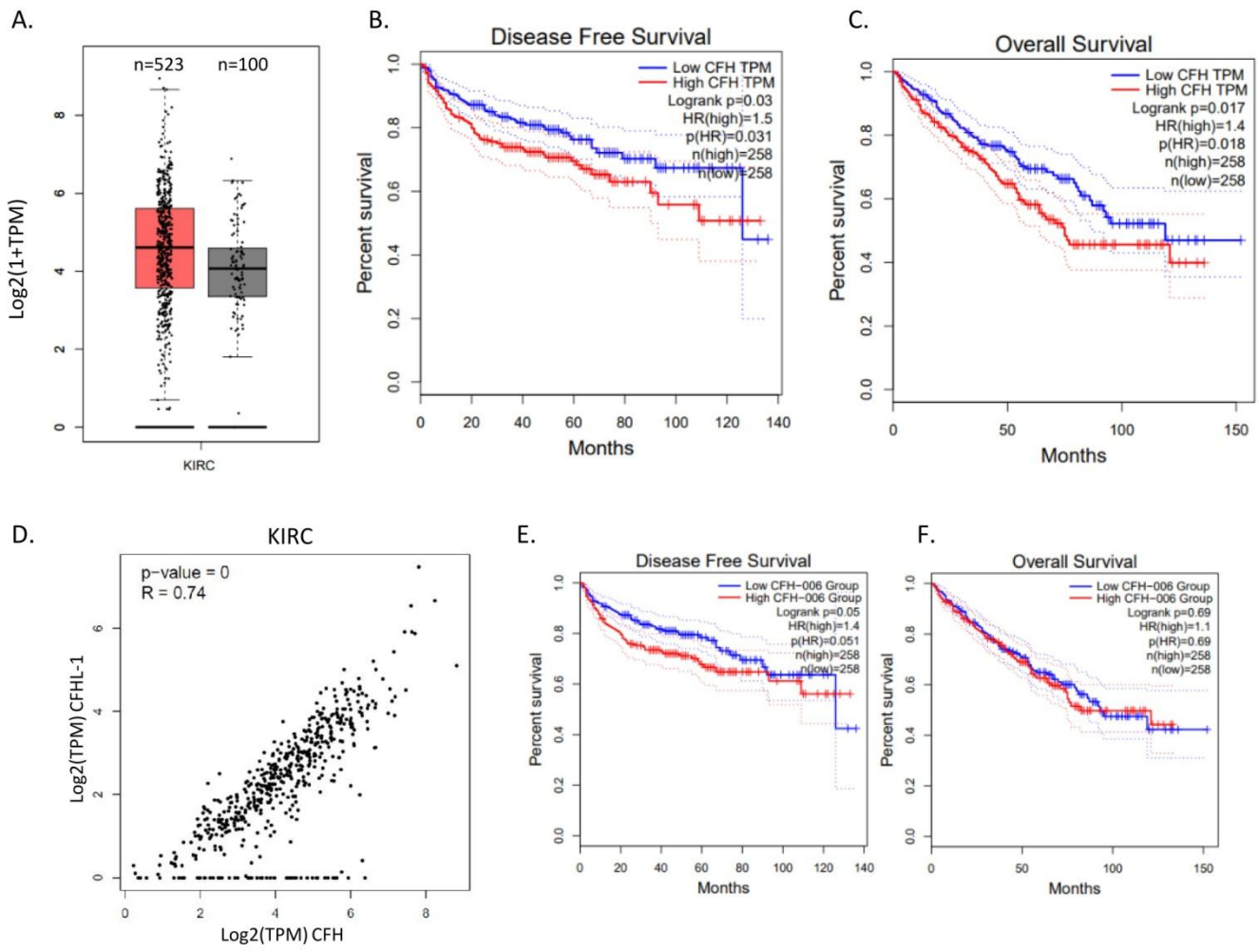
Supplementary Figure 5: Increase complement activation after FH silencing



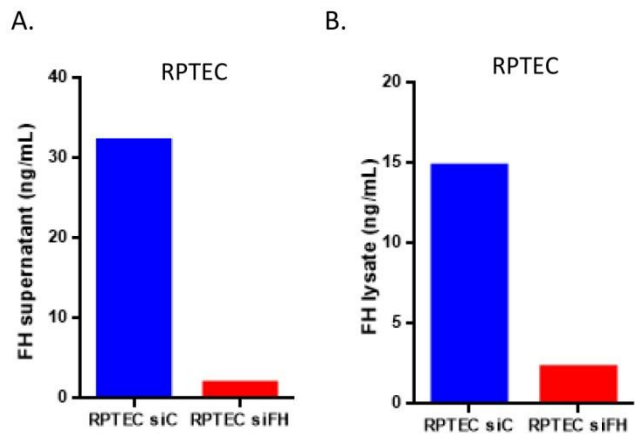
Supplementary Figure 6: Intracellular FH is localized on the mitochondrial fraction of Caki-1 cells



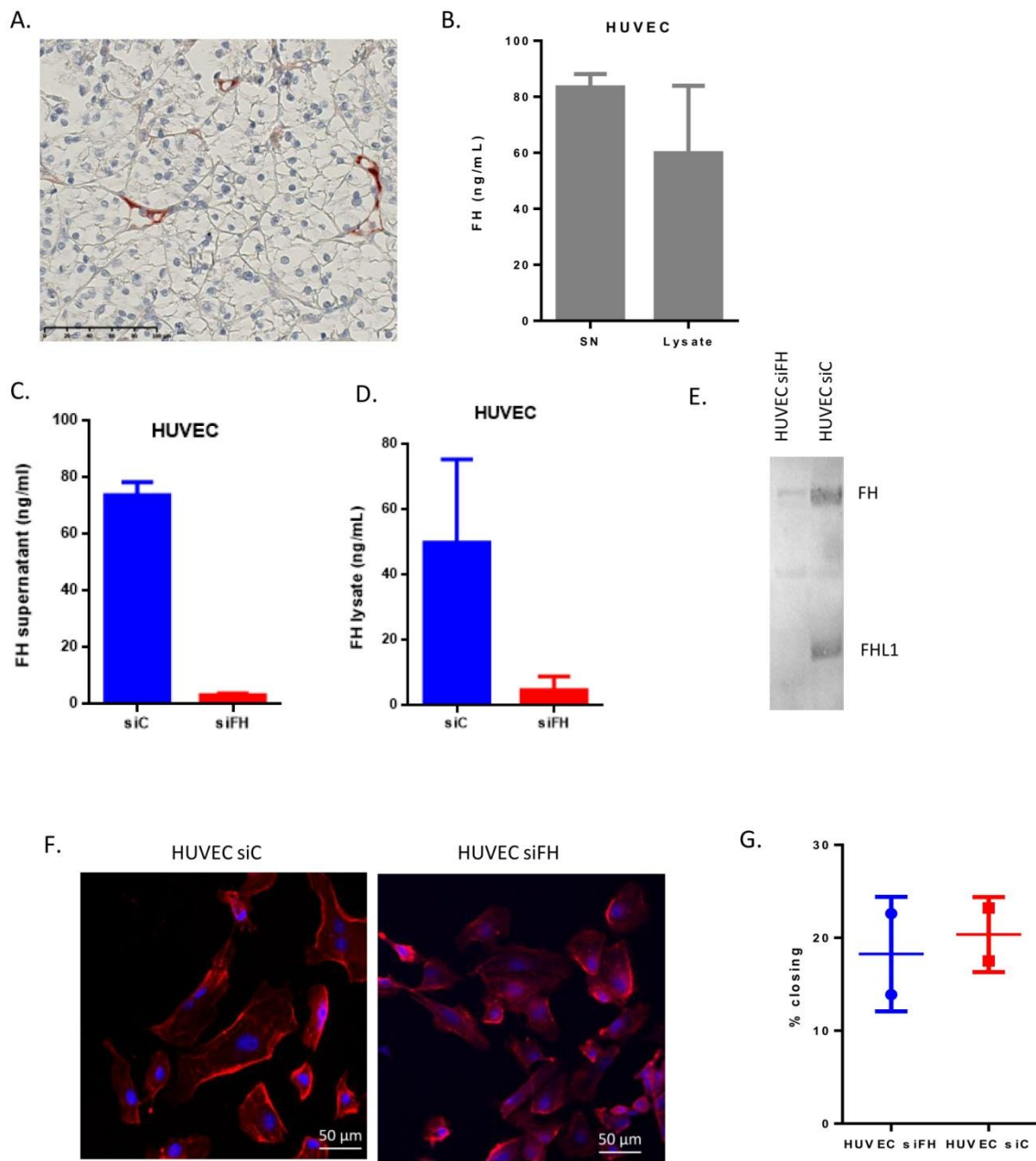
Supplementary Figure 7: Intracellular C3 is mainly localized on the cytoplasmic and nuclei fractions of A498 and Caki-1



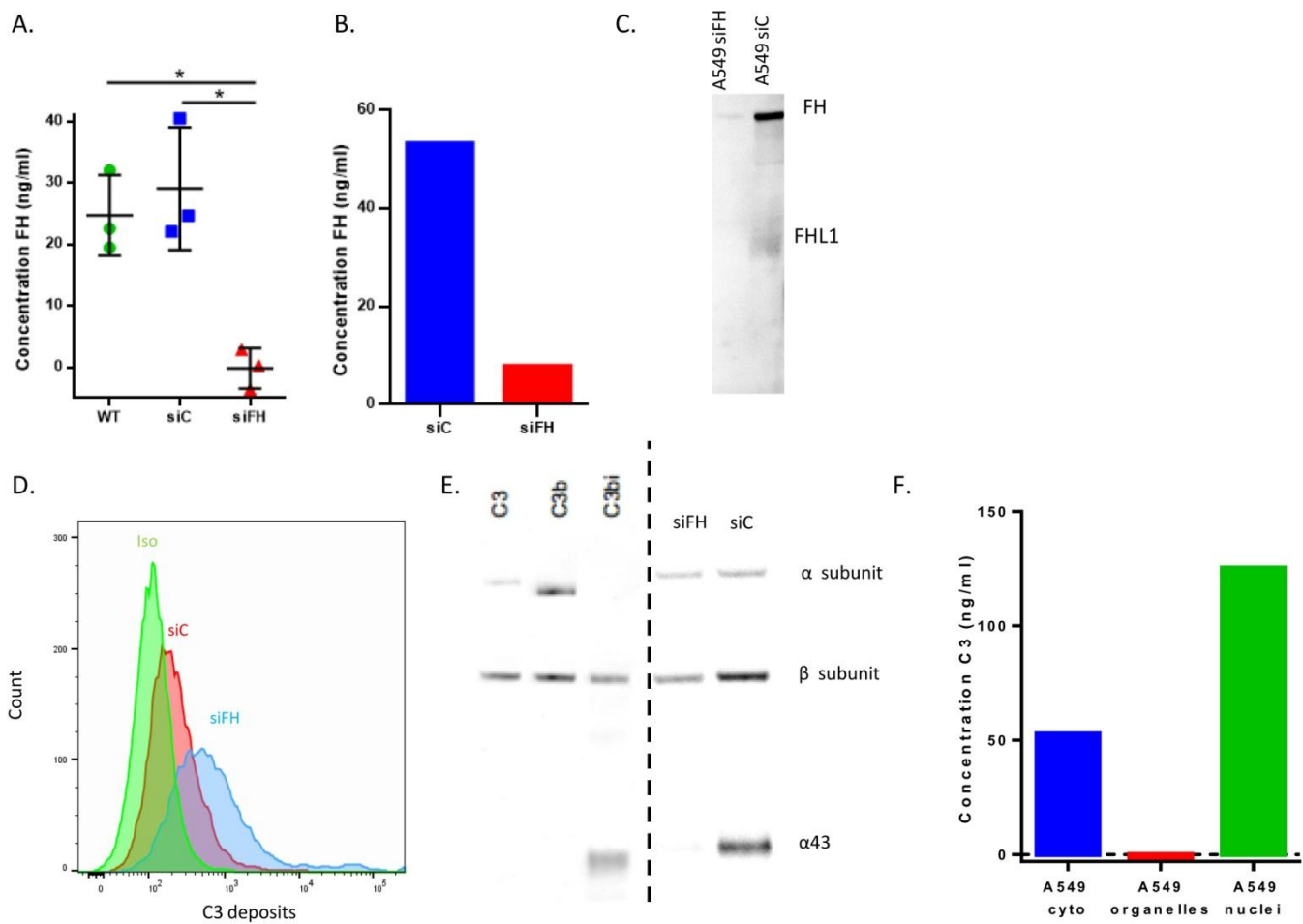
Supplementary figure 8: Prognostic value of CFH and CFHL-1 gene expression in TCGA KIRC cohort



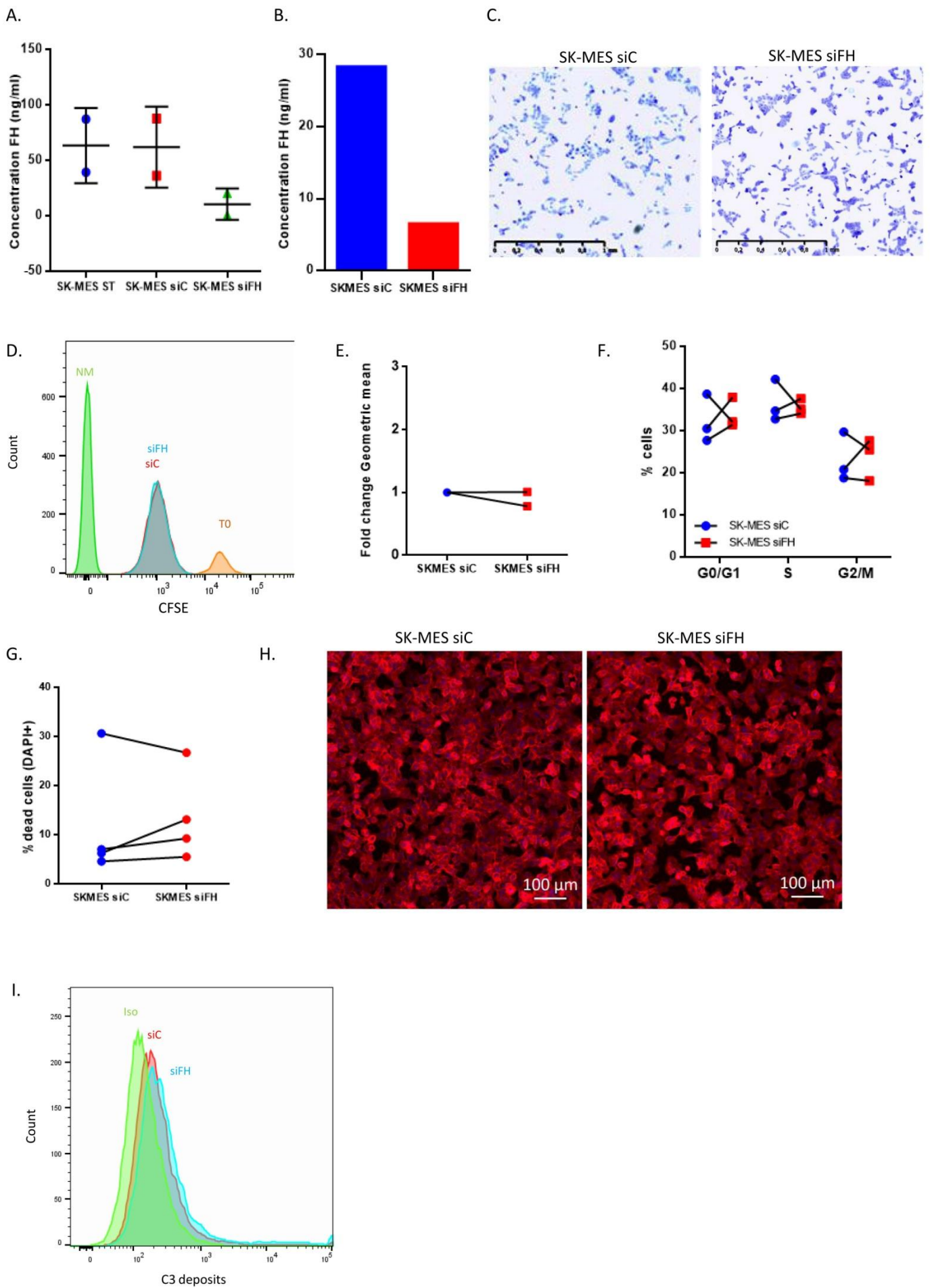
Supplementary figure 9: Efficiency of FH silencing on RPTEC



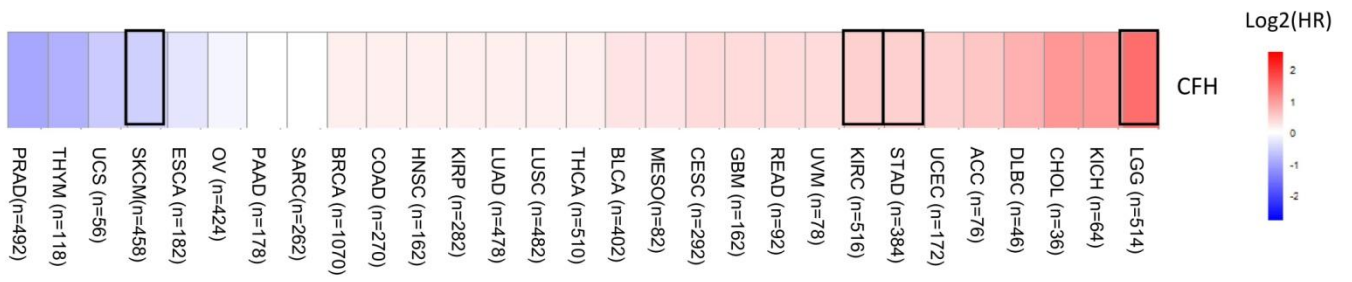
Supplementary Figure 10: No effect of FH produced by HUVEC on their phenotype



Supplementary Figure 11: Increase complement activation after FH silencing on A549



Supplementary Figure 12: No effects of FH silencing on SK-MES cells



Supplementary Figure 13: Pan cancer prognostic impact of FH gene expression

MANUSCRIPT 5: Marie V. Daugan, Margot Revel, ... , Oliver Kepp, Allan Sauvat, Virginie Verkarre, Stephane Marie Oudard, Arnaud Mejean, Pierre Validire, Xavier Cathelineau, Raphael Sanchez-Salas, Diane Damotte, Isabelle Cremer, Catherine Sautès-Fridman, Wolf H. Fridman, Lubka T. Roumenina' " Complement C1s promotes clear cell renal cell cancer progression through classical pathway and non-canonical action on tumor cells " – *In preparation*

Summary and novelty: In the 90's, the role of C1s in malignant transformation was firstly evoked, when C1s knock-in fibroblasts gained tumorigenic capacity. Since then, only few studies have been made to understand the role of C1s in tumor progression. In this manuscript, we show that C1s is mainly produced by tumor cells and fibroblasts in ccRCC and associated with a poor prognosis for patients. The production of C1s enters into a particular, complement-rich phenotype, of the tumor cells, since they express simultaneously other complement components, such as C1r, C3 and C4. This local complement production allows the activation of classical pathway but C1s also exerts non-canonical action by modulating tumor cell phenotype (proliferation, survival). C1s deficient cell lines resulted in decreased tumor growth in a syngeneic mouse model, compared to their parental, C1s-producing counterparts. Thereofre, C1s appears as a key player in the tumor progression, acting both within and outside of the complement cascade.

Complement C1s promotes clear cell renal cell cancer progression through classical pathway and non-canonical action on tumor cells

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Introduction

Human C1s is a glycoprotein with enzymatic activity mostly found in plasma that allows the initiation of the classical pathway of the innate immune complement cascade. It interacts with C1q and C1r to form the C1 complement-initiating complex¹. Hepatocytes constitute the main source of C1s, but within tissues many other cells types participate in C1s production, such as fibroblasts, chondrocytes, dendritic cells², monocytes/macrophages^{3,4} as well as some epithelial cells⁵. This local synthesis can be stimulated by inflammatory cytokines like IFN γ , IFN β 1, TNF α ⁶ or by cell density⁷.

The canonical role of C1s (i.e. its functioning within the cascade) as a complement activator allows an effective control of pathogen invasion⁸. Activation is triggered by the recognition of immune complexes, surface-bound pentraxins or specific ligands on the pathogen surface by C1q and subsequent C1s-mediated cleavage of the substrate components C4 and C2⁹⁻¹². Nonetheless, with accumulating evidences of the last 10 years it becomes evident that the functions of the complement system extend far beyond pathogen clearance¹³ and that it plays a prominent role in tumor progression¹⁴.

Pan-cancer transcriptomic analysis of complement genes revealed that C1s is heterogeneously expressed in many tumor types¹⁴. Like in normal tissues, it has been described that C1s can be produced by fibroblasts in different tumor types such as prostate¹⁵ and colorectal cancer¹⁶. However, the contribution of tumor cells in C1s local concentration is unclear. In urothelial cancer (UC) as well as cutaneous squamous cell carcinoma (cSCC) and melanoma⁷, C1s can be synthesis by tumor cells, especially in advanced stages. Intriguingly, in UC this overexpression predicted poor disease-specific and metastasis-free survival rates but the exact mechanism behind this effect remains to be elucidated.

Classical pathway activation, measured by deposits of C4 activation fragments in tumor sections, is a poor prognostic marker in non-small cell lung cancer (NSCLC)¹⁷ and clear cell renal cell carcinoma¹⁸ (ccRCC, KIRC), implying a canonical role of C1s in this process. Indeed, in RCC, tumor cells that produced C1s and C1r can hijack C1q produced by macrophages to initiate locally classical complement pathway in presence of IgG deposition on tumor cell surface.

Considering that C1s has many substrates exceeding the borders of the classical pathway, a potential action outside the cascade could participate to its effect on tumor progression. Indeed, C1s may cleave components of the extracellular matrix (decorin, type 1 gelatin and type 2 collagens^{19,20} as well as MMP9 in cartilage) and nuclear antigens released in the extracellular space in the context of autoimmunity (HMGB1, nucleophosmin, nucleolin²¹). Moreover, upon C1q binding to Frizzled receptors and C1s activation, the latter cleaves lipoprotein receptor-related protein 6 to trigger aging-associated Wnt receptor signaling. C1s can also cleave MHC class I², the basic fibroblast growth factor²², insulin-like growth factor

binding protein-5²³, etc. Therefore if C1s is available within a tumor, it may exert non-canonical functions in the tumor microenvironment, which are unrelated to the complement cascade. In support of this hypothesis, a recent study has shown that C1s was overexpressed in cSCC tumors, compared to normal skin and its knockdown in cSCC cell lines inhibited the phosphorylation of ERK1/2 and Akt, promoted apoptosis and significantly suppressed growth and vascularization of human cSCC xenograft tumors in vivo. This finding relates to a study from the 90s, showing that mouse fibroblast A31 cells, which do not produce C1s and do not form tumors when injected in vivo, gain tumorigenic capacity when transfected with C1s²⁴. This tumorigenic potential was dependent on the enzymatic activity of C1s²⁵.

Here we show that C1s is strongly expressed by cancer cells in a subset of ccRCC tumors and its high expression confers a negative prognostic impact in patient cohorts. C1s overexpression could be promoted by IFN γ , secreted by tumor-infiltrating CD8 T cells. C1s is produced by tumor cells with a particular, complement-rich phenotype, and it directly affects the cell's transcription and its proliferation capacity. In addition to its canonical functions, related to the classical pathway, we describe a novel mechanism, relying on non-canonical modes of action of C1s. Transcriptomic analysis together with our data on lung cancer suggests that the pro-tumoral effect of C1s in ccRCC is not an isolated finding and can be extended to other tumor types.

Material and methods

Bioinformatic analysis

C1s gene expression, measured as transcripts per million (TPM) was determined by using RNAseq data from 28 TCGA cohorts of patients with different cancer types available on cBioPortal^{26,27}. In this database, TPM values were generated with the RSEM method (RNA-Seq by Expectation Maximization). Tumor types were ranged according to the median expression values. Hepatocellular carcinoma (LIHC) was excluded from the analysis to avoid hiding the potential differences between tumor types linked to the very high expression of complement genes by the liver. The survival analysis was realized with Gene Expression Profiling Interactive Analysis (GEPIA) tool (<http://gepia.cancer-pku.cn/>) and represented the log₂ Hazard ratio of C1s on overall survival with a median cut off calculating with Cox pH Model. A log-rank test was applied to test the difference between the curves; surrounded boxes represented a log-rank p-value < 0.05. Because of the low number of survival events, testicular germ cell tumors cohort (TGCT) was also excluded from the survival study. The heatmaps were generated with R software 3.4.2 and heatmap.2 package. Differential

expression of C1s between tumors versus paired normal tissue from TCGA normal and GTEx normal database (n=100) were investigated with GEPIA. According to the absence of normal tissue for uveal melanoma (UVM), this cancer type was not included in the analysis. The Kaplan-Meier curves showed the overall survival of patients with high (>median) or low (<median) expression of C1s in different TCGA cohort were realized with GEPIA. C1s level expressed in $\log_2(1+TPM)$ in the different TNM stages of clear cell renal cell carcinoma (KIRC = ccRCC) patients were represented by violin plot generated with GEPIA. The Pearson coefficient of correlation between C1s expression (TPM) and the expression of other complement genes in KIRC were represented as heatmap and realized using R software.

Patients

The patients included in this study have signed an informed consent form prior to inclusion. The research was approved (N° CEPAR 2014-001) by the medical ethics board and conducted according to the recommendations in the Helsinki declaration. Two cohort of primary ccRCC patients undergoing nephrectomy were used in this study. The first cohort (cohort 1) is a retrospective cohort of 82 patients operated at Necker-Enfants Malades Hospital (Paris, France) between 1999 and 2003²⁸ and the second cohort (cohort 2) included 38 patients whose tumors were collected at Institut Mutualiste Montsouris (IMM, Paris, France) since 2014. Histologic subtype ccRCC, all TNM stages and tissue with good quality for the analysis constituted the inclusion criteria. Histopathologic features such as Fuhrman grade, TNM stages and size are available for the majority of the patients (Supplementary table 1).

Immunofluorescence(IF)/immunohistochemistry(IHC)

Formalin-fixed paraffin-embedded (FFPE) human ccRCC or NSCLC tumors were cut into 3 μ m thick sections and stained for C1s, C1q, C4, C3, Cytokeratin, α SMA, CD31, CD68, CD163, CD20, CD3, CD8 (Supplementary table 2). Human liver sections were used as positive control for C1s, C4 and C3. Tonsil (Geneticist) is used as positive control for C1q, cytokeratin, α SMA, CD31, CD163, CD20, CD3. First, tissues were deparaffinized with three baths of clearane and rehydrated with ethanol gradient (100%, 90%, 70%, 50%). Second, PT-link (dako) was used for antigen retrieval with heat for 30min at 97°C in an EnVision FLEX Retrieval Solutions (Dako) pH High or Low depending of the antibody. The use of a solution H2O2 3% (Gifrer) allowed the blocking of endogenous peroxidase and non-specific fixation was inhibited by protein block (Dako). The staining conditions were summarized in Supplementary table 2. For IHC, 3-amino-9-ethylcarbazole substrate (Vector Laboratories) was used for the revelation. The slides were then counterstained with hematoxylin (Dako),

mounted with glycerol (Dako) and scanned with NanoZoomer (Hamamatsu). For IF, the nucleus was stained with DAPI, slides were mounted with Prolong Glass antifade reagent and scanned with AxioScan (Zeiss). Calpix software was used to quantify PD-1, CD8, CD163.

Staining specificity. A competition test on liver sections was performed to verify the specificity of C1s staining (Supplementary Figure 2). In this test, a pre-incubation at different molecular ratios between anti-C1s antibody and purified C1s protein (Comptech) during 1 hour at 37°C was performed before putting it on the tissues. Purified C1r (Comptech) served as a negative control.

Patient stratification. The tumors were classified into 3 groups in a semi-quantitative manner according to the number of C1s positive cells; 0 (<5%), 1 (5-30%), 2 (>30%). To avoid errors, the stratification was realized by three independent observers. According to their similar effect on survival, group 0 and 1 was combined to form the “Low” group. The method of C4 and C3 classification was previously described by Roumenina et al¹⁸.

Cell lines

Human lung adenocarcinoma cell line (A549) and human ccRCC cell lines (Caki-1 and A498) were cultured with the appropriate medium in humidified atmosphere of 5% CO₂ at 37°C until 90% confluence. Caki-1 was cultured in McCoy's medium (Gibco) +10% FCS +1X penicillin/streptomycin (Gibco). A498 was cultured in Eagle minimum essential medium (ATCC) +10% FCS +1X penicillin/streptomycin (Gibco). A549 was cultured in Dulbecco's modified eagle medium nutrient mixture F12 (Gibco) +10% FCS +1X penicillin/streptomycin (Gibco) +1X L-glutamine (Gibco) +1X HEPES (Gibco) +1X Non essential amino-acids solution (Gibco). Murine TC-1 cells were cultured in RPMI medium + 10% FCS +1X penicillin/streptomycin (Gibco). All cell lines were routinely tested for *mycoplasma*.

Silencing of C1s

A 30 minutes pre-incubation of 4 siRNAs against C1s (Qiagen Hs_C1S_1_Flexitube siRNA SI00027356, Qiagen Hs_C1S_2_Flexitube siRNA SI00027363, Qiagen Hs_C1S_3_Flexitube siRNA SI00027370 and Hs_C1s_5_Flexitube siRNA SI030322873) or siRNA control (Qiagen AllStars negative control siRNA, SI03650318) at the concentration 50nM with lipofectamine RNAiMax (ThermoFisher, 13778030) in OptiMEM medium (Gibco) was performed before adding this mixture on cells plated in 6-well plate at 50% confluence. The transfection was stopped after 24h by replacing the medium containing siRNA by appropriate medium without antibiotics. Photos and functional assays on silenced cells were performed

72h post-transfection. The cells and supernatants were collected to attest the efficacy of silencing by qRT-PCR and Western blot.

Generation of CRISPR-cas9 stably deficient tumor cell

To generate stably C1s deficient murine tumor cell line, 3 CRISPR-CAS9 plasmids targeting different region of mouse C1s1 from Sigma Aldrich were used. After amplification in E.Coli, plasmids were isolated with ZymoPURE plasmid kit. TC-1 cell were then transfected with the plasmid and a positive selection of cells that have integrated the plasmid had been made with the presence of fluorescent RFP. Each positive cell was isolated with clonal dilution and finally different clones by plasmids were generated.

Experimental procedure

C57BL/6J WT mice were purchased from Charles River. All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with approvals APAFIS by the French Ministry of Agriculture. The mouse TC-1 cells were recovered at 80% confluence, and 400 000 cells for each selected clones were inoculated subcutaneously in the right flank with 200 µl PBS. Tumor size was measured with calipers every 2-3 days for 20.

Western blot

Western blot experiment on silenced cells and Crispr-Cas9 clones supernatant cultured in a serum-free medium for 48h (Opti-MEM, Gibco, 31985070) and previously concentrated with Amicon Ultracel 3K units (UFC, 900324) was performed. NuPAGE LDS sample buffer (4X, Thermofisher Scientific) was added to the concentrated supernatants and then denatured at 80°C for 10 minutes. Proteins were separated with a NuPAGE 10% Bis-Tris gel (Thermofisher Scientific) and transferred to a nitrocellulose membrane. The membrane were blocked with PBS BSA 5% and incubated overnight under a rocking chair platform with the goat polyclonal antiserum human C1s (Quidel, A302) diluted 1:500 in TBS BSA 5%. After washing with TBS Tween 0.1%, a secondary antibody rabbit anti goat HRP 1:1000 (Covalab, lab0048) was added for 1h at RT under agitation. The chemiluminescent signal was read with iBright CL100 (Thermofisher) after incubation with ECL reagent (Pierce, 32106).

qRT-PCR

RNA extraction on silenced cells or cells treated for 48h in medium-free serum with IFN γ (100 or 1000ng/mL) and RNA of CRISPR-Cas9 clones was performed with Maxwell cell 16LEV simplyRNA purification kit (Promega, AS1270). 2100 Bioanalyzer (Agilent) using a Agilent RNA 6000 Nano Assay kit (5067-1511) was used to determine the quality and quantity of RNA. The reverse transcription was realized with Applied Biosystem High-capacity cDNA reverse transcription kit (Applied Biosystem, 4368814). Gene expression of C1s was assessed by Taqman using primers of human C1s (Thermofisher, Hs00156159_m1) or mouse C1s1 (Thermofisher, Mm00663210_mH).

Analysis of differentially expressed genes

For RNASeq analysis, three biological replicate for each conditions (siC1s vs siC) were used. The libraries were prepared with NEBNext Ultra II Directional RNA Library Prep Kit with the following steps; PolyA purification using poly-T oligo attached magnetic beads, 300bp fragmentation with divalent cations under elevated temperature, synthesis of double strand cDNA and amplification by PCR. Sequencing was then carried out on paired-end 75b of Illumina HiSeq 4000 instrument. The quality of FASTQ files was verified by using FASTQC and then aligned to a reference genome using STAR tool. Once the data are mapped on the genome, HTSeqCount was used to count and assign reads to a given exon and generate a count table. Before proceeding to differential analysis, non expressed genes with a count equal to 0 were removed from the analysis. R software and DESeq2 package²⁹ was used for the differential expression analysis between siC1s and siC. Results were considered statistically significant if adjusted $p < 0.05$. The top 50 most significant genes were sorted by adjusted p-value and plotted in volcano plots and heatmap using heatmap.2 and EnhancedVolcano packages in R. Gene ontology enrichment analysis was performed with WEV-based Gene Set Analysis Toolkit (WebGestalt) by using the GO biological process category.

Flow cytometry

Proliferation and mortality. Cells were washed with PBS before the addition of CFSE reagent 1:1000 (Invitrogen, C34554) for 20 minutes at 37°C. The staining was stopper with the addition of complete culture medium. Cells were then washed and cultured on 6-well plate for 72h in presence of complete medium. Supernatants containing dead cells and adherent cells were recovered and stained for DAPI before analysis by flow cytometry (BD Fortessa).

Immunocytofluorescence

Silenced or control cells were cultured for 24h on a round cover glass in the bottom on a 24-well plate in presence of their specific medium. After fixation with PFA 4% for 30min at RT, the cells were stained for their actin cytoskeleton with Phalloidin-IFluor 488 reagent (Abcam, ab176753) and for nuclei with DAPI. The cover glass was recovered and mounted on a microscope slide with Prolong Glass antifade reagent and scanned with AxioScan (Zeiss).

Migration

A wound healing assay was performed on cell monolayer to evaluate their migration capacity. To do this, a scratch was realized with a tip and photos were taken under microscope immediately or after 12h. Wound healing tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool) in ImageJ was used to calculate the percentage of recovery.

Statistical analysis

The package “survival” on R software was used to generate Kaplan-Meier curves censored at 2500 days. Log-rank test was applied to test the difference between curves. Correlation between the distributions of two semi-quantitative variables was assessed by a Chi2 test, correlation between one semi-quantitative variable and one quantitative variable by a Kruskal-Wallis test and correlation between two quantitative variables by Pearson test. The difference between siC1s and siC cells for proliferation and survival was assessed by a paired T-test and unpaired T test is used for cell migration. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

C1s is a poor prognosis biomarker in ccRCC

We previously showed that RCC tumors are particularly sensitive to complement with a negative impact of classical pathway activation¹⁸. Interestingly, C1s expression was increased in ccRCC tumors in comparison to normal kidney (Figure 1D) and especially in advanced stages patients (Stage III and IV) (Figure 1E). We further confirmed this effect on survival at the protein level by immunohistochemistry on two cohorts of ccRCC patients. The specificity of C1s antibody used in this study was verified by a competition test (Supplementary figure 1A) and a western blot with human plasma (Supplementary figure 3C). The patients were classified into three groups according to the number of C1s positive cells within the tumor (Figure 1F). Similarly to gene expression, a correlation between the TNM stage and C1s level was observed as well as with the presence of metastasis or advanced Fuhrman grade (Supplementary figure 1C-E). However, in multivariate Cox model, C1s kept an independent prognostic value from TNM stage and metastasis status but not from Fuhrman grade (data not shown). Importantly, a high expression of C1s (group 2) was associated with a decrease progression free survival and overall survival for ccRCC patients (Figure 1G). This poor prognosis value was further confirmed in a second cohort of ccRCC patients (Figure 1H). The similarity between effect of group 0 and 1 led to combine this two groups to form “low” group (Supplementary figure 1B and 1F).

Tumor cells are the main local producer of C1s in ccRCC

C1s staining of ccRCC tumor sections revealed a intracellular pattern that formed a patchy staining inside the tumor. This staining is localized in tumor cells and in stromal cells (Figure 2A) with either a diffuse staining or more granular one (Figure 2B). Indeed, double staining by immunofluorescence in ccRCC tumors revealed a strong colocalization between C1s and low-cytokeratin (CK) cells (Figure 2C), an epithelial marker that stained tumor cells. Moreover, some stromal cells also expressed C1s within the TME such as some endothelial cells (CD31+), some rare macrophages (CD68+) and some fibroblasts (α SMA) (Figure 2D). Interestingly, the presence of C1s staining in tumor cells correlated with T cells and macrophages infiltration (Figure 2E and 2F) as well as the activation status of T cells with more density of PD-1 (Figure 2G).

Tumor cells that produce C1s also produce other complement proteins

In KIRC, the gene expression of C1s was strongly correlated with the expression of other genes of the complement system and especially C1r, C3, CFB, CFH, SERPING1, C2 and C1QA. At the opposite, the expression of C1s was negatively correlated with expression of the lectin pathway genes (FCN3, MASP1, MASP2, FCN2 and the regulators CD59 and CD46) (Figure 3A). The same observations have been made by immunohistochemistry with a correlation between a high density of C1s staining in tumor cells and the staining for C4 and C3 (Figure 3B-C). Moreover, the cells that produce C1s were the same that produce C4 and C3 suggesting a global complement-rich phenotype of these tumor cells (Figure 3D-G). This global complement production was associated with a poor prognosis value since the simultaneous presence of complement C1s and C4 production was associated with a negative impact on PFS and OS that was less important in the case of an isolated C1s or C4 production (Figure 3H-I).

C1s acts as an initiator of the classical complement pathway locally

The colocalization of C1s and C1q/C4d deposits at the surface of tumor cells (Figure 3J-K) that reflected complement activation suggested that C1s can act as a complement activator locally. Indeed, the majority of the tumors with C4d deposits also display a local production of C1s (Figure 3L). Interestingly, a non-negligible proportion of the tumor present C1s production without C4d deposits suggesting a potential non-canonical role of C1s. To emphasize this hypothesis, Cox multivariate analysis highlighted that the prognostic value of C1s was independent of complement activation markers (C1q, C4 and C3 activation fragments deposits) (Figure 3M).

C1s modulates the phenotype of tumor cells

Considering that the prognostic value of C1s was independent of the complement activation, we investigated its direct effects on tumor cells. After validation of the efficacy of C1s silencing by RTqPCR and Western blot on ccRCC cell lines Caki-1 and A498 (Supplementary figure 2A-B, 2D-E), we performed a RNASeq analysis to compare the differential expression between cells transfected with an siRNA against C1s (siC1s) or an siRNA control against an irrelevant sequence (siC) for Caki-1 and A498 (Figure 4A and Supplementary figure 3A). Among all genes, we identified 56 downregulated genes and 119 upregulated genes in Caki-1 siC1s vs. siC and 41 downregulated genes and 112 upregulated genes in A498 siC1s vs. siC by taking a fold change >2 in absolute values and an adjusted p-value <0.05 (Figure 4B and Supplementary figure 3B). The enrichment analysis with

GO:biological process highlighted that these genes were involved in different key pathways (Figure 4C and Supplementary figure 3C). First, in siC1s cells we observed an upregulation of genes involved in development and negative regulation of growth (Caki-1) and motility (A498) as well as cellular response to erythropoietin and extracellular matrix organization (A498). On the other hand, a downregulation of genes implicated angiogenesis and positive regulation of VEGFR (A498 and Caki-1), endocytosis (A498), negative regulation of cAMP dependent protein kinase activity (A498), chondrocyte differentiation (Caki-1), dopamine metabolic process and bone resorption (Caki-1). Functionally, the silencing of C1s in Caki-1 cells induced an increase of mortality (Figure 4E) and a decrease in proliferation capacity (Figure 4F-5G) but no effect on tumor cell morphology (Figure 4D). These observations were valid for the two ccRCC cell lines Caki-1 and A498 (Supplementary figure 3D-G). Moreover, in vitro this C1s production by tumor cells was enhanced by the addition of IFN γ (Supplementary figure 3H). Furthermore, the expression of C1s by tumor cells was not limited to ccRCC and was also detected on lung tumors (Figure 5A-B) and functionally affects lung cancer cell lines, A549, phenotype (Figure 5C-D). In order to study the effect of C1s silencing in vivo, we generated C1s1 stably deficient murine TC-1 tumor cells through CRISPR-CAS9 technology. C1s expression in the 12 clones obtained were analysed by RTqPCR (Figure 5E) and western blot (Figure 5F). Importantly, some clones presented normal expression level of C1s and other undetectable expression of C1s. For the in vivo experiment, we have selected three clones with low expression of C1s1 (1.16, 2.21 and 3.11). All clones had a slower tumor growth in comparison to WT TC-1 (Figure 5G). For the other clones where C1s protein is physically present, a long PCR is on going to detect the presence of in frame deletions of one or few amino acids which could affect protein functionality.

Pan-cancer analysis of C1s expression and its prognostic value

We found that C1s was involved in the modulation of tumor cell phenotype in two in vitro models of ccRCC and lung tumors and impact the survival of ccRCC patients. To have a global view of the impact of C1s expression in different tumor types, an analysis of C1s expression in 28 cohorts of the TCGA PanCancer study was performed and revealed a heterogeneous expression according to tumor types (Supplementary figure 4A) with almost 100-fold higher expression of C1s in sarcoma (SARC) than in kidney chromophobe (KICH), representing the two extremes. This expression was either down- or upregulated in tumors, in comparison to normal paired tissues (Supplementary figure 4B). Interestingly, the expression of C1s was associated with a dual role as a protective or aggressive factor for patient survival in a context-dependent manner (Supplementary figure 4C-E). Indeed, while in sarcoma (SARC), breast (BRCA) or cutaneous skin melanoma (SKCM) a high C1s gene expression

increased patient survival, in glioblastoma (GBM), papillary renal cancer (KIRP), low grade glioma (LGG), clear cell renal cell carcinoma (KIRC or ccRCC) and uveal melanoma (UVM), the inverse effect was observed, with a poor prognosis value of C1s.

Discussion

Here we describe that complement C1s is a cancer-promoting factor in ccRCC, contributing to chronic complement-mediated inflammation and tumor cell proliferation. The synergy between canonical (cascade-dependent), and local/intracellular effects of C1s described here, could explain its negative prognostic impact in ccRCC. Although ccRCC appears to be a prototypical complement-associated cancer, the non-canonical functions of C1s are not limited to ccRCC. Indeed, we replicated the impact on the proliferation in a lung adenocarcinoma cells.

The overexpression of C1s in the ccRCC tumor tissue, particularly due to production by the tumor cells, is associated with decreased patients' survival. We have recently demonstrated that the classical complement pathway activation, measured by C4 activation fragments deposits on tumor cells and presence of C1q-producing macrophages, occurs in ccRCC and confers poor prognosis¹⁸. Here we provide further evidence for the implication of the classical pathway, through the colocalization of C1q and C1s on the tumor cell surface and the correlation between C1s staining and the presence of deposits of activation fragments of C4. This study supports the notion that in a subset of ccRCC tumors the components of the classical pathway are highly expressed locally, allowing generation of C5a and tumor-promoting signaling through C5aR1^{14,18,30,31}.

In ccRCC, C1s is produced nearly entirely by the tumor cells and to a minimal extend by fibroblasts, while T-cells, B-cells and the large majority of the macrophages and endothelial cells remain negative for C1s at protein level, with the detection limits of the immunofluorescence. The presence of the protein was due to production by the tumoral cells and was less likely to be internalized by the cells from the circulation, since we detected C1s (as well as C1r) mRNA by RNAscope¹⁸. C1s staining in tumor cells was mainly intracellular and strongly correlated with the intracellular staining for C4 and C3. Indeed, the same cells were frequently (but not exclusively) positive for the three proteins. One could hypothesize, that the concomitant presence of these proteins within the same cell may allow the functioning of the complosome i.e. intracellular complement proteins activation steps, resembling the plasmatic cascade³². This hypothesis requires further validation. Nevertheless, combining the data for C1s and C4 positivity allowed us to distinguish a subgroup of patients C1s-high/C4-high, which had a particularly poor outcome, contrary to the double negative

cancers. Besides C4 and C3, the gene expression of C1s also correlated with a large set of complement genes especially from the classical and alternative pathway. We have indeed, detected previously concomitant production of some of these proteins by ccRCC cell lines¹⁸. The ensemble of the gene expression and staining data for the patients' cohort and *in vitro* genes and protein expression suggest that when the tumor cells acquire complement-rich phenotype, it becomes more aggressive, with higher risk for cancer progression and patient death. These complement-rich cells stain also low for CK, suggesting a loss of epithelial and acquisition of a mesenchymal, more aggressive phenotype.

We found that C1s silencing altered the transcriptomic program of the ccRCC cell lines and reduced cell proliferation and survival. This phenotype was independent from the VHL mutation, since it was present both tested cell lines: VHL-wild type (Caki-1) and VHL-mutated (A498). Similar complement-rich phenotype was reported for sSCC³³⁻³⁶, although the differentially expressed genes after C1s silencing were different³⁶. Moreover, the reduced proliferation and increased mortality were shared also by the lung adenocarcinoma cell line A549, suggesting the general relevance of C1s as a proliferation and cell survival promoting gene. Indeed, in a knock-in experiment, introduction of C1s to non-expressing fibroblasts conferred stronger proliferation and tumor-forming capacity²⁴. To study the impact of C1s *in vivo*, we have generated a C1s1-deficient murine lung tumor cancer cell lines (TC-1). In this experiment we revealed the role of tumor derived C1s with a slower tumor growth in mice injected with C1s deficient cells. Therefore, C1s is not just a biomarker for the complement-rich ccRCC phenotype, but an active player in the acquisition of an aggressive phenotype by the tumor cells. Considering the effect of C1s silencing in human tumor cell, a mirror experience in a C1s knock-in model on HCT116 tumor cells lines that expressed very low level of endogenous C1s is currently on going.

However, the stimulus responsible for the induction of C1s expression by tumor cells remains unclear. The generation of pro-inflammatory cytokines such as IFN γ that stimulates C1s and other complement genes expression in a large variety of cells^{6,37,3} and also for the ccRCC cell line. In the tumor microenvironment, immune cells and particularly T cells and NK cells produced IFN γ . In ccRCC we observed a correlation between the level of C1s produced by tumor cells and the density of CD8 that could reflect the capacity of CD8 through inflammatory cytokines secretion to induce the production of C1s by tumor cells as an escape mechanism. Moreover, one of the described substrate of C1s is MHC class I² suggesting a possible interrelation between C1s and the modulation of the immune infiltrate.

These findings make the fraction of complement-overactivating ccRCC patients potential candidates for complement-blocking therapy. Nowadays there is an ever-increasing number of

therapeutic complement inhibitors, targeting different level of the cascade, in clinical trials for various indications³⁸. Considering tumor-complement status, these inhibitors could be adapted for personalized therapy in cancer. Interestingly, a C1s-blocking therapeutic antibody Sutimlimab (=TNT009, BIVV009) showed positive results in a trial with patients suffering from cold agglutinin disease as well as a good safety profile^{39,40}. Therefore, this antibody, as well as the different forms of the already clinically available C1 Inhibitor preparations (Cinryse, Berinert, Ceter, Ruconest), could represent therapeutic options, deserving experimental evaluation. However, to have the most favorable benefit-risk balance, potential unwanted effect of C1s blocking must be evaluated carefully. It has to be noted though, that such therapy will block in part the cell opsonization with C3 activation fragments and complement activation in the tertiary lymphoid structures (TLS) that may affect B cell functionality. Indeed, it has been reported an *in vitro* reduction of proliferation of primary human B cells from patients with rheumatoid arthritis⁴¹ following C1s blockade. Thus, further studies are needed to find out whether complement deposits contribute to the long-term activation of anti-tumoral B cells and the production of tumor cells-recognizing antibodies. Nevertheless, at least in ccRCC, IgG deposited on the tumor cells were the main trigger of the classical pathway, associated with cancer progression. Therefore, in ccRCC C1s blockade may have dual beneficial effect, inhibiting complement and B-cells activation/IgG production. Inhibition of the enzymatic activity of C1s will block not only the complement cascade and will prevent cleavage of the complement-unrelated C1s substrates. The enzymatic activity of C1s is also likely associated with its non-canonical functions, acting on the cell proliferation²⁵. Indeed, the Caki-1 cell line produces an active C1s enzyme in absence of C1q¹⁸. Nevertheless, for A498 we detected only the pro-enzyme form and still the silencing of C1s reduced cell proliferation and increased mortality. Therefore, alternative model of action of C1s could be envisioned, unrelated to its enzymatic activity.

In conclusion, we discovered a tumor-promoting role of C1s in ccRCC, which was dependent on two modes of action: local secretion and canonical classical pathway activation as well as a non-canonical mechanism, resulting in enhancement of tumor cell proliferation. These mechanisms synergize to confer poor prognosis to the patients with C1s-positive tumors. C1s enters into a broader, complement-rich phenotype of a fraction of ccRCC cancers, which are more aggressive. These patients could be potential candidates for C1s-blocking therapy. Further validated in appropriate animal models is necessary in order to select the most appropriate mode of C1s inhibition, having an anti-tumoral efficacy.

Acknowledgements

This work was supported by a grant from the Ligue Regionale Contre le Cancer LTR. This work was also supported by INSERM, University of Paris, Sorbonne University, CARPEM, and the Labex Immuno-Oncology Excellence Program. MVD received a PhD fellowship from La Fondation ARC pour la recherche sur le cancer.

The authors declare no conflict of interest

Legends

Figure 1: C1s is associated with poor prognosis in ccRCC patients

A) Kaplan-meier curves of OS according to C1s gene expression level using median cut-off on KIRC TCGA cohort Log-rank test was used and $p < 0.05$ was considered as significant. **B)** C1s expression (log2TPM) in KIRC tumors versus normal kidney from TCGA normal and GTEx database. **C)** C1s expression (log2TPM) in different TNM stages of KIRC tumors generated by GEPIA. **D)** Classification of C1s cytoplasmic staining into three groups according to the number of C1s positive cells. Group 1: very low number of cells (<5%), group 2: intermediate number of cells (5-30%), group 3: high number of cells (>30%). **E)** Kaplan-Meier curves of PFS and OS according to the C1s classification in three groups in cohort 1. **F)** Kaplan-Meier curves of PFS according to the C1s classification in three groups in cohort 2.

Figure 2: C1s is expressed mainly by the tumor cells in ccRCC.

A) C1s staining on paraffin-embedded sections of ccRCC tumor. Two types of cells were stained inside the tumor core and in the stroma. **B)** C1s intracellular staining can be diffuse or in spots in ccRCC tumor cells. **C-D)** Identification of C1s+ cells in ccRCC by IF double staining. **C)** C1s (green) and CK (epithelial tumor cell marker, red). **D)** FH (green) and CD68 (macrophages marker, red), CD3 (T cell marker, red), CD31 (endothelial cell marker, red), CD20 (B cell marker, red), α SMA (fibroblast marker, red). **E)** Density of CD163+ cells in each groups of C1s classification determined by IHC. **F)** Density of CD8+ cells in each groups of C1s classification determined by IHC. **G)** Density of PD-1+ cells in each groups of C1s classification determined by IHC.

Figure 3: C1s has both canonical (complement cascade-related) and non-canonical (cascade-independent) functions in ccRCC tumors

A) Correlation between CFH gene expression and other complement genes. Colors represents the Pearson coefficient correlation. Red: positive correlation, Blue: negative correlation, White: no correlation. **B)** Correlation between C1s and the presence of C4 production by tumors cells determined by IHC. **C)** Correlation between C1s and the presence of C4 production by tumors cells determined by IHC. **D-F)** C1s, C4 and C3 staining in the same ccRCC tumor area. **G)** Double staining by IF of C1s (green) and C4 (red). **H-I)** Kaplan-Meier curves of PFS and OS according to the combination of C1s and C4 High/Low classification in cohort 1. **J)** Double staining of C1q (green) and C1s staining (red). **K)** Correlation between C1s and the presence of C4 deposits at the surface of tumors cells determined by IHC. **L)** Summary of Cox multivariate analysis between C1s and other complement activation markers

Figure 4: C1s silencing decreases Caki-1 proliferation and survival without affecting morphology and migration

A) Top 50 differentially expressed genes after silencing of with siC1s compared with siC in Caki-1. The most differentially expressed genes (log₂ counts) are selected on adjusted p-value. Expression across each gene have been row scaled with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. Lighter shades and white represent genes with intermediate expression levels. **B)** Volcanoplot depicting the results of differential expression obtained with DESeq2 analysis between Caki-1 siC1s (n=3) and Caki-1 siC (n=3). The volcano plot was generated with R software and EnhancedVolcano package. Red points represented genes with a log₂ fold change >1 and p-value adjusted < 0.05. **C)** Gene ontology analysis of functional annotations (biological process) up- or downregulated by C1s silencing vs. Caki-1 siC. **D)** Immunofluorescence staining of phalloïdin (red) in Caki-1 siC1s (right) or siC (left). **E)** Percentage of DAPI positive dead cells determined by flow cytometry after 72h of culture in Caki-1 siFH or Caki-1 siC in 5 independent experiments. **F)** Histogram representing CFSE intensity on Caki-1 siC1s or Caki-1 siC after 72h of proliferation (green = unstained Caki-1 cells, orange = Caki-1, taken at the time of CFSE staining T₀, red = Caki-1 siC after 72h of proliferation, blue = Caki-1 siC1s after 72h of proliferation). **G)** Fold change of CFSE geometric means in Caki-1 siC1s vs. Caki-1 siC on 4 independent experiments.

Figure 5: Effect of C1s on lung cancer

A) C1s staining on paraffin-embedded sections of NSCLC tumor. Two types of cells were stained inside the tumor core and in the stroma. **B)** Identification of C1s+ cells in ccRCC by IF double staining. **C)** C1s (green) and CK (epithelial tumor cell marker, red). **C)** Fold change of CFSE geometric means in A549 siC1s vs. A549 siC on 3 independent experiments. **D)** Percentage of DAPI positive dead cells determined by flow cytometry after 72h of culture in A549 siFH or A549 siC in 4 or 5 independent experiments. **E)** RTqPCR of C1s1 gene in 12 Crispr-Cas9 TC-1 clones. **F)** Western blot for C1s in TC-1 Crispr-Cas9 clones. **G)** In vivo tumor growth of 3 selected Crispr-Cas9 TC-1 clones and TC-1 WT cells in C57Bl/6 mice injected with 400 000 cells subcutaneously.

Supplementary data

Supplementary tables

Supplementary Table 1: Cohort description

Supplementary Table 2: List of antibodies and staining protocols

Supplemental Figure 1: C1s-positive staining is associated with poor prognosis in ccRCC (binary classification: high vs low)

A) C1s staining specificity validation by competition test on paraffin-embedded liver sections. The tissue were stained with an anti-C1s antibody, isotype control or with a pre-incubated solution of anti-C1s antibody with purified C1s (Comptech, A104) or C1r (Comptech, A102) (1:10 molecular ratio). **B)** Kaplan-Meier curves of PFS and OS according to the C1s classification High/Low in cohort 1. **C)** Correlation between C1s three groups (left) or High/Low classification (right) with clinical and anatomopathological features in cohort 1. **D)** Correlation between TNM stage and C1s High/Low in cohort 1. **E)** Correlation between Fuhrman grade and C1s High/Low in cohort 1. **F)** Kaplan-Meier curves of PFS according to the C1s classification High/Low in cohort 2. **G)** Correlation between TNM stage and C1s High/Low in cohort 2.

Supplemental Figure 2: Validation of the C1s silencing

A) C1s gene expression was determined by RTqPCR after silencing with siRNA against C1s (siC1s) or control (siC) or wild type cells (WT) in A498 cells. **B)** C1s gene expression was determined by RTqPCR after silencing with siRNA against C1s (siC1s) or control (siC) or

wild type cells (WT) in Caki-1 cells. **C)** Western blot of C1s in the supernatant of A498 cells (WT, siC1s, siC, lipofectamine alone) under non reducing conditions at 24h, 48h or 7 days post-transfection. Purified C1s (Comptech, A104) was used as positive control. **D)** Western blot of C1s in the supernatant of Caki-1 cells (WT, siC1s, siC, lipofectamine alone) under non reducing conditions at 24h or 48h post-transfection. Purified C1s (Comptech, A104) was used as positive control.

Supplemental Figure 3: C1s silencing decreases A498 proliferation and survival without affecting morphology and migration

A) Top 50 differentially expressed genes after silencing of with siC1s compared with siC in A498. The most differentially expressed genes (log₂ counts) are selected on adjusted p-value. Expression across each gene have been row scaled with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. Lighter shades and white represent genes with intermediate expression levels. **B)** Volcanoplot depicting the results of differential expression obtained with DESeq2 analysis between A498 siC1s (n=3) and Caki-1 siC (n=3). The volcano plot was generated with R software and EnhancedVolcano package. Red points represented genes with a log₂ fold change >1 and p-value adjusted < 0.05. **C)** Gene ontology analysis of functional annotations (biological process) up- or downregulated by C1s silencing vs. A498 siC. **D)** Immunofluorescence staining of phalloïdin (red) in A498 siC1s (right) or siC (left). **E)** Histogram representing CFSE intensity on A498 siC1s or A498 siC after 72h of proliferation (green = unstained A498 cells, orange = A498 taken at the time of CFSE staining T₀, red = A498 siC after 72h of proliferation, blue = A498 siC1s after 72h of proliferation). Fold change of CFSE geometric means in A498 siC1s vs. A498 siC on 3 independent experiments. **F)** Percentage of DAPI positive dead cells determined by flow cytometry after 72h of culture in A498 siFH or A498 siC in 4 independent experiments. **G)** A scratch on A498 monolayer was performed at T₀ and photo of the scratch area were taken. After 12h, a photo of the same area was taken to compare the migration capacity. ImageJ Wound healing macro was used to determine the area of the scratch at each time point. The percentage of closing of the scratch area was calculated with the following calculation; 100-(scratch area T₀/scratch area t+12h). **H)** C1s gene expression determined by RTqPCR after treatment with IFN γ (0,100 and 1000 ng/mL) in A498 cells.

Supplemental Figure 4: Pan cancer analysis of C1s gene expression and prognostic

A) Analysis of C1s expression in 25 cancer types of the TCGA Pancancer atlas study. C1s is expressed in log₂(1+TPM). Tumor types are classified according to the median expression of C1s. 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. **B)** Gene expression (median TPM) in tumor vs. paired normal tissue in 28 cohorts of the TCGA PanCancer study **C)** Survival analysis representing the hazard ratio of C1s in 28 cancer types from the TCGA PanCancer atlas. Gene Expression Profiling Interactive Analysis (GEPIA) tool⁴² was used to performed the analysis. The hazard ratios are calculated by using overall survival (OS) data and median gene expression cut-off based on Cox PH Model and Log-rank p-value. The heatmap representing the log₂ HR with a scale centered in 0 was generated by using the R package “pheatmap” and cancer types were sorted in ascending order. The surrounded boxes correspond to a log-rank p-value <0.05. **D)** Tumors where C1S gene expression is associated with favorable prognosis. **E)** Tumors where C1S gene expression is associated with poor prognosis

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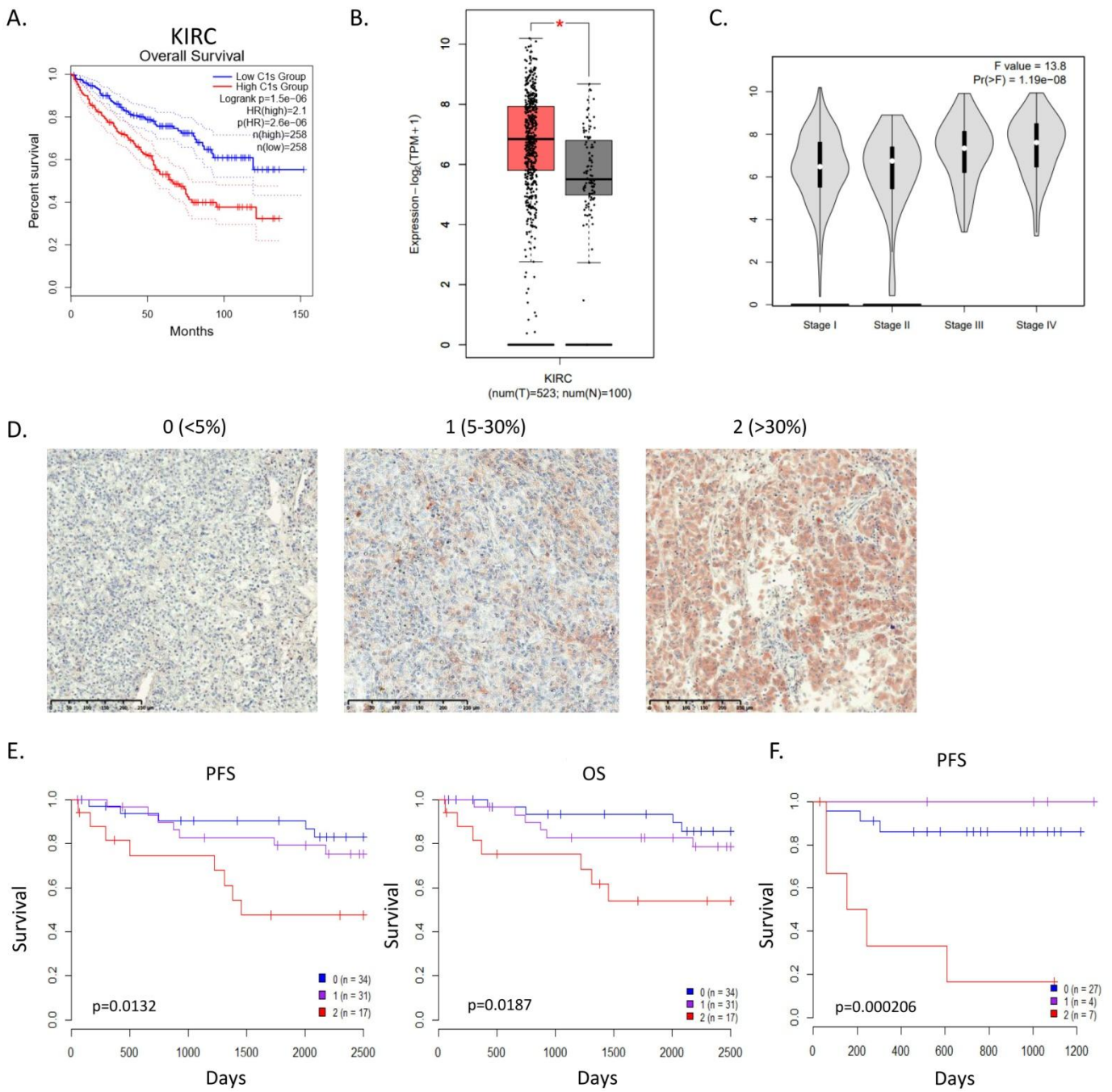


Figure 1. C1s is associated with poor prognosis in ccRCC patients

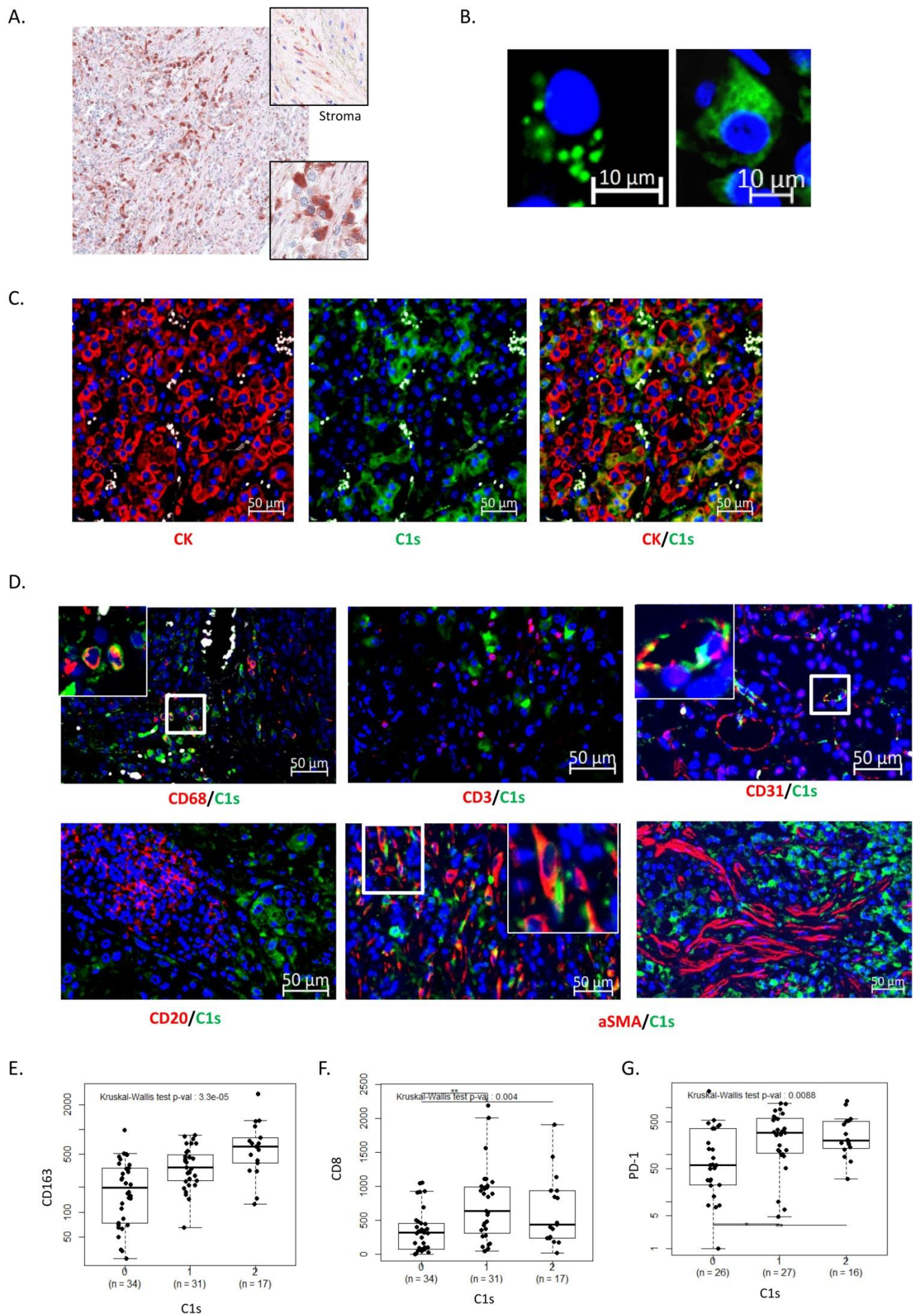


Figure 2. C1s is expressed mainly by the tumor cells in ccRCC.

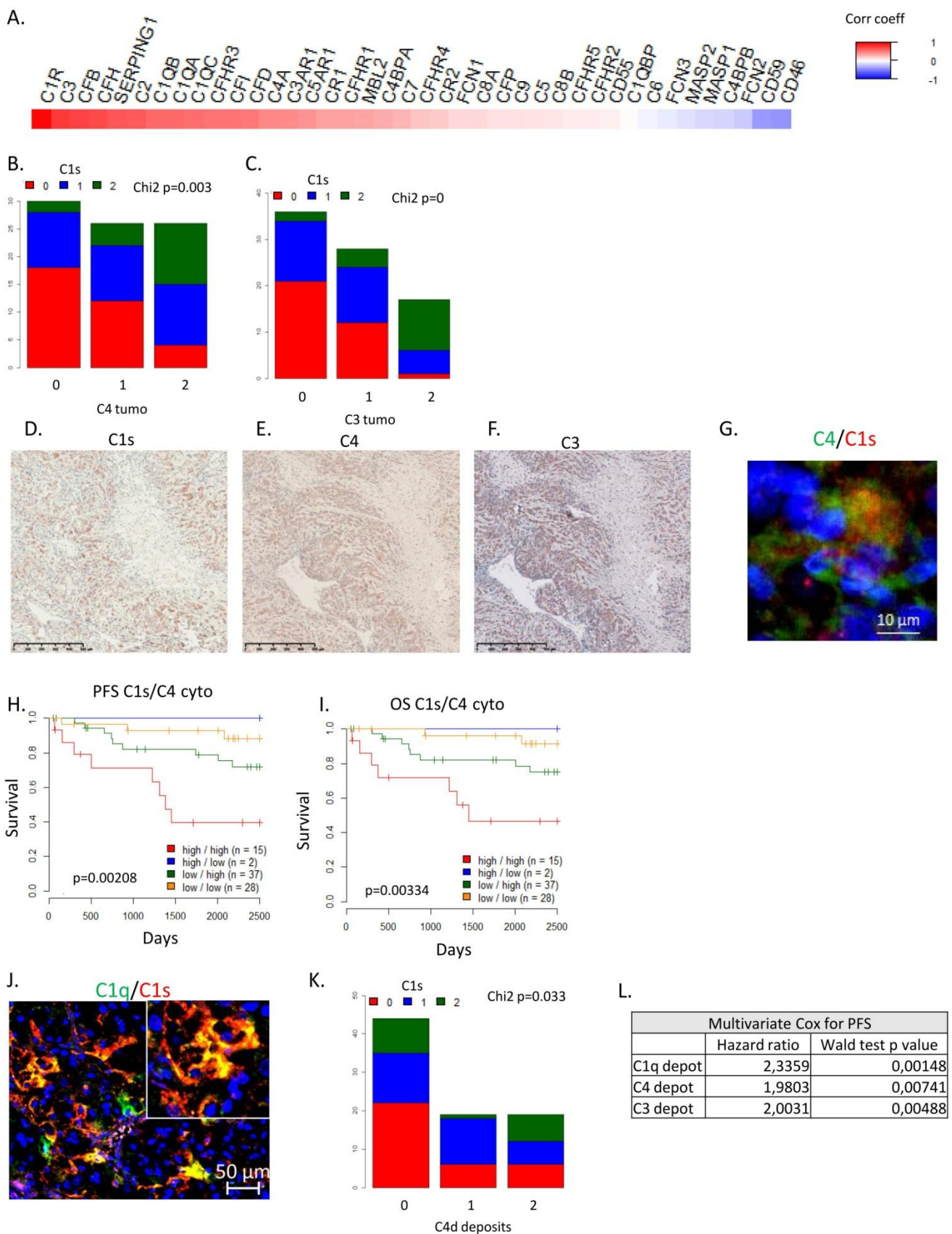


Figure 3. C1s has both canonical and non-canonical role in ccRCC tumors

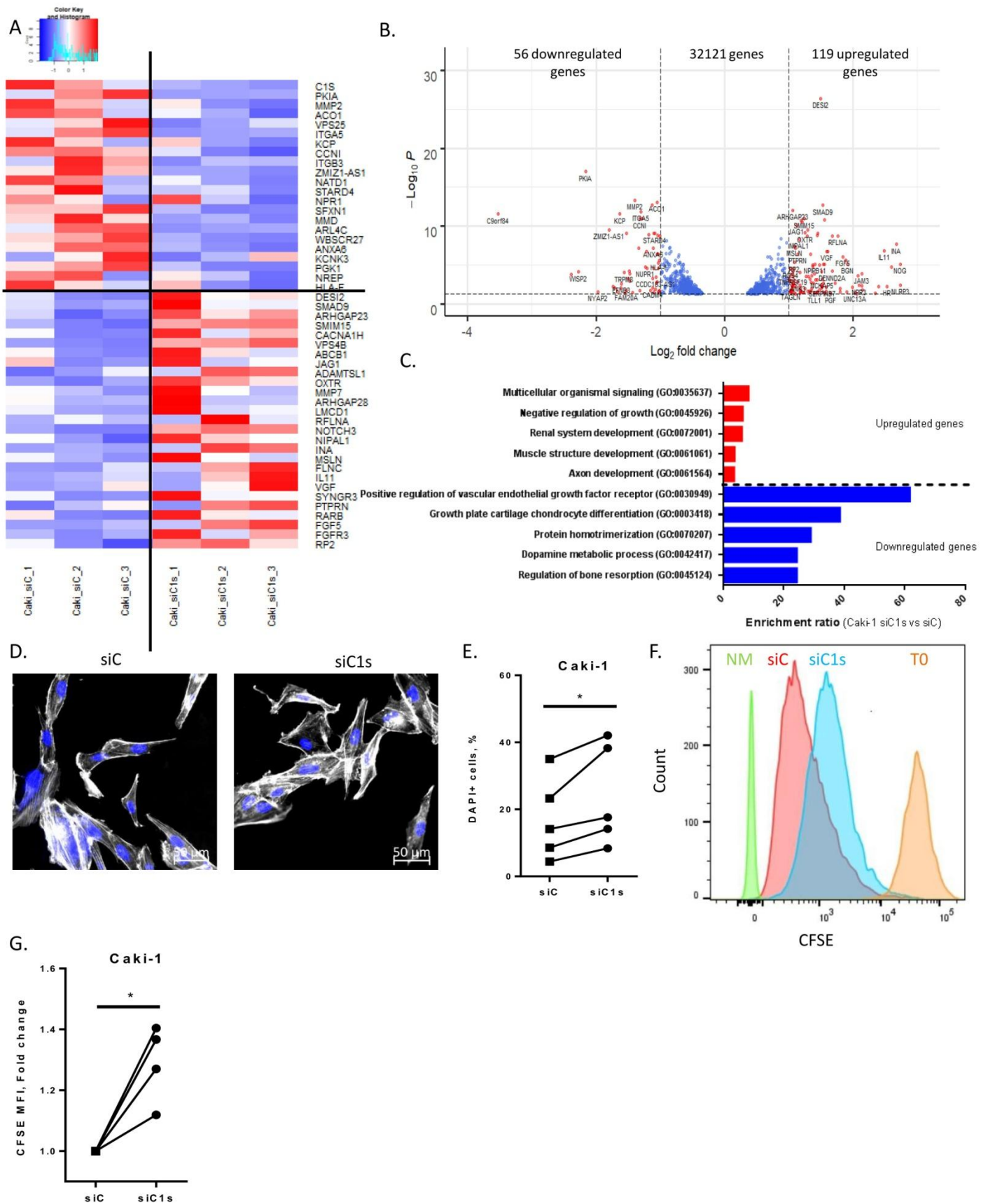


Figure 4: C1s silencing decrease Caki-1 proliferation and survival without affecting morphology and migration

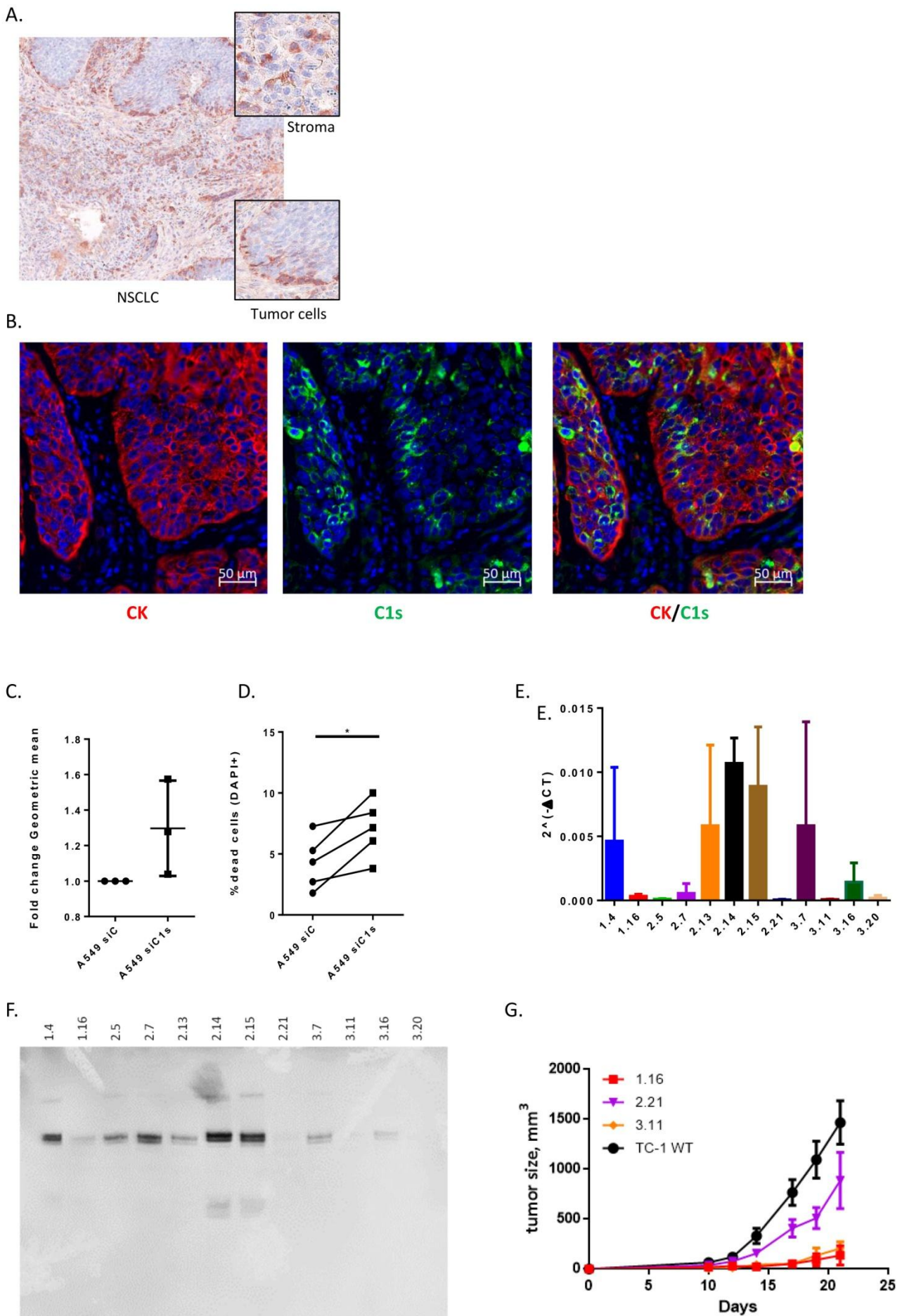


Figure 5: Effect of C1s on lung cancer

Supplementary data

Cohort 1	
Sex (F/M)	19 (23%)/ 63 (77%)
Age (mean)	62
Tumor size (mean)	5,1
TNM stage (I/II/III/IV)	33(40%)/ 5(6%)/ 32(39%)/ 12(15%)
Fuhrman grade (I/II/III/IV/NA)	3(4%)/ 19(23%)/ 47(57%)/ 12(15%)/1(1%)

Cohort 2	
Sex (F/M)	15 (39%)/ 22 (58%)/ 1 (3%)
Age (mean)	69
TNM stage (I/II/III/IV)	26(68%)/ 3(8%)/ 8(21%)/ 0(0%)/ 1(3%)
Fuhrman grade (I/II/III/IV/NA)	1(3%)/ 20(53%)/ 13(34%)/ 3(8%)/1(3%)

Supplementary Table 1: Cohort description

Immunofluorescence (IF)

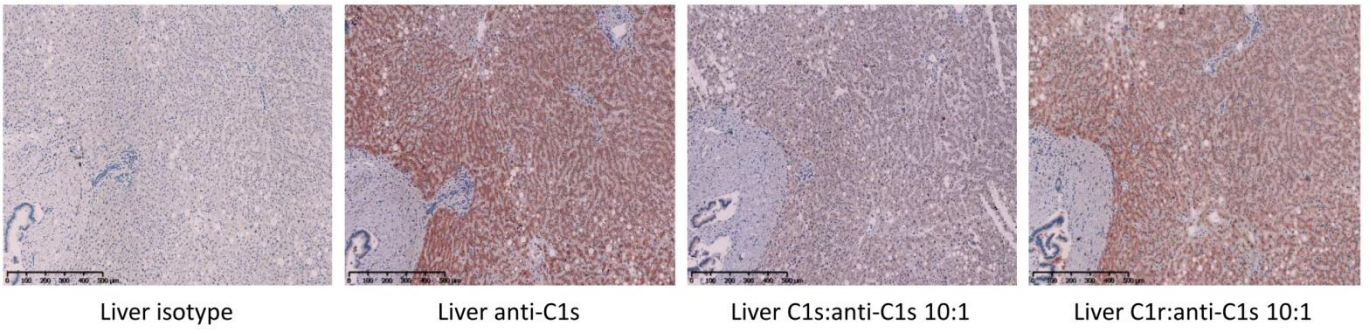
Antigen	Clone	Species	Ref	Antigen retrieval pH	Dilution	Secondary Ab	Tyramide
C1s	EPR9066	Rabbit	Abcam, ab134943		91:200	HRP labelled polymer anti-rabbit, Dako K4002	AF647
C1q	Polyclonal	Rabbit	Dako, A0136		91:2000	HRP labelled polymer anti-rabbit, Dako K4002	AF546
Cytokeratin	AE1/AE3	Mouse	Dako, M3515		61:200	HRP labelled polymer anti-mouse, Dako K4001	AF546
α SMA	1A4	Mouse	Dako, M0851		61:400	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD31	1A10	Mouse	Leica, PA0250		91:400	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD68	KP1	Mouse	Dako, M0814		61:1000	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD20	L26	Mouse	Dako, M0755		61:1000	HRP labelled polymer anti-mouse, Dako K4001	AF546
CD3	2GV6	Rabbit	Ventana, 790-4341		91:200	HRP labelled polymer anti-rabbit, Dako K4002	AF546

Immunohistochemistry (IHC)

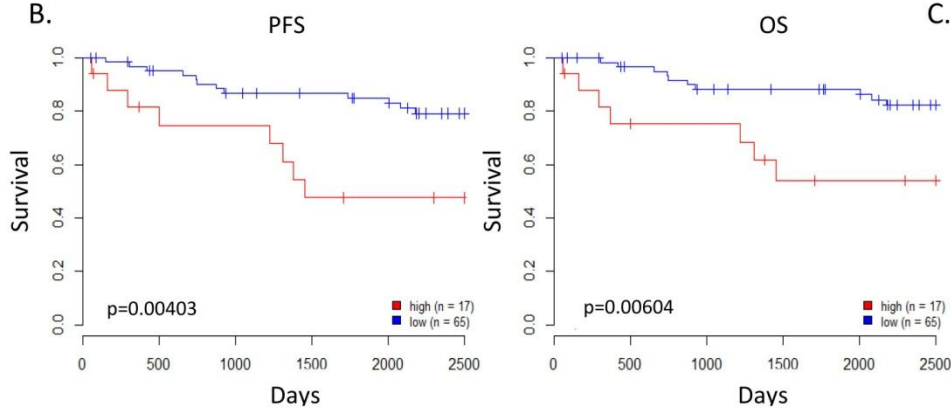
Antigen	Clone	Species	Ref	Antigen retrieval pH	Dilution	Secondary Ab	Substrate
C1s	EPR9066	Rabbit	Abcam, ab134943		91:100	HRP labelled polymer anti-rabbit, Dako K4002	AEC
C4d	A24-T	Rabbit	DB biotech, DB107		91:200	HRP labelled polymer anti-rabbit, Dako K4002	AEC
C3d	Polyclonal	Rabbit	Dako, A0063		61:1000	HRP labelled polymer anti-rabbit, Dako K4002	AEC
CD163	10D6	Mouse	Dako, Mob460		91:200	HRP labelled polymer anti-mouse, Dako K4001	AEC

Supplementary Table 2: List of antibodies and staining protocols

A.



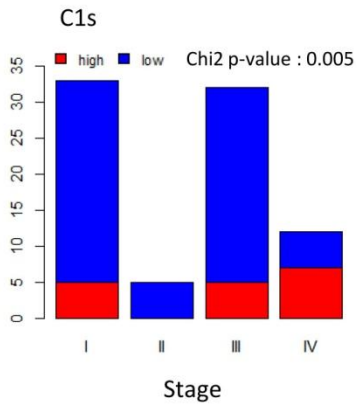
B.



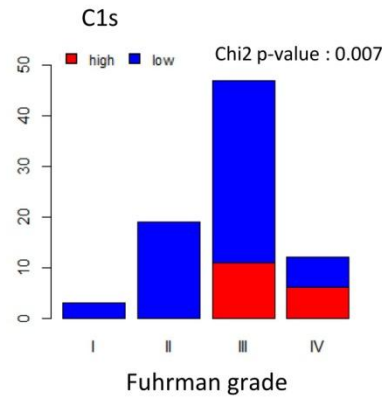
C.

	p-value (Chi2 test)	
	C1s	C1s HiLo
Sexe	0,525	0,777
pT	0,024	0,032
N	0,195	0,768
MET	0,002	0,002
Stage UICC	0,001	0,005
Fuhrman Grade	0,052	0,007
Sarcomatoid	0,43	0,596
Necrosis	0,127	0,872

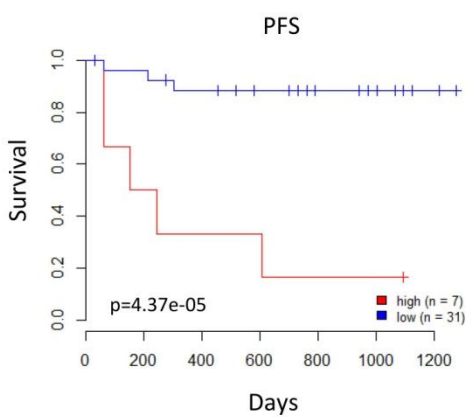
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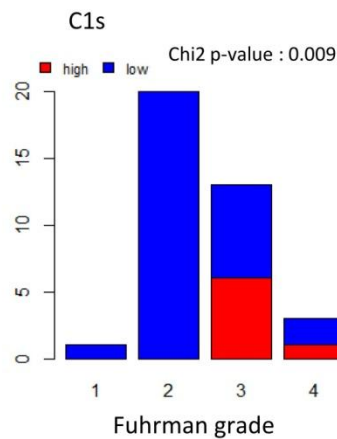
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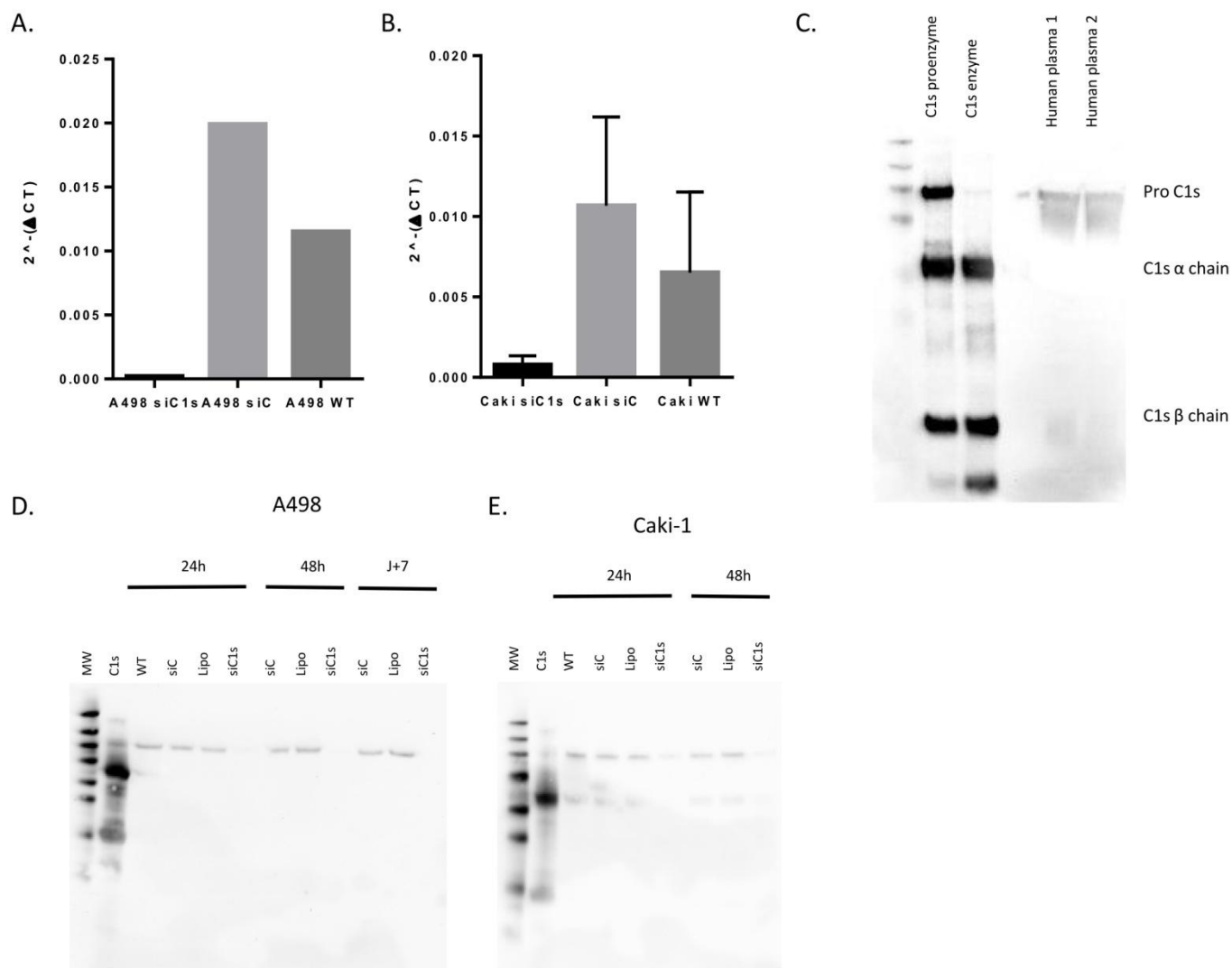
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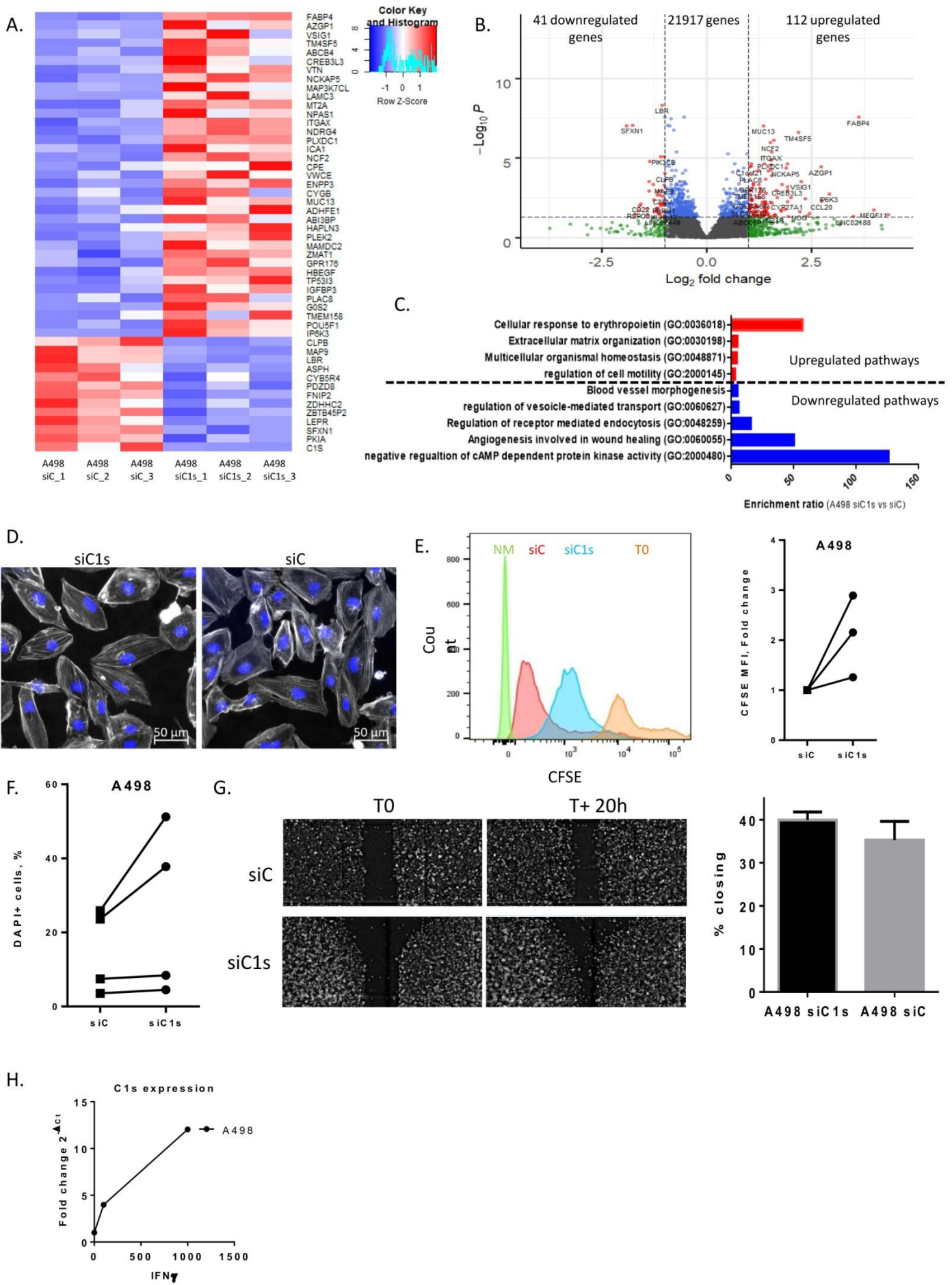
G.



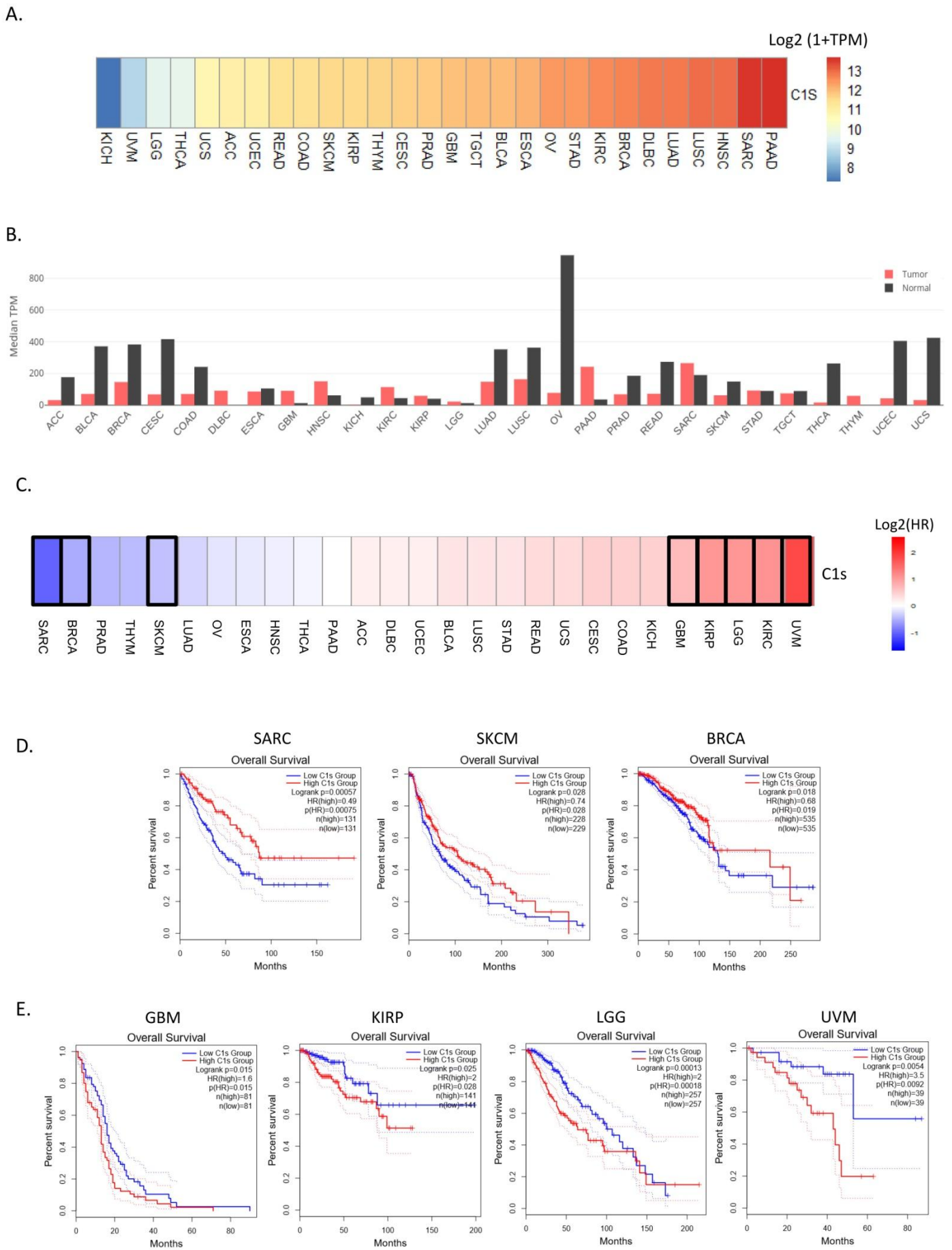
Supplemental Figure 1 : C1s-positive staining is associated with poor prognosis in ccRCC (binary classification: high vs low)=



Supplemental Figure 2: Validation of the C1s silencing



Supplemental Figure 3: C1s silencing decrease A498 proliferation and survival without affecting morphology and migration



Supplemental Figure 4: Pan cancer analysis of C1s gene expression and prognostic

POSTER AND ORAL PRESENTATIONS

The International Cancer Immunotherapy Conference (CICON) (Paris, 2019) : poster, «Canonical and non canonical functions of C1q in cancer progression »

12th International Conference on Complement Therapeutics (Rhodes, Greece, 2019): oral presentation, «Canonical and non canonical functions of C1q in cancer progression », travel award for best oral presentation

AACR Congress (Atlanta, United States of America, 2019): poster « Role of the classical complement pathway in tumor progression »

French Society of Immunology (SFI) Congress (Reims, France, 2017): oral presentation « Implication of the classical complement pathway in the tumor progression of clear cell renal cell carcinoma »

Cordeliers research center Day (Paris, France, 2017): oral presentation « Implication of the classical complement pathway in the tumor progression of clear cell renal cell carcinoma », first prize for oral presentation

PATENTS

- WO2019043138 (A1) - METHOD FOR PREDICTING THE OUTCOME OF A CANCER (2017)
- REF EB19289 – ROLE OF FH IN CANCER PROGRESSION (2019)

ANNEXES

ANNEX 1: Supplemental informations on bioinformatic methodology

ANNEX 2: Comparison of plasma level of C4d in patients vs. healthy donors

ANNEX 3: Comparison of plasma level of different complement proteins in patients vs. healthy donors by Luminex technology

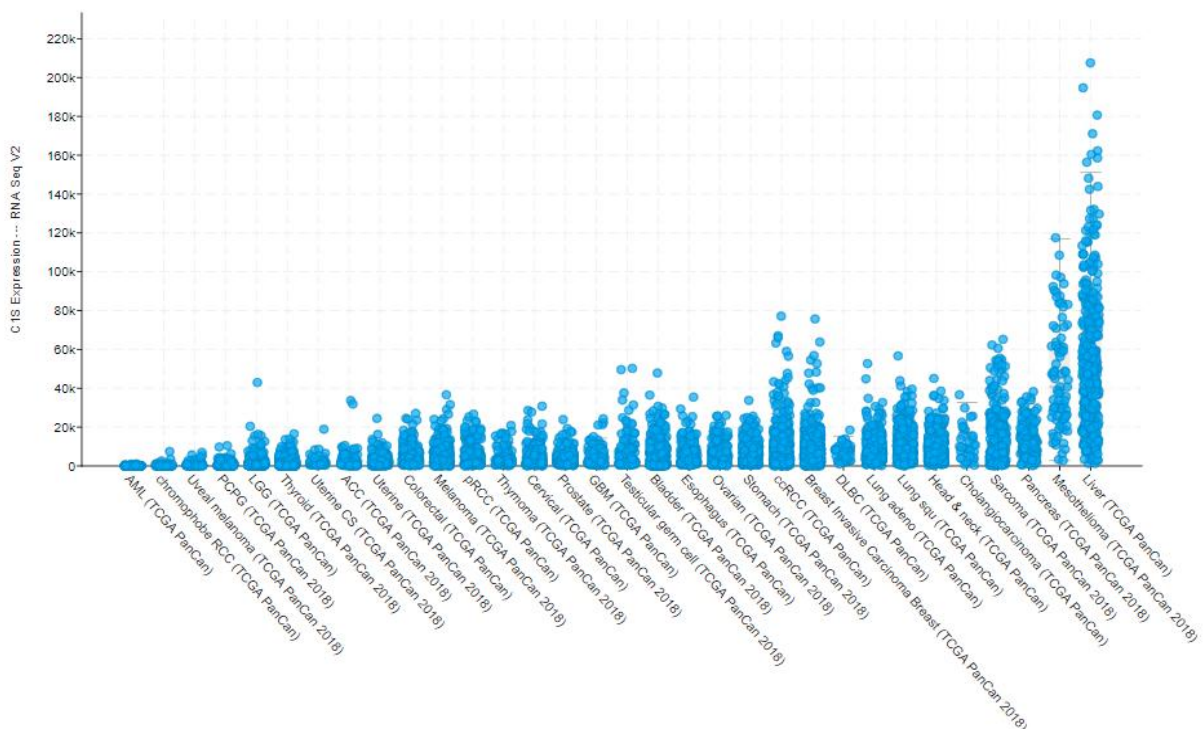
ANNEX 4: Comparison of angiogenesis and microenvironment in C1q ^{-/-} and C4 ^{-/-} mice injected with MCA205 cells

ANNEX 5: Study of the effect of PD-1 treatment in C1q ^{-/-} mice

Annex 1: Supplemental informartions on bioinformatic methodology

Source of transcriptomic data: All the transcriptomic data come from the TCGA (The Cancer Genome Atlas) Pan Cancer Atlas study, a landmark cancer genomic programs that characterized over 11,000 primary cancer from 33 cancer types³⁹². Specific inclusion criteria were applied such as untreated tumor or sample with at least 80% of tumor nuclei. The transcriptomic data are obtained through RNA sequencing that can be downloaded via cBioPortal³⁹³, an open access resource that provide molecular and clinical informations. RSEM algorithm (RNA-Seq by Expectation Maximization) have been used to generate TPM (Transcript Per Million)

Expression analysis: To analyse expression of each gene in each cancer type I have calculated the mean of TPM value for each patients in every cohorts. This method allowed to have a global idea of gene expression but had the inconvenient to hide individual vartiability that can be very important depending on cancer type. For example, we can see that in mesothelioma or liver cancer the expression of C1s can vary a lot from near 0 TPM to 120 000 TPM whereas in diffuse B cell lymphoma (DLBC) the expression of C1s is quite homogeneous (see below).



Because liver is the main producer of almost all complement proteins, we decided to exclude it from the analysis in order to avoid any bias.

Data visualization: To easily compare gene expression level between cancer types, I have represented the level of gene expression by heatmap. In order to facilitate data visualization, a log₂ transformation of TPM data has been applied to limit skewness of the data. In the heatmap, different strategies of normalization can be applied to represent the data. The absence of normalization allows the free comparison of gene and cancer types. At the opposite, the application of normalization by gene or cancer type limits the possibility of comparison but can highlight some little difference that can be hidden.

Tumor versus normal analysis: Median TPM expression for each gene in every cohort for tumor and matched paired sample were downloaded from the GEPIA platform³⁹⁴. To represent the up or downregulation of each gene in tumor vs. normal paired tissue, I have calculated the fold change (medianTPM (tumor)/ medianTPM (normal)). A log₂ transformation was applied for heatmap representation.

Prognostic analysis: Kaplan-Meier curves and log-rank p-value are commonly used to visualize the survival during time. For quantitative variables, a cut off had to be determined to make at least two groups of patients. Different strategies exist to determine a cut off: arbitrary cut-off that is independent of the data or calculation of optimal cut off. Each method has advantages and inconvenients. The use of optimal cut-off allows the maximization of survival the different between groups but can introduce some artificial statistical bias whereas the use of an arbitrary cut-off (e.g median, mean, first quartile...) is more stringent but can hide some statistic difference. To establish the survival map representing the hazard ratio of each gene in the different cancer types, I have calculated the Hazard ratio on overall survival by taking a median cut off. The statistical different between high and low group for one gene in one cancer was assessed with log rank test and a p-value < 0.05 was represented by a black box.

Annex 2: Comparison of plasma level of C4d in patients vs. healthy donors

Background:

In lung cancer, C4d can be used as a diagnostic biomarker since its level increase in the plasma²⁵¹ and bronchial fluid²⁵⁰ of patients in comparison with control group without cancer. In ccRCC patients, I detected that a classical pathway activation can occurred and I sought to investigate if this activation can translate into a modification of the plasmatic compartment.

Goal:

The aim of this study is to compare the plasmatic level of C4d from ccRCC patients with healthy donors. The second objective is to decipher if local complement activation within the tumor can result in a modification of the systemic concentration.

Methods:

Plasma samples of 29 patients with ccRCC were collecting at time of surgery at Hopital Européen Georges Pompidou (Paris, France) since 2017 (cohort 1). Another cohort of 77 patients with ccRCC were collected at time of surgery at Institut Mutualiste Montsouris (cohort 2) (Paris, France). Thirty-one plasma samples from healthy donors were used as a control for C4d quantification by Elisa.

ELISA C4d – A commercial kit was used to measure specifically C4d in the plasma (Svar, ref COMPL, C4d RUO). Preliminary test was performed to compare 1:50 and 1:100 dilutions. A better signal/noise ratio was obtained for 1:50 and this dilution was retained for further experiments. Plasma samples were diluted 1:50 and transferred to the pre-coated microtiter wells with an anti-C4d neoepitope monoclonal antibody for 1hour. After washing, incubation with a secondary antibody labeled with HRP was performed for 30minutes. After washing, the substrate was added for 30minutes and the color was measured by a spectrophotometer.

Nephelometry – Plasma concentrations of the complement components C4 and C3 antigens were measured by nephelometry (Dade Behring, Paris La Defense, France).

Statistical analysis – A Mann-Whitney test was used to test the difference of C4d level between ccRCC patients and healthy donors as well as patients with high or low level of C4d deposits inside the tumor core.

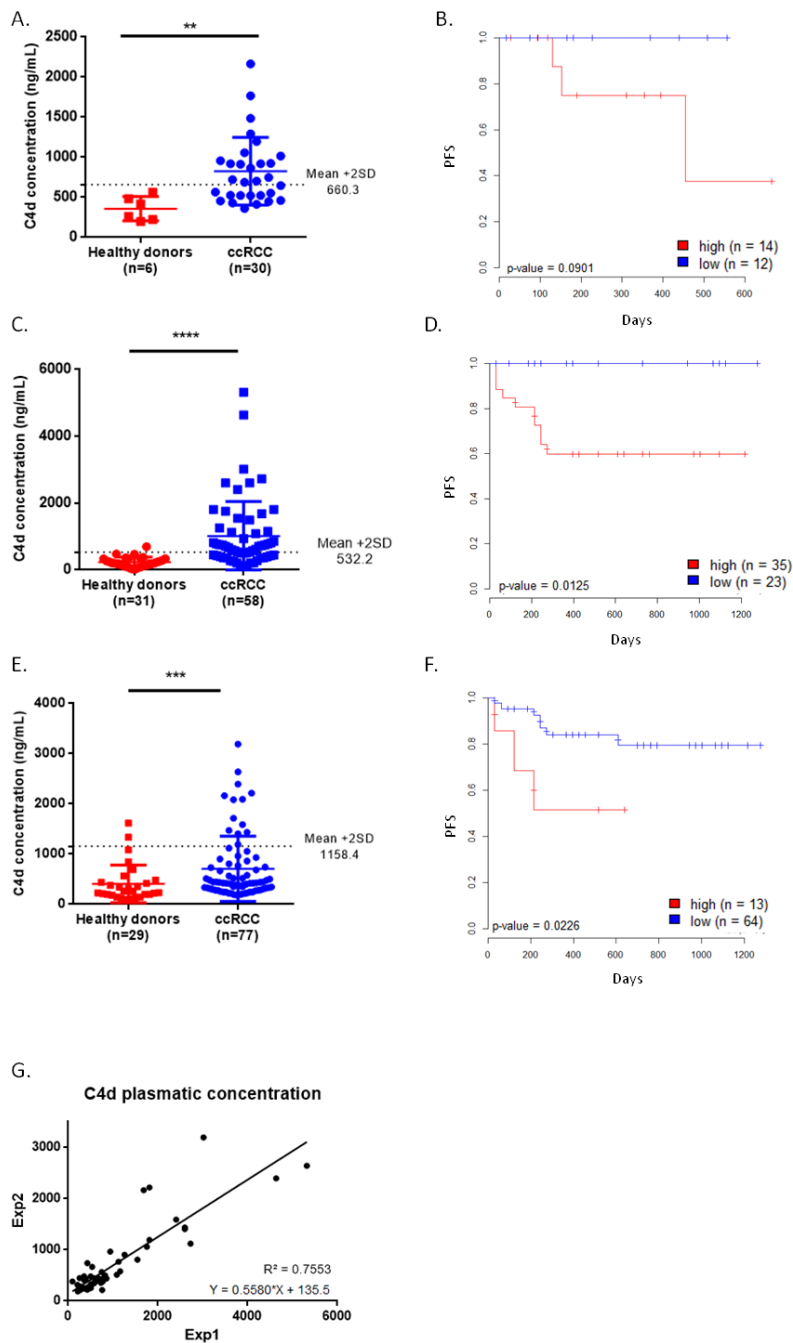


Figure 1: Plasmatic C4d a strong diagnostic and prognostic biomarker. A: Plasmatic C4d concentration determined by ELISA in cohort 1, mean+2SD represents the cut off to determine a High vs. low expression. B: Progression free survival of ccRCC patients according to C4d plasmatic level with mean+2SD cut-of for cohort 1. C: Plasmatic C4d concentration determined by ELISA in cohort 2 first experiment , D: Progression free survival of ccRCC patients according to C4d plasmatic level with mean+2SD cut-of for cohort2 first experiment, E: Plasmatic C4d concentration determined by ELISA in cohort 2 first experiment , F: Progression free survival of ccRCC patients according to C4d plasmatic level with mean+2SD cut-of for cohort2 first experiment, G:Correlation between C4d plasmatic level of common patients of experiment 1 and 2

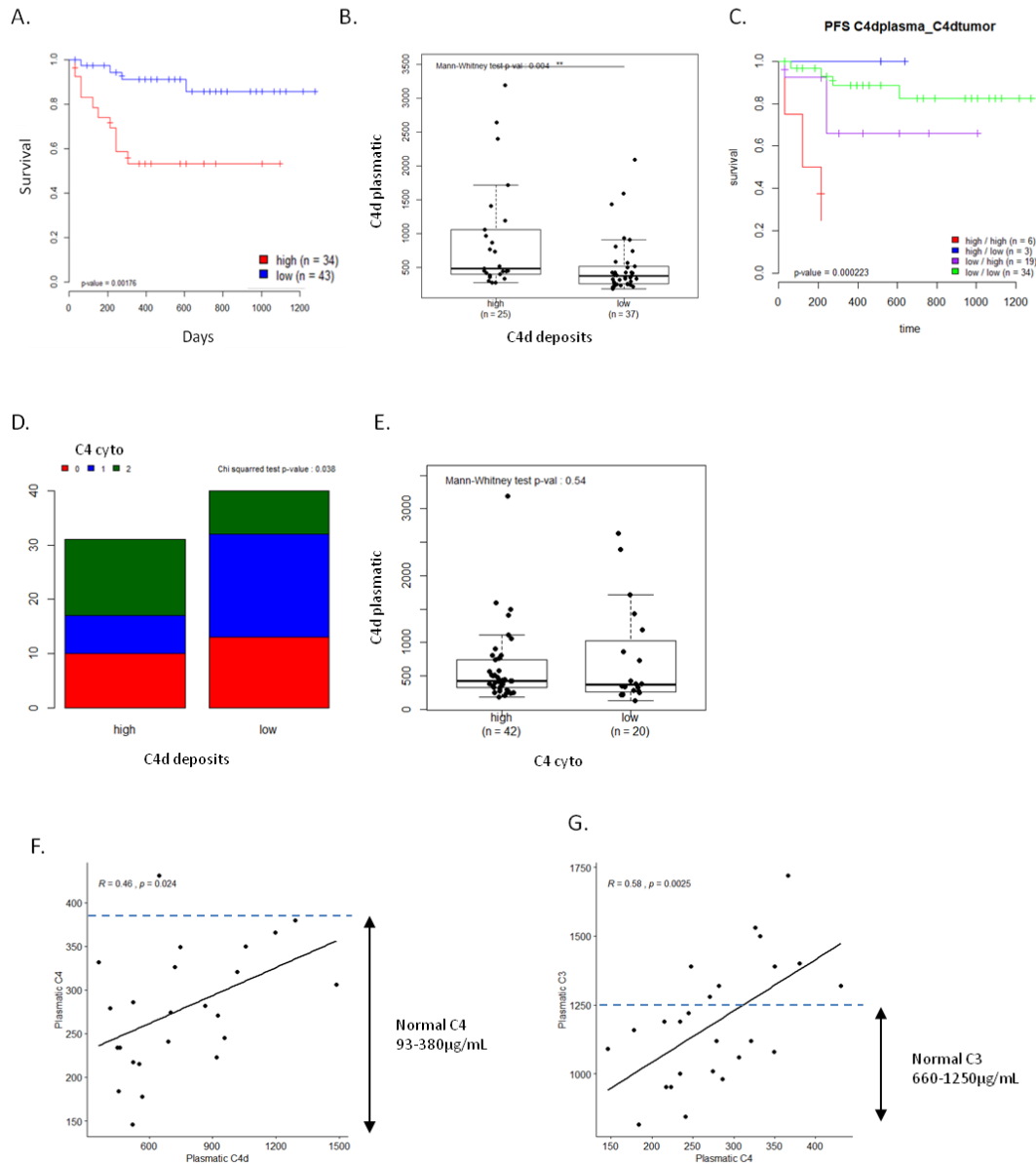


Figure 2: A: Progression free survival of ccRCC patients according to C4d deposition level within the tumor, B: Correlation between C4d plasmatic level and C4d deposits within the tumor, Mann-Whitney test is applied to test the difference between two groups, C: Progression free survival of ccRCC patients according to C4d deposition level within the tumor and the plasmatic level of C4d (cut off = mean + 2SD), D: Correlation between C4 production by tumor cells (C4 cyto) and the number of C4d deposits in situ (C4d deposits), E: Correlation between C4d plasmatic level and C4 production by tumor cells, Mann-Whitney test is applied to test the difference between two groups, F: Correlation between plasmatic C4d and C4, Pearson test is applied to test the difference between two groups, dotted line indicated the upper limit of normal range of C4. F: Correlation between plasmatic C3 and C4, Pearson test is applied to test the difference between two groups, dotted line indicated the upper limit of normal range of C3

Kruskal Wallis p value		Pearson p value	
	C4d plasma		C4d plasma
Sex	0,26	CD163 TC	0,377
Fuhrman	0,31	CD163 IM	0,1389
TNM stage	0,52	CD163 TOT	0,1037
		CD20 TC	0,3386
		CD20 IM	0,6138
		CD20 TOT	0,2061
		CD8 TC	0,3822
		CD8 IM	0,6992
		CD8 TOT	0,4644
		CD3 TC	0,951
		CD3 IM	0,8715
		CD3 TOT	0,9973
		PD-1 TC	0,2836
		PD-1 IM	0,1278
		PD-1 TOT	0,1318
		LAG3 TC	0,9021
		LAG3 IM	0,1604
		LAG3 TOT	0,9027

Mann-Whitney p value	
	C4d plasma
C4 depot	0,004
C4 cyto	0,87
FH depot	0,6
FH cyto	0,46
C1q infiltrate	0,15

Table 15: Correlation between C4d plasmatic level and clinical, immune and complement markers. TC: tumor core, IM: invasive margin, TOT: total

Results:

In the first cohort, the level of plasmatic C4d was significantly higher in ccRCC patients than healthy controls (1A). Moreover, we observed a trend on patient survival with progression events only in the group of ccRCC patients where the level of plasmatic C4d is high ($>\text{mean}+2\text{SD}$) (1B). This cohort has a very short follow-up (starting 2017). To confirm this observation, we took a second cohort of 58 ccRCC patients for which we had a longer follow up (starting 2014) and 31 healthy controls. We confirmed the difference of C4d plasmatic level between patients and healthy controls (1C) and validated the negative prognostic impact on PFS, testing part of the samples after first thawing (1D). The repetition of this experiment with all patients included, but on once freeze-thawed samples, showed the same results (1E,F) with a good correlation between the two experiments (1G). However, the cut-off corresponding to the mean + 2SD varied probably due to an effect of freezing or pre-analytic conditions for the treatment of control samples.

The patients with high number of C4d-deposits positive tumor cells have a decrease PFS comparing to patients with low C4d deposits (2A). Interestingly, the patients that displayed local complement activation inside the tumor revealed by high level of C4d deposition, had a significant increase in plasmatic level of C4d (2B). Moreover, the patients with both high level of C4d in the tumor and the plasma had the worst prognosis. The effect of C4d deposits in the tumor and in the plasma were confirmed by Cox univariate analysis (data not shown) and multivariate Cox analysis with these two variables highlighted that the prognostic value of plasmatic C4d was not independent of C4d deposition in the tumor (data not shown).

We found a correlation between the presence of local production of C4 by tumor cells determined by IHC and the presence of C4d deposition suggesting that the local production was in part responsible for C4d deposits (2D). However, this local production had no impact on the level of plasmatic C4d (2E). Interestingly, we observed that the plasmatic concentration of C4d was directly correlated with C4 plasmatic level (2F) meaning that the systemic compartment also played a role in the elevation of C4d in the plasma. Finally, a strong correlation between the plasmatic level of C4 and C3 was detected (2G). One out of the 29 tested patients had a C4 plasma concentrations above the normal range, while 11/29 (38%) had abnormally high C3.

Interestingly, the increased C4d did not correlate with the available clinical parameters (sex, Furman grade or TNM stage), the immune infiltration and the activation status of the infiltrating immune cells or other complement staining in the tumor. These findings revealed that C4d is independent prognostic marker for poor clinical outcome in ccRCC. Analysis with more patients will be made to confirm this effect.

Conclusion:

Plasmatic c4d was significantly increased in the plasma of ccRCC patients in comparison to healthy donors and, plasmatic C4d and associated with poor prognosis. This result, together with the data for lung cancer²⁵¹ place plasmatic C4d as a novel promising parameter to evaluate the prognosis of cancer patients. However, considering the variability of plasmatic C4d dosage according to freeze/thawing, an increase number of healthy donors with a known status of freezing procedure and standardized pre-analytic conditions are needed to establish a good quality standard, normal range and cut-off.

C4d is also elevated in systemic lupus erythematosus and C4d levels alone could forecast recurrence of future lupus nephritis³⁹⁵. This is a rapid screening test which, upon further validation, could be introduced into the routine screening of complement laboratories.

The elevation of plasmatic C4d correlated with the level of plasmatic C4 but the C4 plasma levels remained in the normal range, although towards the upper limit. Interestingly, the C3 plasma concentration was above the normal range in 38% of the cases, suggesting overall inflammatory context. Thus, the increase of plasmatic C4 could be responsible in part for a higher amount of native C4 available in the tumor microenvironment, which could be cleaved locally to generate C4d. Moreover, the local production of C4 by tumor cells could constitute a second source of local C4 since I found a correlation between C4 production and the presence of C4d deposition.

The origin of the plasma C4d requires further investigation. There is no hypocomplementemia in any of the patients, suggesting that there is no consumption of C4 in the circulation. C4d can be generated via the classical or the lectin pathways, but both require a multivalent ligand (surface) for the activation. Upon C4b generation, it binds covalently to any adjacent surface or remains in the fluid phase with inactivated tioester bond. Therefore, the plasma C4d likely comes from the tumor. Indeed, I detected a correlation between the C4d deposits in the tumor and in the plasma. In addition, the prognostic value of plasmatic C4d was not independent from C4d within the tumor. The plasma C4d could arise due to unsuccessful binding of C4b to the tumor cells and subsequent inactivation in the fluid phase by C4BP and FI, or due to release of a part of membrane-bound C4d through microvesicles. Such process of C4d elimination via tumor microvesicles is described in the context of renal transplantation³⁹⁶, but remains to be proven in cancer.

Annex 3: Comparison of plasma level of different complement proteins in patients vs. healthy donors by Luminex technology

Background:

The concentration of some complement proteins can be modulated in the plasma of cancer patients vs. healthy donors. For example, C1q level was found increased in the sera of glioblastoma patients while FB was decreased²⁴⁵. Many others protein can be up or downregulated in the plasma of cancer patients in a context-dependant manner such as C1s²⁴⁶, C4²⁴⁸, C3²⁴⁸⁻²⁵², C3a²⁵⁵, C5a desArg²⁵⁶, C5a²⁵⁷, FH²⁵⁹⁻²⁷¹, C9²⁶⁴, MBL²⁶⁶, FI²⁶⁹. Thus, we screened the plasma concentrations of a panel of complement proteins in ccRCC using a high throughput method – the multiplex technology.

Goal:

The aim of this study is to compare the plasmatic level of different complement protein from ccRCC patients with healthy donors in a high throughput manner by using the Luminex kit.

Methods:

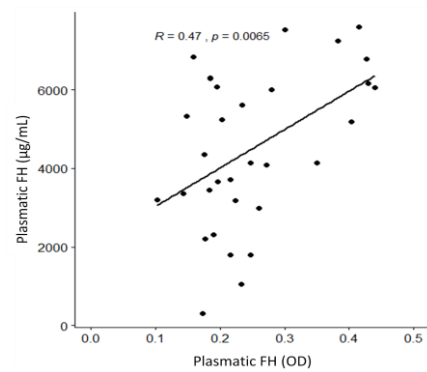
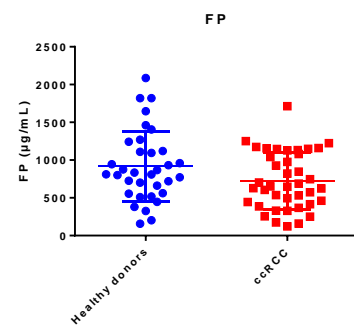
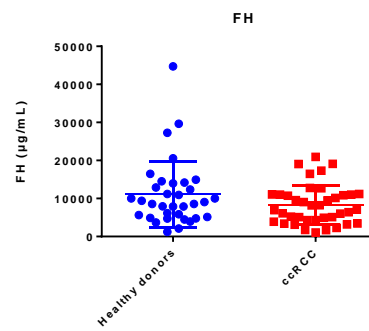
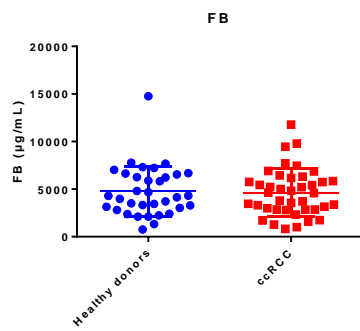
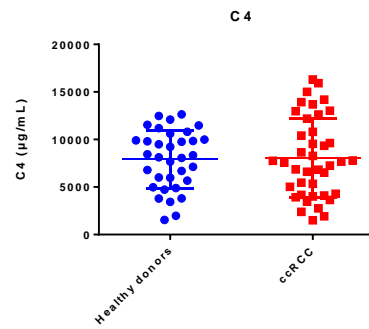
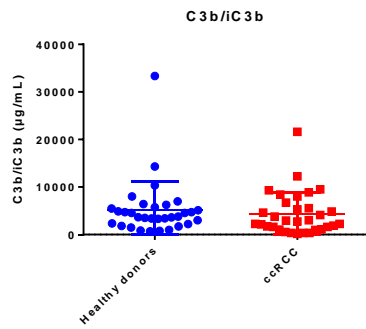
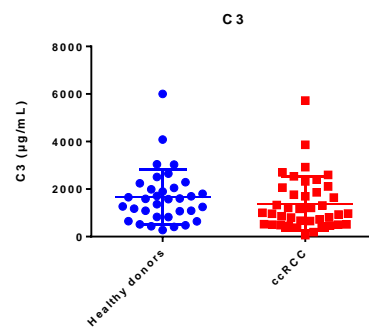
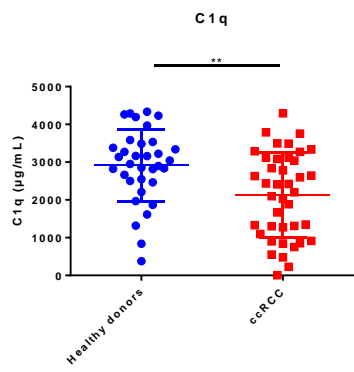
Plasma samples of 40 patients with ccRCC were collected at time of surgery at Institut Mutualiste Montsouris (part of cohort 2 from Annex 2) (Paris, France) and were used for Luminex. 29 plasma samples from healthy donors were also used as a control.

Multiplex Assay for evaluation of the concentration of complement proteins in plasma - The Human Complement Panel 1 Bead-Based Multiplex Assay kit (MILLIPLEX MAP Human Complement Panel 1), using the Luminex xMAP technology, enables the simultaneous analysis of complement proteins and factors in human plasma. Having in mind the minimal amount of plasma from ccRCC patients available (500 µl/patient), we opted for a high throughput evaluation rather than classical routine approaches by nephelometry and ELISA, which require 1 ml of plasma. The same plasma samples as above were tested as indicated in the manufacturer's protocol.

ELISA assay for FH dosage - FH plasma levels were measured by a sandwich ELISA method. Briefly, a polyclonal antibody against FH was purified from goat anti-serum using protein G and left native or was biotinylated to generate a home-made ELISA kit. The native anti-FH was coated on a Nunc Maxisorb ELISA 96-well plate overnight 4°C. After washing with PBS Tween 0.1% and blocking the free reactive sites with PBS BSA 1% during 1 hour at room temperature (RT), the plasma were added to the plate and incubated during one hour at RT.

After washing, plate was incubated with an in-house biotinylated antibody anti-FH (purified from antiserum from Calbiochem) for 1 hour at RT. After additional washings, streptavidine coupled with horseradish peroxidase (HRP) (Dako, P039701-2) was added for 1 hour at RT. After revelation, multiskan Ex (Thermofisher Scientific) was used to read the optical density at 450nm.

Statistical analysis – A Mann-Whitney test was used to test the difference of complement protein level between ccRCC patients and healthy donors.



Plasmatic level of C1q, C3, C3b/iC3b, C4, FB, FH and FP in ccRCC patients vs healthy donors.

Results:

The evaluation of the plasma concentrations of complement proteins using the MILLIPLEX MAP Human Complement Panel 2 kit appeared to be challenging. Indicated dilutions often gave out of standard values, which were for some cases impossible to calculate especially for C3b/iC3b, FH and FP. Moreover, calculated concentrations were far from the expected ones, based on routine approaches. Indeed, while C3 and C4 were little bit more elevated than the normal range determined by nephelometry (C3: 660-1250 μ g/mL, C4: 93-380 μ g/m, the calculated concentrations for FB and FH were very elevated than the normal range (normal range for FB: 90-320 μ g/mL, FH: 65-140 μ g/mL). However, even if the calculated concentrations were not correct, we found a correlation between FH concentration determined by Luminex and FH Optical density determined by ELISA, the standard technique. Nevertheless, comparison of the concentration of the healthy controls and ccRCC patients revealed that there is no difference in the amount of C4 C3, C3b/iC3b, FB FH and FP. The only detected difference was for C1q with a decrease plasmatic concentration in patients in comparison with healthy donors

Conclusion:

It has to be noted that the use of the MILLIPLEX MAP Human Complement Panel 2 kit was challenging due to the inadequate dilutions. Similar problems were encountered by other groups using the same assay³⁹⁷. Although the comparison between the complement concentration in the plasma of the healthy donors and the ccRCC patients allowed us to conclude that there are no differences for C4, C3, C3b/iC3b, FB, FH and FP protein levels. The lack of difference between controls and patients was also confirmed by ELISA for FH (see manuscript 4). Therefore, we agree with the conclusion of Sandholm et al that the MILLIPEX assay less reliable and implies that the company should reconstruct their assay since the results are not on pair with the standard of other techniques used today.

Annex 4: Comparison of angiogenesis and microenvironment in C1q -/- and C4 -/- mice injected with MCA205 cells

Background:

C1q deficiency is associated with an impaired vascularization and a remodeling of the immune microenvironment, that results from either decrease classical complement pathway activation or a C1q cascade-independent effect.

Goal:

In order to study to what extent these effects are C1q specific, I compared the angiogenesis and the immune environment with C4 deficient mice inoculated with MCA205 cancer cells.

Methods:

Study approval - Male and female C1q^{-/-} mice and paired groups of C57BL/6J WT mice were used in this study. C57BL/6J and C4^{-/-} mice were purchased from Charles River, C1qa^{-/-} mice were generated and provided by Pr. Marina Botto (Imperial College, London). All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with approvals APAFIS#34\0-2016052518485390v2 and #9853–2017050211531651v5 by the French Ministry of Agriculture.

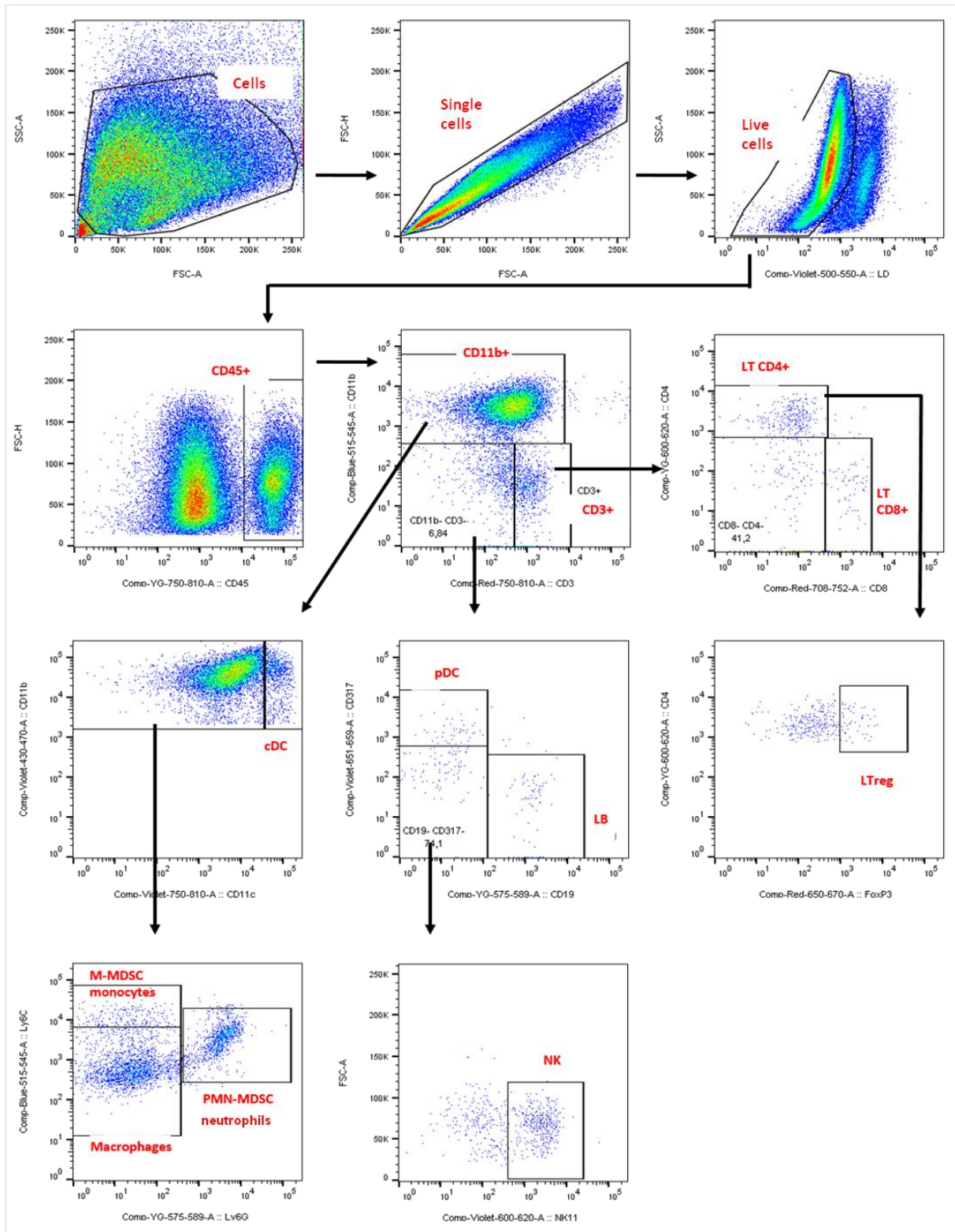
Experimental procedure - The mouse MCA205 cells were recovered at 80% confluence, and 160 000 cells were inoculated subcutaneously in the right flank with 200 µl PBS. Tumor size was measured with calipers every 2-3 days for 25 days or until reaching the ethical endpoint of tumor size approaching 3000 mm³. Tumors were used fresh for flow cytometry analyses or frozen with liquid nitrogen for immunofluorescence experiments.

Flow cytometry - Freshly recovered mouse tumor tissues were dissociated with enzymatic solution (Collagenase I, 200 U/mL and DNase I, 10 U/mL) and mechanically dissociated by using gentleMACS (Miltenyi). The solutions were filtrated with 70 µm and 30 µm nylon membrane filters and washed with PBE (PBS, 0.5% BSA, 2 mM EDTA) to obtain single cell suspensions. The number of total cells was counted. Isolated cells were distributed in V shaped 96-well plates and were incubated with FcBlock (anti CD16/CD32, BD Biosciences) for 20 minutes at 4°C. Between the steps, the cells were washed with PBE. The cells were further incubated with viability marker (Live Dead, Thermo Fisher) and membrane marker antibodies (Supplementary Table 8) diluted in PBE for 30 minutes at 4°C. Then, the cells

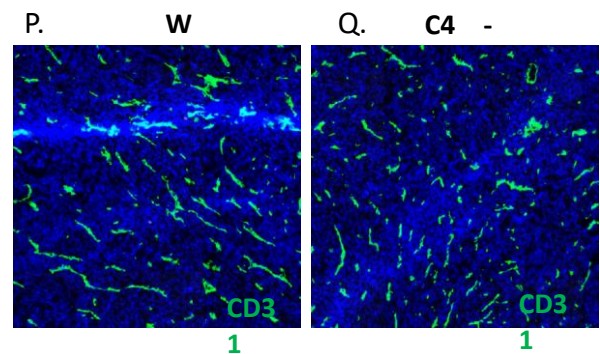
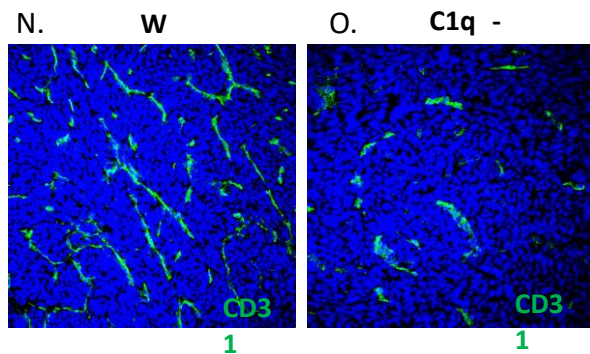
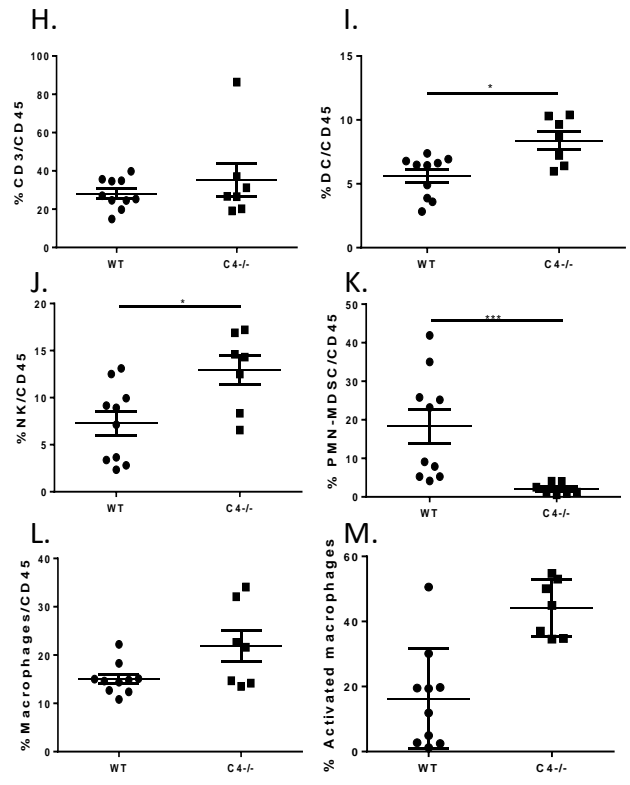
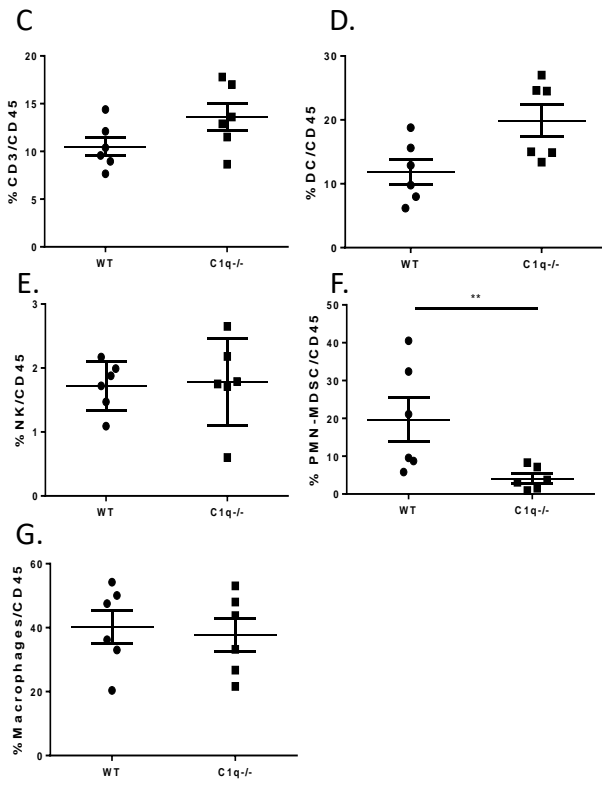
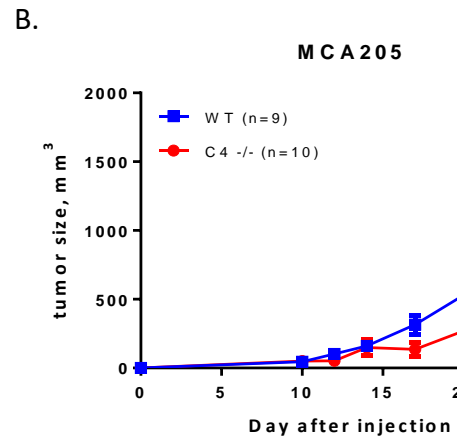
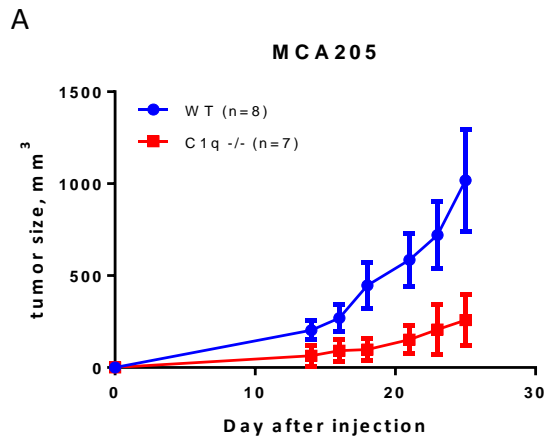
were washed with PBE and suspended in 4% paraformaldehyde (PFA). The gating strategy is presented below.

Antibody	Reference	Clone	Fluorochrome	Supplier	Concentration (µg/ml)
CD11b	553310	M1/70	FITC	BD Biosciences	5
CD11c	563735	HL3	BV786	BD Biosciences	2
CD19	557399	1D3	PE	BD Biosciences	2
CD3	47-0032-82	17A2	APC-eF780	eBioscience	2
CD4	MCD0417	RM4-5	PE Texas Red	Invitrogen	2
CD45	552848	30-F11	PE-Cy7	BD Biosciences	0,5
CD8	56-0081-82	53-6.7	AF700	eBioscience	2
F4/80	MCA497	A3-1	AF700	Bio-Rad	10
Ly6C	553104	AL-21	FITC	BD Biosciences	5
Ly6G	551461	1A8	PE	BD Biosciences	2
NK1.1	563220	PK136	BV605	BD Biosciences	2
IA/IE	563415	M5/114	BV650	BD Biosciences	5

Immunofluorescence staining - Frozen mouse tumors were cut at 6 µm with cryostat (Leica), fixed in acetone and stained for CD31 by using a rabbit polyclonal anti-mouse CD31 at 10µg/mL (Abcam, ab124422) followed by an incubation with the secondary antibody goat anti-rabbit IgG AF647 at 20µg/mL (ThermoFisher, A-21245). Nucleus were stained with DAPI. Slides were mounted with Prolong Glass and scanned with Zeiss Axio Scan.



Gating strategy to define immune populations in the TME. Activated macrophages are defined as IA/IE+ population among macrophages.



Comparison of angiogenesis and immune population in MCA-205 tumors inoculated in C1q -/- or C4 -/- mice. A) Tumor growth of MCA205 cells injected subcutaneously in C1q -/- or WT BL57BL/6 mice. B) Tumor growth of MCA205 cells injected subcutaneously in C4 -/- or WT C57BL/6 mice. C) Percentage of CD3+ cells among CD45+ cells in C1q -/- or WT mice. D) Percentage of DC among CD45+ cells in C1q -/- or WT mice. E) Percentage of NK cells among CD45+ cells in C1q -/- or WT mice. F) Percentage of PMN-MDSC among CD45+ cells in C1q -/- or WT mice. G) Percentage of macrophages among CD45+ cells in C1q -/- or WT mice. H) Percentage of CD3+ cells among CD45+ cells in C4 -/- or WT mice. I) Percentage of DC among CD45+ cells in C4 -/- or WT mice. J) Percentage of NK cells among CD45+ cells in C4 -/- or WT mice. K) Percentage of PMN-MDSC among CD45+ cells in C4 -/- or WT mice. L) Percentage of macrophages among CD45+ cells in C4 -/- or WT mice. M) Percentage of activated macrophages among CD45+ cells in C4 -/- or WT mice. CD31 staining (green) in tumors of WT mice (N) or C1q -/- mice (O). CD31 staining (green) in tumors of WT mice (P) or C4 -/- mice (Q)

Results:

The deficiency of either C1q (A) or C4 (B) was associated with a decreased tumor growth. The composition of the TME was strikingly different between tumors growing in WT and C1q-/- mice with more dendritic cells (D) and less PMN-MDSC (F) that was also observed in C4 deficient mice (J,L). Moreover, macrophages were in a more activated state with more expression of IA/IE (MHCII) (H,N). In terms of angiogenesis, C1q deficient mice displayed smaller vessels with a disrupted architecture (O,P). However, this effect on angiogenesis was not found in C4 deficient mice, which had the same morphology as the wild type mice (Q,R).

Conclusion:

The modulation of the angiogenesis was only found in C1q deficient mice, suggesting that this effect is independent of the complement cascade and specific to the C1q protein. This is in agreement with the study of Bulla et al, showing C1q-specific angiogenesis-promoting effect independent of the cascade, in B16F10 melanoma mouse model¹⁶⁷ as well as with our data on the TC-1 model, where similar results were observed (see Article 3).

The alteration of the immune microenvironment is commonly found in both C1q-/- and C4-/. This suggests that the impact of C1q on tumor microenvironment is potentially relied to complement activation. This is in agreement with the data of Markiewski et al for the TC-1 model, showing that C5a/C5aR1 axis is critical for the immune infiltrate³⁰⁴. On the other hand in the melanoma mouse model B16F10 described by Bulla et al, no difference in the immune infiltrate was detected as well as no activation of the complement cascade.

The ensemble of these results support the conclusion that the impact of C1q on the neoangiogenesis is a non-canonical function, independent on the cascade, while the immune infiltrate modulation depends on the cascade activation and the generation of the anaphylatoxins. Injection of C1 inhibitor could be used to validate that the modulation of immune system in the tumor can be linked to an aberrant classical pathway overactivation and immune infiltration.

Annex 5: Study of the effect of PD-1 treatment in C1q -/- mice

Background:

The concomitant treatment of mice with C5aR1- and anti PD-L1/PD-1 inhibitors showed a synergic effect of this combination in a mouse model of lung cancer³⁹⁸. Moreover, an ongoing clinical trial tests the concomitant inhibition of C5aR1 and PD-L1 solid cancers (STELLAR-001 trial, Innate Pharma, first results to be reported at ESMO2019). We have demonstrated that C1q deficiency alone is associated with a slower tumor growth and we wanted to know if this effect could be amplified by anti PD-1 treatment.

Goal:

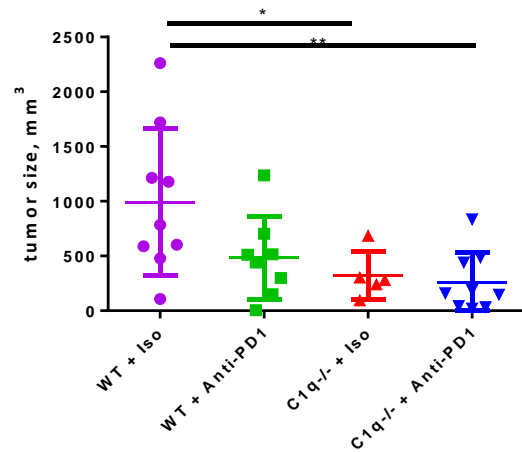
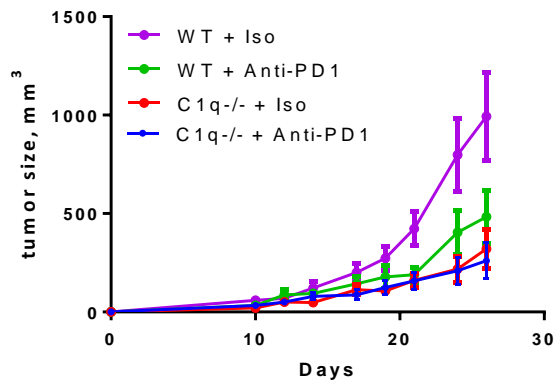
The goal of this study is to evaluate the potential of targeting both C1q and PD-1 by treated C1q deficient mice with anti PD-1 inhibitor.

Methods:

Study approval - Male and female C1q-/- mice and paired groups of C57BL/6J WT mice were used in this study. C57BL/6J mice were purchased from Charles River, C1qa-/- mice were generated and provided by Pr. Marina Botto (Imperial College, London). All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with approval #9853-2017050211531651v5 by the French Ministry of Agriculture.

Experimental procedure - The mouse MC-38 cells were recovered at 80% confluence, and 400 000 cells were inoculated subcutaneously in the right flank with 200 µl PBS. When the tumor volume of 50-100mm³ was reached mice were randomized to homogenize groups and either 100µg of anti PD-1 or control isotype were administrated intraperitoneally every 2-3 days. Tumor size was measured with calipers every 2-3 days for 25 days or until reaching the ethical endpoint of tumor size approaching 3000 mm³.

The MC38 cell line was selected because of the responsiveness of the MC38 tumors to anti-PD-1 treatment³⁹⁹.



Left: tumor growth in C1q or WT mice treated with anti-PD1 or control isotype. Right: tumor size at day 26 in C1q or WT mice treated with anti-PD1 or control isotype.

Results:

Alone anti PD-1 or C1q deficiency delayed tumor growth. Nevertheless, anti-PD1 treatment did not synergize with the C1q deficiency.

Conclusion:

This experiment provides evidence for one more cell line (MC38), for which the tumors formed after sub-cutaneous injection grow slower in C1q^{-/-} mice. The absence of synergistic effect between C1q deficiency and an anti-PD1 treatment shows that this combination is not relevant. A plausible hypothesis is that C1q deficient mice display a less exhausted immune microenvironment, with a decreased expression of PD-1 on T cells, leading to an inability of anti-PD1 antibody to reach its target and to re-invigorate the T cells. Indeed, in human a strong correlation between the level of C1q and the expression of immun checkpoints on T cell like PD-1 or LAG3 was detected. Further experiments, measuring PD1, LAG3 and TIM3 on the tumor infiltrating T cells in the MC38 tumors from C1q^{-/-} versus WT mice could test this hypothesis.

DISCUSSION

During the three years of this thesis, I aimed to find out how the complement system, known for its host protective ability, becomes offensive in the context of cancer. To understand the intricate relationship between complement and cancer, I have taken advantage of different resources including *in silico*, *in vitro*, *in vivo* as well as *in situ* experiments. Firstly, I depicted the global landscape of complement expression in human cancers, showing the context-dependent role of the complement system on tumor progression. Indeed, pan-cancer transcriptomic analysis revealed a heterogeneous expression of complement genes among tumor types associated with either protective or aggressive impact. Using the example of clear cell renal cell carcinoma (ccRCC), which classified in the group of “aggressive complement” cancers, I studied the impact of the canonical (cascade-dependent) and non-canonical (cascade-independent) functions of the complement proteins on tumor progression. In this tumor type, both immunoglobulin-mediated classical complement activation and cascade independent effect of complement proteins such as C1q, C1s or FH were associated with pro-tumoral effect that impact patient survival. I found that C1q could act on immune environment and angiogenesis to promote tumor growth whereas FH and C1s proceeded through an unexpected intracellular way to modify tumor cell phenotype. Moreover, elevated plasmatic C4d appeared to be a non-invasive negative prognostic biomarker in ccRCC. The ensemble of these results highlighted the potential of complement proteins as powerful diagnostic and prognostic biomarker as well as a very promising therapeutic target.

Studies from the last decade allowed to position the complement system as a key actor of the TME that can modulate and influence tumor progression. Besides its ability to opsonize and induce cell death, complement can also interfere with tumor cells proliferation, metastasis, angiogenesis or with the immune microenvironment. Nonetheless, the data from the literature regarding its impact and mechanism of action are often contradictory and do not allow a clear conclusion of its effect on tumor progression. The goal of the present work was to study the context-dependent role of the complement system in cancer. A specific focus on the impact of the complement system in renal cancer has been made due to the negative prognostic impact of complement in this type of tumors and due to the deleterious impact of the complement overactivation particularly within the kidney.

Tumors are a complement-rich environment where the *in situ* presence of complement proteins can result from both systemic compartment and local production. While the bulk of complement components in the circulation comes from hepatocytes synthesis, an extrahepatic production by different cell types can also occur and participate to the local concentration^{68,73}. Thus, in certain tissues such as lung⁴⁰⁰, brain⁴⁰¹ or kidney⁴⁰², complement proteins are highly

expressed. However, after malignant transformation, this local production can be largely affected with either a decrease or an increase synthesis depending on cancer types. For instance, I found that in lung cancer almost all complement genes were downregulated whereas in pancreatic cancer the exact inverse effect was observed. To deal with this heterogeneity, I have applied bioinformatic tools to redefine the complement landscape in tumors (Manuscript 1). By using transcriptomic data from more than 8000 cancer patients available on TCGA database, I was able to establish an overview of complement expression in more than 30 different tumor types. The expression level of complements components undergoes important variations depending on the nature of complement gene, the tumor types and also among patients. In general, I found a high expression of genes from the classical and the alternative pathway as well as complement receptors and regulators. In contrast, low expression of lectin and terminal pathway genes was observed. Nevertheless, some diversity among cancer types as well as tumor-specific effects could be detected. For example, while in the majority of tumor types C8 and C9 genes were not detected, in cholangiocarcinoma there was a high expression of these genes.

The gene expression analyses allow a rapid high throughput analyses, but unfortunately the gene expression data does not always correlate well with the expression at protein level. Therefore, for my study it was critical to evaluate the *in situ* presence of complement components at the protein level using immunohistochemistry/immunofluorescence on tumor sections. Moreover, besides providing data on expression, these techniques give additional important information such as the nature of complement producing cells as well as their spatial distribution within the tumor. Therefore, I have set up an immunohistochemistry staining algorithms, validating the specificity of the antibodies and optimizing the experimental conditions to allow reliable staining and data interpretation (Manuscript 2). The choice of antibodies and staining conditions is critical, since poor sensitivity or sub-optimal antigen retrieval could give false negative (or false positive if background is present) results, hampering the conclusion about the contribution of complement activation in cancer progression. Thus, I was able to identify the cell types that participated in the local production of complement proteins such as tumor cells, fibroblasts, macrophages and endothelial cells. While tumor cells were the main producer of C3, C4 or C1s, C1q was exclusively produced by stromal cells and especially macrophages in ccRCC tumor sections (Manuscript 3). Even though, this synthesis was subject to important variations among patients, ranging from a complete absence to a very high expression of complement proteins. Furthermore, this heterogeneity was also found between the different cancer types. For example, I detected C1s staining of tumor cells in many renal and lung cancer tumors, whereas the production of C1s by the colorectal tumor cells was almost inexistent (Manuscript 5). Many intrinsic and environmental factors are involved in the regulation of complement expression. It is well

known that inflammation stimulates the production of complement proteins by hepatocytes. I showed that *in vitro* IFN γ is a key regulator of complement expression responsible for an increase production of CFH, C1s and C4 by ccRCC tumor cells. Intriguingly, I found that the plasmatic level of CRP in ccRCC patients, a protein that increase sensibly in inflammatory conditions such as cancer, did not correlate with intra-tumoral production of C4 (data not shown) suggesting that others parameters than inflammation are implicated in the regulation of complement protein synthesis.

The complement system consists of a cascade of proteolytic cleavages steps. Such activation of the complement cascade can be evaluated only by the presence of particular split products in plasma or in tissues, which cannot be assessed at transcriptomic level. In this context, a study at the protein level can be very helpful. *In situ* on ccRCC tumors, I detected complement deposits at tumor cells surface such as C4d or C3d that indicated a local activation (Manuscript 3). According to the data from the literature such complement deposits are also found in other cancer types such as lung²⁸¹ and breast cancer²⁹⁷ or astrocytoma²⁸⁰. Interestingly, the early classical complement activation, detected by C4d deposits, was associated with a poor prognosis for the patients and could be used as a powerful prognosis biomarker in ccRCC (Manuscript 3). It is still unclear whether C4d is only a biomarker or a driver of tumor progression. One study showed that C4a binds protease-activated receptor (PAR)1 and PAR4, stimulating thus endothelial permeability⁴⁰³. Neuropilin-1 (NRP1) is now emerging as a novel receptor for C4d (and for C3d and iC3b)⁴⁰⁴. NRP1 is expressed by tumor cells, dendritic cells and Treg and is implicated in tumor vascularization, progression and metastases, including in lung and renal cancer⁴⁰⁵. Therefore, it is tempting to speculate that C4d could exert pro-tumoural effect via NRP1. This hypothesis needs experimental validation. However, regarding the remarkable pro-tumoral actions of C3a and C5a described in the literature, I can speculate that the poor prognosis of complement activation relies, at least in part, on the anaphylatoxins actions generated after complement activation. Indeed, C3a and especially C5a are now considered as major pro-tumoral effectors, which not only promote immunosuppressive microenvironment but also enhance tumor cell proliferation and survival, angiogenesis and metastasis^{314,355}. It is important to note that complement activation in ccRCC tumors is not complete and does not reach MAC formation, since I was unable to detect any C5b9 at the surface of ccRCC tumors cells. Transcriptomic data support this hypothesis that tumor cells escape from complement cytolytic activity with a low expression of terminal pathway genes associated with a high expression of regulators in tumors. This escape process is well described in the literature, especially in the context of resistance to complement cytotoxicity after treatment with monoclonal antibodies³⁷⁶. Taken together, these data suggest that tumors could overcome the cytotoxic effects of complement system while

taking advantage of chronic inflammation mediated by C3a and C5a. Nonetheless, the effect of complement activation on tumor progression is highly dependent on the context: if it is uncoupled from the cytolytic terminal pathway, it can favor tumor progression. But if it is followed by MAC formation and cell killing, it may protect the host.

The elevated complement production as well as the presence of complement activation markers within the tumor could have repercussions on the systemic compartment. Indeed, I found an elevated concentration of C4d in the plasma of ccRCC patients in comparison with healthy donors (Annex 2). Interestingly, the plasmatic C4d tended to correlate with the presence C4d deposition within the tumor. This suggests that the local activation in the tumor may have consequences concerning the level of C4d in the circulation. It is still unclear how this C4d is generated. No C4 hypocomplementemia is detected in the circulation, indicating that there is less likely systemic classical pathway activation. A potential explanation could be that C4d is released by the tumor cells, covalently bound to the membrane of microvesicles. Indeed, tumor cells generate a large amount of microvesicles, including to get rid of complement deposits. Another possibility is that the tumor extracellular vesicles activate complement on their surface. The tumor-derived extracellular vesicles can be found in the circulation and could serve as biomarkers⁴⁰⁶. Further studies are needed to determine whether C4d in the plasma of ccRCC patients is associated with tumor-derived extracellular vesicles.

The method for evaluation of complement components and their split products in plasma has to be rigorously validated, similarly to the staining in tissue sections. The C4d ELISA kit evaluated here for the first time in ccRCC showed that this split product could be a useful non-invasive predictive biomarker for cancer progression. The multiplex analyses of complement components were more problematic and require further optimization. Nevertheless, at least for ccRCC, no detectable variation of the tested components was detected, except for C1q (which needs independent validation). Therefore, the evaluation of the early split products of complement are more promising biomarkers than the native proteins levels, since they give an accurate snapshot of status of the complement activation. Numerous other studies in the literature show an increase of complement components in the plasma of patients compared to their normal.

The results presented until now have highlighted that tumors are complement rich environment, where the complement system can be locally activated to generate cancer-promoting C5a. However, the question of how complement is locally initiated was unclear. The complement system can be activated by three pathways; lectin, classical and alternative pathways. Transcriptomic data indicated a high expression of classical and alternative

pathways genes together with a low expression of lectin pathways genes in most of tumor types. Moreover, *in vivo* and *in situ* evidences from the literature suggest of an activation of complement system mediated by the classical pathway^{304,305}. In ccRCC patients, I provided evidences of classical pathway activation, since C1q as well as C4d deposits but no MBL were present inside the tumors (Manuscript 3). Among the large amount of C1q ligands, immunoglobulins represent a the most likely complement activator in ccRCC. Indeed, I demonstrated that classical pathway can be activated locally through dynamic interactions between macrophages that provided C1q and tumor cells that brought the remaining components necessary for complement activation in presence of immunoglobulins (Manuscript 3). Nonetheless, the source of immunoglobulins as well as their specificity are not elucidated yet. To assess this question, I have already isolated B-cells from ccRCC fresh tumors by FACS- sorting and cultured them in order to recover antibodies. The yield of IgG was poor and the culture needs further optimization. The specificity as well as the capacity of these antibodies to fix and activate complement will be subject to further investigations. Moreover, immunoglobulins were not the only ligands of C1q in the tumors since I found some C1q deposits at the surface of tumor cells that did not colocalize with IgG. In addition, I detected a direct binding of C1q to tumor cell *in vitro* suggesting that classical complement activation could take place independently of immune complex. This result corroborate this different studies showing that C1q can interact with tumor cells through many ligands such as hyaluronic acid³⁴², epithelial discoidin domain-containing receptor 1 (DDR1)¹⁶⁹ or gC1qR³⁴³.

This mechanism of classical pathway activation is a general phenomenon, exceeding the borders of ccRCC. We detected very similar pattern of production of C1q by tumor-associated macrophages and its hijacking by the tumor cells, via the production of the remaining components of the classical pathway, when testing the TC1 mouse tumor model (manuscript 3) and the MCA205 mouse fibrosarcoma model (Annex 4) both as sub-cutaneous injection. The mouse models are excellent tool for mechanistic studies but frequently fail to recapitulate the context of the human tumors. In our case the mouse sarcoma model showed protection in the context of C1q, C4 and C3 deficiency (Annex 4), while the transcriptomic analyses suggested that overexpression of these complement genes confers favorable outcome in human patients (manuscript 1). Similarly, the mouse MC38 colorectal cancer model showed decreased tumor growth in C1q^{-/-} mice, but in human staining for C1q of colorectal cancer patients as well as C1q genes expression did not correlate with survival (data not shown). Therefore, the mouse models should be used with caution when interpreting the contribution of complement for a particular type of cancer. Nevertheless, they remain a useful tool for *in vivo* exploration of given biological functions, such as the mechanism of complement activation described here.

Complement can also be activated independently from the three well established conventional (intrinsic) pathways via the action of proteases. This process is called extrinsic activation. In the literature different proteases have been described to cleave C3 or C5 in the tumor outside the complement cascade^{89,311,312}. Even if I have no direct evidences of a protease cleavage in ccRCC, this hypothesis cannot not be excluded. Interestingly, I detected an active form of C1s and complement cleavage fragments of C4 and C3 in the serum-free supernatant of ccRCC tumor cells. C1s could have been cleaved by auto-activated C1r. The capacity of activated tumor cells-derived C1s to cleave C4 in absence of C1q has to be proven by further experiments. Indeed, I detected a concomitant production of C1r, C1s, C4 and C3 by the ccRCC cells. This complement-rich phenotype conferred particularly poor prognosis. Further analyses are necessary to find out the compartment, in which the activation of C3 and C4 takes place.

To make the situation even more complex, complement proteins could also exert an unconventional mode of action, independent from complement cascade. These last properties are called non-canonical functions and can explain the sporadic prognostic impact of complement protein observed with the transcriptomic data. Experiments performed in C1q^{-/-} mice highlighted this duality of action. In this model I observed a retardation of tumor growth initiated by s.c. injection of different cell line (TC-1, MCA205 and MC38). It is in agreement with the data of Bulla et al for B16F10 melanoma¹⁶⁷. This impact can be explained by both canonical function of C1q as complement initiator and non-canonical functions. Indeed, in these mice, there was a significant decrease of C4 deposits on tumor cell surface meaning that local complement activation was impaired. This impairment was accompanied by a modulation of the immune microenvironment with more dendritic cells and activated macrophages and less PMN-MDSC also found in C4 deficient mice (Annex 4). In parallel, on human ccRCC tumors, I found a correlation between macrophages infiltration and C1q level. Using macrophages sorted from ccRCC fresh tumors I showed that the macrophages that highly expressed C1q also expressed high level of PD-L2 and LAIR1. Such phenotype could correspond to the M5 subtypes of macrophages described by Chevrier et al. which is associated with T cell exhaustion. Interestingly, C1q levels correlated with PD-1 and LAG3 at both transcriptomic and proteomic level. Nevertheless, other modifications found in C1q^{-/-} mice seem to rely on C1q functions outside the cascade. For example, I found that C1q could act on the tumor vasculature independently from complement cascade. Indeed, C1q deficient mice but not C4 deficient mice display an alteration of vascular architecture with shorter vessels presenting a disrupted organization in both TC1 and MCA205 models. The disorganization of the vascular network in the TC1 tumors of the C1q^{-/-} mice was associated with a strong modification of angiogenic genes expressions such as VEGFR2 or VEGFC.

This C1q effect on angiogenesis has been also described in the literature *in vitro* with a promoting effect on permeability, proliferation and migration of endothelial cells¹⁶⁵ and *in vivo* with a modulation of the vasculature in melanoma tumors. Nonetheless, this effect seems cancer dependent since an opposite effect is found in Her2/neu breast cancer model³³⁷. It has to be noted that the 3 tumor models for which impaired neoangiogenesis was detected in C1q^{-/-} mice are induced by subcutaneous injection of tumor cells, while the Her2/neu breast cancer model is a spontaneous one.

The role of C1q for the neoangiogenesis was demonstrated in models of skin wound healing¹⁶⁵ and in the mouse placenta¹⁶⁸. Further studies are needed to evaluate whether the C1q affects angiogenesis only in the skin and selected organs, or this is a more general effect. Observations in C1q deficient patients will be of interest here, but no evidence from the literature suggest that these people have impaired wound healing. They are too few (about 80 described worldwide)⁴⁰⁷ to conclude on their pre-disposition to cancer. According to the very high level of vascularization in ccRCC tumors, it is difficult to evaluate an impact of complement proteins in this specific tumor type. Moreover, no correlation between VHL mutation status and production of complement proteins (C1q, C4) was found (data not shown). In this context, less vascularized tumors like NSCLC tumors could be used to correlate C1q with the vascularization level in human.

These non-canonical effects of C1q are also true for other complement proteins. I demonstrated that FH and C1s have a direct impact on lung and ccRCC tumor cells phenotype with a strong modulation of their proliferative and survival capacity (Manuscript 4 and 5). In the literature, this mitogenic effect is also described for FB³³⁹, FI³⁴⁴ and sublytic MAC³⁴⁷ through activation of Erk1/2 pathway. On the other hand, properdin exerts anti-tumoral effects through upregulation of the pro-apoptotic factor DDIT3³⁵⁴.

Taken together, our data suggest that complement proteins are multitasking effectors exerting their actions on different key parameters of the tumor such as angiogenesis, immune microenvironment or tumor cells themselves. These actions may result from both complement activation and cascade independent effects. Moreover, many factors can influence the effect of the complement system such as the local concentration of complement components or the nature of the molecular partner. For example, in a mouse model of lymphoma, C5a exert a dose-dependent effect. While a high level of C5a promotes tumor growth through an immunosuppressive action, a low dose of C5a is associated with anti-tumoral T cell response³²⁸. Important modifications of complement protein level are also detected according to the sex. For example, female C57BL/6 mice have lower serum level of C6 and C9 as well as a decrease of C9 functional activity⁴⁰⁸. These sex- difference is also observed in human

with a lower alternative pathway activity and reduce level of terminal components in females compared to males⁴⁰⁹. Furthermore, depending on the binding partner/receptor of complement proteins, the resulting effect can be very different. In the case of C1q, it can promote the proliferation and migration of tumor cells when it binds to acid hyaluronic³⁴² or gC1qR³⁴³ but it can also induce cell cycle arrest and apoptosis when it interacts with the tumor suppressor WWOX³⁵³. This complexity leads to some heterogeneity of complement effects depending on tumor types.

At the cellular level, complement proteins were detected with two localizations; in the intracellular compartment (Int-C) and/or the extracellular space (Ext-C). The intracellular detection of complement proteins reflects their local production. The presence of complement components in the extracellular compartment can result from both systemic recruitment and secretion of locally produced proteins. Indeed, even if hepatocytes are the main source of complement in the circulation at the tissue level, many cells types synthesize and secrete complement proteins. Nevertheless, it is very difficult to discriminate the proportion of each source on the extracellular complement content. In the model of hepato-FH^{-/-} mice, where the plasmatic concentration of FH was decrease by 80%⁴¹⁰, the *in situ* presence of FH was also decrease suggesting that the systemic compartment played an important role to provide FH in the tumor (Manuscript 4). Intriguingly, depending on the origin of complement proteins, their effects can be different. A recent study has demonstrated that complement and especially C3a, coming from the circulation or produced by the tumor, does not display the same effect since the activation state is different³⁷⁰. Indeed, in the systemic compartment the activation of C3 is tightly regulated by soluble and membrane-bound complement regulators in order to avoid complement attack on the host cells, whereas such regulation did not interfere with intracellular production and activation of C3 by proteases on tumor cells. Moreover, intracellular cleavage of C3 by proteases only generated C3 fragments whereas systemic activation by the conventional pathway lead to the generation of multiple bioactive fragments such as C5 cleavage products that display various effects notably on the TME. Thus, the origin of complement products is an important feature to take into consideration when we look at its effect on tumor progression.

To address the question of the implication the intracellular vs. the extracellular compartment on tumor progression, I have developed a semi-quantification method by immunohistochemistry able to discriminate the presence of complement protein within the cells from the presence of complement deposition in the extracellular space (Manuscript 2). This method is based on visual quantification by human eyes since we failed to educate the machine to discriminate extra vs. intracellular complement. Intriguingly, the localization of

complement proteins greatly affects their prognostic value. First, I was able to detect C1s within tumor cells on ccRCC tumor sections (Manuscript 5). This intracellular staining may reflect the local production by tumor cells. In order to exclude the hypothesis of an uptake from the extracellular space, an RNAscope technique to detect mRNA was performed (Manuscript 3). I confirmed the capacity of tumor cells to produce complement by the detection of mRNA of C1s (and C1r) inside them. Otherwise, only limited C1s staining was localized in the extracellular compartment. On contrary, C4 was present in both compartments. While intracellular staining with C4a-positivity was mainly detected within tumor cells and may reflect the synthesis of the native C4, extracellular C4d+ staining was found at the surface of tumor cells and indicated complement activation (Manuscript 3). Interestingly, the two types of staining were associated with poor prognostic meaning that both local production and complement activation had a negative role on patient survival which can be even worse in the case of both staining are present in the same time. The same method was set up for C3 but in this case, the presence of C3d+ deposits at tumor cells surface had no prognostic value contrary to the local production by tumor cells which correlated with poor prognosis. Multiple hypotheses could explain these contradictory effects between C4d and C3d depositions. First, the antibody that we used in this study detects all forms of C3 (except of C3a and the C3c cleavage fragment) and the extracellular staining of C3d may also reveal other C3 fragments (C3b, iC3b, C3dg) which have different functions. Moreover, a recent study have shown that C3d can exert anti-tumoral effect by increasing T cell infiltration and decreasing their exhausted phenotype³²⁴. These specific properties of C3d could hide the effect of complement activation. Finally, I have also correlated the presence of intracellular FH with a decrease survival in ccRCC and lung adenocarcinoma patients (Manuscript 4). FH was also detected at the surface of tumor cells and colocalized with C3b suggesting that extracellular FH played its role as complement regulator. Nonetheless, this last property was not associated with an effect on patient survival. Thus, the pro-tumoral effect of FH synthesis by tumor cells that I observed in ccRCC may not result from its ability to protect cells from complement cytolytic effect, as suggesting in a previous study in mouse model of lung cancer³⁶³.

Even if this local production can be followed by a secretion, complement proteins can also remain within the cells. However, the effects of intracellular complement (Int-C) have been under-estimated and poorly studied and the majority of the studies mainly focused on its action as extracellular effectors. Emerging data aim to redefine this intracellular complement, named “complosome” since a seminal study have shown that intracellular C3a is a key effector of T cell survival and metabolism^{72,112}. Few years later, novel evidences of Int-C role have emerged. For example, C3 in the cytosol of pancreatic beta cells is required for efficient autophagic turnover and may be considered as a protective factor during diabetes¹⁵⁶. Int-C is

also involved in the resistance of cancer immunotherapy. Indeed, the deletion of intracellular C3 on tumor cells enhanced efficacy of anti-PD-L1 treatment³⁷⁰. In ccRCC, I found that the main source of complement protein (e.g C4, C3, C1s, FH) come from tumor cells but their intracellular role remains to be addressed.

Having shown that the silencing of FH modulates ccRCC and lung tumor cell phenotype by acting on proliferation, migration, survival and morphology, I sought to decipher the role of intracellular FH (Int-FH) in this process (Manuscript 4). I found that FH was present intracellularly in a specific subtype of cancer cells that displayed mesenchymal marker. Furthermore, supplementation of the supernatant of tumor cells with FH did not reverse the effect of FH silencing and consolidate the hypothesis of an intracellular role.

Similar effects were also observed with C1s on tumor cells with an alteration of proliferation and survival. Regarding the permanent expansion of C1s substrateome¹⁸¹, a possible mechanism to explain C1s pro-tumoral effect is the intracellular cleavage of an essential target implicated in tumor growth. Further studies are needed to re-define the C1s substratome. These non-canonical substrates are often described in a single study in the past and not necessarily reproduced later by an independent team. Recently cleavage of HMGB-1 by C1s has been described¹⁸⁷. HMGB1 participates in the chromatin organization. In the nucleus it interacts with nucleosomes, transcription factors, and histones and regulates transcription⁴¹¹. C1s silencing modified the transcriptional program of the renal cancer cells. Further studies are needed to find out whether it is related to its interaction with HMGB1.

Contrary to C1s which display protease activity, FH had to bind to a partner to exert its pro-tumoral effects. In the extracellular space, FH binds to C3b in order to regulate the alternative pathway of complement. Nevertheless, in the intracellular space, C3 and FH are not localized in the same compartment suggesting that the FH bind to another protein to exert its pro-tumoral effects. In this context, further investigations to find FH intracellular partner need to be addressed. Optimizations of tumor cells lysate conditions are currently on going in order to identify FH binding partner by mass spectrometry experiments.

To evaluate the specificity of these effects toward tumor cells, FH silencing was performed on renal proximal tubular epithelial cells (RPTEC), the cell type from which ccRCC is derived (Manuscript 4). Interestingly, I reported no effect of FH on these cells suggesting that FH exerted tumor specific action. Moreover, while FH exerted a pro-tumoral effect on ccRCC and lung adenocarcinoma tumor cells, it had no effect on squamous cell lung cancer cells. Thus, additional studies are required to understand the source of the heterogeneity of FH effect in order to clearly delimitate the tumor types where FH can represent a potential therapeutic targets. Two hypotheses may be relevant to explain this differential effect of FH

between tumor and normal cells. First, Int-FH can regulate key pathway essential for tumor progression that are less active and relevant for normal cells. For example, the uncontrolled proliferation and resistance to apoptosis constitute two major hallmarks of cancer that can be made possible through an inhibition of tumor suppressor genes like p53. Interestingly, I found that the silencing of FH resulted in an increase expression of activated p53 that can explain its effects on tumor cells proliferation and survival without affecting normal cells that are not dependant towards p53 signaling pathway. The second hypothesis is based on a difference of FH functions between tumor and normal tissue. Unexpectedly, two studies on lung cancer have reported some specificity of FH in a tumoral context. First, people with FH H402 polymorphism have more risk to developed cancer lung cancer²⁹³. Second, in lung tumor, a specific reduced form with free thiols is detected^{412,413}. Intriguingly, this reduced form present alternative functions and has been described to lose its regulatory activity through a reduction of FI-cofactor activity⁴¹⁴. This last finding could explain the specific role of FH on tumor cells phenotype that is absent in normal cells. This reduced FH migrates are higher apparent molecular weight^{273,412}. We have not detected such band in our tumor cell lines. This protein was detected in the plasma of early stage lung cancer patients and was a target for anti-FH autoantibodies, which interacted with the reduced but not the native FH⁴¹². It was suggested that these auto-antibodies may exert protective effect, deregulating complement on the surface of the tumor cells and promoting their killing. In my experiments blocking of FH (by a blocking antibody and by silencing) indeed increased C3 activation fragments deposits, but this was not associated with a massive complement overactivation on the surface of renal and lung cancer cell lines when exposed to serum. This could be explained by the redundant action of the membrane regulators CD46, CD55 and CD59, which are expressed at high level on these cells. To gain further insight on the presence and potential function of reduced FH in ccRCC, I will perform a western blot on patients' plasma to search for it and to evaluate its correlation with clinical data and complement activation in situ and in the circulation.

During my thesis I focused mostly on ccRCC. Nevertheless, the tumor-promoting impact of the classical pathway activation and the non-canonical and intracellular functions of the complement proteins that I have described are not limited to this type of cancer. I have shown experimentally that lung ADK shares similar features in terms of negative impact of complement. These results, together with the data from the literature, suggest that in a fraction of ccRCC and lung ADK patients, complement inhibition may have a therapeutic effect. Interestingly, at least for the impact of FH, lung SCC showed no impact, contrary to lung ADK and ccRCC, suggesting a context-dependent action of the complement proteins. The fact that the overexpression of a given gene or a combination of genes, confers favorable or pejorative prognosis or has no impact depending on the cancer type, suggests that it is not the

complement protein(s) *per se* that drive pro-or anti-tumoral effect, but their complex interaction with the TME as well as the other proteins, produced by the tumor cell itself. The presence of the complement cascade activators within the tumor, the level of expression of the complement factors and regulators or the concomitant presence of the complement proteins and their intracellular partners in the same time in the same compartment will all affect the action of complement in cancer.

To grasp the overall picture of the impact that complement may have in tumor progression, I have performed a bioinformatics analyses of the prognostic impact of each of the complement genes among 30 cancer types, for which the transcriptomic data was available in the TCGA database. The analyses of the prognostic impact revealed a highly context-dependent impact of the expression of different complement genes. Based on the prognostic impact of the ensemble of the genes of the classical and alternative pathway we proposed a novel classification of the cancer types, where complement could be either aggressive, or protective or of uncertain significance (manuscript 1). ccRCC classified as a “complement aggressive” tumor and my experimental findings fully support this classification. This classification, upon extensive validation by staining at protein level, may help the selection of patients for precision medicine targeting with anti-complement drugs.

In conclusion, the work during my thesis revealed that ccRCC is a tumor type, in which the canonical complement activation and the non-canonical action of the complement proteins outside of the cascade promote tumor growth. In a fraction of patients, the ccRCC tumor-infiltrating macrophages of a particular phenotype, associated with T cells exhaustion, produce C1q. The tumor cells hijack this C1q and activate complement thanks to the local production of C1r, C1s, C4, C3 and C5 by the tumor cells. I defined this concomitant expression of multiple complement proteins by the same tumor cells as a “complement-rich” phenotype. Intratumoral immune complexes serve as an initiator of the classical pathway, thanks to the binding of in situ formed C1 complex and subsequent cleavage and deposits of C4 and C3 activation fragments on the surface of the tumor cells. This promotes chronic inflammation, driving tumor progression. Intratumoral staining for C1q, C4 production and deposits, C1s and FH are strong candidates for prognostic biomarkers. My data show that plasmatic C4d at the time of surgery could serve as a non-invasive prognostic biomarker.

I have discovered that this complement activation in ccRCC is paralleled by novel “moonlighting” functions of the complement proteins within the tumor cells. FH and C1s control the transcriptomic program of the cell, modifying its proliferation and survival. These functions of FH are context-dependent, unique for a subset of cancers and not shared by the tested normal human cells. These findings broaden the scope of the impact of complement

proteins and cancer. It is not limited to the generation of the anaphylatoxins, the opsonization and the MAC formation. These novel functions have to be further explored and taken into account in the mechanistic studies and in the search of novel therapeutic agents. Our novel classification of the tumor types depending on the co-expression of complement genes, upon validation at protein level, could serve as a guide for precision medicine for targeting the right complement protein in the right type of cancer.

PERSPECTIVES

The results obtained during these three years highlighted the complexity of the complement system that act through a context-dependent manner. Each of the 50 components that compose it, displays either a protective, an aggressive or no effects depending on tumor types. Mechanistically, these proteins exert multifaceted actions through modulation of different cells in the TME ranging from tumor cells to immune or endothelial cells in an extracellular or an intracellular dependant manner. Moreover, these effects may result from a local activation of the complement or an unconventional non-canonical role, independent from complement cascade. This complexity raises many questions about the mechanisms by which the complement effectors and each protein independently of the cascade, modulate the TME. Tumor cells are the main local producer of many complement proteins in ccRCC and lung tumors. Interestingly, tumor-derived complement proteins can remain within the cells or can be secreted in the extracellular space. In the case of FH, I demonstrated that the Ext-FH had no effect on ccRCC tumor cells phenotype whereas the intracellular counterpart exerted an unexpected pro-tumoral effect by enhancing survival, proliferation and migration capacity (Manuscript 4). However, further studies are required to understand the mechanism besides the intracellular action of FH. Which are the molecular partners of FH within the tumor cells, what are the biochemical reactions, resulting in alteration of the transcriptional program, the cell morphology, proliferation, migration and survival? Why FH silencing has no impact on non-malignant in the normal cells? These questions could be answered in part by co-immunoprecipitation of FH from cell lysates, followed by mass spectrometry. This type of analysis will reveal not only the state of the thiol groups and the glycosylation of the Int-FH but also its molecular partners. Hopefully these experiments will help me to reveal the FH interactome within the ccRCC cell lines and to oppose it to the RPTEC or within lung ADK cell lines and to oppose it to the lung SCC cells. This comparison of the affected vs non-affected cells upon FH silencing will potentially reveal hints to understand what is the molecular mechanism besides the action of FH and why it is operational only in a fraction of malignant cells.

Similar approach will be applied to C1s. The difficulty for C1s is that it is an enzyme and at least part of its action may pass via its enzymatic activity. The enzymatic act is usually very rapid and the intermediate complex between C1s and its substrate may not be detected by co-immunoprecipitation and mass spectrometry. To get further insight to what extend the enzymatic activity is important, I am now testing the C1s knock in model, where C1s producing plasmid is introduced into C1s negative HCT116 colorectal cancer cell line. If a gain of phenotype is observed (i.e. enhanced proliferation), similar transfection will be performed with mutant C1s, without enzymatic activity. The tumor growth of these stably transfected cell lines will be tested in vivo, in immunodeficient mice. Similar knock in model is under optimization for FH as well.

Even if the ext-FH coming from the systemic compartment seems to have no effect on tumor growth as revealed with the FH hepato deficient mice, it is not excluded that the ext-FH coming from tumor cells exert a different effect. Indeed, it has been reported that depending on the origin of the complement proteins (systemic vs. tumor), their structure and functions can be modified. For instance, secreted C3a generated intracellularly inside tumor cells exert a different effect on immune microenvironment than C3a coming from the circulation³⁷⁰. Intriguingly, a distinct conformational form of FH with free thiols has been described in the tumor microenvironment of lung cancer patients but not in normal tissues⁴¹². The specific presence of this reduced form can be due to a more reducing environment in tumor with the presence of thioredoxin than in normal tissues. To this end, I will test the presence of such reduced FH in the plasma of ccRCC patients by western blot, as already published for lung cancer⁴¹². Moreover, the effects of tumor-derived FH on stromal cells need to be evaluated. To address this question, I will compare the *in vivo* tumor growth after subcutaneous injection of FH deficient or sufficient tumor cells into WT mice. To this end, I have already set up a lentiviral technology in order to stably silenced FH on tumor cells. The staining of Ki67 on tumor frozen sections, a proliferation marker, will indicate the direct intracellular effect of FH on tumor cells as it occurs *in vitro*. On the other hand, the potential modulation of immune infiltrate will be investigate using multicolor flow cytometry and the impact of angiogenesis will be study through endothelial cell staining on tumor sections. If I detect an effect of tumor derived FH on immune microenvironment, the same cell lines will be injected in immunodeficient mice in order to discriminate the contribution of each effects on tumor growth. Besides FH, I also demonstrated that C1s could modulate the proliferation and survival capacity of tumor cells. Thus, the same experiments will be made with C1s sufficient or deficient cells lines that I have already created with the Crispr-Cas9 technology as well as for the knock in cell lines described above.

Moreover, the differential effect of ext-FH vs. int-FH on ccRCC tumor cells raised novel question about the specific intracellular action of complement proteins and to what extend this effect can be relevant in other cell types in the TME. Concerning C3, I found that independently from its production level by tumor cells, C3 deficient mice displayed a complete inhibition of tumor growth (Manuscript 3). Cumulating with the recent discoveries about intracellular C3 action on T cells¹¹², this remarkable effect of C3 deficiency, much stronger than C4 deficiency, could be explain by an impact of C3 production in stromal cells. Nevertheless, further studies are needed to evaluate the impact of Int-C on these cells. In addition to immune environment, a modulation of angiogenesis is a common effect of complement proteins. However, our preliminary data tended to demonstrate that the silencing of FH or C1s did not modify the phenotype of endothelial cells (HUVEC). In the TME,

fibroblasts are another important source of complement. A recent study show that specific a subtype of fibroblast, associated with T cell infiltration and exhaustion, produce C1s^{327,326}. Thus, the particular intracellular impact of C1s on fibroblasts phenotype remains to be addressed.

The increasing number of studies regarding the role of complement in physiopathology together with the arrival of eculizumab on the market, the first drug that specifically target complement, are responsible for a major gain of notoriety of the frequently forgotten complement system. This expansion is associated with an increase number of clinical development related to complement drugs.

The complexity of complement impact in cancer, ranging from pro- to anti-tumoral, raises many questions about its potential use as a therapeutic target in cancer. How the monoclonal antibodies that activate complement should be designed to efficiently kill the target tumor cells? Shall the complement be activated or inhibited? What pathway/protein must be targeted? Is it better to target the plasmatic components or to design agents that penetrate the cell and inhibit the intracellular functions of the complement components?

In the last decades, the complement system has been used for this lysis property as an adjunctive component that promotes the effects of anti-cancer monoclonal antibodies. In this context, many improvements have been made to increase complement cytotoxic effects. However, the emergence of pro-tumoral effects of complement changes the game. Even if it remains a good therapeutic option, the use of monoclonal antibodies in cancer treatment must be done with more cautions. Indeed, tumor cells have a phenomenal capacity to adapt and develop resistance mechanism to the cytolytic effect of complement system through overexpression of regulators while benefiting from the inflammation generated by early complement activation. In this context, the activation of the complement system induced by monoclonal antibodies must be strong enough to overcome the effects of regulators and induce an efficient cell death otherwise complement activation can fuel tumor growth.

The rationale underlying the choice of the target among protein of the complement system is very challenging. Both efficiency and potential adverse effect as to be take into account in order to have the better risk/benefit balance. Indeed, every modulation of complement system toward either a deficient or an overactivation can caused undesirable actions. For instance, the major side effect of eculizumab, an anti C5 antibody is the development of severe infections such as meningococcal meningitis. In the other hand, an inhibition of a complement regulator such as FH can induce renal toxicity.

In the case of ccRCC, I have demonstrated that classical complement activation that occurred in a fraction of patients was associated with poor prognosis. In this case, the use of an

inhibitor can represent a promising therapeutic strategy. Nonetheless, many options exist to block complement activation like inhibition of complement cascade at different level or prevention of regulation. In one hand, considering that the major effectors are the anaphylatoxins C3a and C5a, an inhibition of these proteins seems to be a logical approach. To comfort this choice, the use of C5a or C5aR inhibitors give good results in different mouse models of cancer. Moreover, an anti-C5aR monoclonal antibody is currently under clinical development in human for the treatment of advanced solid tumors³⁷¹. On the other hand, targeting classical pathway proteins earlier in the cascade can also be a good alternative. In this context, the blockade of C1q could result in decrease complement activation by the classical pathway while leaving intact the other two pathways and thereby limit adverse effects like severe infections. Moreover, I found that in addition to its activity as complement initiator, C1q had independent pro-tumoral functions in ccRCC and thereby amplify its therapeutic potential (Annex 4). To investigate this strategy, we collaborate with an industrial partner to develop anti-C1q antibodies (Laboratoire Pierre Fabre). The evaluation of their ability to inhibit tumor growth is currently on going in mouse models and further studies are needed to investigate which parameters of the TME are modulated.

Furthermore, I found that FH exerted a strong pro-tumoral effect in ccRCC and lung ADK, which questions the hypothesis that FH will block complement cascade and will hence down-regulate the tumor-promoting anaphylatoxins generation. Considering that the impact of FH is due to modulation the phenotype of tumor cells in ccRCC through an intracellular mechanism of action, Int-FH represents a promising target. In this context the development of inhibitors, able to penetrate into the cell and to modulate the function of proteins that are inaccessible to conventional antibodies, is required. This can be achieved by the use of small molecules or recombinant antigen-binding proteins antibody fragments. To this end, we built collaboration with Pr. Andersen and Dr. Larsen in Denmark who have a strong expertise in the development of nanobodies and could give us access to already available library of anti-FH nanobodies. The advantages of these nanobodies remain on their reduced size and stability allowing intracellular delivery while keeping their specificity^{416,417}. Nevertheless, targeting complement proteins can be very challenging. First, all complement proteins are present in high amount in the circulation. Thus, the administration of a complement inhibitor can result in an early neutralization in the systemic compartment even before reaching the tumor. To limit this process, a modulation of the pharmacokinetic parameters to redirect the biodistribution toward the tissular compartment can be considered as well as an intratumoral mode of administration. Second, complement proteins are not specific for the tumor and also exist in normal tissue. Thereby, off-targets effects can be a major problem. For instance, inhibition FH may cause renal toxicity since the presence of auto-antibodies against FH has been associated with renal thrombotic microangiopathy (aHUS)⁴¹⁸. To overcome this adverse

effect, a targeted delivery to the tumors is required. To this end, the use of bispecific antibodies against both tumor cell and complement protein represents a promising strategy⁴¹⁹. Moreover, an antibody directed against the tumor antigen Epcam and complement regulator CD55 are currently under preclinical development^{387,388}. In the same logic, the use of tumor cell penetrating peptides like CREKA can offer a promising strategy for specific intratumoral drug delivery⁴²⁰. Moreover, besides inhibiting the protein, the use of tumor cell-targeted gene-editing techniques can be a powerful approach to specifically affect tumor intracellular FH as I have shown *in vitro* with siRNA or shRNA experiments. In all case, the potential complement overactivation that can result from FH inhibition must be evaluated carefully in order to avoid unwanted pro-tumoral effects linked to the anaphylatoxins actions.

Regarding the complexity of the tumor, combination strategies between complement inhibitor and others anti-cancer drugs may offer synergic actions to effectively and durably affect tumor progression. Indeed, different mice studies have reported an enhancement of PD-L1 efficacy when combined to anti C3aR and C5aR blockade^{117,362,398}. Moreover, a phase I clinical trial is currently on going to evaluate the potential of anti-C5aR in combination with anti PD-L1 treatment. This synergistic effect is not limited to immunotherapy since C5aR blocking also improves the efficacy of paclitaxel chemotherapy⁹⁶. However, the rational of drug combination with complement inhibitors must be clearly evaluated because it can also bring some detrimental effects as for instance with radiotherapy³⁶⁹. Having in mind these findings, I hypothesized that C1q inhibition could synergize with anti PD-1 treatment. To address this hypothesis, C1q deficient or WT mice were randomized and treated with an anti PD-1 antibody when the tumor volume reached 100mm³. Intriguingly, anti PD-1 treatment did not improve the protective effect of C1q deficiency (Annex 5). Thus, additional studies are necessary to understand this effect.

In conclusion, this work opens short term and long term perspectives. The short term ones are related to the future experiments in which we will aim to uncover the mechanisms of action of intracellular complement proteins and to design nanobodies with therapeutic potential to target FH within the cell. Moreover, we aim to validate plasmatic C4d as a non-invasive prognostic biomarker. The global perspectives are related to the therapeutic targeting of complement cascade and its individual proteins in the clinical practice. My results may provide a rational for personalized medicine and stratification of the patients, who may benefit the most from such approach.

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ATTACHEMENTS

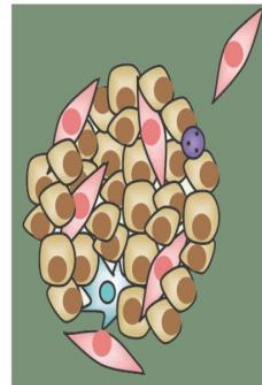
Review: Daugan, M., Noe, R., Herman Fridman, W., Sautes-Fridman, C. & Roumenina, L. T. [The complement system: a double edge sword in tumor progression]. *Med. Sci.* MS 33, 871–877 (2017).

► Le système du complément est un composant du système immunitaire inné qui joue un rôle clé dans l'élimination des pathogènes et dans l'homéostasie. Par ses propriétés, ce système a longtemps été considéré comme participant à la réponse anti-tumorale. Cependant, de récentes études ont permis de repositionner le complément en révélant ses effets pro-tumoraux, plus particulièrement des anaphylatoxines C3a et C5a, dans une grande variété de cancers. Ces protéines agissent en effet à différents niveaux de la progression tumorale, que ce soit au sein des cellules tumorales, sur l'angiogenèse ou sur le microenvironnement immunitaire. ◀

Le système du complément

Une épée à double tranchant dans la progression tumorale

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Les voies d'activation du complément

La voie classique

L'activation de la voie classique est initiée à la suite de la fixation à une cible du complexe C1 constitué d'une molécule de reconnaissance, le C1q, et deux sérine protéases, C1r et C1s¹ [3]. Le C1q peut se lier à une grande variété de cibles, dont les complexes immuns, par les régions constantes des immunoglobulines de classe M ou G, mais également des ligands endogènes comme les pentraxines [4] et d'autres molécules qui sont exposées à la surface des cellules apoptotiques [5].

La voie des lectines

La voie des lectines est initiée à la suite de la reconnaissance de résidus glycaniques par le complexe constitué de l'association de lectines, comme la MBL (*mannan-binding lectin*) ou les ficolines, avec des sérine estérases, les MASP (*MBL-associated serine proteases*) [6].

La voie alterne

La voie alterne agit comme un système de surveillance. Elle maintient un faible niveau d'activation des cellules par le système du complément. Elle peut subir une boucle d'auto-amplification dans un contexte infectieux [1].

Le système du complément (SC) a été découvert à la fin des années 1890. Il est alors décrit comme un composant thermolabile du sérum, qui possède des propriétés antimicrobiennes, capable de compléter l'action des anticorps. Depuis, les connaissances ont évolué et il est désormais établi que ce système fait partie intégrante du système immunitaire inné. Le SC fait référence à un ensemble de protéines sériques qui jouent un rôle primordial dans l'immunité innée : elles permettent à l'hôte de se défendre contre les pathogènes ; elles participent à l'élimination de ses propres cellules en condition d'apoptose [1]. À la surface des cellules apoptotiques ou du pathogène, le système du complément peut être activé en cascade selon trois voies : la voie classique, la voie des lectines ou la voie alterne (Figure 1). Ces trois voies convergent pour aboutir à l'activation de la protéine C3 (C pour complément) avec pour résultat la formation du complexe d'attaque membranaire (CAM). Le CAM est à l'origine de la lyse des pathogènes ou de l'activation de certaines cellules de l'hôte. En dehors de leur action directe, chacune de ces voies peut induire des réponses inflammatoires et moduler les réponses immunitaires innée et adaptative conduisant ainsi à l'élimination des pathogènes, des complexes immuns et des cellules apoptotiques [2].

Vignette (Photo © Alain Puisieux).

¹ Les protéases sont associées en un tétramère (C1s-C1r-C1r-C1s) qui s'assemble à la protéine de reconnaissance C1q, formant ainsi le complexe C1.

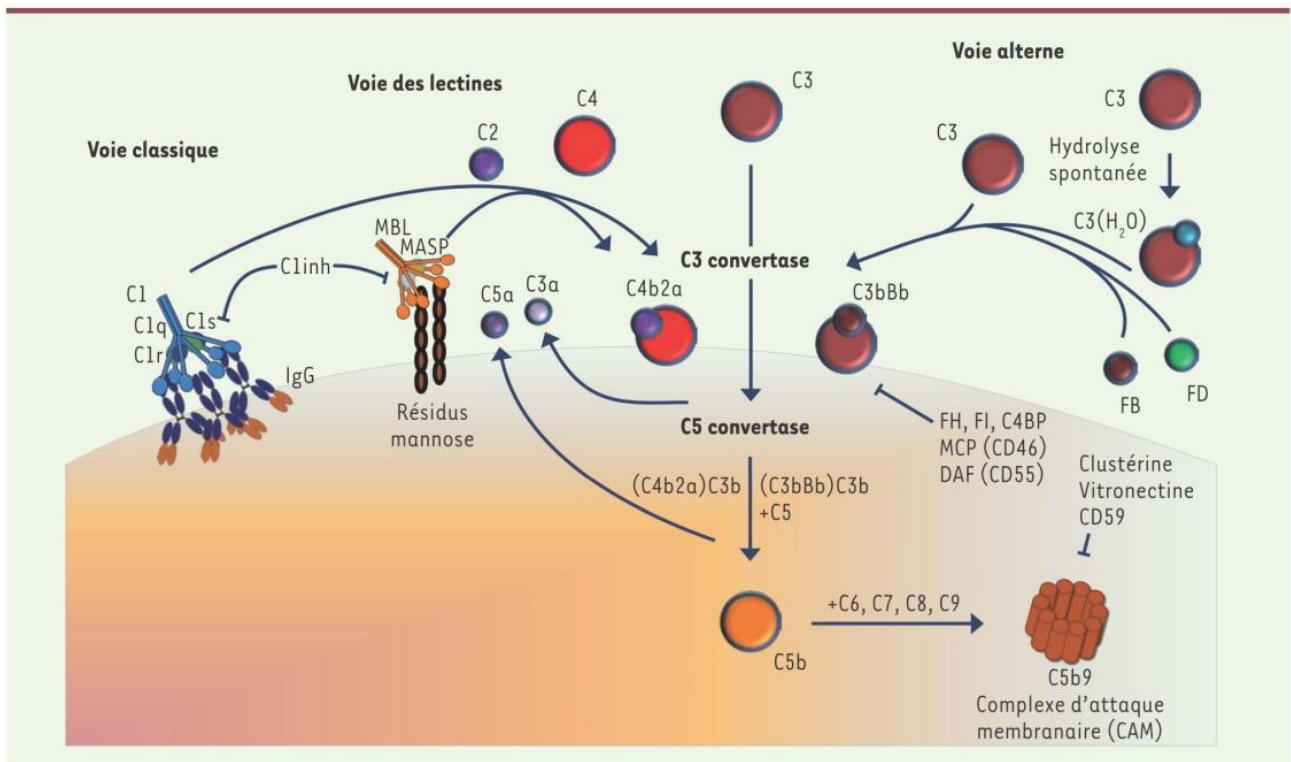


Figure 1. Le système du complément. La voie classique est initiée par la fixation du complexe C1 à des immunoglobulines ou à des ligands endogènes. Ce complexe va pouvoir cliver le C4 et le C2 pour former la C3 convertase classique (C4b2a). La voie des lectines (analogue à la voie classique) est activée par la fixation d'un complexe MBL-MASP à la surface des pathogènes. Ce complexe, lié au pathogène, permet de cliver le C4 et le C2 de façon identique à ce qu'il advient lors de l'activation par la voie classique. Ces clivages forment ainsi une C3 convertase (C4b2a) identique à celle formée dans la voie classique. La voie alterne agit comme un système de surveillance en maintenant un faible niveau d'activation du système par un processus connu sous le nom de « tick-over ». La tick-over est l'hydrolyse spontanée du C3 en C3(H₂O), forme bio-active du C3, dans la phase fluide. Le C3(H₂O) peut se fixer au facteur B, qui est ensuite clivé par le facteur D pour former la C3 convertase alterne en phase fluide : C3(H₂O)Bb. À la surface des pathogènes, le facteur B, fixé au C3b, pourra ensuite être clivé par le facteur D pour former la C3 convertase alterne (C3bBb). Ces trois voies conduisent à la formation d'une C3 convertase permettant de cliver le C3 en C3a, une anaphylatoxine, et en C3b à l'origine de la formation de la C5 convertase (C4b2a3b ou C3bBb3b). Le C5 est ensuite clivé en C5a, une anaphylatoxine, et en C5b qui va initier l'assemblage de la voie terminale du complément. Cette voie terminale conduit à la formation du complexe d'attaque membranaire (CAM, C5b-9) et permet de lyser les pathogènes. Le système du complément est hautement régulé par des protéines sériques (C1inh, FI, C4BP, FH, clusterine, vitronectine) et membranaires (CR1, MCP, DAF, CD59). MBL-MASP : *mannan binding protein- mannan-binding lectin serine protease* ; C1inh : C1 inhibiteur ; C4BP : *C4 binding protein* ; MCP : *membrane cofactor protein* ; DAF : *decay accelerating factor*.

Les voies effectrices du complément

Les trois voies d'activation du complément aboutissent à la formation d'une C3 convertase (classique ou alterne) qui est responsable du clivage du C3 en deux fragments, le C3a et le C3b [7], puis d'une C5 convertase qui clive la protéine C5 en deux fragments, le C5a et le C5b. Ces deux convertases (C3 et C5 convertases) sont des composants essentiels pour la lutte contre les pathogènes. En générant le C3b, elles permettent en effet l'opsonisation de la cible (le C3b la recouvrant, il favorise sa reconnaissance par le système immunitaire inné). Elles sont également à l'origine d'une réponse inflammatoire induite par les anaphylatoxines C3a et C5a. Elles initient enfin l'assemblage du CAM : le C5b qu'elles génèrent se lie aux protéines C6, C7, C8 et à plusieurs

molécules de C9 qui s'ancrent à la membrane de la cellule permettant de former un pore lytique [8].

Les régulateurs du complément

Afin de limiter les dommages potentiels qu'il peut induire sur les tissus sains, le système du complément est finement régulé. Cette régulation s'effectue à trois niveaux : (1) l'inhibition de l'activité des protéases qui sont impliquées dans la cascade d'activation du complément ; (2) la facilitation de la dégradation de ces protéases ; et (3) le contrôle de la formation du complexe d'attaque membranaire. Les régulateurs intervenant sur le système

du complément peuvent être solubles, comme le C1 inhibiteur, le facteur I et le facteur H, ou membranaires, comme le CD35 (ou *complement receptor 1*, CR1), le CD46 (ou *membrane cofactor protein*, MCP), le CD55 (*decay-accelerating factor*, DAF) et le CD59 [1].

Implication du système du complément en physiopathologie

La régulation du système du complément est essentielle afin d'assurer l'homéostasie de l'hôte. Une déficience de certains de ses composants peut en effet être à l'origine d'une susceptibilité accrue aux infections, notamment aux méningocoques, et aux maladies auto-immunes comme le lupus [2]. À l'inverse, la suractivation du système du complément peut avoir des conséquences délétères et être responsable de dommages tissulaires. C'est notamment le cas dans les C3 glomérulopathies [9], le syndrome urémique hémolytique atypique, ou dans la dégénérescence maculaire liée à l'âge [2, 10].

Récemment, des études ont suggéré un rôle du système du complément dans le cancer. La question de son intérêt comme biomarqueur, ou cible thérapeutique, dans un contexte tumoral a ainsi été évoquée.

Protéines du complément et contexte tumoral

Le rôle anti-tumoral du complément semblait acquis depuis plusieurs années en raison de son activité lytique, de ses propriétés de chimiotactisme et d'opsonisation des cellules stressées. Cependant, de récentes études semblent contredire ce paradigme en montrant que le système du complément peut être relié à des effets pro-tumoraux. L'expression anormale de protéines du complément a été révélée dans de nombreux types de tumeurs, notamment dans des tumeurs du poumon [11], du sein [12], du pancréas [13], du rein [14] et urothéliales (vessie et haut appareil urinaire) [15]. Parmi ces protéines, le C1q, le C1s, le C3, le C4, les anaphylatoxines C3a et C5a, et leurs récepteurs, ainsi que les protéines régulatrices comme le facteur B, facteur H, facteur I, CD46, CD55 et CD59, sont le plus souvent surexprimées. La possibilité d'une activation locale du complément au sein de ces tumeurs a été suggérée, ce qui soulève la question de son rôle possible dans la progression tumorale. Les cellules tumorales sont capables de s'adapter à leur environnement. Elles peuvent profiter des effets pro-inflammatoires induits par le complément, tout en s'affranchissant de ses activités cytotoxiques qui reposent sur le CAM. Cette adaptation implique la surexpression des protéines régulatrices du système comme le facteur H, le facteur I, le CD46, le CD55 et le CD59 [16, 17].

Une certaine contradiction sur le rôle du complément dans le cancer subsiste donc selon les données de la littérature. En fonction du type de cancer considéré, l'impact du système du complément peut en effet varier en ce qui concerne les protéines impliquées, mais également en termes de valeur pronostique. Dans la majorité des cancers, comme le cancer du rein [14], du sein [12], du poumon [11], de l'ovaire [18] et urothéliales [15], les protéines du complément favorisent la croissance tumorale. Dans d'autres, comme le cancer de la prostate [19], ces protéines n'ont aucune influence sur la croissance tumorale, elles peuvent même la ralentir.

Mécanismes impliqués dans les effets du complément sur la progression tumorale

Effets du complément sur les cellules tumorales

Dans différents types de tumeurs comme le cancer de l'ovaire, du poumon, du rein, du sein et du foie, l'activation du complément par la génération des anaphylatoxines C3a et C5a, est associée à un mauvais pronostic. En effet, le C3a et le C5a, connus pour être de puissants médiateurs de l'inflammation, induisent la croissance tumorale en activant des voies de signalisation pro-tumorales (Figure 2). Cet effet repose sur l'interaction des anaphylatoxines avec leurs récepteurs respectifs, le C3aR et le C5aR, deux récepteurs couplés aux protéines G qui peuvent être exprimés à la membrane des cellules tumorales. Parmi les voies de signalisation activées par la stimulation du C3aR ou du C5aR, les voies ERK (*extracellular signal-regulated kinases*) et PI3K (*phosphoinositide 3-kinase*)/Akt (protéine kinase B) [20, 21] favorisent la prolifération, la survie et l'invasion des cellules tumorales. Cette activation pourrait résulter, en partie, d'une action autocrine des anaphylatoxines sur leurs récepteurs. En effet, il a été montré, dans des modèles cellulaires de cancers de l'ovaire et du poumon, que C3 et C5 sont sécrétés par les cellules tumorales [22] puis clivés respectivement par les convertases C3 et C5, ce qui permet la production des anaphylatoxines C3a et C5a. Elles exercent ainsi une action autocrine via leurs récepteurs qui sont exprimés par les cellules tumorales, activant des voies de signalisation comme la voie PI3K/Akt.

Le C5a est également associé à la formation de métastases. Dans un modèle murin de cancer colorectal, une diminution du nombre de métastases hépatiques est observée chez des souris déficientes pour le gène codant le récepteur C5aR, par rapport aux souris sauvages [23]. Ce potentiel métastatique aurait pour origine une modulation par le C5a de la capacité invasive des cellules. De même, dans un modèle *in vitro* de cancer gastrique, la stimulation du récepteur du C5a favorise la motilité cellulaire, via une activation de RhoA (*ras homolog gene family, member A*), et donc, la capacité invasive des cellules tumorales [24]. Dans des cellules de différentes lignées tumorales du côlon et des voies biliaires, qui expriment le C5aR, le C5a peut induire un réarrangement du cytosquelette des cellules, modifier leur motilité, et induire la sécrétion de métalloprotéinases (MMP) qui favorisent l'invasion par ces cellules [25].

Bien que les principaux effets du complément sur la croissance tumorale reposent sur l'activité du C3a et du C5a, un rôle pour les autres protéines du complément ne

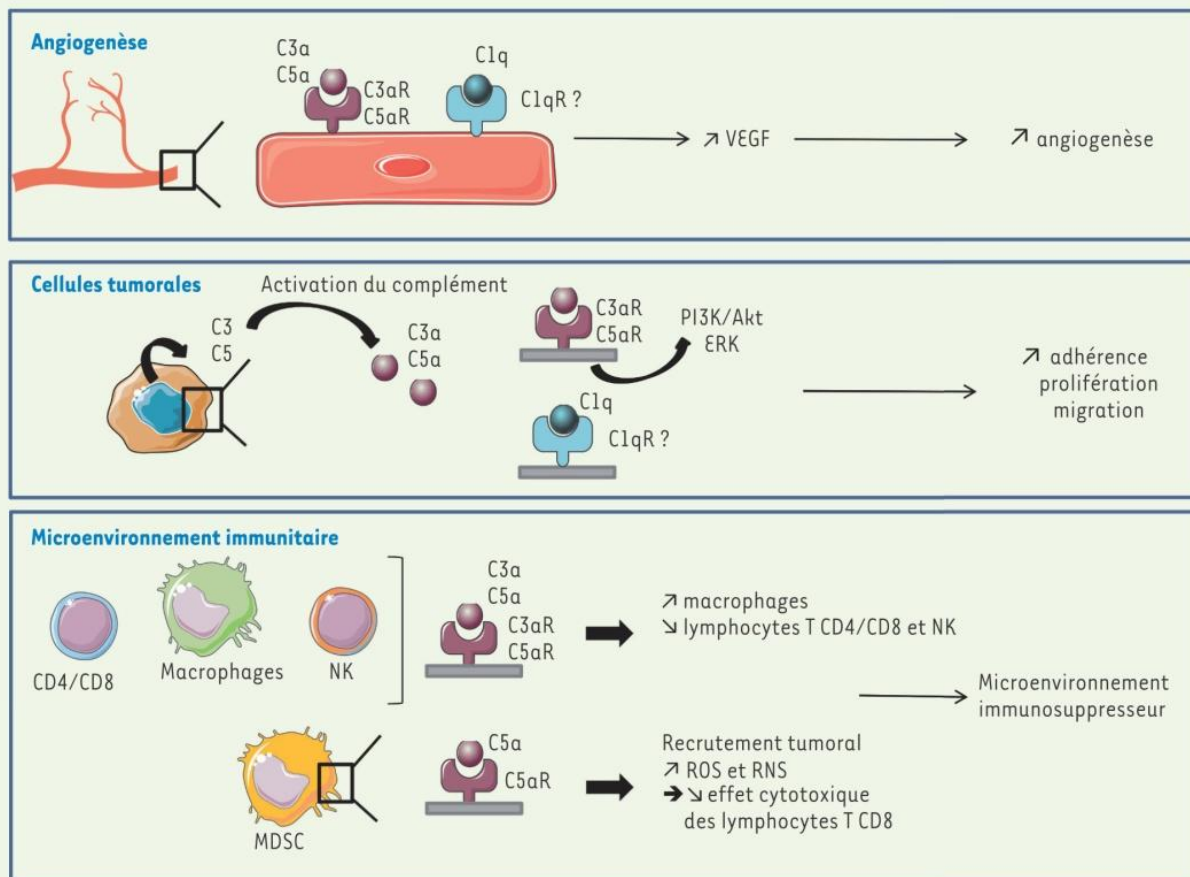


Figure 2. Effets pro-tumoraux du complément. Le système du complément est responsable d'effets pro-tumoraux dans de nombreux cancers en agissant au niveau de différentes étapes clés de la progression tumorale. Au niveau de l'angiogenèse, le C1q et les anaphylatoxines C3a et C5a, stimulent l'adhérence, la prolifération et la migration des cellules endothéliales, notamment, via l'induction de la production de VEGF (*vascular endothelial growth factor*). Au niveau des cellules tumorales, le C1q et les anaphylatoxines induisent l'activation de voies de signalisation impliquées dans la survie et la prolifération des cellules comme PI3K (*phosphatidylinositol-3-kinase*)/Akt ou ERK (*extracellular-signal-regulated kinase*). Enfin, les anaphylatoxines possèdent des propriétés inflammatoires qui permettent le remodelage des populations immunitaires présentes, ainsi que leur fonctionnalité. Parmi, ces populations, les macrophages, les cellules *natural killer* (NK), les MDSC (*myeloid-derived suppressor cells*) et lymphocytes T CD4 et CD8 semblent être les plus impliquées dans l'axe C5a/C5aR. ROS : *reactive oxygen species* ; RNS : *reactive nitrogen species*.

peut être écarté. De récentes études ont en effet montré que le C1q et le CAM possédaient également des propriétés pro-mitogéniques et pro-métastatiques. Ainsi, dans le modèle murin syngénique de mélanome B16/F10², la croissance tumorale observée chez des souris déficientes pour le gène codant C1q est ralentie et la survie des animaux est prolongée [26]. Le C1q agit directement sur les cellules tumorales dont il augmente l'adhérence, la migration et la prolifération. Il interagit également avec certaines MMP, dont MT1-MMP (*membrane-type matrix metalloproteinase 1* ou *matrix metalloproteinase-14*), des protéines impliquées dans la dégradation de la matrice extracellulaire et l'acquisition par les cellules, d'un phénotype invasif [27].

² La lignée B16 est une lignée établie à partir d'un mélanome murin. B16F10 dérive de la lignée B16 et est métastatique.

Malgré ces propriétés cytotoxiques anti-tumorales, le CAM peut également favoriser la croissance tumorale en stimulant des voies de signalisation pro-tumorales comme ERK1/2, PI3K/Akt, et p70 S6 kinase. Il inhibe également l'apoptose des cellules en bloquant FLIP (*FLICE-inhibitory protein*) et la caspase-8 [28]. D'un point de vue thérapeutique, cette dualité des effets du CAM sur la progression tumorale nécessite une meilleure compréhension afin d'optimiser l'utilisation des anticorps monoclonaux activant le complément.

Les effets pro-tumoraux du complément peuvent s'observer dans la majorité des cancers. Cependant, dans certains cas, il peut présenter des effets anti-tumoraux (Figure 3). Le C1q est exprimé au niveau de la pros-

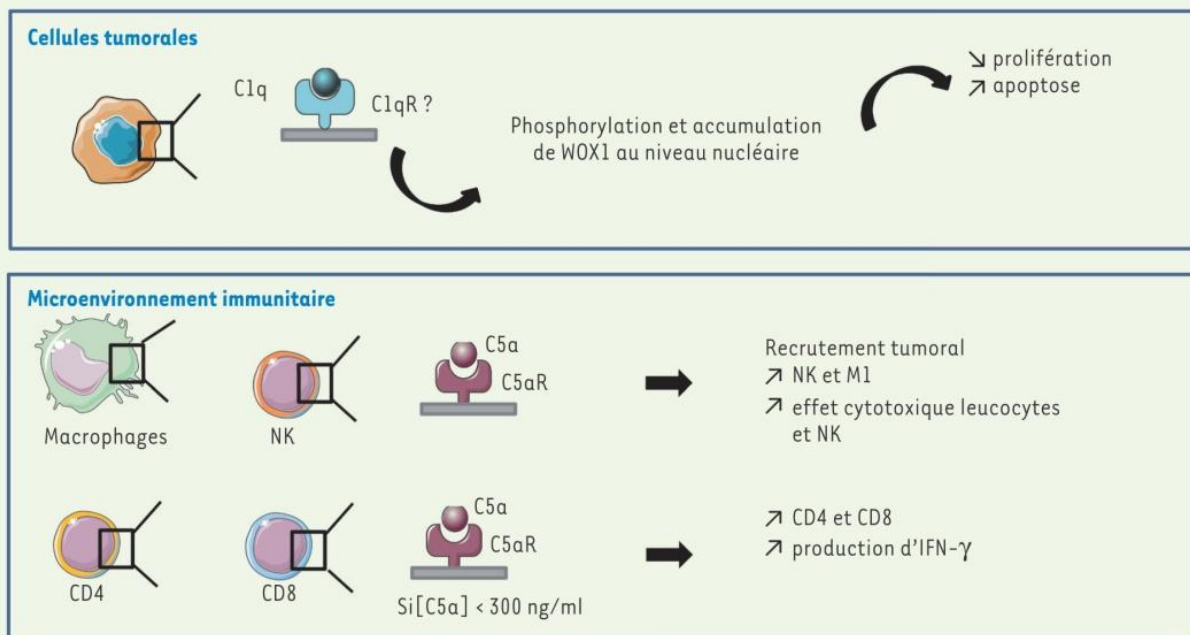


Figure 3. Effets anti-tumoraux du complément. Dans certains types tumoraux tels que le cancer de la prostate ou du sein, le système du complément peut exercer des effets pro-tumoraux. D'une part, le C1q peut induire la voie de signalisation WWOX/WOX1 limitant ainsi la prolifération et favorisant l'apoptose des cellules tumorales. D'autre part, les anaphylatoxines peuvent selon le type tumoral et la concentration locale, favoriser le recrutement et l'action de cellules cytotoxiques et notamment les lymphocytes T, NK (*natural killer*) et les macrophages de type 1 (M1). WWOX/WOX1 : *WW domain-containing oxidoreductase* ; IFN- γ : interféron gamma.

tate. Son expression protéique est significativement diminuée en cas d'hyperplasie bénigne ou de cancer de la prostate [19]. Indépendamment de son rôle dans l'activation de la voie classique du complément, le C1q peut induire une apoptose des cellules tumorales de prostate en activant le gène suppresseur de tumeur *WWOX/WOX1* (*WW domain-containing oxidoreductase*) et en déstabilisant l'adhérence cellulaire. *In vitro*, le C1q conduit à l'accumulation rapide dans le noyau de WOX1 sous sa forme phosphorylée, qui est responsable de signaux antiprolifératifs et proapoptotiques. À noter que cette association entre C1q et WOX1 a également été observée dans d'autres types de cancers comme le cancer du sein ou le neuroblastome [19]. L'effet du C1q sur l'apoptose des cellules tumorales a aussi été observé sur une lignée de cancer de l'ovaire. Il repose dans ce cas sur un mécanisme impliquant le TNF- α (*tumor necrosis factor- α*) [29].

Effets du complément sur le microenvironnement immunitaire

Dans un contexte d'inflammation chronique comme celui produit par la transformation cellulaire, les anaphylatoxines, C3a et C5a, participent à l'instauration d'une tolérance qui favorise une croissance plus rapide de la tumeur.

Dans le modèle murin de mélanome, la délétion du gène codant le récepteur du C3a, le C3aR [30], ou une déficience en C3 [31], sont à l'origine d'une croissance tumorale ralentie qui est associée à un remodelage du microenvironnement immunitaire. Cela se traduit par

une augmentation du nombre de neutrophiles et de lymphocytes T CD4, associé à une diminution du nombre de macrophages intra-tumoraux et une amélioration de la réponse T cytotoxique.

L'anaphylatoxine C5a exerce également des effets immunomodulateurs. En effet, dans un modèle de cancer cervical, la génération de C5a dans le microenvironnement tumoral induit un chimiotactisme touchant les cellules myéloïdes suppressives (*myeloid-derived suppressor cell*, MDSC) qui, activées, sécrètent des espèces réactives de l'oxygène (*reactive oxygen species*, ROS) et de l'azote (*reactive nitrogen species*, RNS) à l'origine d'une immunosuppression des lymphocytes T CD8 [32]. Cet effet sur le recrutement des MDSC par chimiotactisme a également été observé dans un modèle de cancer pulmonaire dans lequel l'ajout d'un antagoniste du C5aR conduit à une diminution de l'infiltrat de MDSC et une réduction de l'expression des protéines immunomodulatrices ARG1 (*arginase 1*), CTLA-4 (*cytotoxic T-lymphocyte antigen 4*), IL(interleukine)-6, IL-10, LAG3 (*lymphocyte-activation gene 3*) et PD-L1 (*programmed death-ligand 1*). Chez les patients atteints de cancer pulmonaire non à petites cellules, une augmentation de la concentration sérique de C5a a été

également rapportée [33]. Cependant, cet effet immunomodulateur des anaphylatoxines sur le microenvironnement immunitaire reste, encore une fois, controversé en fonction des données de la littérature, particulièrement selon le type de tumeur concerné et la concentration des anaphylatoxines.

Dans différents modèles murins de sarcome du sein, de cancer de l'ovaire et de lymphome [34], l'expression par les cellules tumorales du C5a ralentit la croissance tumorale, en comparaison à celle observée chez des souris n'exprimant pas cette anaphylatoxine. Plusieurs hypothèses ont été émises afin d'expliquer cet effet anti-tumoral de la protéine. Le C5a rendrait les cellules plus susceptibles à l'apoptose, limitant ainsi leur capacité de prolifération. Il pourrait aussi participer à l'élimination des cellules tumorales en attirant les macrophages et les granulocytes par chimiotactisme. Dans le modèle de lymphome, en particulier, la concentration locale de C5a aurait un effet direct sur la croissance tumorale [34] : les tumeurs sécrétant de fortes quantités de C5a voient leur croissance accélérée alors qu'une quantité moindre de la protéine lui confère des propriétés anti-tumorales. Dans ce modèle, les effets anti-tumoraux du C5a sont principalement liés à une augmentation de la production d'IFN- γ (interféron gamma) par les lymphocytes T CD4 et CD8 dans la rate et les ganglions.

Effets du complément sur l'angiogenèse

L'angiogenèse est un mécanisme clé de la carcinogenèse. Il est relié directement à l'agressivité des tumeurs [35]. Certaines protéines du complément comme le C1q et les anaphylatoxines C3a et C5a, peuvent participer à ce processus. Ainsi, dans le modèle de mélanome B16/F10 [26], les souris déficientes pour le gène codant le C1q présentent une croissance tumorale ralentie qui peut être reliée, notamment, à une densité du réseau vasculaire des tumeurs diminuée. Cet effet du C1q sur la vascularisation a également été rapporté dans des modèles *in vitro* utilisant des cellules endothéliales, mais également *in vivo*, dans le cadre de la pré-éclampsie [36] ou de la cicatrisation des plaies [37]. De même, dans un modèle de cancer épithélial ovarien murin, la déficience en C3 ou en C5aR est à l'origine d'une diminution de la vascularisation tumorale en lien avec une réduction de l'expression du VEGF (*vascular endothelial growth factor*) [38]. Cet effet pro-angiogénique des anaphylatoxines n'a cependant pas pu être mis en évidence dans d'autres modèles de cancer murins comme le cancer cervical [32,33].

Le complément en immunothérapie par anticorps monoclonaux

Lorsque l'utilisation des anticorps monoclonaux a été proposée pour la lutte contre les tumeurs, des stratégies visant à activer le mécanisme de cytotoxicité dépendant du complément (CDC), fondée sur la capacité d'opsonisation du complément et l'activité du CAM sur la mort cellulaire, ont été développées. Le mécanisme de CDC repose sur l'activation de la voie classique du complément par les immunoglobulines (*via* leur fragment Fc) qui, *in fine*, conduit à la formation du CAM à la surface des cellules ciblées par les anticorps, provoquant leur mort

[39]. Certaines méthodes permettent d'amplifier cet effet cytotoxique : le blocage des mécanismes de résistance des cellules au CDC, en utilisant des inhibiteurs des protéines régulatrices (CD46, CD55 et CD59), ou des techniques d'ingénierie des anticorps afin de potentialiser leur capacité à activer le complément [40], notamment en jouant sur la cinétique de dissociation ou encore le site de liaison de l'anticorps. La modulation des activités du complément nécessite cependant quelques précautions en raison, comme nous l'avons exposé, de cette capacité qu'il a à induire des réactions pro-tumorales. Son activation n'est bénéfique que dans le cas où elle est suffisamment importante pour entraîner la formation des complexes d'attaque membranaire à la surface de la cellule afin de la déstabiliser et induire sa mort. À l'inverse, s'ils n'induisent pas une activation suffisante pour permettre la formation des complexes d'attaque membranaire et la mort des cellules, les anticorps pourraient favoriser la génération d'anaphylatoxines à l'origine d'une inflammation chronique permettant ainsi la progression tumorale.

Conclusion

Les études récentes que nous rapportons ont permis de repositionner le rôle du système du complément dans la progression tumorale et révèlent son importante implication dans de nombreux cancers. Elles pourraient conduire à l'identification de nouveaux biomarqueurs diagnostiques ou pronostiques ainsi qu'à de nouvelles cibles thérapeutiques. Le complément ayant des effets divergents selon les types de cancer, il reste néanmoins essentiel de considérer le type de tumeur afin de développer ces stratégies. \diamond

SUMMARY

The complement system: a double edge sword in tumor progression

The complement system is a key component of the innate immunity, playing a role in pathogen elimination and in host homeostasis. The complement system has been considered for long time as an anti-tumoral element. However, recent studies showed a pro-tumoral effect of complement and particularly of the anaphylatoxins C3a and C5a in a large variety of tumor types. Complement proteins act on different levels of tumor progression, affecting the tumor cells, the angiogenesis and the immune microenvironment. The impact of the complement system on tumor progression seems to be cancer type-dependent and this has to be taken into account in the establishment of potential biomarkers and development of therapeutic strategies. \diamond

REMERCIEMENTS

Ce travail a été soutenu par le financement de : SIRIC-CARPEM and ARC PJA 2014201954 pour LR, ARC aide doctorale pour MD and by Inserm. Les dessins des figures sont inspirés de : <http://servier.fr/smart/banque-dimages-powerpoint>.


LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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