

Dissertation

submitted to the
Combined Faculty of Natural Sciences and Mathematics
of the Ruperto Carola University Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

Presented by

Xiaobo Liu, Master of Medicine

Born in: Hebei, P.R. China

Oral examination: 19th of December, 2019

Membrane attack complex activated neutrophils increase the permeability of tumor blood vessels

Referees: Prof. Dr. rer. nat. Viktor Umansky

Prof. Dr. med. Stefan W. Schneider

Table of Contents

Abstract.....	1
Zusammenfassung.....	2
1 Introduction.....	3
1.1 The complement system.....	3
1.2 Terminal complement complex as a trigger for inflammation.....	5
1.3 Complement in cancer.....	6
1.4 Therapeutic regulation of the complement.....	8
1.5 Neutrophil in the tumor microenvironment	9
1.6 Tumor endothelium	12
1.7 Aims of study	13
2 Methods.....	15
2.1 Chemicals and tissue culture materials	15
2.2 Antibodies	15
2.3 Kits	16
2.4 Cell lines and cell culture	16
2.5 Mouse procedures	17
2.6 Human malignant melanoma patients' plasma and tissue	17
2.7 Tissue immunofluorescence staining	18
2.8 Immunofluorescence cell staining.....	18
2.9 ELISA assay.....	19
2.10 RNA extraction and qPCR	19
2.11 Neutrophil isolation.....	20
2.12 Complement activation on ECs and co-culture with neutrophil	22
2.13 <i>In vitro</i> sublytic MAC formation and detection on neutrophil.....	22
2.14 ROS detection	22

2.15 DNA-Histone fragments detection	23
2.16 Neutrophil stimulation by C5a	24
2.17 MACs and NETs staining	24
2.18 EC layer integrity staining.....	24
2.19 Electric Cell-substrate Impedance Sensing (ECIS).....	24
2.20 Human melanoma cells transmigration assay	25
2.21 Complement assay.....	25
2.22 Statistical analysis	25
3 Results	27
3.1 The complement system is activated in human malignant melanoma	27
3.2 Complement activation fragments deposit in human melanoma tissue	28
3.3 MACs deposit on neutrophils in murine tumors	30
3.4 Complement activation occurs locally in tumor	32
3.5 The lectin pathway is predominantly involved for the complement activation	33
3.6 MACs form on neutrophils in close proximity to the tumor.....	36
3.7 MACs on neutrophil leads to ROS production	38
3.8 MAC on neutrophil induces NETs.....	39
3.9 C5a can not induce MAC formation and neutrophil activation	42
3.10 Neutrophils in close proximity to the tumor blood vessel wall.....	44
3.11 MAC-activated neutrophils contribute to endothelial barrier disruption <i>in vitro</i>	46
3.12 Perivascular neutrophils increase the endothelium permeability in tumor tissue	47
3.13 Disruption of the MAC formation protects the vascular integrity	49
3.14 Undetectable complement activation and less MAC-neutrophils in human non- metastatic skin tumors	51
3.15 LMWH blocks complement activation <i>in vitro</i>	52
3.16 Tinzaparin blocks MAC-related neutrophil activation <i>in vitro</i>	53
3.17 Tinzaparin block complement activation <i>in vivo</i>	55

4 Discussion.....	58
4.1 Complement activation in melanoma microenvironment	59
4.2 MAC formed on neutrophils but not on EC	62
4.3 MAC on neutrophil lead to ROS production and NETs	63
4.4 Perivascular activated neutrophils contribute to endothelial barrier disruption.....	64
4.5 Tinzaparin blocks complement-related neutrophil activation <i>in vitro</i> and <i>in vivo</i>	66
4.6 Outlook.....	68
References	70
Abbreviations	79
Acknowledgments	81

Abstract

Increased activation of the complement system has been measured in various malignancies. Previous studies indicated that the complement system activates endothelial cells (ECs) and neutrophils. However, in the context of tumor progression, knowledge on the crosstalk between the vascular endothelium, the complement system and the neutrophil associated innate immunity is still scarce. Here, we report the tumor-specific complement activation in patients suffering from malignant melanoma. Using mouse and human tumor tissue samples, we showed that accumulation of complement effectors such as C3 fragments and C5a around tumor blood vessel walls were increased. Moreover, we detected high levels of the mannose binding lectin (MBL) at the endothelium suggesting the involvement of the lectin pathway as main trigger of the melanoma mediated complement activation. However, the complement cascade terminated by the formation of the membrane attack complex (MAC) not on the endothelium but on perivascular neutrophils. *In vitro* experiments with human ECs and neutrophils confirmed this complement mediated crosstalk. Further *in vitro* experiments demonstrated that MAC positive neutrophils released reactive oxygen species (ROS) and neutrophil extracellular traps (NETs). In close proximity to the endothelium, complement activated neutrophils were able to increase the vascular permeability allowing the transmigration of melanoma cells. MAC deposition on tumor-associated neutrophils was also found in human melanomas but not in rarely metastasizing basal cell carcinomas, keratoacanthoma, or non-metastatic nevocytic nevi. Interference with the deposition of complement factors on the EC surface through the low-molecular weight heparin tinzaparin prevented MAC formation and thus ROS and NETs release from neutrophils. Moreover, tinzaparin treatment stabilized the vascular permeability and might contribute to a reduced metastasis as previously published. In summary, we discovered a triangular communication between the complement system, neutrophils and the vascular endothelium mediating NETosis, endothelial dysfunction and subsequently melanoma cells extravasation. Therefore, targeting complement activation envisions a new therapeutic strategy for the treatment of malignant melanoma.

Zusammenfassung

Eine verstärkte Aktivierung des Komplementsystems konnte bereits in verschiedenen Tumorerkrankungen nachgewiesen werden. Vorangegangene wissenschaftliche Arbeiten weisen darauf hin, dass das Komplementsystem Endothelzellen (EZ) und neutrophile Granulozyten aktiviert. Dennoch ist das Wissen hinsichtlich der Interaktion zwischen dem Gefäßendothel, dem Komplementsystem und dem mit den Neutrophilen assoziierten Immunsystem, im Kontext der Tumorprogression, begrenzt. Hier berichten wir über die tumorspezifische Komplementaktivierung bei am malignen Melanom erkrankten Patienten. In von der Maus stammenden und humanen Gewebeproben haben wir eine vermehrte Anreicherung der Komplementeffektoren C3b und C5a um Tumorblutgefäßwände gefunden. Darüber hinaus haben wir hohe Spiegel des Mannose bindenden Lektins (MBL) am Endothel festgestellt, welche auf eine Beteiligung des Lektin Weges, bei der Melanom vermittelten Komplementaktivierung hinweist. Jedoch endete die Komplementkaskade in der Formation des Membran Angriff Komplexes (MAK) nicht auf dem Endothel, sondern auf den perivaskulären Neutrophilen. In *in vitro* Experimenten mit humanen EZ und Neutrophilen wurde ihre Komplement vermittelte Wechselwirkung bestätigt. *In vitro* Experimente haben auch gezeigt, dass Neutrophile reaktive Sauerstoffspezies (ROS) und neutrophile extrazelluläre Fallen (NEF) ausschütten. Komplementaktivierte neutrophile Granulozyten waren daher in der Lage, im eng benachbarten Endothel die vaskuläre Permeabilität zu erhöhen. Ein Mechanismus, der die Transmigration von Melanomzellen erleichtert. Die Ablagerung des MAK auf Tumor assoziierten Neutrophilen wurde in humanem Melanom gefunden, aber nicht im selten metastasierenden Basalzellkarzinom, Keratoakanthom, oder nicht metastasierenden nävozytischen Nävi. Das niedermolekulare Heparin Tinzaparin interferiert mit der Ablagerung der Komplementfaktoren auf der EZ Oberfläche und verhindert die MAK Bildung und somit in der Folge die Freisetzung von ROS und NEF aus Neutrophilen. Darüber hinaus stabilisierte Tinzaparin die vaskuläre Integrität und reduzierte die Metastasierung des Melanoms. Zusammenfassend beschreiben wir hier die trianguläre Kommunikation zwischen dem Komplementsystem, neutrophilen Granulozyten und dem vaskulären Endothel im Mikromilieu des Melanoms. Aufgrund der hier aufgezeigten Bedeutung der Komplement vermittelten NEF-Bildung und der damit einhergehenden endothelialen Dysfunktion und Extravasation von Melanomzellen, erscheint die Inhibition der Komplementaktivierung als eine neue therapeutische Strategien bei der Behandlung des malignen Melanoms besonders sinnvoll.

1 Introduction

1.1 The complement system

Complement system is a major part of innate immunity and has been traditionally considered as the first line of defense against microbial infections. Complement activation can be initiated by three distinct pathways: the classical, lectin and alternative pathways (Figure 1)¹. IgM or IgG binds to the surface of microbial intruders to create immune complexes, which are recognized by C1q. C1q serves as the initiator of classical pathway activation and it subsequently activates C1r and C1s serine protease subunits, which cleave complement components C4 and C2, resulting in the formation of C4bC2a, the C3 convertase in the classical pathway.

The activation of lectin pathway can be triggered by the aberrant carbohydrates or glycoproteins exposed on the surface of pathogens or damaged cells². The pattern-recognition molecules of the lectin pathway are mannose-binding lectin (MBL), ficolins and collectins¹. Collectins have carbohydrate recognition domains, which are able to bind sugar patterns. MBL belongs to the collectin family and it can recognize microbial polysaccharides or glycoconjugates rich in mannose or N-acetylglucosamine². Similarly, Ficolins contain the C-terminal recognition fibrinogen-like domains and can recognize acetyl groups on the surface of bacteria. After binding, these pattern-recognition molecules will form a complex with MBL-associated serine proteases (MASP1 and MASP2), cleaving C4 and C2 to form the C3 convertase C4bC2a, which is same with classical pathway route.

The alternative pathway initiates by spontaneous hydrolysis of C3 to form C3(H₂O); a process also known as the “tickover” of C3¹. C3(H₂O) binds to factor B, and then factor B can be cleaved by factor D and results in the formation of the C3 convertase C3(H₂O)Bb, which is the initial C3 convertase of the alternative pathway. This proteolytic complex can convert C3 into reactive C3b. C3b can bind Factor B and Factor D forming the predominant alternative pathway C3 convertase C3Bb. Although this convertase is a short lived complex, its association with properdin (also known as factor P) can enhance C3Bb stability.

All of the complement activation pathways lead to the cleavage of C3 into C3a and C3b. C3b binds either to the invading pathogen, abnormal cells or to the C3 convertases to generate C5 convertases. The C5 convertase cleaves C5 into C5a and C5b. In the classical and lectin pathways the C5 convertase is composed of C3bC4bC2a whereas the alternative pathway

convertase is formed by C3bBbC3b. C5b recruits complement components C6, C7, C8 and multiple C9 to form the terminal pathway component C5b-9, also known as membrane attack complex (MAC).

The complement system is strictly controlled at several steps by complement regulators, which have been grouped into two categories: soluble and membrane-bound regulators¹. C1 inhibitor (C1INH) binds and inactivates C1r, C1s and MASP-2 in the classical and lectin pathway. Complement receptor 1 (CR1, also known as CD35), C4-binding protein (C4bp) and decay-accelerating factor (DAF, also known as CD55) dissociate the C3 and C5 convertases. Moreover, factor H, membrane cofactor protein (MCP, also known as CD46), DAF and CR1 serve as cofactors for factor I mediated cleavage of C4b and C3b to their inactive fragments iC4b and iC3b. CD59 is expressed on most of cell types and blocks the assembly of MAC on cell membrane³. In addition, Factor P stabilizes the C3 convertase and is therefore the only known positive regulator of the complement system.

As a central part of innate immunity, complement plays a key role in pathogens elimination and clearance of damaged cells. The process of covalent deposition of complement effectors such as C1q, C3 fragments (C3b/iC3b) and C4b on the target cell surface is called opsonization¹. Opsonization accelerates the amplification loop of the complement pathways and promotes the phagocytosis of the opsonized cell (e.g. tumor cells, pathogens, apoptotic cells) by phagocytic cells such as neutrophils and macrophages. Complement anaphylatoxins, C3a and C5a, trigger the inflammatory response by activating immune cells via C3a receptor (C3aR) and C5a receptor (C5aR)⁴. Moreover, C3a and C5a play a critical role in the recruitment of immune cells with phagocytic properties.

Complement activation terminates in the formation of the lytic MAC on the surface of invading pathogens, and is thus considered as a hallmark of the complement attack. In order to avoid accidental host cell damage, MAC formation is tightly regulated. For example, nucleated cells are protected from lysis by expression of CD59, which is known as the most important regulatory factor for MAC formation. For many years, the classical view of the MAC is the formation of membrane pore. However, lytic effects of MAC on nucleated cells are counteracted through the combination of complement surface regulators and activated recovery processes, resulting in the activation of host cells and proinflammatory signal transduction^{5,6}.

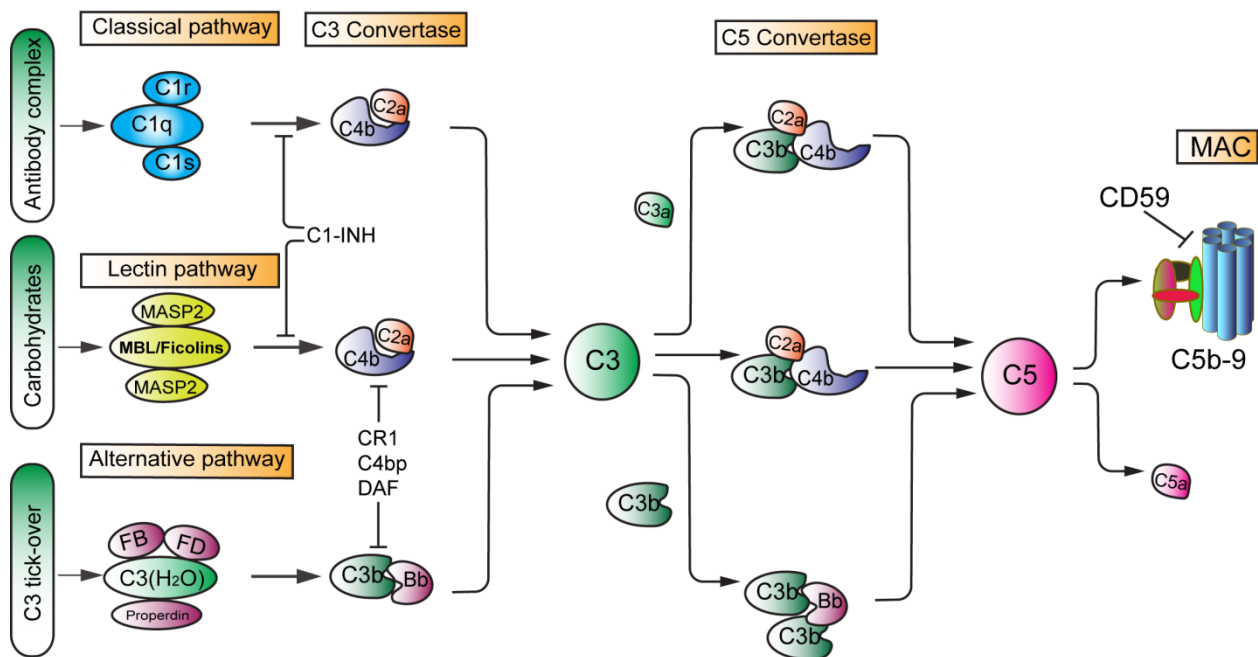


Figure 1. Complement activation cascade.

The complement system is activated by three distinct pathways: the classical pathway, the alternative pathway and the lectin pathway. The classical pathway is activated through antigen-antibody complexes; the lectin pathway begins with the binding of MBL or Ficolins to carbohydrate ligands on the surface of pathogens and the alternative pathway initiates by spontaneous hydrolysis of C3. All complement activation pathways converge on the formation of C3 convertase and the subsequent generation of C5 convertase, which then cleaves C5 to anaphylatoxin C5a and C5b. C5b forms a membrane attack complex with C6, C7 C8 and multiple of C9. C1-INH inhibits the function of C1r, C1s and MASP2. CR1, C4bp and DAF can work as a co-factor for factor I to inactive C3b or C4b. Moreover, those factors can directly inhibit the C3 convertase function to negatively regulate complement activation. CD59 can disassemble the MAC formation.

1.2 Terminal complement complex as a trigger for inflammation

In principle, deposition of critical amounts of MACs disrupts the bilayer of the cell membranes, leading to cell lysis. However, next to gram-negative bacteria, only aged erythrocytes are susceptible to MAC induced cell lysis under physiological conditions⁶. MAC formation on nucleated cell membrane is tightly regulated and can be easily eliminated. CD59 is a 20 kDa glycolipid anchored protein which is expressed on most nucleated cells, and it binds tightly with C5b-8 to inhibit further recruitment of C9, consequently preventing MAC formation. Vitronectin is a 80 KDa glycoprotein and binds to the nascent C5b-7 to

produce water soluble C5b-7 (SC5b-7)⁷. This SC5b-7 is unable to further bind on the cell membrane but can bind C8 or three C9 molecules to form soluble C5b-8 or C5b-9, thereby limiting the generation of MAC pore on the membrane. Similarly, clusterin is another 70 kDa glycoprotein and it can interact with C7, C8 and C9b to block the assembly of C5b-7, C5b-8 and C5b-9 respectively⁸. Moreover, ion pumps can counteract the lytic effects of MAC. For example, in rat oligodendrocytes, non-lethal complement attack led to reversible cell injury, recovery following a transient rise in intracellular calcium and fall in ATP⁹. Nucleated cells can also escape MAC lysis through endocytosis or membrane shedding¹⁰.

Growing evidence indicates that sublytic levels of MAC create a variety of effects in different cell types^{5, 11}. In Schwann cells, MAC has been shown to activate the small G-protein Ras and PI3K/AKT pathway to regulate cell cycle specific genes and promote cell proliferation¹². Some research also reported that sublytic C5b-9 can trigger neutrophil and macrophage to synthesis and secrete inflammatory cytokines⁵. Furthermore, in retinal epithelial cells, MAC stimulates them to release interleukin-6 (IL-6), IL-8 and vascular endothelial growth factor (VEGF)¹³. MAC formed on human ECs can be rapidly internalized through clathrin-mediated endocytosis, and it can further activate proinflammatory functions via noncanonical nuclear factor- κ B (NF- κ B) signaling^{14, 15}.

Collectively, nucleated cells avoid MAC-mediated killing by diverse resistance mechanisms. Moreover, sublytic concentrations of MAC could activate pro-inflammatory signaling in many cell types^{5, 9, 10, 13, 16}.

1.3 Complement in cancer

The complement system belongs to the innate immunity and is thus part of the classical pathogen defense^{1, 6}. In that context, complement activation is triggered by pathogen-associated molecular patterns and culminates in the formation of MACs on the surface of the invading pathogen^{17, 18, 19, 20}. Nowadays, complement activation has been reported to enhance tumor progression and to increase metastasis suggesting its contribution beyond pathogen elimination^{6, 21, 22}.

Soluble complement factors are mainly produced in the liver and located in the blood. However, some cell types, like endothelial cell (EC), immune cells and fibroblast cells can produce complement proteins^{21, 23}. Furthermore, it has been reported that complement

proteins produced by tumor cells can act in an autocrine manner to stimulate tumor growth through C3aR and C5aR mediated PI3K/AKT pathway²⁴. During inflammatory and pathological conditions, deposition of complement fragments in tissue due to a boost complement production can be detected.

Danger signals in tumor microenvironment for triggering complement activation have been reported by several studies recently. C1q has been shown to deposit along the tumor vessels and bind directly to the phospholipids in A549 and H157 lung cancer cells²⁵. The damage associated molecular patterns (DAMPs) including modified glycolipids, glycoproteins and mucins exposed on the surface of tumor cells can trigger the complement activation in the tumor microenvironment^{21, 26}. Natural or adaptive IgM antibodies against tumor associated antigens could also induce the complement activation^{21, 27}. Surace *et al.* reported that radiotherapy induces local complement activation which is mediated by the IgM binding to necrotic tumor cells²⁸. Moreover, in the tumor microenvironment, serine proteases like plasmin and thrombin cleave C3 and C5 directly, enhancing the complement terminal pathway activation via generation of the C3/C5 intermediates (e.g. C3a, C5a)^{29, 30, 31}.

Complement activation promotes chronic inflammation and supports an immunosuppressive tumor microenvironment. Markiewski *et al.* reported that C5a in the tumor microenvironment recruits myeloid derived suppressor cells (MDSCs) and induces the release of reactive oxygen species (ROS), resulting in the suppressing of antitumor CD8⁺ T cell-mediated response³². Consistent with these findings, C3a has also been reported to be implicated in tumorigenesis. In a spontaneous intestinal tumorigenesis mouse model, lipopolysaccharide, leaking from the intestine, was able to induce the complement activation and upregulation of C3aR on neutrophils³³. C3aR-mediated signaling activation further polarizes neutrophils toward a pro-tumorigenic phenotype and triggers neutrophil extracellular traps (NETs)³³. Recently, Wang *et al.* reported that in CD8⁺ tumor-infiltrating lymphocytes, autocrine complement C3 inhibits IL10 production via C3aR and C5aR, inhibiting IL10-dependent T cell mediated antitumor immunity²³. In accordance with the idea of an immunosuppressive role of the complement, a combined blockade of the immune checkpoint molecule PDL1 and of complement receptors might be beneficial³⁴.

Apart from fuelling inflammation, a wealth of research has provided compelling evidence regarding the contribution of complement to the tumor growth. For example, C3a/C5a activate PI3K/AKT pathway to promote tumor cell proliferation²⁴. Deposition of sublytic

levels of MAC on tumor cells induces cell cycle progression^{35,36}. Furthermore, C1q has been shown to support angiogenesis in the melanoma mouse model³⁷. In line with that, complement activation correlates with poor outcome in cancer patients^{38, 39, 40, 41}. Pharmacological blockade of complement related mediators or the deficiency of complement factors (e.g. C1q, C3) correlates with decreased tumor growth in experimental mouse models^{23, 32, 33, 41}.

1.4 Therapeutic regulation of the complement

Complement activation mediates multiple roles in tumor progression, including promotion of chronic inflammation⁴², the establishment of an immunosuppressive tumor microenvironment^{4, 21, 32} and the activation of cancer growth signaling pathways^{21, 24}. In this context, therapeutic manipulation of complement activation in tumor microenvironment has great potential.

Eculizumab, an antibody against complement C5, is the first complement specific drug approved by the US Food and Drug Administration for the treatment of paroxysmal nocturnal hemoglobinuria (PNH)⁴³ and atypical hemolytic uremic syndrome⁴⁴. However, there are only very few clinical reports about the treatment with Eculizumab in cancer. Additionally, C5a has been shown to be a potent proinflammatory mediator in tumor microenvironment and contributes to the tumor growth²¹. C5aR antagonists, including PMX-53²² and AON-D21³⁴, have been reported to inhibit tumor growth effectively in mice. Furthermore, combined blockage of PD-1/PD-L1 and C5a improves the antitumor immune responses and results in a substantial improvement in the efficacy of anti-PD1 antibody against lung cancer metastasis^{23, 34}.

Many complement factors interact with proteoglycans on the cell membrane or in the extracellular matrix and these interactions can be used for intervention of complement activation⁴⁵. Notably, heparin and heparin related derivatives can be used for this purpose. Heparins are strongly related to the naturally occurring heparin sulfate, a major component of the endothelial glycocalyx. Low molecular weight heparins (LMWHs) are derived from unfractionated heparin by enzymatic or chemical depolymerization and used widely for the anticoagulant treatment. Coagulation and complement systems are evolutionarily related enzymatic cascades^{29, 30, 31}. Interestingly, heparin is also demonstrated to have the anti-

complement effects by many previous studies. For example, Girardi *et al.* showed that LMWH prevents antiphospholipid antibody induced fetal loss by inhibiting complement activation, rather than by their anticoagulant effects⁴⁶. In the early studies, Wardle *et al.* confirmed that short-term administration of heparin could decrease 25% complement activation in nephritic patients⁴⁷. Another study by Weiler *et al.* showed that treatment with heparin or N-acetyl heparin are effective to reduce cobra venom factor induced complement activation in guinea pigs⁴⁸. A more recent research reported the inhibition of classical pathway via the interaction of LMWH with C1q in pregnant women^{49,50}. All of those studies showed the use of heparins as potential modulators of the complement system.

Previous studies identified several anti-complementary effect mechanisms by heparin. For example, a lot of complement factors were found to be associated with the alteration of glycan profiles on the inflamed endothelium^{45,51}. Heparins can block those interactions and thus inhibit complement activation. Apart from this, heparin can inhibit the binding of C1q to immune complex and interfere the interaction of C4 with C1s and C2, blocking the initiation of classical pathway activation^{46, 48, 52}. Furthermore, it has been reported that LMWH can inhibit the alternative pathway convertase formation by blocking the binding of C3b and factor B^{46,53}. LMWH could also inhibit complement mediated lysis of PNH red blood cells by the inhibition of C5b-6 binding to red blood cell membranes *in vitro*^{46,54}.

Altogether, heparins can interact with multiple complement factors and a large body of research supports the use of heparin as inhibitor for complement activation. Imbalanced complement activation in the tumor microenvironment plays plenty of functions in tumor progression. Heparin based drugs targeting the complement envisions new therapeutic strategies in cancer therapy.

1.5 Neutrophil in the tumor microenvironment

Neutrophils, accounting for almost 50-70% of the white blood cells, are the first responders of defense against pathogens infection and tissue damage. Targeting microorganisms, neutrophils directly mediate host defense through phagocytosis and intracellular killing. In addition, neutrophils can also release granular antimicrobial protease and ROS or form NETs to trigger the innate immune response. Although neutrophils are well known for the host

protection, uncontrolled neutrophils activation potentiates chronic inflammation and tissue damage⁵⁵.

Many proinflammatory signals mediate neutrophil recruitment to damaged tissue or tumor site, including DAMPs, lipid mediators like LTB₄, chemokines like CXCL1/2, cytokines like tumor necrosis factor α (TNF- α), IL17 and hydrogen peroxide^{55, 56}. In addition, in the context of complement activation, immune cells such as neutrophils are recruited to the site of inflammation. The attraction of neutrophils is directly triggered by the chemoattractant anaphylatoxins C3a and C5a cleaved from the complement factors C3 and C5 as by-products of the complement cascade. Moreover, accumulation of the C3 fragments (C3b/iC3/C3c) on the endothelial surface promotes a rapid adhesion of blood-circulating neutrophils to the vessel wall⁵⁷.

Emerging evidence indicates a positive correlation between enhanced level of neutrophils and poor human tumor patient outcome^{55, 56}. In the last years, a growing body of evidence indicated that neutrophils were educated by primary tumors in order to support the formation of metastasis in distant organs^{55, 56, 58}. Importantly, neutrophil produced factors can directly enhance tumor proliferation and invasion, stimulate angiogenesis and induce immunosuppressive effects^{55, 56}. For example, it has been reported that tumor related neutrophils can release granules containing neutrophil elastase and matrix metalloproteinase 8/9 (MMP8/9) to remodel the extracellular matrix or modulate the inflammation in tumor microenvironment, supporting tumor progression and dissemination^{55, 56, 59}. In addition, neutrophils can also release ROS to enhance tumorigenesis by inducing DNA damage and genetic instability^{55, 56}.

The tumor related microenvironment mediates activation of neutrophils and triggers the activation of oxidative burst, during which their NADPH oxidase gets activated to yield large amounts of superoxide^{55, 60}. ROS contain superoxide, hydrogen peroxide and hydroxyl radical, which are generated by the reduction of O₂ with a single electron, two electrons and three electrons in mitochondria respectively⁶¹. ROS not only mediate antimicrobial activity directly but also participate in stress signaling in normal cells⁶¹. In cancer cells, ROS amplify the tumorigenic phenotype by oxidizing nuclear or mitochondria DNA⁶⁰. The released ROS are essential for the regulation of inflammatory response⁶¹. For example, ROS have been shown to regulate cell signaling pathway or trigger stress response in immune system to exacerbate or dampen inflammatory response⁶¹. Recent studies reported that neutrophil

released ROS inhibit CD8 T cell functions to create an immunosuppressive microenvironment⁶². It is interesting to note that those oxidant released from neutrophils can modify extracellular target and affect the function of surrounding cells, causing cytotoxicity⁶³. ROS induce Ca²⁺ entry across the EC membrane and promote the formation of interendothelial gaps⁶⁴.

Tumor or complement mediated activation of neutrophil triggers also the release of NETs^{55, 65, 66}, which are large web-like extracellular structures composed of histones and neutrophil granule constituents that assembled on a scaffold of decondensed chromatin. This process, commonly called NETosis, occurs in response to infectious stimuli, sterile inflammation and cancer^{65, 67} and the release of the chromatin by neutrophils is dependent on the generation of ROS. ROS stimulate myeloperoxidase (MPO) to trigger the activation and the translocation of neutrophil elastase from granules to nucleus, resulting in histone degradation and DNA decondensation⁶⁸.

The molecular impact of those NETs is versatile and their functions not only limit to defense against pathogens but also contribute to cancer progression. There is evidence that NETs formed in the circulation provide a scaffold to promote cancer associated thrombosis⁶⁵. In mouse models of cancer, NETs adhere to the endothelium and favor the arrest of circulating tumor cells, hence allowing tumor metastasis^{33, 66, 67}. Moreover, NETs-associated histones and proteases can also be toxic for EC, contributing to vascular injury^{59, 69}. Importantly, during NETosis, the released protumor factors, including MMP9, elastase, ROS can directly stimulate tumor cell proliferation and invasion^{70, 71}.

Complement and neutrophil are critical parts of the humoral and blood cell mediated innate immunity and many studies indicate the crosstalk between them³¹. C3a and C5a can recruit neutrophils to inflammatory site. Complement C3b opsonization on pathogens not only induces neutrophil phagocytosis but also facilitates NETs formation. Neutrophils in either C3 or C3aR knock-out mice were not able to form NETs, suggesting the importance of the complement system for NETosis³³.

In addition, activated neutrophil can sever as a platform for complement activation³¹. Neutrophils activation under the treatment of LPS, TNF- α or PMA mediates the release of properdin, which binds to the membrane of neutrophil and initiates the alternative pathway activation⁷². Complement activation can also occur on released NETs⁷³. Numerous complement factors, including properdin, C3, factor B have been reported to be deposited on

NETs^{31, 74}. Additionally, granular proteases MPO and elastase that are present on NETs are able to directly cleave C3 or C5 into their activated fragments³¹.

1.6 Tumor endothelium

Complement activation and Netosis can trigger EC activation and may be involved in vessel diseases such as vasculitis, thrombotic microangiopathy or tumor progression. However, the influential role of the crosstalk between complement and neutrophil on tumor endothelium dysfunction remains poorly understood and only few recent publications address this issue.

Tumor cells rely on vascular network to get nutrition and oxygen, supporting their growth and dissemination⁷⁵. In healthy tissue, endothelial monolayers are quiescent and the vascular endothelium maintains the regular blood flow by providing a barrier to plasma and cell extravasation⁷⁵. However, in the tumor microenvironment tumor cells secrete a variety of growth factors, especially VEGF, to stimulate EC proliferation and migration⁷⁶. In addition, tumor ECs and the tumor cells utilize MMP9 to modulate the degradation of extracellular matrix, allowing tumor cells migration⁷⁷. Multiple proinflammatory factors, like TNF, interleukin, ROS and complement related effectors can stimulate EC activation. Upon activation, ECs can upregulate cell adhesion molecules to capture or arrest circulating immune cells. For example, Albrecht *et al.* reported that C5a stimulates Human Umbilical Vein Endothelial Cells (HUVEC) showing progressive increases in gene expression for cell adhesion such as E-selectin, intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and related receptors (VEGFR, IL-6, IL-18R)⁷⁸. Similarly, the research from Wu *et al.* revealed that the complement component C3a activate phosphorylation of p38 mitogen-activated protein kinase (MAPK) and NFκB to induce murine primary cerebral ECs activation and the expression of adhesion molecules, such as E-selectin and VCAM-1⁷⁹. In addition, tumor associated changes of the endothelium have previously been linked to an altered proteoglycan expression at the EC surface^{80, 81}. It has been reported that the expression of biglycan, a small leucine-rich-repeat proteoglycan, was specifically upregulated in highly metastatic tumor-derived EC^{81, 82}. Interestingly, proteoglycans are able to accumulate different complement factors at the blood vessel wall and they are therefore also able to support localized complement activation⁴⁵.

A prerequisite for metastasis formation is the change of vascular permeability, allowing tumor cells to cross the barrier. On the one hand, tumor cells can produce several factors to induce interendothelial junction disassembly, such as VEGF, IL-6, IL-8 etc. On the other hand, through binding on the adhesion molecules expressed on activated EC, activated neutrophil can mediate barrier dysfunction through the generation of ROS. A large body of literature indicated the oxidant induced endothelial structural alteration. Cytoskeletal remodeling by ROS leads to the disruption of endothelium barrier integrity and formation of paracellular gap. For example, HUVEC treatment with H₂O₂ results in the remodeling of the actin microfilament network, from submembranous bundles into long transcytoplasmic stress fibers⁸³. ROS can increase the level of intracellular Ca²⁺, which subsequently cause phosphorylation of myosin light chain, resulting in actin polymerization and enhanced endothelial permeability^{84, 85}. Furthermore, MAPK and protein kinase signal pathway also take part in the oxidative stress mediated reorganization of the actin cytoskeleton⁸⁶.

ROS are also able to promote the release of NETs. NETs associated endothelial dysfunction is reported in many diseases, such as atherosclerosis, venous thromboembolism and systemic lupus erythematosus⁸⁷. Netting neutrophils secrete variety cytotoxic mediators that have been implicated in increased EC permeability. Neutrophil elastase and cathepsin G can digest VE-cadherin and lead to the increase of EC permeability *in vitro*^{88, 89}. Moreover, Carmona-Rivera *et al.* reported that MMP2/MMP-9 released from NETs specifically impaired murine aortic endothelium-dependent vasorelaxation and induced endothelial cell apoptosis⁹⁰.

1.7 Aims of study

The role of the complement in protection against invasive pathogens through direct lysis and mobilization of adaptive immunity has been extensively studied. However, recent discoveries provide new perspectives on additional physiological roles of the complement in the tumor microenvironment. Complement activation derived effectors in tumors can promote tumor progression by creation of an immunosuppressive environment and by triggering tumor growth and metastasis.

Melanoma is one of the most aggressive forms of skin cancer. High capacity of metastases is malignant melanoma's main characteristics and melanoma is considered to be an immunogenic tumor. Although recent data demonstrate the contribution of several

complement effectors (such as C3a, C5a) to melanoma initiation^{91,92}, the distinctly different function for complement activation that might promote melanoma metastasis has not yet been explored. Therefore, the present study aims to investigate the activation status of the complement system in melanoma and the impact of activated complement on malignant behavior of melanoma.

Complement system and neutrophil are two major effectors of humoral and blood cell mediated innate immunity. Neutrophils are the first responder recruited to the site of inflammation under a variety of signals including lipid mediators, chemokines and C3a and C5a. Released from activated neutrophils, ROS and NETs-associated histones and proteases can be toxic for EC, resulting in vascular injury. A growing body of evidence indicates the crucial involvement of neutrophil in tumor cell dissemination. A prerequisite for metastasis formation is the dysfunction of the endothelium, followed by melanoma cells extravasation. Notably, many reports suggest a connection between complement and neutrophil activation. However, so far, data on the triangular crosstalk between the complement, neutrophils and the endothelium within the tumor microenvironment are scarce. Here, we used distinct mouse models and human tumor tissues to study complement activation, the functional relevance of complement activation on neutrophils and ECs and the impact on melanoma cell dissemination.

The activity of many complement factors is controlled by their interaction with heparan sulfate exposed on the EC surface⁴⁵. Intervention of these interactions can attenuate complement activation and heparin sulfates related LMWH has previously been used for this purpose⁴⁵. Additionally, our previous work has shown that tinzaparin treatment attenuated tumor progression and metastatic burden in murine animal models^{93,94}. Therefore, our study also investigated the impact of LMWH on complement activation and the crosstalk between neutrophils and the endothelium.

2 Methods

2.1 Chemicals and tissue culture materials

All general chemicals are commercially available analytical reagents and were purchased from Sigma, Roth, Merck, Neolab and Biozym. Cell culture reagents were purchased from Invitrogen, PAA, Lonza and Sigma. Cell culture materials were from BD. The information about important reagent is shown in the following method description.

2.2 Antibodies

Product Description	Company	Catalog NO.
mouse anti-human C3b/iC3b	Hycult	HM2286
rabbit anti-human VWF	Dakocytomation	A0082
mouse anti-human CD15	Abcam	Ab188610
rabbit anti-human TCC-FITC	Hycult	HM2167F
rat anti-mouse C3b/iC3b/C3c	Hycult	HM1065
rat anti-mouse CD31	BD Bioscience	550274
rabbit anti-mouse C5a	Biorbyt	Orb10213
rat anti-mouse ly6g	BD Bioscience	551459
rabbit anti mouse C5b-9-FITC	Biobyte	Orb102206
rabbit anti-human Histone H3	Abcam	Ab5103
GAPDH loading control antibody	Thermo	MA5-15738
goat anti-mouse IgG-HRP	Santa cruz	Sc2005
rat anti-mouse C1q	Hycult	HM1044
Mouse anti-human C9	Santa cruz	Sc390000
Rat anti-mouse F4/80	BD Bioscience	565409
Hamster anti-mouse CD11c-APC	Biolegend	117310
Rat anti-mouse CD3	BD Bioscience	555273
Rabbit anti-mouse Factor B	Lifespan	114998
Rat anti-mouse C4	Hycult	HM1046
Rat anti-mouse MBL-C	Hycult	HM1038
Goat anti-mouse SDC1	R&D	AF3190

FITC-conjugated goat anti-rat	BD Pharmingen	554016
FITC-conjugated goat anti-rabbit	BD Pharmingen	554020
Alexa® 555-conjugated goat anti-rat	Invitrogen	A21434
Alexa® 555-conjugated goat anti-rabbit	Invitrogen	A21428
Alexa® 555-conjugated goat anti-mouse (IgG)	Invitrogen	A21422
Alexa® 555-conjugated goat anti-mouse (IgM)	Invitrogen	A21426
Alexa® 647-conjugated goat anti-rabbit	Invitrogen	A21244

2.3 Kits

Product Description	Company	Catalog NO.
Human C5a ELISA Kit	R&D	DY2037
Human C3a ELISA Kit	Hycult	HK354
Human C3 ELISA Kit	Abcam	Ab108822
Human C3b ELISA Kit	Abcam	Ab195461
Pierce® BCA protein assay kit	Thermo	23225
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo	34580
RNeasy Mini Kit	QIAGEN	74106
Reverse Transcription System	Promega	A3500
GoTaq® qPCR Master Mix	Promega	A6001/2
Cell Death Detection ELISA	Roche	11920685001

2.4 Cell lines and cell culture

The human melanoma cell lines (WM9, MV3 and IGR37) were cultured in DMEM (Gibco, Life Technologies) with 10% FBS. Human melanoma cell lines (BCL, WM3211, Mel-6, SB-CL-2, BLM, WM4511 and G361) were cultured in RPMI-1640 (Gibco, Life Technologies) with 10% FBS. Mouse melanoma cell lines Ret were cultured in RPMI-1640 (Gibco, Life Technologies) with 10% FBS, 1% non-essential amino acids (Sigma) and 1% L-Glutamine. Mouse melanoma cell lines B16 were cultured in DMEM (Gibco, Life Technologies) with 10% FBS, 1% NEAA (Sigma) and 1% L-Glutamine.

HUVEC were isolated from donor umbilical cord according to the ethical regulation (Ethics committee, University medical Centre Mannheim, Germany) and were cultivated in the Endothelial Cell Growth Medium (Promocell, C-22010) supplemented with SupplementMix (Promocell, C-39215). The HUVEC isolation was done using our lab established protocol.

All cell lines were cultured in humidified incubator at 37°C in a humidified 5% CO₂ atmosphere. The cells were passaged at a confluency of about 90% according to stander protocol.

2.5 Mouse procedures

All animal experiments were approved by the government animal care authorities and mice were maintained under specific pathogen free conditions. C57BL/6J wild type and C5-deficient mice were purchased from the Jackson Laboratory, backcrossed as previously reported⁹⁵. 7.5×10^5 *ret* transgenic melanoma cells in 100 µl PBS per mouse were i.d. injected into 8-12 week old mice. After 15 days, mice were sacrificed and tumors were embedded in Tissue Tek[®] for cryosectioning. 5×10^5 B16F10 melanoma cells in 100 µl PBS were intravenously injected into the tail veins of mice to induce lung metastases. The mice were sacrificed after 14 days, and the metastatic lungs were dissected and photographed.

For heparin treatments, Tinzaparin (600 IU/kg) dissolved in 100 µl NaCl were subcutaneously injected into the mice to check the effect of anti-complement on tumor progression. The application took place the first day before the injection of melanoma cells, then daily until the day of scification (2 weeks). Control mice were injected with 100 µl of physiological saline. For anti-NETs therapies, Cl-amidine (10mg per kg body weight; Millipore) or PBS was injected intraperitoneally on days 12, 13, 14 and 15. The intradermal tumor was excised and immediately embedded in in Tissue Tek[®], placed on dry ice and snap-frozen at -80 ° C.

2.6 Human malignant melanoma patients' plasma and tissue

EDTA-plasma samples and melanoma tumor tissues were obtained from stage UICC IV malignant melanoma patients. Skin tumors tissue samples were obtained from the department of human pathology, UKE, Germany. This study received the approval from the ethics

committee of the Medical Faculty of Mannheim, Heidelberg University and the University Medical Center Hamburg-Eppendorf.

2.7 Tissue immunofluorescence staining

10 µm frozen sections were prepared with a microtome, and the sections were stored at -20 °C until staining. This staining was a two-step method: First, a primary antibody binds specifically to the structure to be detected. Then, a second antibody labeled with a fluorescent dye binds to species-specific epitopes on the primary antibody. As a result, the cell structures were stained indirectly.

Tissue cryosections (10µm) were fixed in 4% PFA for 15min, and then washed with PBS and blocked with 10% goat serum for 30 min. After a single wash with PBS, the cryosections were incubated with primary antibody for two hours in room temperature or 4 °C overnight. Negative controls sections were incubated only with PBS-T to recognize non-specific binding of the secondary antibody. After washing three times with PBS-T, the slides were incubated with secondary antibody diluted in 10% goat serum for 1 hour in room temperature. In order to stain the second cell structure, the sections were washed again three times with PBS-T, blocked for 30 min, and then the above procedures were repeated with corresponding antibodies. Nuclei were stained with DAPI (Sigma). FITC–conjugated wheat germ agglutinin (WGA, sigma) were used to stain glycocalyx. Finally, the tissue sections were washed thoroughly with PBST, and slides were mounted with mowiol-DABCO (0.5ml Mowiol4-88 + 25mg DABCO) (Sigma) by coverslips. The staining was viewed with Zeiss Axiovert 200 microscope. Images were processed with Axio Vision software and Image J.

2.8 Immunofluorescence cell staining

Prior to immunofluorescence staining, ECs were seeded on 10% gelatin coated coverslips in a 12-well plate. This was followed by stimulation of the ECs according to the procedure described below. Subsequently, the cells were washed with PBS and fixed in 4% PFA for 10 min, and then washed with PBS and blocked with 10% goat serum for 30 min. Appropriate antibodies were used for the following staining procedures, which were same according to

tissue immunofluorescence staining protocol described above. After the final washing step, coverslips were mounted on glass slides and viewed with microscope.

2.9 ELISA assay

ELISA was performed in accordance with the instructions of the manufacturer. BioTek PowerWave XS2 Photometer was used to determine the optical density of each well at the wavelength of 450 nm. The relative amounts of C3, C3 fragments, C3a and C5a were calculated basing on the standard curve generated according to the respective manufacturer' protocols.

2.10 RNA extraction and qPCR

RNA was extracted by using RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized by using Reverse Transcription System (Promega). qPCR reactions (GoTaq® qPCR Master Mix, Promega) were performed using a real-time PCR system (Light cycler 96 system, Roche). The expression of each gene was normalized to its housekeeping gene GAPDH, calculated by $2^{-(\Delta\Delta Ct)}$. The expression profile of the different cell populations was shown in a heat map. Primers can be found in attached table.

CD46-FP (Gene ID:4179)	AAGTGGTCAAATGTCGATTTCCA
CD46-RP (Gene ID:4179)	TCGAGGTAAAAACCCTTATCGC
CD55-FP (Gene ID:1604)	AGAGTTCTGCAATCGTAGCTGC
CD55-RP (Gene ID:1604)	CACAACAGTACCGACTGGAAAAT
CD59-FP (Gene ID:966)	CAGTGCTACAACCTGTCCTAACC
CD59-RP (Gene ID:966)	TGAGACACGCATCAAAATCAGAT
FactorP-FP (Gene ID:5199)	TGCTCTGCTTCACCCAGTATG
FactorP-RP(Gene ID:5199)	CCACTACGTTTCTGGTAGGCA
GAPDH-FP (Gene ID:2597)	GGAGCGAGATCCCTCCAAAAT
GAPDH-RP (Gene ID:2597)	GGCTGTTGTCATACTTCTCATGG

2.11 Neutrophil isolation

Blood was drawn from healthy donors into EDTA coated tube. Neutrophil isolation was performed by using Histopaque-1119 (Sigma) and Percoll Plus (GE Healthcare) gradients as described⁹⁶, which cause minimal neutrophil activation during isolation⁹⁷. 6 ml Histopaque-1119 was added to a 15 ml Falcon tube and 7 ml whole blood was carefully added on the top of Histopaque. After that, tube was centrifuged for 20 min at 800g without braking. Attached Figure 1 shows the tube after centrifugation and we can find four different layers from up to down - plasma, PBMC, neutrophil mixed with erythrocytes and erythrocytes layer.

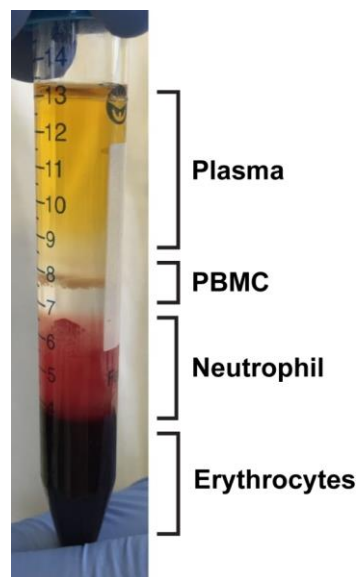


Figure 1. Falcon tube after the first time of $800 \times g$ centrifugation.

7 ml whole blood was carefully added on the top of 6 ml Histopaque and the tube was centrifuged for 20 min at $800 \times g$ without braking. After centrifugation, four different layers can be observed.

Next, the plasma and PBMC layers were discarded and only the reddish neutrophil layer was transferred into a fresh Falcon tube. Cells were washed with 10 ml PBS and mixed by inverting the capped tube 5 times, and then centrifuged for 10 min at 300 g. At the meantime, 2 ml $10 \times$ PBS was added to 18ml 100% Percoll and mixed it by inverting the capped tube several times. 85 %, 80 %, 75 %, 70 % and 65 % Percoll were prepared according to following table.

	85 % Percoll	80% Percoll	75% Percoll	70% Percoll	65% Percoll
Mixed Percoll	3.4ml	3.2ml	3ml	2.8ml	2.6ml
1 × PBS	0.6ml	0.8ml	1ml	1.2ml	1.4ml

After that, Percoll gradient was performed in a new Falcon tube by layering 2 ml of different percentage Percoll on top of each other in decreasing order. This procedure should be done carefully and slowly. Cell pellets after 300g centrifugation were resuspended in 4 ml of PBS and then 2 ml of the cell resuspension was added on the top of Percoll gradient carefully. After that, centrifuge for 20 min at $800 \times g$ without braking. Attached figure2 shows the tube after centrifugation and neutrophils rich layer was found at the 80% Percoll phase (red arrow). After centrifugation, discarded top and 65% layer and collected remaining layers until 85% layer into new tube. Neutrophils were washed with 10 ml PBS and mixed by inverting the capped tube 5 times, and then centrifuged for 10 min at 300 g. After centrifugation, the supernatant was removed and 2 ml RPMI was used to resuspend the sedimented neutrophils. Normally, we can isolate 1×10^7 neutrophils from 7 ml blood. RPMI was used as the basic medium for following neutrophil *in vitro* experiments.

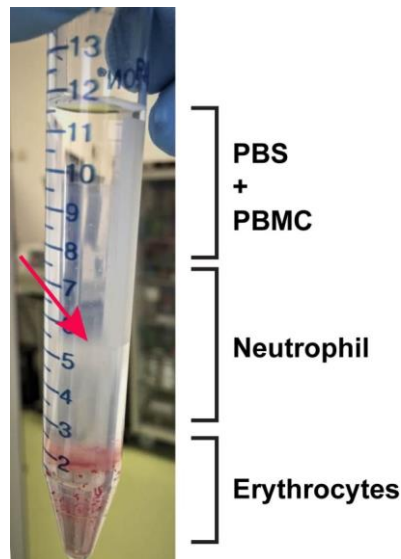


Figure 2. Falcon tube after the second time of $800 \times g$ centrifugation.

2 ml of the neutrophil resuspension (in PBS) was added on the top of Percoll gradient carefully and the tube was centrifuged for 20 min at $800 \times g$ without braking. Red arrow showed the neutrophil rich layer after centrifugation.

2.12 Complement activation on ECs and co-culture with neutrophil

HUVEC were seeded on 10% gelatin coated coverslips in a 12-well plate. After the cells reached to subconfluency, cells were sensitized by CD31 antibodies (DAKOCytomation, 1:50 dilution, 20 min). Antibody-labeled HUVECs were washed with PBS and then exposed to 10% normal human serum (NHS) for 15 min. After that, non-sensitized neutrophils (1×10^5) co-cultured with HUVEC. After 30 min incubation, MAC formation was analyzed by immunofluorescence cell staining.

2.13 *In vitro* sublytic MAC formation and detection on neutrophil

The induction of sublytic MACs on neutrophils was performed as described by *B.P. Morgan*¹⁰. Briefly, anti-human CD15 antibody (Abcam, 1:100 dilution, 15 min) was used to sensitize neutrophils and then neutrophils were washed with PBS and exposed to 10% NHS to induce MACs formation. Heat inactivated serum (56°C for 30min; HIS) and C5-depleted serum were used as negative controls.

For the detection of *in vitro* MAC formation on neutrophils, antibody sensitized neutrophils were exposed to 10% of NHS or HIS for 30 min first and then cells were washed 3 times with PBS. For western blot detection, neutrophils were resuspended in cold lysis buffer containing CompleteTM protease inhibitor cocktail (Roche). 20µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot was performed according to standard protocols using monoclonal antibody to C9 (Santa Cruz, 1:50) for detection.

For FACS detection, cells were washed with PBS and then incubated with rabbit anti-human TCC-FITC antibody (Hycult, 1:100) for 30 min and acquisition was done using FACS Canto II (BD bioscience). FACS was performed according to standard protocols.

2.14 ROS detection

MAC mediated intracellular ROS production was measured with dichlorofluoresceindiacetate (DCFDA, Sigma). Neutrophils were stained with 20 µM DCFDA at 37°C for 30 min. Then

neutrophils were washed once, and the dichlorofluorescein (DCF) level was evaluated by microscope (Axiovert 200, Zeiss) or flow cytometer (FACS Canto II, BD).

Luminol chemiluminescence was used to monitor the kinetics of the MAC induced ROS production. Prior to Luminol-Assay, the following reagents were prepared.

- Luminol (Sigma) stock solution, in DMSO: 0.25 mol/L (50×)
- P-coumaric acid (Sigma) stock solution, in DMSO: 0.09 mol/L (50×)
- Neutrophil suspension in Medium RPMI (Gibico): 2×10^6 /ml
- Mastermix for stimulation, based on medium RPMI.
 - Luminol: 5 mmol/L
 - P-coumaric acid: 1.8 mmol/L

PMA (Sigma) was used as positive control. Luminescence measurement was performed on 96 white wells plate (BRANDplates®). 50 µl neutrophil suspensions was added in each well first and then incubated with 50 µl Mastermix in each well. After adding the Mastermix, luminescence was measured immediately on Tecan M200 microplate reader.

Final concentrations per well (100 µl) during stimulation:

- Luminol: 2.5 mmol/L
- P-coumaric acid: 0.9 mmol/L
- PMA 0.1 µg/ml (Positive control)
- 1×10^5 neutrophils per well

2.15 DNA-Histone fragments detection

Antibody sensitized neutrophils were treated with 10% NHS, HIS, C5 deplete serum and NHS with the addition of tinzaparin (100IU/ml) for 3 hour. The supernatants were collected and centrifuged to remove cell debris. Histone-associated DNA fragments were quantified by the ELISA Kit (Roche) following the manufacturer's protocol. Citrullinated histone H3

(H3Cit) in supernatants was evaluated by Western Blot using the antibody from Abcam. Western Blot was performed according to standard protocols.

2.16 Neutrophil stimulation by C5a

Isolated neutrophils were incubated with human recombinant C5a (100ng/ml, R&D) for 30mins and then FACS was used to check the changes for neutrophil morphology and ROS production. C5a stimulated neutrophils were treated with HIS or NHS for 1 hour. MAC formation was checked by FACS and the neutrophil supernatants were collected for the analysis of NETs.

2.17 MACs and NETs staining

Neutrophils treated with NHS, HIS or NHS with the addition of tinzaparin (100IU/ml) for 30 min. Cytospin (Shandon[®]) was used for the preparation of neutrophil for microscopy. Neutrophils were fixed with 4% PFA for 10 min. Anti-human MAC-FITC antibody (Hycult) and anti-human anti-citrullinated histone H3 antibody (Abcam) were used for the MACs and NETs immunofluorescence staining. DAPI was used to stain the nuclei and the released DNA.

2.18 EC layer integrity staining

HUVEC were seeded on 10% gelatin coated coverslips in a 12-well plate one day before the experiment. After the HUVECs reached to 100% confluence, HUVEC co-cultured with MAC positive neutrophils and control neutrophils (5×10^5 per well) for 6 hours. After one time wash with PBS, HUVEC was fixed with 4% PFA for 10 min, and blocked with 2% BSA for 20 min. Mouse anti-human CD31 (DAKO Cytomation) and DAPI were used for following staining.

2.19 Electric Cell-substrate Impedance Sensing (ECIS)

The trans-endothelial electrical resistance (TEER) of an ECs monolayer was measured by ECIS, using established protocols⁹⁸. Briefly, HUVECs were grown to confluence on gelatin coated gold electrodes, and then co-cultured with MAC positive neutrophils (1×10^5 per well) or untreated control neutrophils as described above. The real time changes in TEER were measured, and data was presented as change in HUVECs layer resistance normalized to value at the beginning time for the co-culture. For the inhibition of NETosis, MAC-neutrophils were co-cultured with HUVECs in the media containing Tinzaparin (100IU/ml) or DNase I (100U/ml, sigma) or Cl-amidine (50 μ M, Millipore).

2.20 Human melanoma cells transmigration assay

Melanoma cells transmigration assay was performed as described previously⁹⁹. MAC positive neutrophils or control neutrophils (1×10^5 per well) co-cultured with confluent HUVECs for 6 hours in the cell culture inserts (24 well plate, Greiner Bio-one) and then the neutrophils were removed by washing with PBS. Human melanoma MV3 (1×10^5 per well) was labeled by Celltrace calcein green (Invitrogen) and then added to the upper chambers. After 12 hours, microscope was used to detect the migrated labeled cells.

2.21 Complement assay

The effect of tinzaparin on the haemolytic activity of the classical pathway (CH50) and the alternative pathway (APH50) was assessed according to the described procedures by Wehling¹⁰⁰ and Joiner¹⁰¹ *et al.* The CH50 tests the total hemolytic activity of the classical and terminal pathways by human serum, and this method depends upon lysis of sheep red blood cells pre-coated with rabbit anti-sheep red blood cell antibody. Like the CH50 assay, the APH50 assay measures the total alternative pathway hemolytic activity to lyse unsensitized chicken erythrocytes by human serum. Results were showed in reference to a pool of normal human serum as standard (100%). The reagents and devices were provided by Prof. Dr. Kirschfink and this experiment was done in their lab (Institute for Immunology, University of Heidelberg, Heidelberg, Germany).

2.22 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software and significance was tested by Student's t-test. All results are presented as the means \pm standard error as indicated in the legend. $P < 0.05$ was considered as significant difference.

3 Results

3.1 The complement system is activated in human malignant melanoma.

To investigate the activation status of the complement system in melanoma patients (stage IV), we measured the levels of C3 in blood samples by ELISA (Figure 1A). In healthy controls, the mean plasma level of C3 was $2210 \pm 81.75\mu\text{g/ml}$. In cancer patients C3 was significantly decreased to $1665 \pm 155.3\mu\text{g/ml}$. This result suggests the consumption of C3 due to the activation of the complement system. C3 plays a central role in the complement cascade activation and its activation is required for both classical and alternative pathways. Therefore, we further analyzed the C3 derived fragments C3a and C3b (Figure 1B-C). The concentration of C3a in healthy control was $263.3 \pm 9.6\text{ng/ml}$, this was significantly increased to $301.3 \pm 9.3 \text{ ng/ml}$ in melanoma patients (Figure 1B). Similarly, the mean plasma level of C3b in healthy controls was $1059 \pm 24.7\mu\text{g/ml}$ and increased to $1175 \pm 24\mu\text{g/ml}$ in melanoma patients (Figure 1C). C3a is an inflammatory mediator and recruits immune cells to inflammatory sites. The increased concentrations of C3 cleavage products in the plasma of cancer patients confirm the melanoma-related complement activation. In both classical and alternative pathways, C3b binds to the C3 convertases (C4b2a and C3bBb) to form C5 convertases, which cleave C5 to produce C5a and C5b. To further prove the activation course of the complement cascade, we measured the plasma levels of C5a (Figure 1D). In line with the supposed activation of the complement system, C5a levels were significantly elevated to $1354 \pm 137.8\text{pg/ml}$ in melanoma patients compared with $989.9 \pm 88.9\text{pg/ml}$ for the controls. C5a is the downstream product of the C3b-catalyzed cleavage of C5 during the complement activation. C5a plays critical role in supporting inflammation by the mobilization of immune cells and activation of cells expressing C5a receptors.

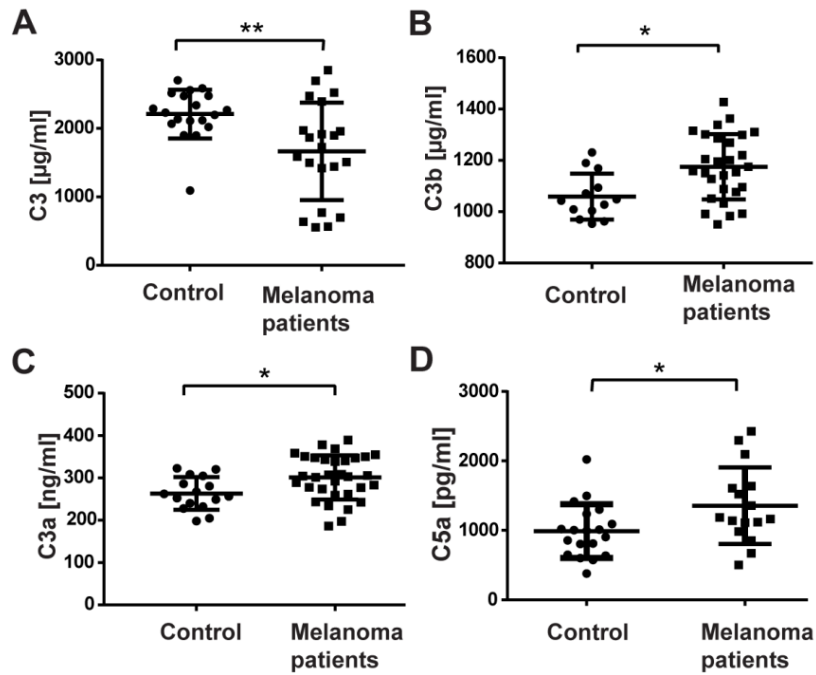


Figure 1. Quantification for complement effectors in plasma samples from control donors and melanoma patients.

Systemic complement effector levels in plasma samples from control donors and stage IV malignant melanoma (MM) patients (A-D) were analyzed by ELISA. C3 levels in blood samples of MM patients were decreased compared with healthy control donors (A). In contrast, C3 activation fragments C3b and anaphylatoxins C3a and C5a were increased in the plasma of cancer patients (B-D). * $P < 0.05$, ** $P < 0.005$.

3.2 Complement activation fragments deposit in human melanoma tissue.

To support our ELISA data, we evaluated the presence of C3 fragments C3b/iC3b in cryosections of human malignant melanoma tissues (n=5, stage IV). Healthy skin was used as control tissue. To this end, tumor cryosections were stained for C3b/iC3b and Von Willebrand factor (VWF), and VWF was used as endothelial cell marker. Immunofluorescence analysis revealed that C3b/iC3b colocalized with the endothelium of tumor blood vessels suggesting the deposition of C3 fragments on the vessel wall and thus complement activation around the vasculature (Figure 2A, right). C3 fragments deposition was scarcely present in healthy skin, which was used as control tissue (Figure 2A, left), indicating the quiescent complement activation in healthy tissue. The corresponding quantification revealed that only 4.9% of vessels in healthy skin contained C3 fragments, and

this was significantly increased to 12.1% in the human melanoma tissue (Figure 2B). Because the complement cascade terminates in the formation of the MAC, we further analyzed the presence and localization of this terminal complex in the tumor tissue. To our surprise, we could not detect significant levels of MAC at the endothelium (date not shown); however we discovered that about 52% of all tumor-related neutrophils were MAC positive (Figure 2C-D). In healthy skin, not so many neutrophils were found and most of those neutrophils were MAC negative.

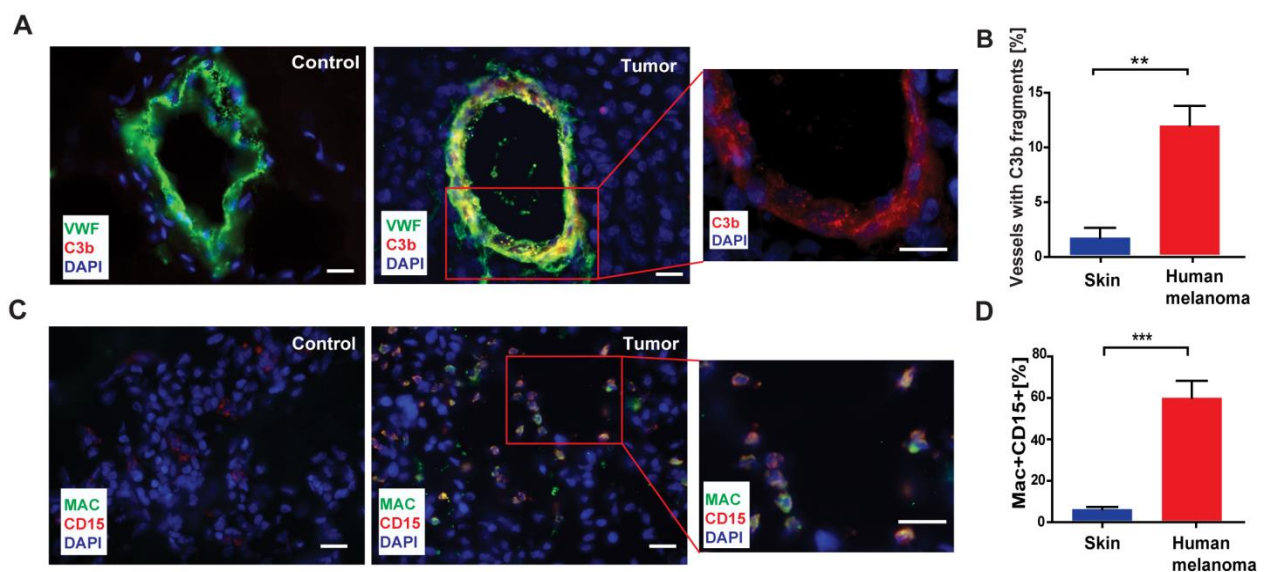


Figure 2. Complement activation in human malignant melanoma patients.

Immunofluorescence analysis of C3b/iC3b (A) or CD15 and MAC (C) in cryosections (scale bars=20 μ m) of human MM tissues (stage IV, n=5) and healthy skins was performed; VWF was used as blood vessel marker. Nuclei were stained with DAPI. Representative images of melanoma tissue show the blood vessel associated deposition of C3b/iC3b whereas analysis of healthy skin indicates the absence of C3 fragments (A). Quantification revealed significantly increased numbers of vessels with C3b/iC3b deposition in tumors compared with healthy skin (B). MACs were detectable on neutrophils in tumor tissue whereas no MACs were formed on healthy skin neutrophils (C). Neutrophils with or without MACs were quantified and the number of MAC positive neutrophils were significantly increased compared with healthy skin (D). Bars indicate the mean \pm SD, **P< 0.005, ***P< 0.0005.

3.3 MACs deposit on neutrophils in murine tumors.

The results from the human samples suggest that the melanoma microenvironment promotes complement activation. Moreover, the finding of strongly elevated levels of MAC+ neutrophils in the tumor tissue indicates a crosstalk between the humoral complement system and the cell mediated neutrophil innate immunity. To further investigate the impact of the complement system on melanoma progression and the potential connection to neutrophil activation, we checked the levels of different complement factors by immune fluorescence in tissue sections of primary tumors generated by the intradermal injection of human *ret* transgenic murine melanoma cells into the dorsal skin of mice.

In line with the human melanoma tissue, C3 cleavage products (C3b, iC3b and C3c) were extensively deposited along almost 30% of the tumor vessels, indicative of an activate complement environment around tumor vessels, whereas deposition of those C3 fragments was absent in control skin (Figure 3A-B). Recently, it was shown that blood vessels in early staged melanomas (1 week tumor) retained only neglectable amounts of C3 fragments. We confirmed these previous findings in our melanoma model, indicating that complement factor deposition and complement activation is related to advanced melanomas (Figure 3C). The anaphylatoxin C5a, a potent immune mediator and neutrophil attractant protein was also elevated in the proximity of tumor blood vessels when compared to healthy skin (Figure 3D-E). In correlation with the increased C5a levels, we also found the recruitment of neutrophils to C5a rich areas (Figure 3F). In addition, we analyzed the staining of MACs, and in analogy to our human tissue analysis, we found MACs deposited not on the endothelium but on neutrophils (Figure 3G). Quantification revealed almost 85.1% of tumor-associated neutrophils exhibited positive MAC (Figure 3H). In summary, our findings suggest that the complement cascade is initiated on the endothelium, whereas the terminal MACs is established on adjacent neutrophils.

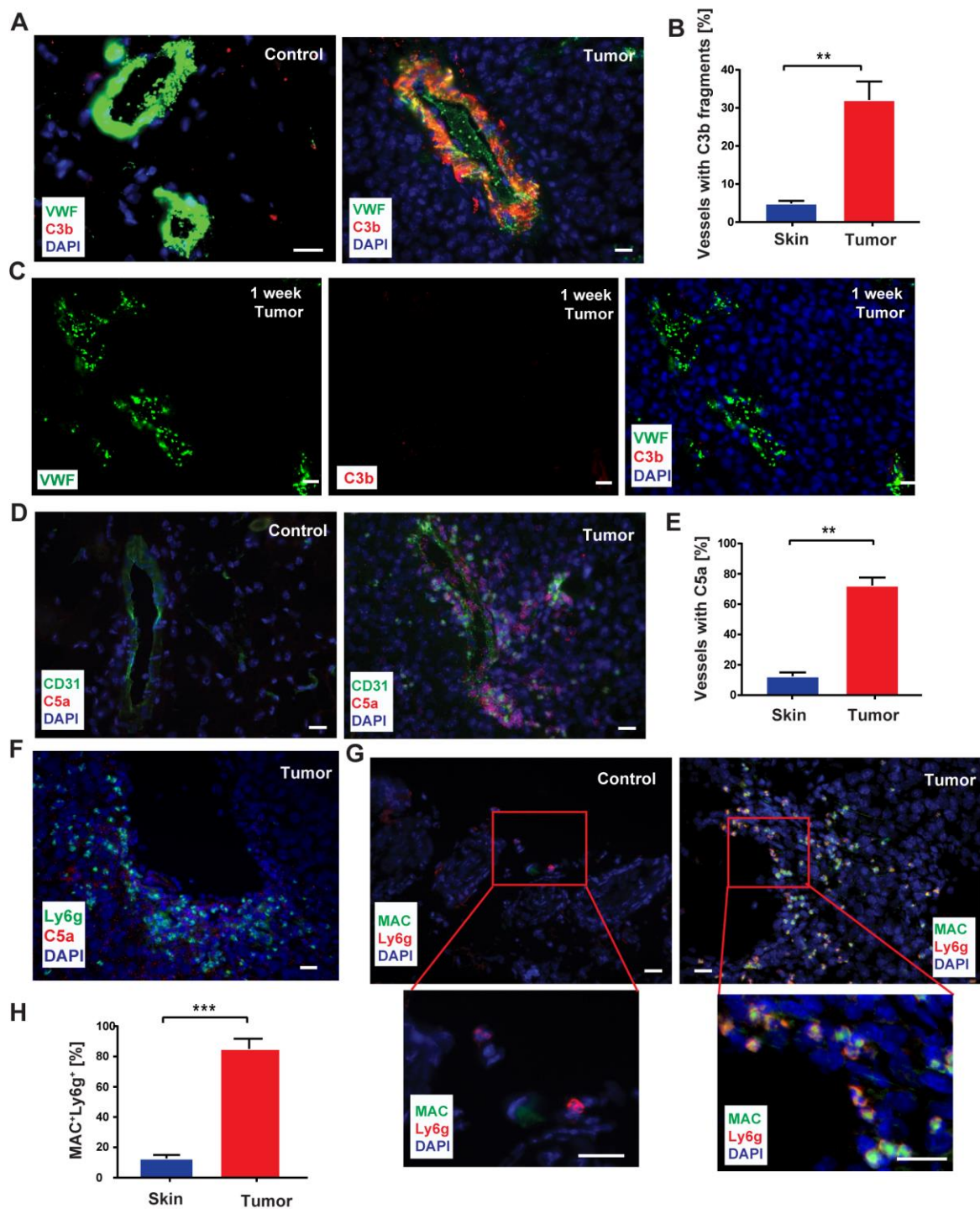


Figure 3. Immunofluorescence analysis of complement components in mouse engrafted tumor.

Cryosections (n=5, scale bars=20 μ m) from mice primary tumors were stained for VWF and C3 cleavage products (C3b, iC3b and C3c) (A, C) or CD31 and C5a (D) or Ly6g and MAC (G). Nuclei were stained with DAPI. In healthy mouse control skin, no complement effectors (C3 fragments, C5a, MAC) were detected, indicating a quiescent complement milieu (A, D, G left). Representative images of mice melanoma show that C3 cleavage products were deposited along the tumor vessels (A). Quantitative analysis demonstrates an increased number of tumor blood vessels with C3 fragment

deposition, as compared with healthy skin control (B). Immunofluorescence staining show that no obvious C3 cleavage products (C3b/iC3b) deposit on the vessels walls in cryosections of 1 week mouse melanoma tumors (C). C5a shows a punctuate staining in the proximity of blood vessels (D) and quantification revealed a significantly increased number of vessels with C5a accumulation (E). Neutrophils were recruited to C5a rich areas of the tumor (F, Scale bar=50 μ m) and MACs deposited on tumor associated neutrophils (G-H). Bars indicate the mean \pm SD, * P < 0.05, ** P < 0.005, *** P < 0.0005.

3.4 Complement activation occurs locally in tumor.

To further investigate whether the complement activation occurs systemically or only locally, we checked the complement activation status in the peripheral organs of tumor bearing mice. We were not able to detect an enhanced C3b/iC3b deposition in the vasculature of other tissues (e.g. kidney, lung and liver; Figure 4A) except in tumors. So, local tumor-specific complement activation appeared to be evident. In further control experiments regarding MACs location, we detected MAC deposition neither on tumor associated macrophages, dendritic cells and T Cells (figure 4B) nor on neutrophils in mouse peripheral organs such as lung, liver and kidney (figure 4C), confirming that the MAC forms only on tumor-associated neutrophils.

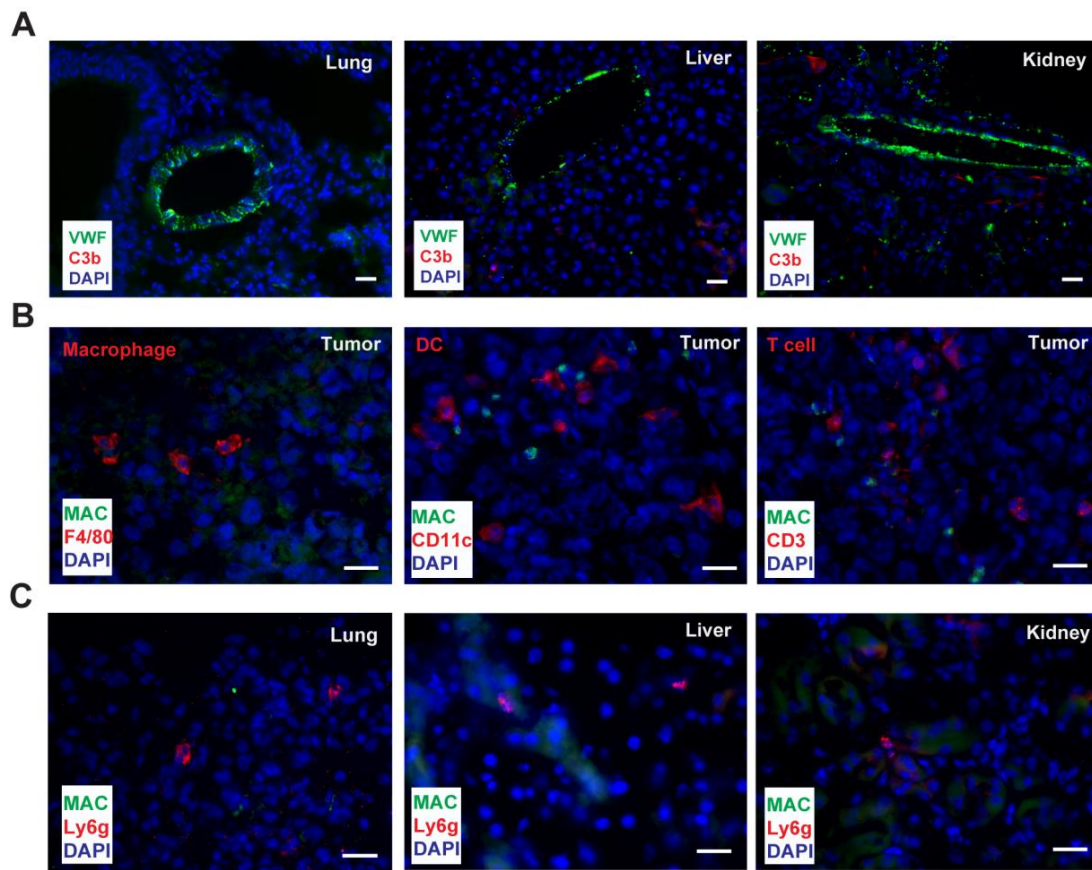


Figure 4. Complement factor deposition in murine tissues.

Kidney, lung and liver tissues were obtained from melanoma bearing animals (n=4 each). Cryosections were stained for VWF (green) and C3 cleavage products (C3b/ iC3b, red) (A) or MAC (green) and Ly6g (red) (B, C). No C3 cleavage product deposition and MAC positive neutrophils were observed in the peripheral organs (A, C). MAC was neither formed on tumor associated macrophages (F4/80 staining, red), T cells (CD3 staining, red) nor in DC (CD11c staining, red) (B).

3.5 The lectin pathway is predominantly involved for the complement activation.

To clarify which of the complement pathways contributed to the measured production of anaphylatoxins and the formation of MACs, we compared the endothelial expression of C1q, MBL and factor B (Figure 5A). Lack of factor B deposition on the tumor endothelium suggested a negligible contribution of the alternative pathway. In line with the previous work published by Bulla *et al*³⁷, we were able to detect C1q at the blood vessel wall. However, we also detected a considerable amount of blood vessels positive for MBL. Through quantification of tissues sections of 5 different melanomas we found that about 25% of the blood vessels were positive for MBL whereas only 12% of the blood vessels were C1q

positive indicating that the lectin pathway is mainly responsible for the complement activation (Figure 5A). Conform to the supposed activation of the classical and especially the lectin pathway we found the deposition of C4 as well (Figure 5A). The MBL recognizes pathogen-associated carbohydrate structures rich in mannose, N-acetylglucosamine or glucose². Although those carbohydrates are not exposed by healthy mammalian cells, a growing body of evidence indicates that under pathophysiological conditions an aberrant EC glycosylation allows the binding of MBL^{2, 102}. Interestingly, the function of various complement factors is tuned by their interaction with carbohydrates. For instance, the binding of factor P to specific epitopes of heparan sulfate has been shown to initiate alternative pathway activation⁵¹. To further clarify whether the endothelial glycosylation controlled the tumor blood vessel wall restricted complement activation, we correlated the C3b/iC3b deposition with the endothelial expression of N-acetylglucosamine, a major component of heparan sulfate. N-acetylglucosamine was stained by wheat germ agglutinin (WGA). As shown in Figure 5B, deposition of C3b/iC3b along the blood vessel wall co-localize with the WGA staining. Quantifications reveal that significant more C3b/iC3b deposited on WGA positive blood vessels. In line with the WGA staining, deposition of C3b/iC3b correlated also with increased levels of the heparan sulfate exposing proteoglycan syndecan 1 (SDC1) (Figure 5C). This data suggest that an aberrant glycosylation of the tumor EC may trigger complement activation.

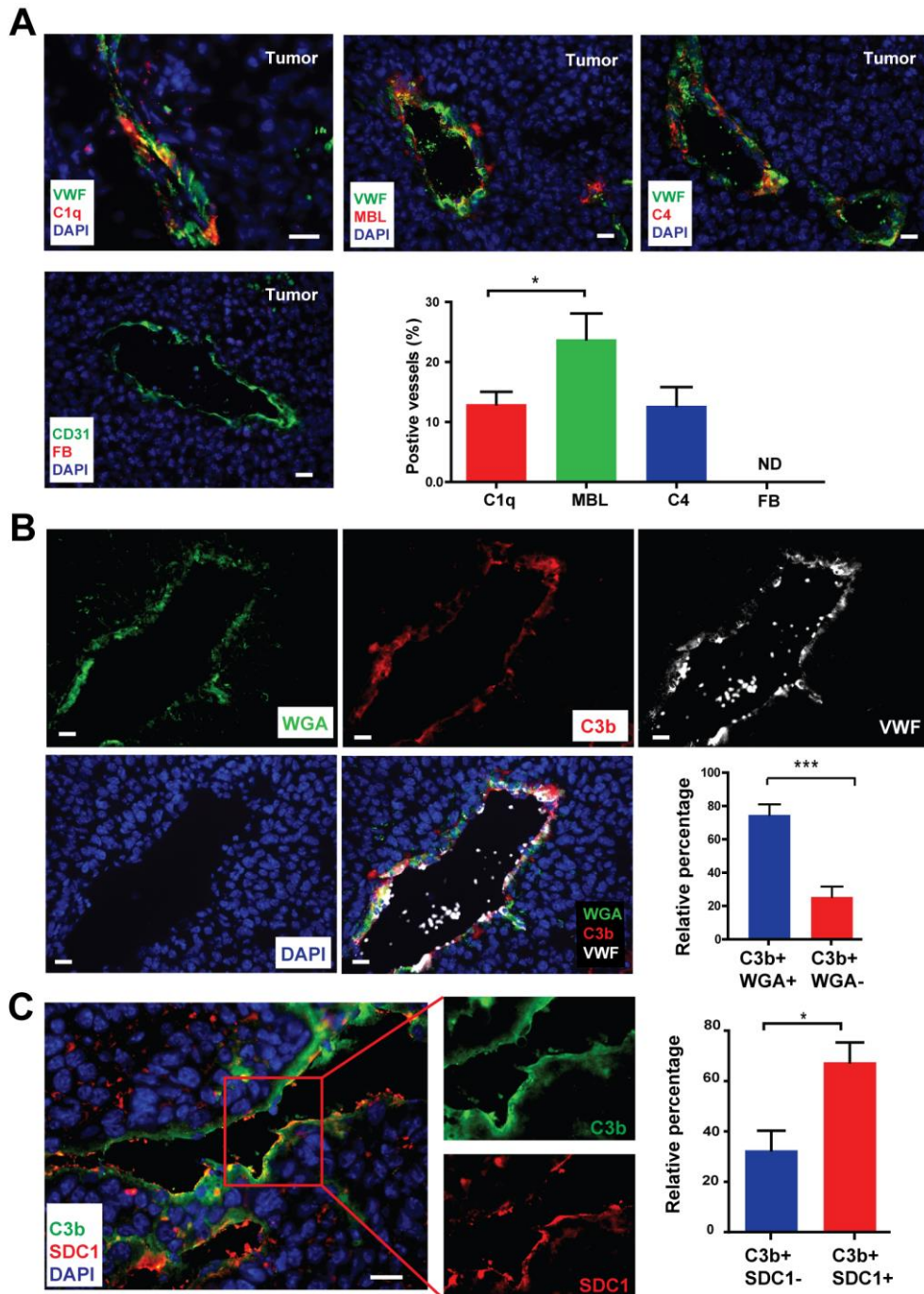


Figure 5. Proteoglycan induces the restricted complement activation on tumor vessel walls.

Immunofluorescence stainings of early complement components (C1q, MBL, FB (Factor B) and C4) were performed (scale bars=20 μ m). Quantification indicates a comparable high abundance of MBL positive vessels. Less are positive for C1q. Blood vessel wall deposition of factor B was not detectable (A). Cryosections of primary tumors were stained for WGA (green), VWF (white), C3 cleavage products (C3b/ iC3b, red) and nuclei were stained with DAPI (blue) (B). Representative image and quantification showed C3 fragment deposition on blood vessels with high levels of N-

acetylglucosamine (B). Immunofluorescence staining of SDC1 (red) and C3b/iC3b (green) in cryosections obtained from mouse melanoma tumors (C, scale bars=20 μ m, n=4). Quantification shows that higher levels of SDC1 expression were accompanied by enhanced C3b/iC3b deposition (C). Bars indicate the mean \pm SD, * P < 0.05, ** P < 0.005.

3.6 MACs form on neutrophils in close proximity to the tumor.

Our *ex vivo* data demonstrated the association between complement activation (C3b deposition) and the glycosylation (WGA staining and SDC-1 expression) of the tumor endothelium. Even more indicative for a local complement activation was the discovery of neutrophils that began to gain MAC deposition at the interface of the blood vessels (Figure 6A-B yellow arrows), while neutrophils without physical contact to the endothelium remained MAC negative (Figure 6A-B, white arrow). To further prove whether the initiation of the complement system on ECs could finally lead to MAC deposition on neutrophils which were in direct physical contact with the endothelium, we aimed to mimic our *in vivo* findings by an *in vitro* approach. To enable the initiation of complement on HUVECs, we sensitized the ECs with antibodies directed against CD31. Antibody-labeled HUVECs were then exposed to 10% normal human serum (NHS) and non-treated neutrophils. Finally, MAC formation was analyzed by immune fluorescence microscopy. Although complement activation was intentionally induced on HUVECs, we discovered MAC formation exclusively on neutrophils (Figure 6C). This result is further supported by expression analysis of the complement regulatory proteins, CD46, CD55, CD59 and factor P in HUVEC, neutrophil and several human melanoma cell lines (Figure 6D). CD46 serves as a cofactor for factor I-mediated proteolysis of C3b/C4b and CD55 accelerates the decay of the C3 convertase¹. CD59 prevents recruitment of C9 to the C5b-8 complex and inhibits the final step of MAC formation¹. Interestingly and in agreement with our assumption that the complement cascade is initiated on the endothelium but terminated on neutrophils, HUVECs were found to express even 3.5 time higher levels of CD59 than tumor cells, which are known to highly express CD59 as indicated by previous studies^{21, 42}. Therefore, HUVECs appears to be able to prevent MAC deposition on their plasma membrane, whereas low levels of CD46 and CD55 suggest vulnerability to the deposition of the early complement factors C3b and the C3 convertase. In contrast, neutrophils showed high levels of CD46 and CD55, but only low levels of CD59 enabling MAC formation. Moreover and in contrast to HUVECs and melanoma cells, neutrophils expressed comparable high levels of factor P. This is also in good agreement with

the expression of factor P by neutrophils which we found in close proximity to the tumor endothelium (Figure 6E).

Neutrophils are the most abundant circulating leukocytes and recently neutrophils have been shown to alter the tumor microenvironment to support tumor progression^{55, 56}. Our finding that MAC formed on neutrophils in both human and mice tumor tissues suggest that MAC positive neutrophils contribute to melanoma metastasis.

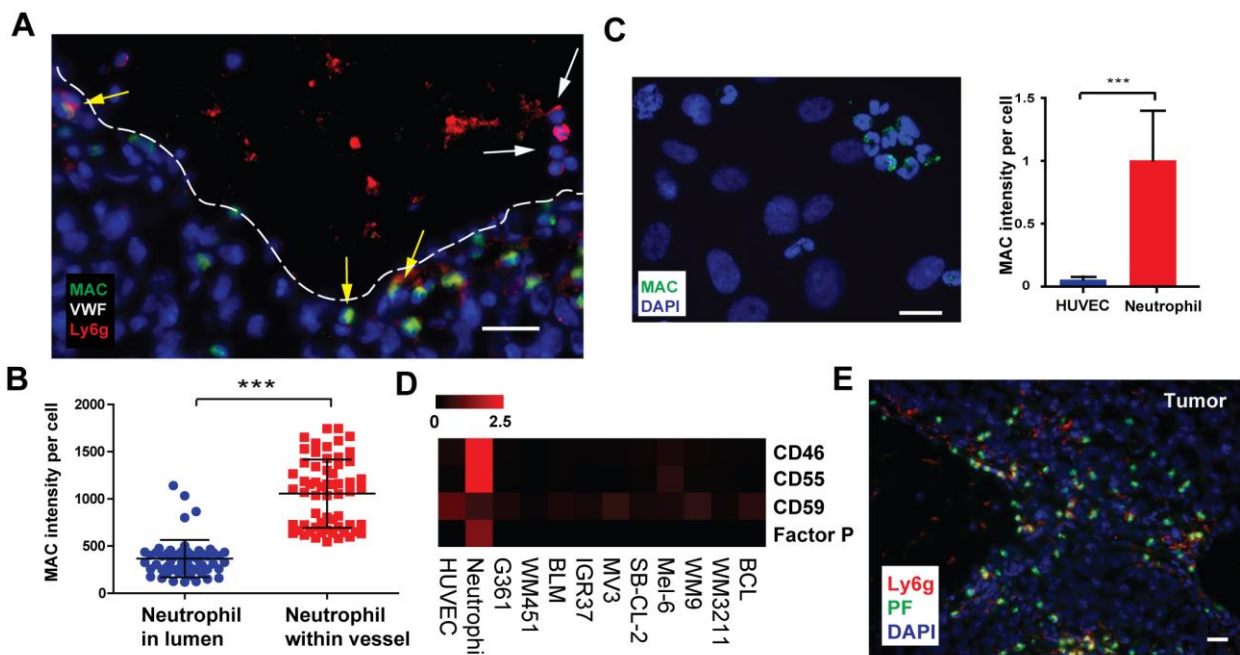


Figure 6. MACs deposit on tumor associated neutrophils but not on EC.

Cryosections of primary tumors were stained for MAC (green), VWF (white), Ly6g (red) and nuclei were stained with DAPI (blue). Representative image of luminal MAC negative neutrophils distant to the blood vessel wall (A, white arrow, scale bars=20µm) and MAC positive neutrophils upon contact with the endothelium (A, yellow arrows, dotted line reflecting endothelial-luminal interface). The MAC intensity on neutrophils in lumen or attached with endothelium was quantified (B). Representative microscope image of neutrophils co-cultured with CD31 antibody sensitized HUVECs in 10% NHS (C). MAC deposition was detected by immunofluorescence (C, scale bar=20µm). HUVECs and neutrophils were distinguished by the shape of their nuclei (HUVEC: oval nucleus; neutrophils: polymorphous nucleus). Quantification of fluorescence intensities revealed that MACs formed only on neutrophils but not on the endothelial cells (C). Gene expression of complement regulatory proteins (CD46, CD55, CD59 and factor P) in human melanoma cell lines (BCL, WM3211, WM9, Mel-6, SB-CL-2, MV3, IGR37, BLM, WM451 and G361), HUVECs and neutrophils suggest

that neutrophils are susceptible to MAC deposition (D). Representative image shows that factor P is expressed by tumor associated neutrophils (E, scale bars=20 μ m). Data are shown as the mean \pm SD, **P< 0.005, ***P< 0.0005.

3.7 MACs on neutrophil leads to ROS production.

To gain insight into the pathophysiological function of MAC on neutrophil, we adapted the aforementioned *in vitro* model to induce the deposition of MAC on neutrophil. In contrast to the experiment with HUVEC, we directly sensitized neutrophils with CD15 antibodies¹⁰. Sensitized neutrophils were treated with either NHS (10%) as a complement source or heat inactive serum (HIS) as a control. FACS analysis confirmed that NHS treated neutrophil can lead to the MAC deposition on neutrophil (Figure 7A). For the MAC formation, multiple copies of C9 need to interact with C5b-8. After three times washes with PBS, NHS and HIS treated neutrophils lysates were collected and measured by western blot. As expected, C9 deposition was detected in NHS treated neutrophils, but not in HIS treatment control group (Figure 7B), confirming the MAC formation in our *in vitro* model. One of the hallmarks of neutrophil activation is the oxidative burst. We asked whether MAC deposition could also trigger the neutrophil activation. To answer this question, we measured the release of ROS by different techniques. DCFH-DA is a fluorogenic dye that measures intracellular ROS activity. After cell uptake, DCFH-DA is deacetylated by cellular esterases and later oxidized by ROS into 2'-7'-dichlorofluorescein (DCF), which can be detected by FACS or fluorescence microscopy. As shown in figure 7C, in comparison to the HIS treated control group, neutrophils treated with NHS produced elevated intracellular ROS levels as indicated by the increased oxidation of the intracellular fluorescent probe DCFHDA. The corresponding quantification revealed almost threefold increased DCF intensity in MAC positive neutrophils, compared with that in control neutrophils. These data were further confirmed by corresponding flow cytometric experiments. Intracellular oxidant was detected in NHS treated neutrophils, but not in control cells (Figure 7E). ROS react with luminol causing the light to be emitted, which can be detected with luminescence plate reader. So, the kinetics of the ROS generation can be monitored using this luminol-dependent chemiluminescence assay. In our study, we also verified that MAC+ neutrophil released increased levels of ROS by this assay (Figure 7D).

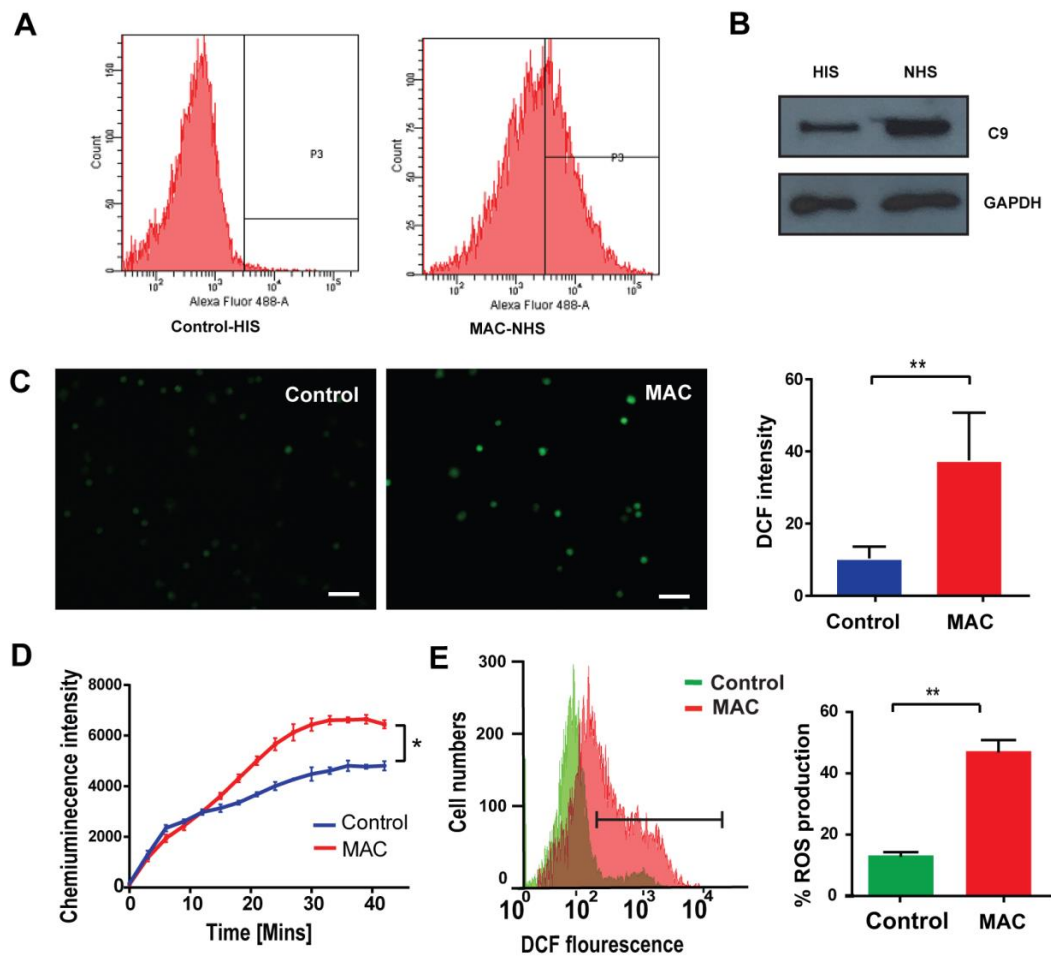


Figure 7. MACs form on neutrophils *in vitro* and promote ROS production.

Antibody sensitized neutrophils were treated with 10% NHS or HIS for 30 min and then washed with PBS. FACS results show the formation of MAC on NHS treated neutrophils (A). Neutrophil lysates were electrophoresed and immunoblotted using anti-C9 antibody. The amount of C9 deposition is higher on NHS treated neutrophils, compared with HIS group (B). Intracellular ROS was measured by the staining of DCFDA (20 μM, 30 min, scale bar=20μm) (C). Quantification of the microscope based detection of the intracellular ROS levels (C). Flow cytometric analysis of MAC mediated intracellular ROS production (E). Luminol chemiluminescence was used to monitor the kinetics of the MAC induced ROS production (D). Data are shown as the mean ± SD, *P< 0.05, **P< 0.005.

3.8 MAC on neutrophil induces NETs.

Neutrophil can undergo NETosis, a process of antimicrobial infection and implicate in inflammatory disease and tumor progression⁶⁵. Because oxidative stress is a strong promoter of NETosis, we next analyzed the effect of MACs on NETs formation. Sensitized neutrophils

were treated with NHS, HIS or C5 depleted serum for 3 hours and the respective supernatants were collected. ELISA analyses demonstrated a strongly increased concentration of DNA-Histones fragments in supernatants of NHS treated neutrophils, but not in HIS and C5 depleted serum treated supernatants (Figure 8A). Citrullination of the histone 3 (CitH3) is used as a common marker for NETosis. Accordingly, a robust western blot signal of citrullinated Histone3 was detected in MAC⁺ neutrophil supernatants. In contrast, no CitH3 was found in the HIS and C5 depleted serum control groups (Figure 8B). Immunofluorescence images of NHS treated neutrophils show that externalized DNA structures co-localized with the NET-associated CitH3. Neither DNA release nor a positive CitH3 staining was detectable in the HIS treated control group (Figure 8C-D). To prove those *in vitro* findings, we then examined the release of CitH3 from MAC positive neutrophils in murine (Figure 8E) and human tumor tissues (Figure 8F). In agreement with our *in vitro* results, CitH3 and MAC staining was in close proximity in both human and mouse tissue.

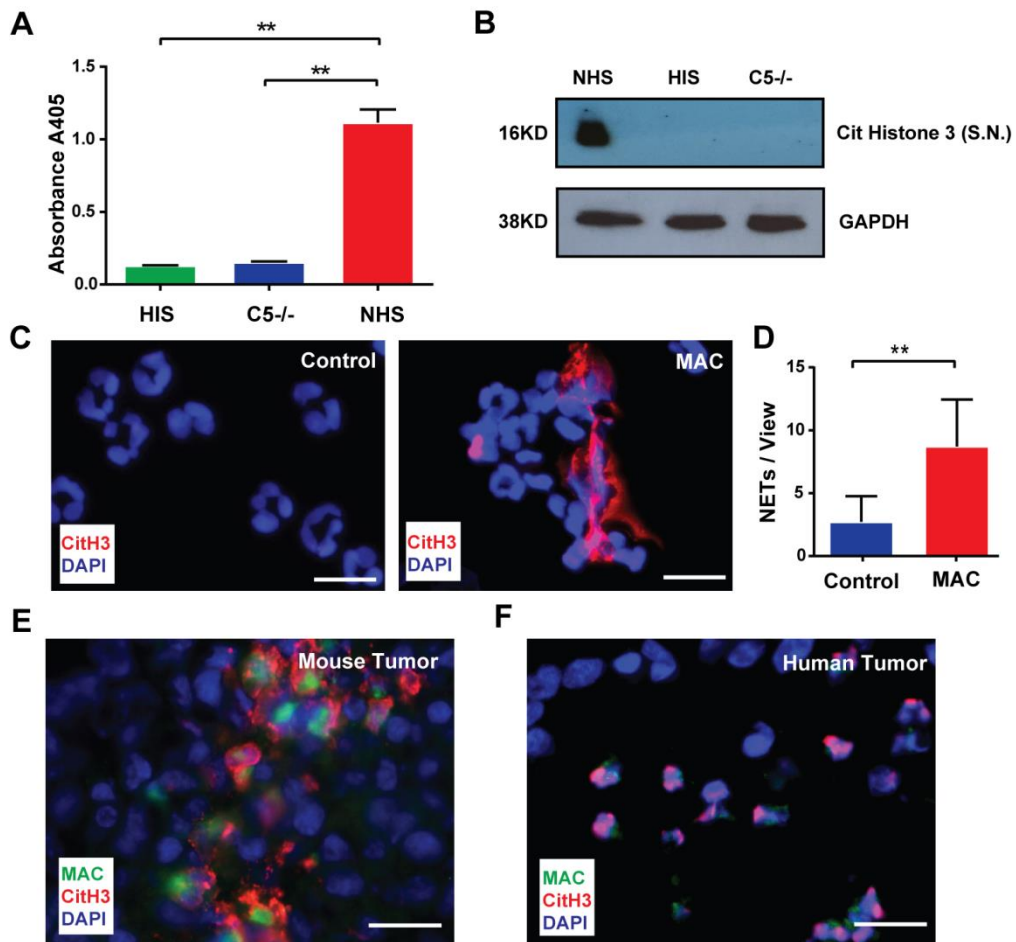


Figure 8. MACs promote NETs formation.

Sensitized neutrophils were treated with 10% NHS for 3 hours to induce NETosis. HIS or C5 depleted serum were used as control. ELISA was used to analyze the release of DNA-Histones fragments in the supernatants. NHS treated neutrophils released significantly more DNA-Histones fragments, compared with control groups (A). Citrullinated Histone3 (CitH3) was detected in NHS treated neutrophil supernatant by western blot (B). NHS treated neutrophils and HIS treated control neutrophils were stained with an anti-CitH3 antibody and DAPI. Fluorescence microscopy revealed externalized DNA structures co-localized with the NETs-associated CitH3 in MAC positive neutrophils but not on control neutrophils (C, scale bar=20 μ m). For quantification, NETs were counted on the whole slide and expressed as NETs per view (D). Representative immunofluorescence staining of MACs (green) and CitH3 (red) in the cryosections (n=5, scale bars=20 μ m) of mice melanoma tumor (E) and human MM tissues (F) showing the close association between CitH3 and MAC. Bars indicate the mean \pm SD, *P< 0.05, **P< 0.005.

3.9 C5a can not induce MAC formation and neutrophil activation.

Generated after complement activation, C5a is a potent chemoattractant for pro-inflammatory leukocytes and recruits neutrophils to areas of inflammation. As an immune mediator, it plays an important role in the initiation and regulation of inflammatory response. Because neutrophil activation might not only be related to MAC deposition but also be induced by C5a, in further experiments, we analyzed the impact of C5a on neutrophil activation (ROS production, NETosis) and MAC formation. Neutrophils were stimulated by recombinant C5a (100ng/ml) and FACS was used to measure the changes in neutrophil morphology, ROS release and MAC formation. In line with previous studies^{103, 104, 105}, we found that stimulation of neutrophils with C5a promoted cell swelling but failed to induce ROS generation and MAC deposition (Figure 4A-C). The C5a induced increase in cell size has recently been reported and might represent the reorganization of the cytoskeleton necessary for chemotaxis¹⁰³. Also the stimulation of neutrophils with recombinant C5a was not able to induce the release of DNA-histone fragments (Figure 9D), suggesting that not C5a stimulation but MAC formation is highly relevant for NETosis.

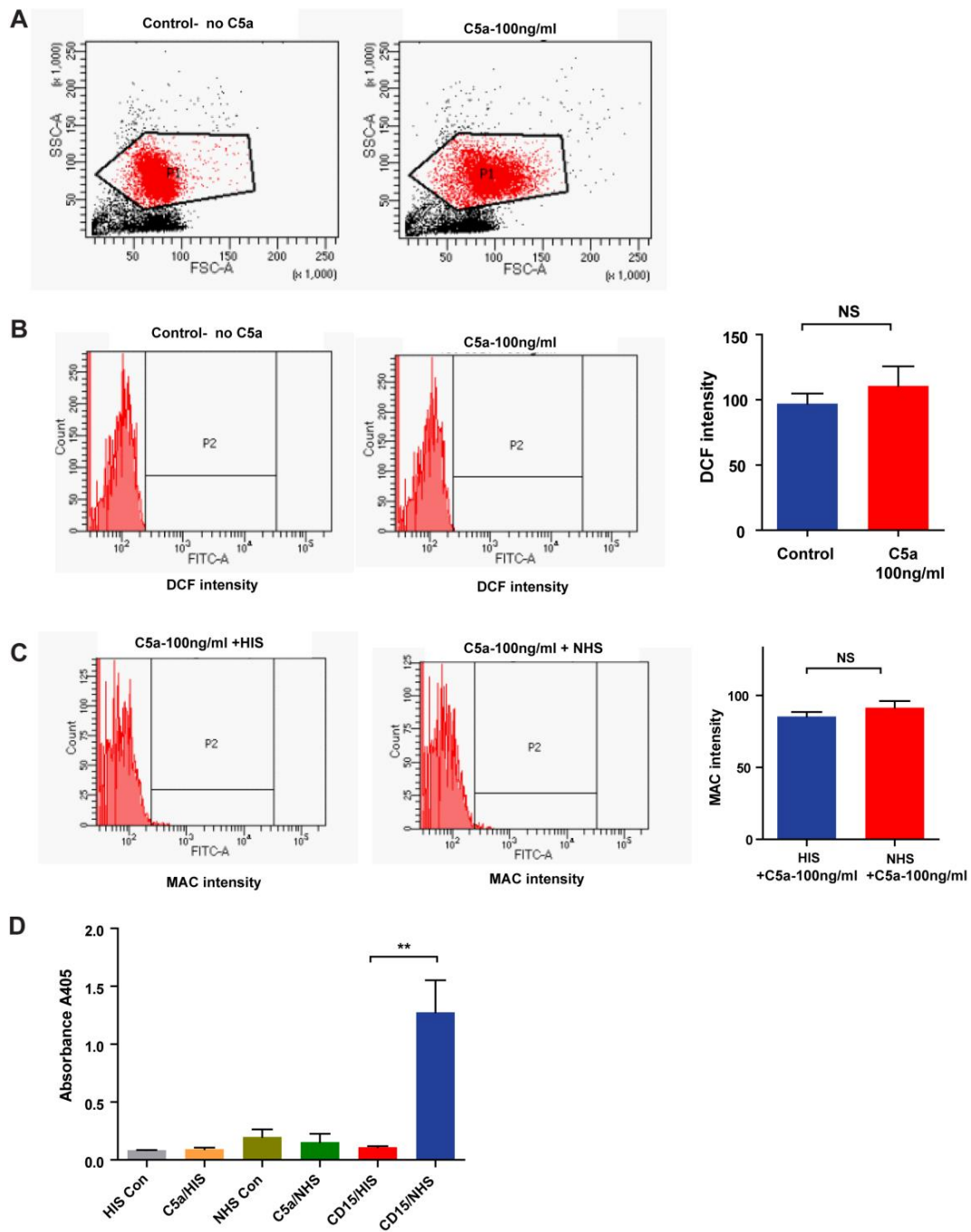


Figure 9. C5a is not able to induce neutrophil ROS release, MAC and NETs formation.

FACS showed that stimulation of neutrophils with recombinant C5a (100ng/ml) induced cell swelling (A) but failed to induce ROS generation (B). C5a (100ng/ml) stimulated neutrophils were further treated with 10% NHS for 30 min to induce MAC formation on neutrophils, 10% HIS was used as a control. MAC formation was measured by FACS and quantification indicates the failure of MAC formation upon stimulation with C5a (C). C5a stimulation was neither able to induce the release of DNA-Histone fragments in the HIS nor the NHS treatment group. As a positive control, CD15

antibody primed neutrophils released significantly increased levels of DNA-Histones fragments after incubation with NHS (D). Bars indicate the mean \pm SD, *P< 0.05, **P< 0.005.

3.10 Neutrophils in close proximity to the tumor blood vessel wall.

Although C5a stimulation was not potent to induce ROS production or NETosis in neutrophils, the presence of C5a around the tumor vasculature (Figure 3D-E) and the complement activation fragments deposition on the vessel wall (Figure 3A-B) suggest that neutrophils might be recruited around the tumor vessel. We hypothesized that neutrophils recruited around tumor vessel mediate EC dysfunction. To this end, we first checked the distribution of neutrophils in mice and human melanoma. Human and mouse cryosections were stained for neutrophil and VWF. In human tissue, CD15 was used as a marker for neutrophil, while Ly6g was used as mouse neutrophil marker. Within the tumor, numerous neutrophils marginated along or penetrated the wall of blood vessels in both human (Figure 10A) and mouse tumor tissue (Figure 10B), which is also conform with the C5a induced cell swelling (Figure 9A).

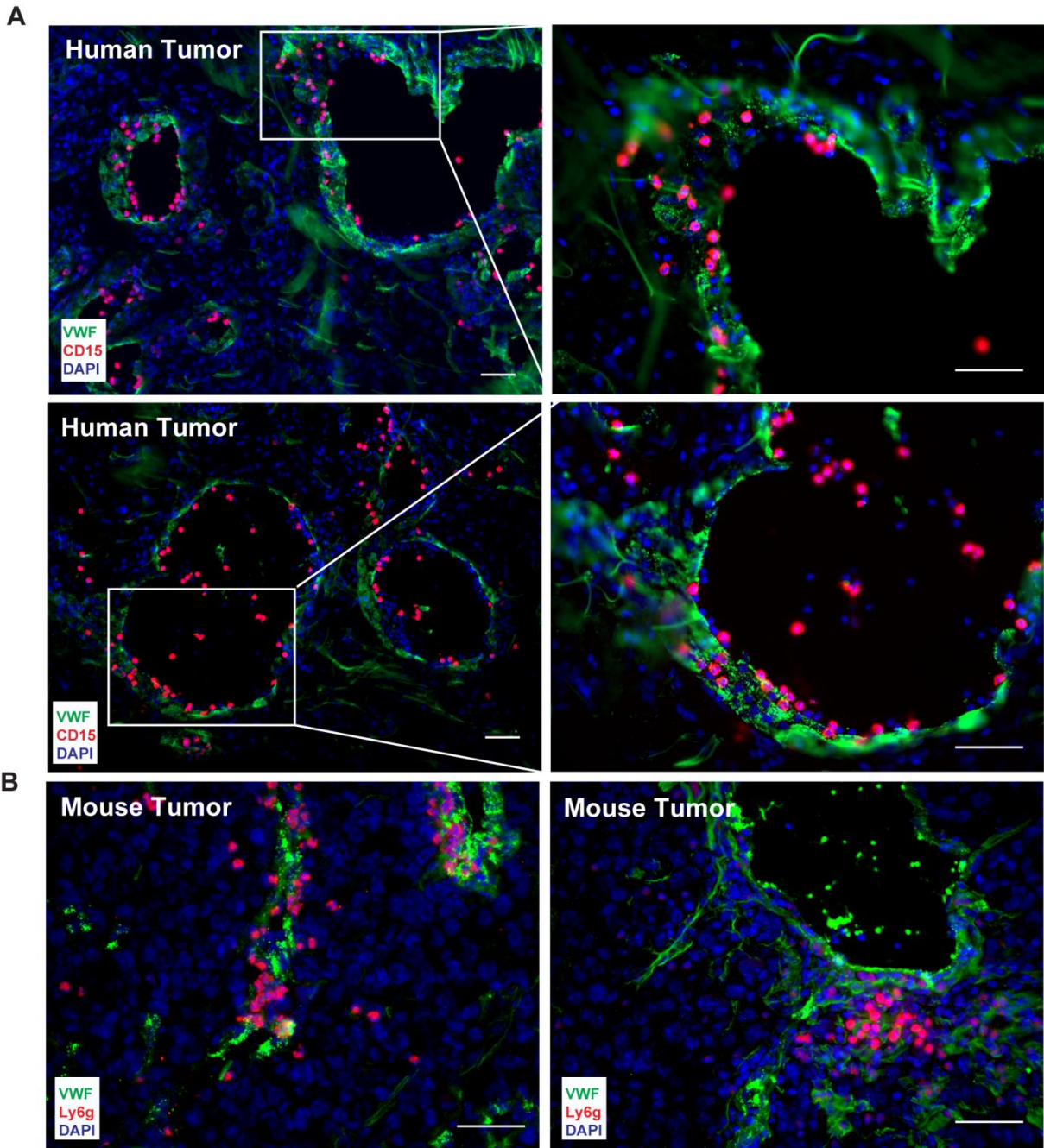


Figure 10. Neutrophils marginate along or penetrate the blood vessel walls in human and mice tumor.

Cryosections of mice and human malignant melanoma tissue were analyzed by immunofluorescence staining for the distribution of neutrophils. Nuclei were stained with DAPI. VWF antibody was used to identify tumor vessel. Ly6g antibody was used to stain mouse neutrophil (A) and human neutrophil was stained with CD15 antibody (B). Within the tumor, numerous neutrophils marginate along or penetrate the wall of vessel (A-B, n=5, scale bar=20 μ m).

3.11 MAC-activated neutrophils contribute to endothelial barrier disruption *in vitro*.

We have shown that MAC on neutrophil mediated neutrophil ROS release and NETs formation (Figure 7-8). ROS as well as NETs could potentially be toxic for ECs^{59, 64, 69}. Therefore, we postulated that in close contact with EC, MACs activated neutrophils could induce the dysfunction of ECs. To study neutrophil-EC crosstalk, we co-cultured HUVEC with NHS treated neutrophils *in vitro*. As shown in Figure 11A, MAC positive neutrophils induced gap formation in a confluent EC monolayer. The mean level of relative gap areas in co-culture with MAC+ neutrophils was almost twofold increased compared with control group (Figure 11A). Electric Cell-substrate Impedance Sensing (ECIS) is a real time impedance based method to study the barrier function of ECs. The trans-endothelial electrical resistance (TEER) of a ECs monolayer can be measured by ECIS. ECs at confluence exhibit a high TEER, reflecting the integrity of the EC monolayers. When the permeability of the monolayer is disrupted, the electrical resistance will dramatically decrease. Data from ECIS experiments further confirmed that co-culture with MAC positive neutrophils decreased the TEER of an endothelial monolayer suggesting an increased vascular permeability (Figure 11B).

To further prove the NETs formation is the cause of the disrupted vascular barrier, we performed additional ECIS experiments with the treatment of NETosis inhibitors. Blockage of NETosis by the protein arginine deiminase 4 (PAD4) inhibitor CI-amidine prevented the neutrophil induced breakdown of the endothelial resistance (Figure 11C). Also the treatment with DNase was able to slightly counteract the endothelial destructive effect of the NETs. However the effect was statistically not significant indicating that histones or other NETs related cytotoxic factors remained unaffected by DNase treatment (Figure 11C).

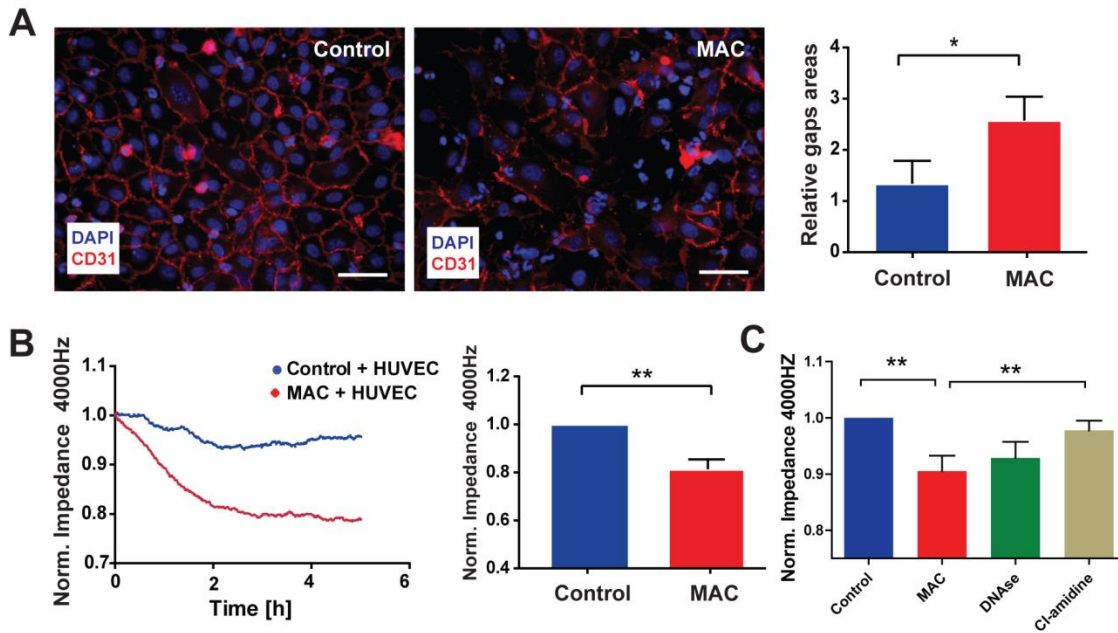


Figure 11. Activated neutrophils provoke EC dysfunction.

Integrity of a HUVEC layer after 6 hours of treatment with MAC positive neutrophils or control neutrophils was analyzed by immunofluorescence staining of CD31 and DAPI (A, scale bar=50µm). HUVECs and neutrophils were distinguished by the shape of their DAPI stained nuclei (HUVEC: oval nucleus; neutrophils: polymorphous nucleus). Gap areas in the HUVEC monolayer were measured and corresponding quantifications show increased relative gap areas after co-culture with MAC positive neutrophils (A). Permeability of HUVEC was determined using Electric Cell-substrate impedance sensing (ECIS). Co-culture with MAC neutrophils (1×10^5 per electrodes) decreased the impedance of the endothelial monolayer, indicating an increased vascular permeability (B). The PAD4 inhibitor CI-amidine (50µM) prevented the MAC neutrophil induced breakdown of the endothelial resistance. Treatment with DNase (100U/ml) was less effective (C). Bars indicate the mean \pm SD, *P< 0.05, **P< 0.005.

3.12 Perivascular neutrophils increase the endothelium permeability in tumor tissue.

We intended to measure this supposed permeability increase across the endothelial barrier also in our tumor tissue sections. For this purpose, we analyzed the leakage of IgG from the blood vessel into the adjacent tissue by immune fluorescence analysis. Normally endothelial monolayers are quiescent and vascular endothelium stabilization keeps the regular blood flow and provide barrier to plasma and cell extravasation. The leakage of IgG from the blood vessel can be used as a marker for disrupted endothelial barrier. To this end, tumor cryosections were stained for Ly6g, VWF, anti-mouse IgG and DAPI. In the absence of

perivascular neutrophils, IgG located only along the blood vessel wall (Figure 12A, arrowheads); however, IgG were found in the tissue beyond the blood vessel in neutrophil rich region (Figure 12A arrows). Quantitative analysis revealed significant more IgG leakage from vessels disrupted by neutrophil accumulation (Figure 12B). This suggests that neutrophils in the vicinity of the blood vessels disrupt the ECs monolayer barrier and increase the permeability. To better attribute the increased leakage of the endothelial barrier to neutrophil activation we treated tumor-bearing mice with the PAD4 inhibitor CI-amidine. We cannot exclude that the impairment of the neutrophil activation through PAD4 inhibition. Therefore, to allow a reliable quantification, we compared the IgG leakage in vessels with or without neutrophils. The results show that impaired neutrophil activation (Figure 12C-D) abolished the ability of the neutrophils to compromise the endothelial barrier.

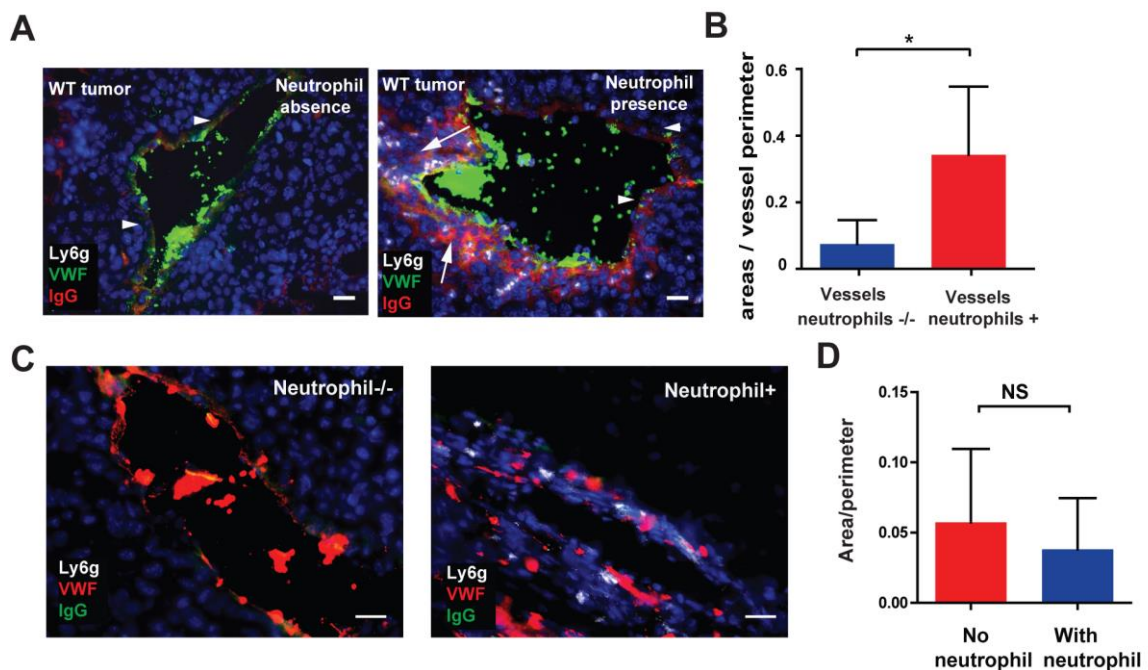


Figure 12. IgG leakage from the blood vessel into the adjacent tissue by immune fluorescence analysis.

For the *in vivo* proof of our findings, immunofluorescence staining of Ly6g (white), VWF (green) and IgG (red) in cryosections of mouse primary tumors were performed to measure the blood vessel integrity *in vivo* (A, scale bar=20 μ m). Nuclei were stained with DAPI (blue). In the absence of perivascular neutrophils, IgG locates only along the blood vessel wall (arrowheads). In neutrophil rich regions, IgG leaks out into the tissue and was detected beyond the blood vessel border (arrows, A). IgG leakage areas to the vessel perimeter ratio were quantified. Vessels with perivascular

accumulation of neutrophils exhibit a higher ratio compared with the vessels without neutrophils recruitment (B). Immunofluorescence staining of Ly6g (white), VWF (red) and IgG (green) in cryosections of CI-amidine (10mg/kg, n=5) treated mouse tumors were performed to measure the blood vessel integrity (C). Quantitative analysis revealed no significant difference of IgG leakage between vessels with or without neutrophils (D). Scale bars = 20 μ m. Bars indicate the mean \pm SD, *P< 0.05.

3.13 Disruption of the MAC formation protects the vascular integrity.

The tight physical contact between the MAC-activated neutrophils and the tumor endothelium induced the disruption of the tumor blood vessel barrier. To confirm these findings at the functional level, the relevance of the complement system for endothelial barrier disruption was tested in C5^{-/-} mice. In mouse models of cancer, complement deficiencies (C3, C4 or C5aR) and treatment with complement inhibitors have been associated with impaired tumor growth and suppressed metastasis^{23, 24, 32, 33}, whereas C5 deficiency did not affect the growth of the primary tumor³⁷. Our experiments with C5^{-/-} mice confirmed these results (Figure 13A-B). In addition, the recruitment of neutrophils due to a lack of C5a in the C5^{-/-} mice affected the total amount of neutrophils within the tumors tissue (Figure 13D). Therefore, to allow a reliable quantification, we compared the IgG leakage in vessels with or without neutrophils in tumor tissues of C5^{-/-} mice. The results show that the disruption of the complement cascade (Figure13C) abolished the ability of the neutrophils to compromise the endothelial barrier. To prove whether an impaired endothelial barrier may promote tumor cell extravasation we measured the transmigration of human melanoma cells by transwell assays. In line with an increased vascular permeability, the number of transmigrated melanoma cells through an endothelial monolayer was almost twofold increased in the presence of MAC positive neutrophils (Figure 13E). To further investigate the impact of complement activation and MAC formation in melanoma metastasis, we performed a group of lung metastasis experiments by intravenous injection of melanoma into WT and C5 deficient mice. As shown in the figure 13F, we detected less lung metastasis in C5 deficient mice. Taken together, our data indicate that perivascular MAC positive neutrophils increase the EC barrier permeability, promoting tumor cells transmigration *in vitro*.

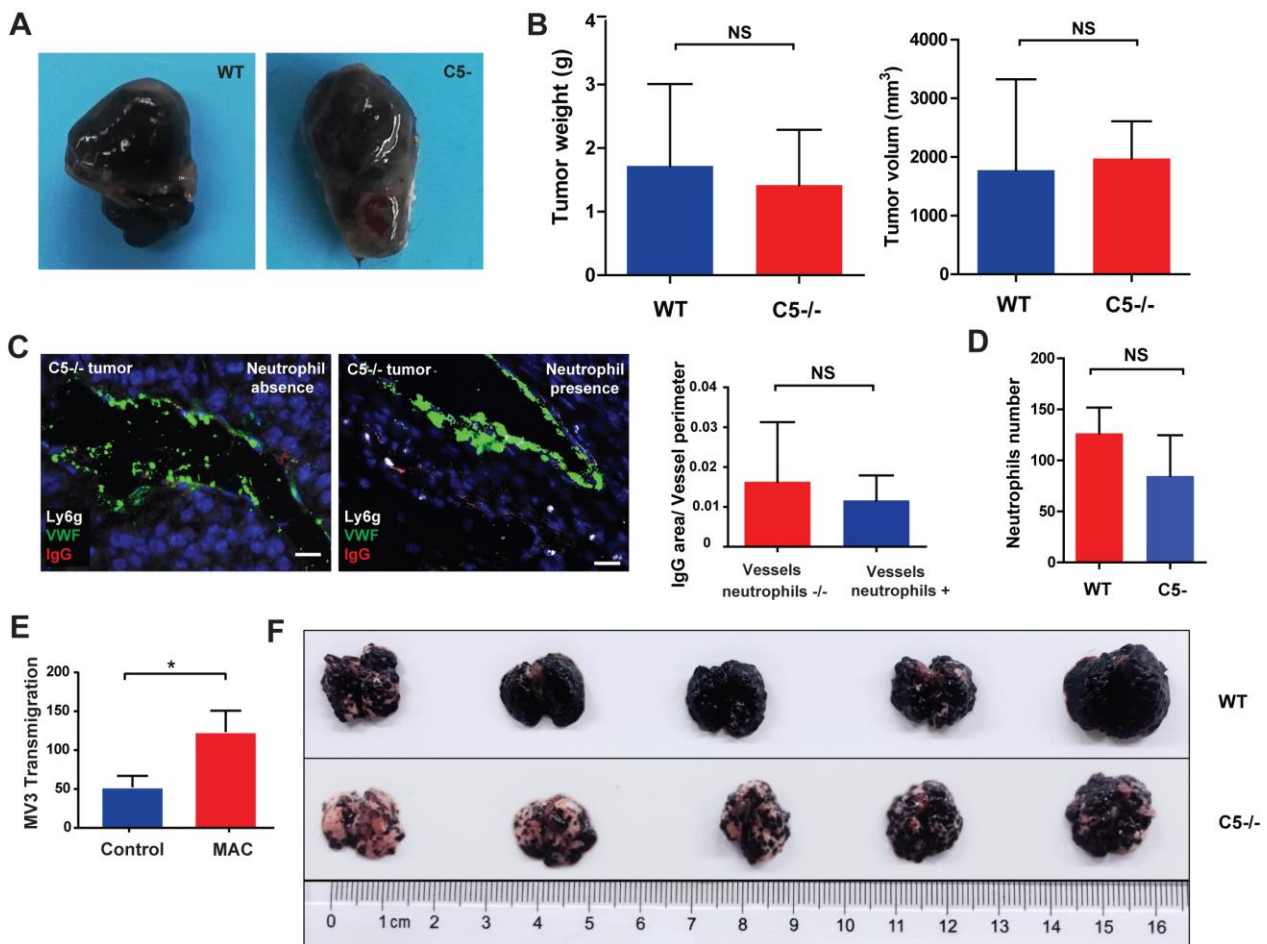


Figure 13. Effect of C5 deficiency on tumor growth and metastasis.

Wild type mice (n=5) and C5^{-/-} mice (n=5) received an intradermal injection of 7.5×10^5 ret transgenic melanoma cells. After 15 days, mice were sacrificed and tumors weight and volume were measured. C5 deficiency did not affect the primary tumor growth (A-B). Immunofluorescence staining of Ly6g (white), VWF (green) and IgG (red) in cryosections of C5 deficient mouse tumors were performed to measure the blood vessel integrity (C, scale bar=20 μ m). The IgG leakage in vessels with or without neutrophils was quantified and not obvious IgG leakage was observed around vessel with neutrophils (C). Compared with wild type mice, the total amount of neutrophils was decreased but not significantly in C5 deficient mice (D). Transmigration of the human melanoma cells MV3 through confluent HUVEC layers co-cultured for 6 h prior to the tumor cell challenge with MAC positive neutrophils or HIS treated control neutrophils (E). 5×10^5 B16F10 melanoma cells in 100 μ l PBS were intravenously injected into the tail veins of wild type mice (n=5) and C5^{-/-} mice (n=5) to induce lung metastases. Lung metastasis was decreased in C5 deficiency mice (F). Bars indicate the mean \pm SD, NS: not significant, *P< 0.05.

3.14 Undetectable complement activation and less MAC-neutrophils in human non-metastatic skin tumors.

The crosstalk between complement and neutrophil affects the permeability of tumor blood vessels, which further potentially impact the tumor metastasis. To further confirm the pathophysiological relevance of this crosstalk for metastasis in humans, we compared four different types of human skin tumors. The selected tumor entities differ in their potential to metastasize. Malignant melanomas are highly metastatic skin cancer. Human basal cell carcinoma (BCC) and keratoacanthoma (KA) are two rarely metastasizing, semi-malignant skin tumors. Nevocytic nevi (NCN) are non-metastatic, benign skin tumors. We compared the complement activation status (C3b/iC3b deposition at the blood vessel wall, Figure 14A) and the presence of MAC-neutrophils (Figure 14B). Highly conform to the metastatic property of those four human skin tumors, we detected a very limited number of C3b/iC3b deposited in vessels (<5%) of BCC, KA and NCN tissues; in contrast 12% of melanoma vessels are positive for C3b/iC3b staining (Figure 14A). In comparison to 52% MAC positive neutrophils in melanoma, we also found less MAC positive neutrophils in BCC (16%) and KA (12%). No MAC-neutrophils were detectable in NCN (Figure 14B). These results indicate a positive correlation between complement activation and the ability of human skin tumors to metastasize.

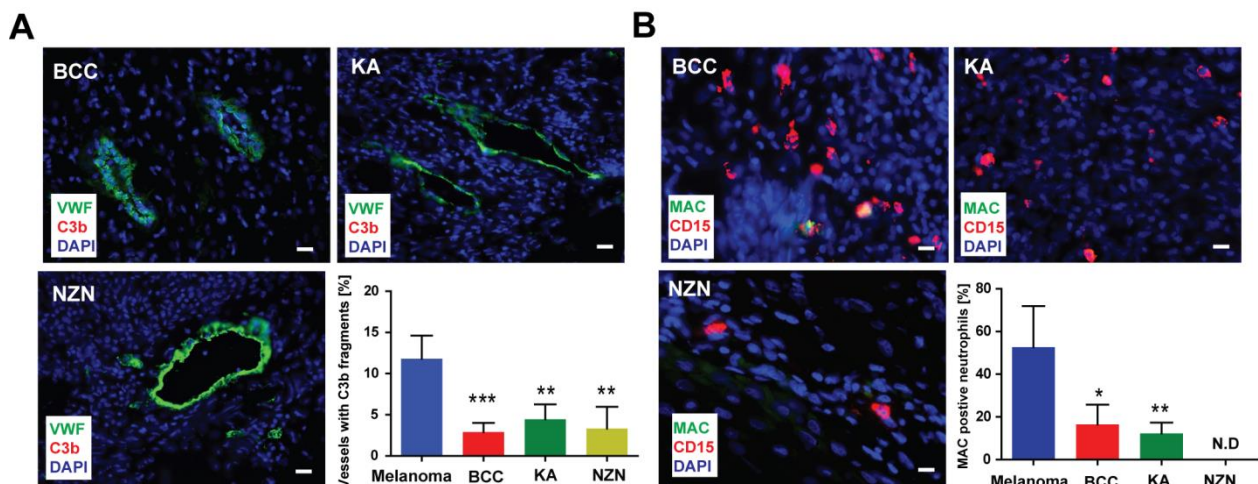


Figure 14. complement activation status in human skin cancers.

Human basal cell carcinoma (BCC, n=6), keratoacanthoma (KA, n=5) and Nevocytic nevi (NCN, n=5) tissue samples were stained for VWF and C3 cleavage products (C3b/ iC3b) (A) or CD15 and MAC (B). Nuclei were stained with DAPI. Quantification showed a very limited number of C3b/iC3b

deposited in vessels (<5%) of BCC, KA and NCN tissues in comparison to melanoma (A). In addition, less MAC positive neutrophils were detected in BCC (16%), KA (12%) and non-detectable MAC-neutrophils in NCN compared to 52% MAC positive neutrophils in melanoma (B). Bars indicate the mean \pm SD, *P< 0.05, **P< 0.005.

3.15 LMWH blocks complement activation *in vitro*.

The observed correlation between the glyocalyx related C3b deposition (Figure 5B-C) followed by the recruitment and activation of neutrophils and the disruption of the tumor vasculature (Figure 10-12), prompted us to assess whether LMWHs are able to prevent tumor related complement activation. Heparins are strongly related to the naturally occurring heparin sulfate, a major component of the endothelial glyocalyx. *Zaferani et al.*⁴⁵ showed that various complement factors have heparin binding sites enabling their storage within the endothelial glyocalyx and thus their deposition at the blood vessel wall. To better understand the anti-metastatic properties of heparins and the impact of heparin on tumor-associated complement activation, we performed further *in vitro* and *in vivo* experiments.

In the first set of experiment, we compared the two LMWHs, tinzaparin and N-acetylheparin (NAH, a heparin without anticoagulant properties). The complement inhibitory activity of both LMWHs was assessed by analyzing the haemolytic activity of the classical pathway (CH50) and the alternative pathway (APH50). The CH50 assay tests the total hemolytic activity of the classical and terminal pathways, which depends upon lysis of sheep red blood cells pre-coated with rabbit anti-sheep red blood cell antibody by human serum. Similarly with the CH50 assay, the APH50 assay measures the total alternative pathway hemolytic activity to lyse unsensitized chicken erythrocytes by human serum. Both CH50 and APH50 assay results were showed in reference to a pool of normal human serum as standard (100%). Treatment with tinzaparin significantly blocked the classical (Figure 15A) and the alternative (Figure 15B) complement pathway by 80% and 95% respectively. NAH were also able to decrease the CH50 and APH50 values; however tinzaparin was significantly more efficient than NAH (Figure 15A-B). In the next set of experiments, we analyzed whether treatment with tinzaparin could also inhibit MACs formation on neutrophils. Sensitized neutrophils were treated with NHS, HIS or NHS with the supplement of tinzaparin (100IU/ml) for 30 min. Anti-human MAC-FITC antibody and anti-human citrullinated histone H3 antibody were used for the MACs and NETs immunofluorescence staining. As shown in Figure 15C,

MACs presented on NHS treated neutrophils whereas administration of tinzaparin was sufficient to completely inhibit MACs formation. Quantification also revealed a significant reduction of the intensity of MAC on neutrophils for tinzaparin treatment.

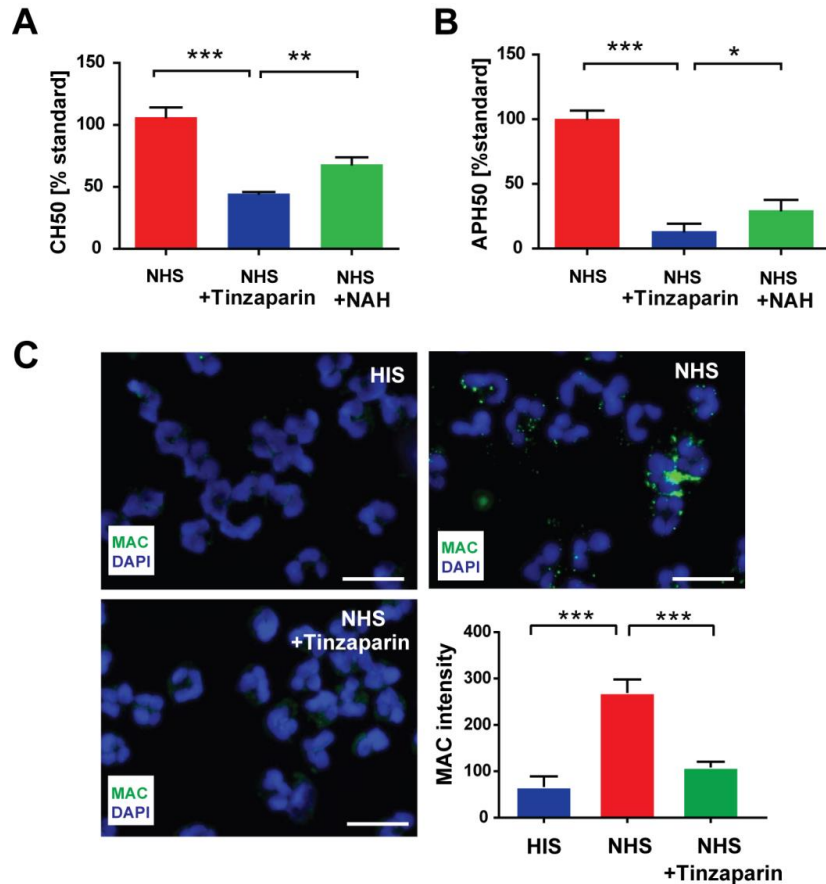


Figure 15. Tinzaparin inhibits complement activation *in vitro*.

Haemolytic complement function of the classical pathway (CH50) and the alternative pathway (APH50) was significantly reduced by the LMWH tinzaparin and NAH. Tinzaparin was more efficient than NAH (A-B). Representative immunofluorescence images of antibody sensitized neutrophils treated with 10% NHS or with 10% NHS supplemented with tinzaparin (100IU/mL). The speckled MAC staining on NHS treated neutrophils was significantly abolished by tinzaparin (C, scale bar=20µm). Bars indicate the mean ± SD, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

3.16 Tinzaparin blocks MAC-related neutrophil activation *in vitro*.

We have shown that MAC is a potent activator of neutrophil activation, resulting in oxidative burst and formation of NETosis. We next analyzed the impact of tinzaparin treatment on

MAC-induced neutrophil activation. As shown in Figure 16A, compared with NHS treated neutrophils group, DNA-histone fragments were significantly reduced in the supernatant of tinzaparin-NHS treated neutrophils. Western blot results further proved that tinzaparin could significantly prevent the release of CitH3 into the supernatant (Figure 16B). In comparison to the NAH, tinzaparin was again more efficient to block NETosis (Figure 16A-B) suggesting that tinzaparin was more applicable for further experiments. The effect of tinzaparin on ROS production was measured by chemiluminescence assay. Compared with the NHS group, tinzaparin treatment reduced the MACs induced ROS release from neutrophils (Figure 16C). As MAC positive neutrophils reduce the endothelial barrier function, we checked whether tinzaparin was sufficient to protect the endothelium. ECIS measurement showed that tinzaparin effectively prevented MAC induced break down of the electrical resistance (Figure 16D). Taken together, our *in vitro* results indicate that tinzaparin significantly inhibited MACs formation and prevented ROS release, NETs formation and thereby attenuated MAC activated neutrophil mediated EC dysfunction.

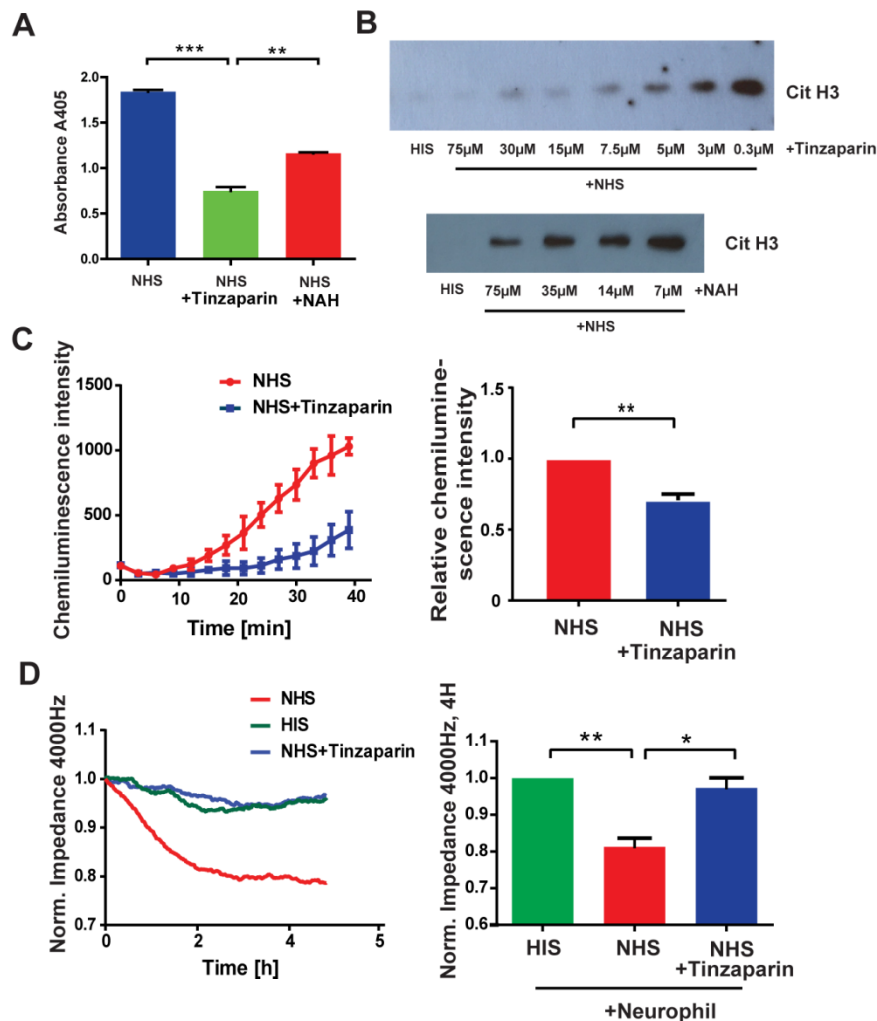


Figure 16. Tinzaparin treatment blocks ROS release and NETosis formation *in vitro*.

To analyze the tinzaparin treatment effect for neutrophils activation, we detected the NETs formation and ROS release. Tinzaparin was more efficient than NAH to block NETosis in NHS-treated neutrophils as indicated by the significantly reduced release of DNA-histone fragments (Tinzaparin, 8 μM; NAH, 8 μM) (A) and citrullinated histone3 (B). Luminol chemiluminescence assay shows a reduction of MAC-induced ROS release in the presence of tinzaparin (C). TEER measurement reveals that supplementation of NHS with tinzaparin (100IU/mL) counteracts the NHS induced ECs dysfunction (D). Bars indicate the mean ± SD, *P< 0.05, **P< 0.005, ***P< 0.0005.

3.17 Tinzaparin block complement activation *in vivo*.

We selected tinzaparin also for our following *in vivo* experiments because this LMWH has frequently been demonstrated to possess anti-complement effects^{45, 46, 52}. In addition, our own previous work showed that the treatment with tinzaparin attenuated tumor progression and

metastatic burden in murine animal models^{93,94}. To prove of our findings *in vivo*, we treated *ret* tumor engrafted mice with tinzaparin. The complement activation status in tinzaparin treated primary tumor were measured by immunofluorescence. Daily intradermal administration of tinzaparin to *ret* tumor engrafted mice prevented the deposition of the C3 fragment C3b/iC3b at tumor blood vessel walls (Figure 17A-B). In agreement with our *in vitro* experiments, MAC formed on neutrophils in primary control tumor; however, blocking complement activation by tinzaparin decreased MACs formation on tumor neutrophils (Figure 17C). Quantification also revealed a significant reduction of MAC intensity by 75% in tinzaparin treated tumors, compared with non-treated tumors (Figure 17D). Taken together, these results confirmed the complement inhibition effect by LMWH *in vivo*. A schematic model of current study is present in Figure 18.

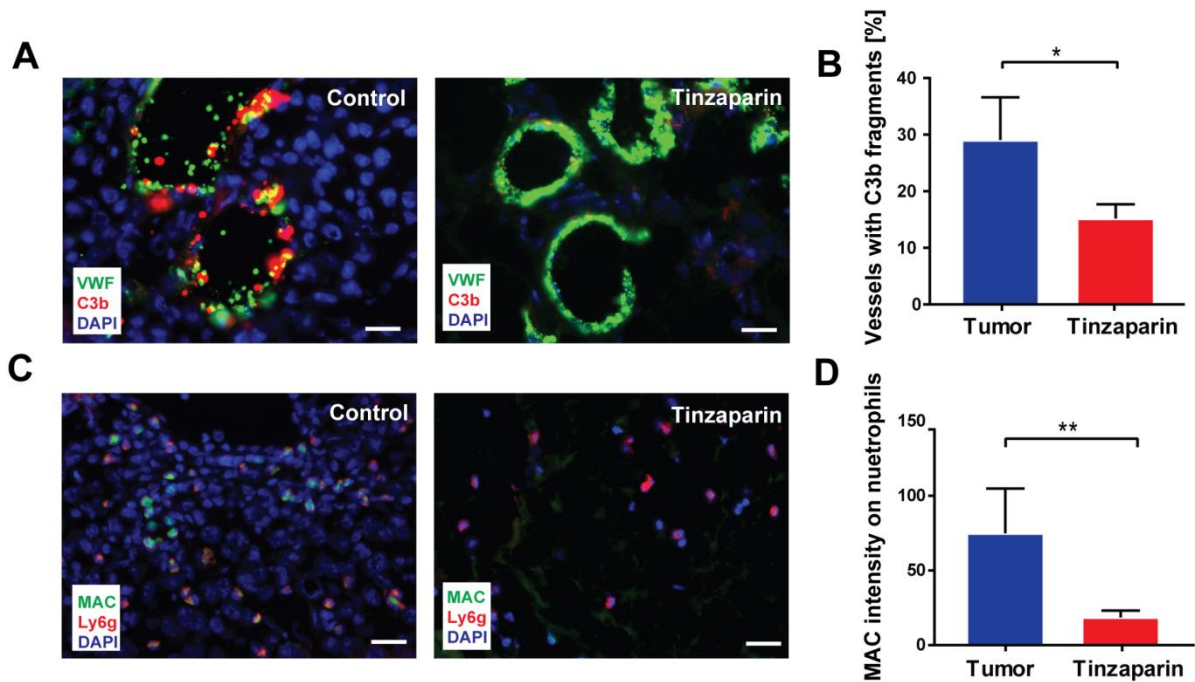


Figure 17. Tinzaparin blocks complement activation in primary tumor.

Tumor bearing mice were treated with vehicle or tinzaparin and cryosections of primary tumors were analyzed by immunofluorescence staining (n=5, scale bar=20µm). Tinzaparin treatment reduced the deposition of C3 fragments along the vessel wall compared with control tumors (A). Quantification revealed a significant reduction of C3 fragments deposition after tinzaparin treatment (B). MAC positive neutrophils are showed in the in tumors of control mice. In contrast, treatment with tinzaparin results in a decreased MAC formation on neutrophils (C). Quantification showed a significant reduction of MAC intensity on neutrophils after tinzaparin treatment (D). Data are shown as mean ± SD, *P< 0.05, **P< 0.005.

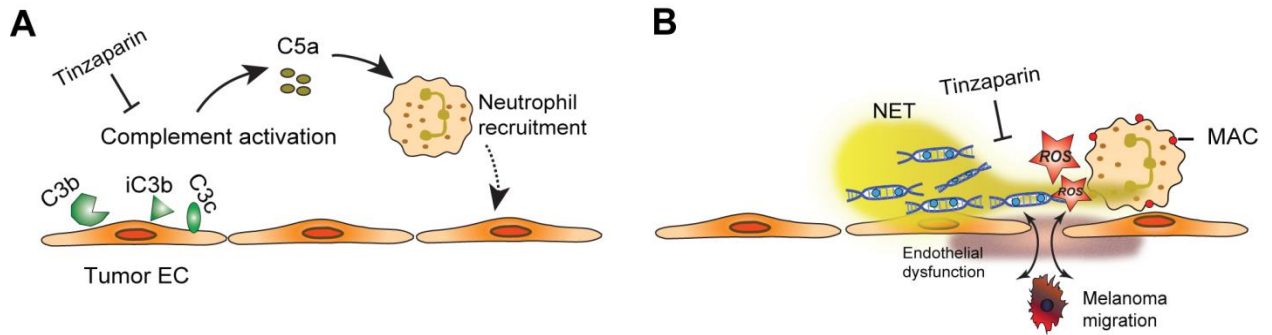


Figure 18. Schematic model of current study.

The schematic overview shows the crosstalk between the complement system, neutrophils and ECs in the tumor microenvironment (A-B). Complement activation at the endothelial-blood interface leads to the C5a mediated recruitment of neutrophils. Upon contact with the endothelium, MACs are formed on neutrophils, which mediate neutrophil activation represented by ROS release and NETs formation. Complement activated neutrophils increase the permeability of the endothelium allowing the transmigration of melanoma cells. Treatment with tinzaparin can block complement activation and thus tumor cell transmigration.

4 Discussion

The complement system is a major effector of the innate immunity and its role in pathogen defense has been extensively studied^{1, 4}. High capacity of metastases is the malignant melanoma's main characteristics and several complement effectors (such as C3a, C5a) contribute to melanoma progression^{91, 92}. In the frame of the complement activation, neutrophils are recruited to the inflamed site and manipulated by the primary tumor to participate in various steps of the metastatic cascade^{55, 56, 77}. The aim of our study was to investigate the involvement and crosstalk between complement, neutrophils and the tumor endothelium in the melanoma microenvironment.

To study the role of complement in malignant melanoma, we analyzed tumor biopsies of melanoma patients, we applied murine melanoma models and we used several *in vitro* experiments. In summary, we showed that tumoral ECs recruit complement factors from the blood to their surface to provide a platform for the initiation of the complement cascade, especially the lectin pathway activation. The associated production of the complement C3 fragments and C5a correlated with neutrophil attraction. In close proximity to the blood vessel wall, these recruited neutrophils are further activated by the complement-derived MAC. Profound neutrophil activation, characterized by an oxidative burst and the NETs formation, therefore increased the permeability of the tumor endothelium which in turn enables extravasation of tumor cells. We were able to block this effect by chemical inhibition of the MAC, and likewise the metastatic spread of melanoma cells was prevented in C5 deficient mice, which were unable to form MAC. MAC deposition on tumor-associated neutrophils was also found in human melanomas but not in rarely metastasizing basal cell carcinomas or non-metastatic keratoacanthoma and nevocytic nevi. Interestingly, administration of the LMWH tinzaparin to melanoma bearing mice prevented complement associated neutrophil activation and stabilized the vascular barrier explaining the efficient blockage of tumor cell metastasis.

The complement system and its crosstalk with the cells of innate and adaptive immune responses is an emerging field of research in life sciences, as evidenced by several recent articles in high-impact journals^{22, 42, 106}. Our work describes the first functional analysis of the MAC and its impact on neutrophil activation during the progression of melanoma (Figure 18). Moreover, our results provide a sound explanation of a triangular molecular crosstalk

wherein the imbalanced complement activation in tumors promotes neutrophil activation followed by endothelial dysfunction and thus increased metastasis.

4.1 Complement activation in melanoma microenvironment

A growing body of data show that imbalanced complement activation in the tumor microenvironment participates in chronic inflammation and suppressive immune responses^{23, 32}. In addition, clinical data demonstrated complement activation in cancer patients. Levels of complement effectors such as C3a, C5a, C3 and MBL in patients' plasma/serum sample or tumor tissues positively correlate with promoted tumor growth and poor outcome in patients suffering from lung cancer^{38, 107}, ovarian cancer³⁹, colorectal cancer⁴⁰ and chronic lymphatic leukemia¹⁰⁸.

Melanoma is one of the most aggressive forms of skin cancer and exposure to sun or ultra violet (UV) radiation has been accounted for the major cause of melanoma^{109, 110}. UV radiation contributes to the DNA damage and leads to immunosuppression, which is a major risk factor for melanoma development¹⁰⁹. Previous reports showed that C3 fragments C3b and C3d were deposited on epidermal cells after the exposure of the skin to UVB¹¹¹. Moreover, UVA is a potent oxidizer of cellular molecules, and complement can be triggered in to action by UVA-altered cell lipids and other cell structure¹¹². Accordingly, recent evidence indicated that radiation induced tumor cell damage could induce transient complement activation and up regulate the release of C3a and C5a, subsequently leading to DC and CD8 anti-tumor response²⁸. Based on these observations, we postulate that melanoma progression is associated with complement activation.

C3 plays a key role in the classical and alternative pathways of the complement system. In non-small cell lung cancer, low C3 level is related to shorter overall survival and disease-free survival rates³⁸. Apart from cancer, anti-neutrophil cytoplasmic antibody associated vasculitis (AAV) is a group of autoimmune disease and complement activation is crucial for the development of AAV. Accumulating evidence has shown that low serum C3 level at diagnosis was associated with severity and poor out-comes of AAV¹¹³. In this study, ELISA results show decreased C3 level in stage IV melanoma patients' plasma (Figure 1A). Several studies suggested the use of complement as biomarkers for cancer diagnosis and prognosis. Increased levels of C3 fragments (e.g. C3b/C3c and C3f) have been detected in serum of a

variety of cancer patients²¹, including breast cancer, pancreatic cancer, leukaemia and lymphoma. Moreover, C3a and C5a are potent proinflammatory mediators. Increased levels of C3a have been reported in the serum of patients with lymphoma, oesophageal cancer and colorectal cancer²¹. At higher concentration, C5a recruits MDSCs into tumor and exert immunosuppressive effects by ablating CD8 T cell response³². C5a could also stimulate angiogenesis and promote cancer cell invasion and migration^{22, 91}. Inhibition of the binding of C5a to its receptors was demonstrated to be associated with a decreased tumor growth in experimental mouse models^{91, 114}. In the present study, we show that the complement effectors C3b, C3a and C5a are elevated in human melanoma blood samples, indicating systemic complement activation in melanoma patients (Figure 1B-D). Thus, the determination of complement related proteins may be potentially useful in the melanoma screening and tumor staging.

To support our ELISA data, we evaluated the presence of complement effectors in cryosections of human and mice malignant melanoma tissue. In our study, C3 cleavage products deposit along the tumor vasculature in both human and mice tumor tissues, indicating that activation of complement proteins occur at the blood vessel interface (Figure 2A and 3A). In agreement to a previous study, we detected no complement activation in murine melanomas at an early stage (Figure 3C) but only in advanced tumors suggesting a stage-dependent complement-promoting environment. The work by Marks *et al.*⁵⁷ reported that enforced deposition of complement factors at EC is a potent and rapid stimulus for neutrophil-endothelial adhesion. The binding of neutrophils to the vascular endothelium is an early event in their recruitment into acute inflammatory lesions. In strong accordance with the notion of tumor blood vessel restricted complement activation, we also detected high levels of C5a in close proximity to blood vessels (Figure 3D). C5a is a potent chemoattractant for pro-inflammatory leukocytes and recruits neutrophils to areas of inflammation and tissue damage¹ (Figure 3F). Thus, the enrichment of complement effector C3 fragments and C5a along the vasculature is a sound explanation for the observed recruitment of neutrophils around tumor vessels (Figure 10). However, C5a is not able to induce ROS formation and NETosis by its own (Figure 9) further emphasizing the potential relevance of MAC as a potent physiological activator of neutrophils. Additionally, we found no evidence of C3b deposition on the vasculature of the lung, liver and kidney of tumor bearing animals (Figure 4), indicating the complement activation is specific in the tumor microenvironment. Accordingly, MAC deposition was not found on tumor-associated macrophages, dendritic

cells or T cells, or on the neutrophils of peripheral organs, suggesting MAC forms only on tumor associated neutrophils.

To better understand the initiation of the complement cascade in our animal model, we quantify the accumulation of early complement components (Figure 5A; C1q, C4, MBL and Factor B). Our experiments suggest the predominant involvement of the lectin pathway. However, the tight molecular interconnection of the complement system does not exclude the contribution of the other activation routes. For instance, previous studies suggested that coagulation and fibrinolytic cascade related proteases such as thrombin and plasmin are able to trigger complement activation through the direct cleavage of C5²². In the earlier study, we showed that melanoma cells are able to induce coagulation through the formation of thrombin via tissue factor or through the release of VEGF^{115, 116}. In the present study we also investigated the potential crosstalk between coagulation and the complement; however we were not able to find a significant correlation between blood vessel occlusion and complement activation suggesting that in the melanoma microenvironment hypercoagulation is less relevant for complement activation.

MBL deposition and the presence of C3 cleavage products along the tumor vasculature, suggest that activation of complement proteins occurred at the blood vessel interface in a subset of tumor blood vessels. The MBL can recognize ECs with an aberrant glycosylation at their surface¹⁰² and evidence for the activation of lectin pathway has been reported in patients suffering from colorectal¹¹⁷ and ovarian cancer³⁹. In this study we discovered that the degree of C3b/iC3b deposition was related to the endothelial glycosylation and the surface expression of SDC1 suggesting that tumor ECs exhibit a disease-related phenotypes (Figure 5B-C). Interestingly, the expression of endothelial SDC1 (a highly glycosylated proteoglycan) has previously been linked to enhanced complement activation⁵¹. So, the altered expression of SDC1 and the changed glycosylation of the surface structures may explain the prevailed involvement of the lectin pathway^{118, 119, 120, 121}. This is also supported by the recent findings from Talsma *et.al* documenting that a modified heparan sulfate synthesis in ECs is associated with an increased deposition of the MBL¹⁰². However, more details about altered surface structures of tumor ECs and the crosstalk between the glycosylation and complement activation should be further explored.

In addition, SDC1 is involved in several physiological processes such as angiogenesis¹²². Removal of SDC1 from the endothelial surface is a common marker for endothelial cell

damage^{123, 124}. In this study, we found a positive correlation between SDC1 expression and C3b/iC3b deposition indicating that the initiation of the complement is not triggered by damaged ECs but dependent on an intact endothelium.

4.2 MAC formed on neutrophils but not on EC

Classical, alternative and lectin pathways converge on a common terminal pathway: the formation of the MAC. To investigate the distinguish role of complement in melanoma environment, we further analyzed the presence and localization of this terminal complex in the tumor tissue by immunofluorescence staining. We have already found C3 fragments deposit around tumor vasculature, so we hypothesized the deposition of MAC along the tumor vessel. To our surprise, we could not detect significant levels of MAC at the endothelium; however, we discovered that about 60% of all tumor-related neutrophils were MAC positive in both human and mice primary tumor tissue (Figure 2C and 3G).

One of the main functions of complement system is to directly kill invading pathogens through the formation of MAC. In principle, deposition of critical amounts of MACs disrupts the bilayer of the cell membranes, leading to cell lysis. However, most nucleated cells, including neutrophil, have several protection mechanisms to counteract complement-mediated cellular lysis. For example, through a combination of ion pumps, complement regulators (CD59) and active recovery processes (endocytosis), most nucleated cells actively remove the MAC^{5, 10, 16}. Neutrophils can also protect themselves against lytic killing by transient cell swelling and metabolic depletion^{5, 10}. At sublytic levels, MAC deposition permits Ca²⁺ influx and interacts with other signaling molecules involving G-protein-coupled receptors and NF- κ B^{5, 15, 16, 125}. MACs are able to induce many different effects that have been associated with altered cell proliferation, induction or inhibition of apoptosis and inflammasome activation in various cell types, for example, macrophage, microglia, epithelial cell and some tumor cells^{5, 16, 125}.

In this study, we further analyzed the localization of MAC negative and MAC positive neutrophils within the tumor vasculature. We observed that in mice tumor tissues most of non-attached luminal neutrophils remained MAC negative, whereas neutrophils attached to the tumor endothelium gain the MAC complex (Figure 6A-B). This result suggests that the deposition of the MAC assembly on the neutrophils occurs at surface of the blood vessel wall

during neutrophils recruitment and transmigration from the blood through the endothelium into the tumor tissue.

To test whether the initiation of the complement system on ECs could finally lead to MAC deposition on adjacent neutrophils, we intentionally induced the complement activation on HUVECs prior to the co-culture with neutrophils. MAC *in vitro* staining shows that MACs exclusively form on neutrophils but not on HUVEC (Figure 6C), indicating MAC is *de novo* formed on neutrophils but not transferred from EC surface. Moreover, Morgan *et al.* reported that neutrophils can remove MAC by endocytosis¹²⁶. Our staining result also shows that MAC can be detected both on the neutrophil surface and in the cytoplasm. To further understand the mechanism of MAC formation exclusively on neutrophils, we next analyzed the expression of the complement regulatory proteins CD46, CD55, CD59 and factor P in HUVEC, neutrophil and several human melanoma cell lines (Figure 6D). Our data reveals that in contrast to CD59, neutrophils strongly express factor P. Factor P can recruit the C3(H₂O) and C3b to the cell surface and serves as the platform for alternative pathway activation¹. Furthermore, Factor P can also stabilize the C3 convertase and bind on the pathogens or activated cells surface to promote the complement activation. Neutrophils can further amplify the complement cascade e.g. by the release of factor P which lead to a positive feedback loop of neutrophil activation⁷². Our results also show that neutrophils are almost the only cells expressing factor P, suggesting neutrophils tend to mediate complement related disease (Figure 6E). Expressed on most of nucleated cell, CD59 is the most important negative regulator of the MAC formation. CD46 acts as a cofactor for the inactivation of C3b and C4b. CD55 dissociates the C3 convertase. Therefore, CD46 and CD55 are not able to inhibit MAC formation directly. In agreement with our assumption that the complement cascade is initiated on the endothelium but terminated on neutrophil, weak expression of CD59 by neutrophils (Figure 6D) suggests that they could not sufficiently prevent the formation of the MAC. In contrast to neutrophils, ECs express high levels of CD59 suggesting their protection against MAC formation whereas low levels of CD46 and CD55 may promote the deposition of early complement factors.

4.3 MAC on neutrophil lead to ROS production and NETs

To gain further insights into the pathophysiological function of MAC on neutrophil, we induced the MAC formation on neutrophils *in vitro* and further confirmed the MAC

formation by FACS and western blot (Figure 7A-B). One of the hallmarks of neutrophil activation is the oxidative burst, so, we first checked the ROS release from MAC positive neutrophils. We measured the release of ROS by different techniques, including DCF fluorescence staining, FACS and chemiluminescence assay. Using these methods, we confirmed that MAC on neutrophils leads to ROS production (Figure 7C-E). ROS can mediate antimicrobial activity and participate in stress signaling in cells. Moreover, ROS cause cell death and are associated with tissue damage. For example, ROS initiated Ca^{2+} signaling plays an important role in regulating endothelial barrier function⁸⁵. Interestingly, neutrophil-derived ROS have been reported to directly activate the complement in fluid phase¹²⁷.

Oxidative stress is a strong promoter of NETosis¹²⁸, which involves the secreted nuclear chromatin and the release of granule proteins. We therefore further investigated MAC induced NETs formation (Figure 8). Histone citrullination is a decisive event during NETosis and thus used as molecular marker. As expected, a robust western blot signal of citrullinated Histone3 (CitH3) and the release of DNA-histone fragments were detected in MAC+ neutrophil supernatants. We can also find the externalized DNA structures co-localized with the NETs-associated CitH3 in NHS treated neutrophils. For the *in vivo* proof of our findings, we evaluated cryosections of tumor tissues for the MAC associated CitH3 staining. In agreement with our *in vitro* results, CitH3 and MAC staining were in close proximity in both human and mouse primary tumor tissues. Notably, NETs can activate the alternative complement pathway¹²⁹ and NETs may influence different steps of the tumor development, including tumor growth, metastasis and cancer associated thrombosis^{65, 67, 130}.

Taken together, our results show that in human and murine melanoma tissue the complement system is activated at the endothelial-blood interface. Complement effector molecules C3 fragment and C5a are produced and neutrophils are thereby recruited and activated. The terminal formation of MAC at the neutrophils surface drives further neutrophil activation characterized by ROS production and release of NETs.

4.4 Perivascular activated neutrophils contribute to endothelial barrier disruption

Neutrophils are the most abundant immune cells in human immune system and many proinflammatory factors contribute to the recruitment of neutrophils to the inflammatory

sites. Our study indicates an imbalanced complement system resulting in MAC formation in the tumor microenvironment. In the course of the complement cascade chemoattractant C5a is formed and deposited around tumor vessels suggesting neutrophil recruitment to the tumor blood vessel. Neutrophils have varied functions in the tumor microenvironment and increasing body of evidence suggests that neutrophils are educated by primary tumors in order to support the formation of metastasis in distant organs⁵⁸. Tumor metastasis depends on increased permeability of the endothelium. Therefore, we hypothesize that imbalanced complement cascade mechanistically links neutrophil activation with dysfunction of endothelium and subsequent melanoma cells extravasation.

In human and mice tumor tissues, numerous neutrophils marginated along or penetrated into the wall of tumor blood vessels (Figure 10). Because MAC deposition on neutrophils promotes an oxidative burst (Figure 7) and induces NETosis (Figure 8), these neutrophils can locally induce or amplify the permeability of the endothelium¹³¹. For example, ROS interact with ECs and increase the intracellular oxidative stress, which may provoke cellular damage and transformation⁶⁴. NETs-associated cytotoxic histones and proteases could injure the surrounding endothelium, leading to vascular leakage^{59, 69}.

To study the neutrophil-EC crosstalk, we co-cultured HUVEC with MAC positive neutrophils *in vitro*. MAC positive neutrophils induce gap formation in a confluent EC monolayer (Figure 11A). ECIS assay can be used to detect the integrity of EC monolayer, and in this study the results show that co-culture with MAC positive neutrophils decreases the TEER of an endothelial monolayer, suggesting an increased vascular permeability (Figure 11B). *In vivo*, tumor blood vessels with increased levels of neutrophils are characterized by IgG leakage from the blood into the adjacent tissue suggesting increased vascular permeability (Figure 12A-B).

To highlight the fundamental role of neutrophils, we blocked neutrophil activation (NETs formation) by inhibiting PAD4 in additional *in vitro* and animal experiments (Figure 11C and 12C-D). The results indicated that PAD4 inhibitor treatment prevent the neutrophil induced break down of the endothelial resistance *in vitro* and IgG leakage *in vivo*, suggesting neutrophil activation is needed to increase tumor blood vessel permeability. To confirm these findings at the functional level, we performed *in vivo* experiments in C5 deficient mice, which are unable to generate the MAC (Figure 13). We found that lack of C5 reduced neutrophil recruitment into the tumor tissue underlining the high relevance of C5a for the

recruitment neutrophils. In accordance with our conclusion, blood vessel permeability was not decreased even in blood vessels with a comparable high amount of neutrophils, indicating that neutrophils without MAC are not able to increase the permeability of the tumor blood vessel.

To study the influence of the impaired endothelial barrier on tumor cell extravasation, we measured the transmigration of human melanoma cells by transwell assays. As expected, we found that the MAC induced increase of the vascular permeability promoted tumor cell transmigration (Figure 13E). Moreover, we conducted additional lung metastasis experiments in C5 knockout mice (Figure 13F). In agreement with our hypothesis, the inability to generate the MAC correlated with a significantly reduced rate of metastasis. We also analyzed human skin tumors differing in their metastatic potential to gain further insight into the impact of complement activation and the role of MAC formation (Figure 14). In accordance with this clinical phenotype and our hypothesis that complement activation contributes to metastasis, we detected significantly weaker complement activation and less frequent MAC deposition on neutrophils in non-metastatic skin cancers compared to melanoma. This result highlights the correlation between complement activation, MAC deposition on neutrophils and cancer cell dissemination. It also provides insight into the mechanisms of melanoma metastasis and emphasizes the relevance of our findings for the clinical phenotyping of melanoma.

Taken together, these results demonstrate MAC+ neutrophils play an important role in controlling EC paracellular permeability allowing the transmigration and thus metastasis of melanoma cells.

4.5 Tinzaparin blocks complement-related neutrophil activation *in vitro* and *in vivo*

Given the pro-tumor effect of the activated complement system, the idea of complement inhibition as anti-cancer treatment is gaining recognition^{6, 21, 41}. Previous studies identified LMWH as potent inhibitor of the complement cascade. Different modes of action have been reported including inhibition of C1q binding to immune complexes, blockade of the C3 convertase formation and inhibition of the MAC assembly^{45, 52, 132}. Various complement factors and complement regulators react with polysaccharide glycosaminoglycan (GAG) on cellular membranes⁴⁵. Accordingly, in our study we observed a positive correlation between complement activation (C3b/iC3b deposition) and the glycosylation of the tumor endothelium

(WGA staining and syndecan-1 expression, Figure 5B-C). These interactions might serve as a potential target for blocking complement activation. Heparan sulfate related GAGs especially LMWHs can be used to intervene in the interaction of complement factors with proteoglycans on the cell membrane or in the extracellular matrix to limit the complement activation⁴⁵. In addition, we previously showed that tinzaparin treatment attenuated tumor progression and metastatic burden in murine animal models^{93, 94}. So, we selected LMWH for further complement inhibition studies.

Tinzaparin is produced by chemical or enzymatic depolymerization of porcine unfractionated heparin and it is a heterogeneous carbohydrate mixture with an average molecular weight of 6.5 kDa¹³³. Therefore, tinzaparin is not a single compound, but a mixture of glycosaminoglycan chains with different chain lengths and structure features^{133, 134}. We first checked the complement inhibition effect by tinzaparin *in vitro* (Figure 15A-B). The CH50 and APH50 assay were applied to detect the impact of LMWHs on the complement system activation. The results show that treatment with tinzaparin significantly blocks the classical and alternative pathway activation. Additionally, treatment with the LMWH tinzaparin blocked MACs formation on neutrophils (Figure 15C). Taken together, those *in vitro* experiments clearly confirmed the complement inhibitory effects of tinzaparin.

Because MACs on neutrophils are a strong trigger for NETs and ROS production, we next analyzed the blocking effect of tinzaparin (Figure 16). DNA-histone fragments are significantly reduced or even absent in the supernatants of tinzaparin treated neutrophils. Western blot results further prove that tinzaparin could significantly prevent the release of CitH3. We next checked the tinzaparin induced complement inhibition efficiency. Western blot results confirmed that 10 U/ml is the minimal working concentration.

To measure the complement inhibition efficiency of non-coagulative heparins we analyzed commercially available N-acetylheparin (NAH) lacking anti-coagulative properties but still owing anti-complement activity¹³⁵. In our analysis we found that NAH was able to efficiently block the classical and alternative pathway as measured by CH50 and APH50 assays (Figure 15A-B). NAH was also able to prevent NETosis. However at equimolar concentrations, NAH was significantly less efficient than Tinzaparin, especially the inhibitory effect on NETosis (Figure 16A-B).

In line with the result of NETs detection, treatment with tinzaparin decreases the ROS release, which was measured by a chemiluminescence assay (Figure 16C). As MAC positive

neutrophils could induce EC dysfunction, we next checked the treatment effect of tinzaparin in a neutrophil/HUVEC co-culture system. The TEER result shows that tinzaparin effectively abolishes the EC dysfunction through by inhibiting the complement activation effects on neutrophils (Figure 16D).

To further investigate the treatment effect of tinzaparin *in vivo*, we treated ret tumor engrafted mice with tinzaparin, and followed by analysis of complement activation (Figure 17). As expected, the C3 fragments deposition along vessel wall in tumor is decreased in tinzaparin treated tumor tissue. Furthermore, inhibition of complement activation by tinzaparin blocks the MAC deposition on neutrophils.

LMWHs are used as clinical antithrombotic agents. Indeed, heparin is not a specific blocker of the complement but it can also interfere with coagulation and the action of growth factors and chemokine⁴⁵. Therefore, clinical usage of heparins to attenuate complement activity should be done carefully, and heparins that specifically block complement activation should be selected to minimize the cross reactivity with coagulation and non-complement mediated inflammation⁴⁵. Within this context we tested the complement and NETs inhibitory activity of NAH, a heparin with reduced anti-coagulative properties. However, it was less efficient than tinzaparin. Further research is required to optimize potential heparins and heparin related molecules to identify subtypes of heparins with precisely defined inhibitory properties.

4.6 Outlook

Malignant melanoma is an immunogenic tumor. To our knowledge, our manuscript reports for the first time the formation of the MAC on tumor-associated neutrophils and considers the pathophysiological consequences in melanoma (Figure 18). The complex crosstalk between the complement system and immune cells during tumor progression is an emerging field of research because many of the pathophysiological mechanisms remain unclear^{21, 42}. Notably, extensive evidence obtained in experimental models suggests that the complement can modulate T cell responses in a variety of inflammatory diseases^{21, 42}. However, the impact of MAC positive neutrophils on adaptive immune responses in the tumor microenvironment is still poorly understood and need further exploration. Recently, Ajona.D *et al.*³⁴ reported the blockade of C5a resulted in a substantial improvement in the efficacy of anti PD-1 antibody against lung cancer. In this study, we highlight the potential use of LMWH as an anti-

complement reagent. For the treatment of melanoma, a combined blockade of complement signaling by tinzaparin with other immunotherapy might be an alternative therapeutic strategy. As discussed above, although LMWH can block the complement activation efficiently, it is still not a specific inhibitor. So, it would be also worth to investigate the subtypes of heparins with precisely defined inhibitory properties. A better understanding of heparins and related derivatives will reveal new therapeutic options in the future. Another interesting finding is the association between glycosylation and complement activated factor deposition in tumor microenvironment. However, the precise biological role of aberrant glycosylation on tumor endothelial surface triggering complement activation requires further clarification.

Apart from tumor, complement, neutrophil and EC are also the main participators of many immunological, hematological and dermatological related disease. We are convinced that our findings and proposed mechanisms (complement factor deposition, neutrophil activation and the loss of blood vessel integrity) not only improve the understanding of this triangular crosstalk in the context of melanoma progression, but are transferable to other human diseases such as vasculitis¹³⁶, thrombotic microangiopathy¹³¹, immunothrombosis¹³⁷.

References

1. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Frontiers in immunology* **6**, 262 (2015).
2. Swierzko AS, Kilpatrick DC, Cedzynski M. Mannan-binding lectin in malignancy. *Molecular immunology* **55**, 16-21 (2013).
3. Zhang R, Liu Q, Liao Q, Zhao Y. CD59: a promising target for tumor immunotherapy. *Future oncology (London, England)* **14**, 781-791 (2018).
4. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. *Frontiers in immunology* **6**, 257 (2015).
5. Morgan BP. The membrane attack complex as an inflammatory trigger. *Immunobiology* **221**, 747-751 (2016).
6. Ricklin D, Reis ES, Lambris JD. Complement in disease: a defence system turning offensive. *Nat Rev Nephrol* **12**, 383-401 (2016).
7. Preissner KP, Podack ER, Muller-Eberhard HJ. SC5b-7, SC5b-8 and SC5b-9 complexes of complement: ultrastructure and localization of the S-protein (vitronectin) within the macromolecules. *European journal of immunology* **19**, 69-75 (1989).
8. Tschopp J, Chonn A, Hertig S, French LE. Clusterin, the human apolipoprotein and complement inhibitor, binds to complement C7, C8 beta, and the b domain of C9. *Journal of immunology (Baltimore, Md : 1950)* **151**, 2159-2165 (1993).
9. Scolding NJ, Houston WA, Morgan BP, Campbell AK, Compston DA. Reversible injury of cultured rat oligodendrocytes by complement. *Immunology* **67**, 441-446 (1989).
10. Morgan BP. Non-lethal complement-membrane attack on human neutrophils: transient cell swelling and metabolic depletion. *Immunology* **63**, 71-77 (1988).
11. Morgan BP, Boyd C, Bubeck D. Molecular cell biology of complement membrane attack. *Seminars in cell & developmental biology* **72**, 124-132 (2017).
12. Hila S, Soane L, Koski CL. Sublytic C5b-9-stimulated Schwann cell survival through PI 3-kinase-mediated phosphorylation of BAD. *Glia* **36**, 58-67 (2001).
13. Lueck K, *et al.* Sub-lytic C5b-9 induces functional changes in retinal pigment epithelial cells consistent with age-related macular degeneration. *Eye (London, England)* **25**, 1074-1082 (2011).
14. Jane-wit D, *et al.* Complement membrane attack complexes activate noncanonical NF- κ B by forming an Akt+ NIK+ signalosome on Rab5+ endosomes. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 9686-9691 (2015).
15. Fang C, *et al.* ZFYVE21 is a complement-induced Rab5 effector that activates non-canonical NF- κ B via phosphoinositide remodeling of endosomes. *Nature communications* **10**, 2247 (2019).
16. Cole DS, Morgan BP. Beyond lysis: how complement influences cell fate. *Clin Sci (Lond)* **104**, 455-466 (2003).

17. Serna M, Giles JL, Morgan BP, Bubeck D. Structural basis of complement membrane attack complex formation. *Nature communications* **7**, 10587 (2016).
18. Spicer BA, *et al.* The first transmembrane region of complement component-9 acts as a brake on its self-assembly. *Nature communications* **9**, 3266 (2018).
19. Parsons ES, *et al.* Single-molecule kinetics of pore assembly by the membrane attack complex. *Nature communications* **10**, 2066 (2019).
20. Heesterbeek DA, *et al.* Bacterial killing by complement requires membrane attack complex formation via surface-bound C5 convertases. *The EMBO journal* **38**, e99852 (2019).
21. Reis ES, Mastellos DC, Ricklin D, Mantovani A, Lambris JD. Complement in cancer: untangling an intricate relationship. *Nature reviews Immunology* **18**, 5-18 (2018).
22. Medler TR, *et al.* Complement C5a Fosters Squamous Carcinogenesis and Limits T Cell Response to Chemotherapy. *Cancer cell* **34**, 561-578.e566 (2018).
23. Wang Y, *et al.* Autocrine Complement Inhibits IL10-Dependent T-cell-Mediated Antitumor Immunity to Promote Tumor Progression. *Cancer Discov* **6**, 1022-1035 (2016).
24. Cho MS, *et al.* Autocrine effects of tumor-derived complement. *Cell Rep* **6**, 1085-1095 (2014).
25. Ajona D, *et al.* Investigation of complement activation product c4d as a diagnostic and prognostic biomarker for lung cancer. *Journal of the National Cancer Institute* **105**, 1385-1393 (2013).
26. Kohl J. Self, non-self, and danger: a complementary view. *Advances in experimental medicine and biology* **586**, 71-94 (2006).
27. Diaz-Zaragoza M, Hernandez-Avila R, Viedma-Rodriguez R, Arenas-Aranda D, Ostoa-Saloma P. Natural and adaptive IgM antibodies in the recognition of tumor-associated antigens of breast cancer (Review). *Oncology reports* **34**, 1106-1114 (2015).
28. Surace L, *et al.* Complement is a central mediator of radiotherapy-induced tumor-specific immunity and clinical response. *Immunity* **42**, 767-777 (2015).
29. Amara U, *et al.* Molecular intercommunication between the complement and coagulation systems. *Journal of immunology (Baltimore, Md : 1950)* **185**, 5628-5636 (2010).
30. Schmidt CQ, Verschoor A. Complement and coagulation: so close, yet so far. *Blood* **130**, 2581-2582 (2017).
31. de Bont CM, Boelens WC, Puijn GJM. NETosis, complement, and coagulation: a triangular relationship. *Cell Mol Immunol*, (2018).
32. Markiewski MM, *et al.* Modulation of the antitumor immune response by complement. *Nature immunology* **9**, 1225-1235 (2008).
33. Guglietta S, *et al.* Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. *Nature communications* **7**, 11037 (2016).
34. Ajona D, *et al.* A Combined PD-1/C5a Blockade Synergistically Protects against Lung Cancer Growth and Metastasis. *Cancer Discov* **7**, 694-703 (2017).

35. Vlaicu SI, *et al.* Role of C5b-9 complement complex and response gene to complement-32 (RGC-32) in cancer. *Immunologic research* **56**, 109-121 (2013).
36. Towner LD, Wheat RA, Hughes TR, Morgan BP. Complement Membrane Attack and Tumorigenesis: A SYSTEMS BIOLOGY APPROACH. *The Journal of biological chemistry* **291**, 14927-14938 (2016).
37. Bulla R, *et al.* C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation. *Nature communications* **7**, 10346 (2016).
38. Lin K, *et al.* Complement component 3 is a prognostic factor of nonsmall cell lung cancer. *Molecular medicine reports* **10**, 811-817 (2014).
39. Swierzko AS, *et al.* Mannose-Binding Lectin (MBL) and MBL-associated serine protease-2 (MASP-2) in women with malignant and benign ovarian tumours. *Cancer immunology, immunotherapy : CII* **63**, 1129-1140 (2014).
40. Habermann JK, *et al.* Increased serum levels of complement C3a anaphylatoxin indicate the presence of colorectal tumors. *Gastroenterology* **131**, 1020-1029; quiz 1284 (2006).
41. Pio R, Ajona D, Lambris JD. Complement inhibition in cancer therapy. *Semin Immunol* **25**, 54-64 (2013).
42. Reis ES, Mastellos DC, Hajishengallis G, Lambris JD. New insights into the immune functions of complement. *Nature Reviews Immunology* **19**, 503-516 (2019).
43. Hill A, DeZern AE, Kinoshita T, Brodsky RA. Paroxysmal nocturnal haemoglobinuria. *Nature reviews Disease primers* **3**, 17028 (2017).
44. Legendre CM, *et al.* Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *The New England journal of medicine* **368**, 2169-2181 (2013).
45. Zaferani A, *et al.* Heparin/heparan sulphate interactions with complement--a possible target for reduction of renal function loss? *Nephrol Dial Transplant* **29**, 515-522 (2014).
46. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* **10**, 1222-1226 (2004).
47. Wardle EN, Uldall PR. Effect of heparin on renal function in patients with oliguria. *British medical journal* **4**, 135-138 (1972).
48. Weiler JM, Edens RE, Linhardt RJ, Kapelanski DP. Heparin and modified heparin inhibit complement activation in vivo. *Journal of immunology (Baltimore, Md : 1950)* **148**, 3210-3215 (1992).
49. Regal JF, Gilbert JS, Burwick RM. The complement system and adverse pregnancy outcomes. *Molecular immunology* **67**, 56-70 (2015).
50. Oberkersch R, Attorresi AI, Calabrese GC. Low-molecular-weight heparin inhibition in classical complement activation pathway during pregnancy. *Thrombosis research* **125**, e240-245 (2010).
51. Zaferani A, *et al.* Identification of tubular heparan sulfate as a docking platform for the alternative complement component properdin in proteinuric renal disease. *The Journal of biological chemistry* **286**, 5359-5367 (2011).

52. Rossi V, *et al.* Functional characterization of the recombinant human C1 inhibitor serpin domain: insights into heparin binding. *Journal of immunology (Baltimore, Md : 1950)* **184**, 4982-4989 (2010).
53. Weiler JM, Yurt RW, Fearon DT, Austen KF. Modulation of the formation of the amplification convertase of complement, C3b, Bb, by native and commercial heparin. *The Journal of experimental medicine* **147**, 409-421 (1978).
54. Ninomiya H, Kawashima Y, Nagasawa T. Inhibition of complement-mediated haemolysis in paroxysmal nocturnal haemoglobinuria by heparin or low-molecular weight heparin. *British journal of haematology* **109**, 875-881 (2000).
55. Powell DR, Huttenlocher A. Neutrophils in the Tumor Microenvironment. *Trends Immunol* **37**, 41-52 (2016).
56. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nature reviews Cancer* **16**, 431-446 (2016).
57. Marks RM, Todd RF, 3rd, Ward PA. Rapid induction of neutrophil-endothelial adhesion by endothelial complement fixation. *Nature* **339**, 314-317 (1989).
58. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature* **528**, 413-417 (2015).
59. Pieterse E, *et al.* Neutrophil Extracellular Traps Drive Endothelial-to-Mesenchymal Transition. *Arteriosclerosis, thrombosis, and vascular biology* **37**, 1371-1379 (2017).
60. Moloney JN, Cotter TG. ROS signalling in the biology of cancer. *Seminars in cell & developmental biology* **80**, 50-64 (2018).
61. El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidal MA, Dang PM. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunological reviews* **273**, 180-193 (2016).
62. Siska PJ, *et al.* Mitochondrial dysregulation and glycolytic insufficiency functionally impair CD8 T cells infiltrating human renal cell carcinoma. *JCI insight* **2**, (2017).
63. Weinberg F, Ramnath N, Nagrath D. Reactive Oxygen Species in the Tumor Microenvironment: An Overview. *Cancers* **11**, 1191 (2019).
64. Mittal M, *et al.* Neutrophil Activation of Endothelial Cell-Expressed TRPM2 Mediates Transendothelial Neutrophil Migration and Vascular Injury. *Circulation research* **121**, 1081-1091 (2017).
65. Demers M, Wagner DD. NETosis: a new factor in tumor progression and cancer-associated thrombosis. *Semin Thromb Hemost* **40**, 277-283 (2014).
66. Park J, *et al.* Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci Transl Med* **8**, 361ra138 (2016).
67. Erpenbeck L, Schon MP. Neutrophil extracellular traps: protagonists of cancer progression? *Oncogene* **36**, 2483-2490 (2017).
68. Bjornsdottir H, *et al.* Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Free radical biology & medicine* **89**, 1024-1035 (2015).

69. Fuchs TA, Bhandari AA, Wagner DD. Histones induce rapid and profound thrombocytopenia in mice. *Blood* **118**, 3708-3714 (2011).
70. Cedervall J, Zhang Y, Olsson AK. Tumor-Induced NETosis as a Risk Factor for Metastasis and Organ Failure. *Cancer research* **76**, 4311-4315 (2016).
71. Demers M, *et al.* Priming of neutrophils toward NETosis promotes tumor growth. *Oncoimmunology* **5**, e1134073 (2016).
72. Camous L, *et al.* Complement alternative pathway acts as a positive feedback amplification of neutrophil activation. *Blood* **117**, 1340-1349 (2011).
73. Yuen J, *et al.* NETosing Neutrophils Activate Complement Both on Their Own NETs and Bacteria via Alternative and Non-alternative Pathways. *Frontiers in immunology* **7**, 137 (2016).
74. Wang H, Wang C, Zhao MH, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. *Clinical and experimental immunology* **181**, 518-527 (2015).
75. Cedervall J, Dimberg A, Olsson AK. Tumor-Induced Local and Systemic Impact on Blood Vessel Function. *Mediators of inflammation* **2015**, 418290 (2015).
76. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4-10 (2005).
77. Hurt B, Schulick R, Edil B, El Kasmi KC, Barnett C, Jr. Cancer-promoting mechanisms of tumor-associated neutrophils. *American journal of surgery* **214**, 938-944 (2017).
78. Albrecht EA, *et al.* C5a-induced gene expression in human umbilical vein endothelial cells. *The American journal of pathology* **164**, 849-859 (2004).
79. Wu F, *et al.* Complement component C3a plays a critical role in endothelial activation and leukocyte recruitment into the brain. *Journal of neuroinflammation* **13**, 23 (2016).
80. Brand C, *et al.* NG2 proteoglycan as a pericyte target for anticancer therapy by tumor vessel infarction with retargeted tissue factor. *Oncotarget* **7**, 6774-6789 (2016).
81. Maishi N, Hida K. Tumor endothelial cells accelerate tumor metastasis. *Cancer science* **108**, 1921-1926 (2017).
82. Maishi N, *et al.* Tumour endothelial cells in high metastatic tumours promote metastasis via epigenetic dysregulation of biglycan. *Scientific Reports* **6**, 28039 (2016).
83. Huot J, Houle F, Marceau F, Landry J. Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circulation research* **80**, 383-392 (1997).
84. Kvietys PR, Granger DN. Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. *Free radical biology & medicine* **52**, 556-592 (2012).
85. Di A, Mehta D, Malik AB. ROS-activated calcium signaling mechanisms regulating endothelial barrier function. *Cell calcium* **60**, 163-171 (2016).
86. Xu Q, Huff LP, Fujii M, Griendling KK. Redox regulation of the actin cytoskeleton and its role in the vascular system. *Free radical biology & medicine* **109**, 84-107 (2017).

87. Qi H, Yang S, Zhang L. Neutrophil Extracellular Traps and Endothelial Dysfunction in Atherosclerosis and Thrombosis. *Frontiers in immunology* **8**, 928 (2017).
88. Ma Y, Yang X, Chatterjee V, Meegan JE, Beard RS, Jr., Yuan SY. Role of Neutrophil Extracellular Traps and Vesicles in Regulating Vascular Endothelial Permeability. *Frontiers in immunology* **10**, 1037 (2019).
89. Hermant B, *et al.* Identification of proteases involved in the proteolysis of vascular endothelium cadherin during neutrophil transmigration. *The Journal of biological chemistry* **278**, 14002-14012 (2003).
90. Carmona-Rivera C, Zhao W, Yalavarthi S, Kaplan MJ. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. *Annals of the rheumatic diseases* **74**, 1417-1424 (2015).
91. Darling VR, Hauke RJ, Tarantolo S, Agrawal DK. Immunological effects and therapeutic role of C5a in cancer. *Expert review of clinical immunology* **11**, 255-263 (2015).
92. Nabizadeh JA, *et al.* The Complement C3a Receptor Contributes to Melanoma Tumorigenesis by Inhibiting Neutrophil and CD4⁺ T Cell Responses. *The Journal of Immunology* **196**, 4783-4792 (2016).
93. Bauer AT, *et al.* von Willebrand factor fibers promote cancer-associated platelet aggregation in malignant melanoma of mice and humans. *Blood* **125**, 3153-3163 (2015).
94. Goertz L, *et al.* Heparins that block VEGF-A-mediated von Willebrand factor fiber generation are potent inhibitors of hematogenous but not lymphatic metastasis. *Oncotarget* **7**, 68527-68545 (2016).
95. Wang Q, Wang N, Zhang X, Hu W. A simple PCR-based method for the rapid genotyping of inherited fifth complement component (C5)-deficient mice. *Experimental animals* **64**, 261-268 (2015).
96. Brinkmann V, Laube B, Abu Abed U, Goosmann C, Zychlinsky A. Neutrophil extracellular traps: how to generate and visualize them. *J Vis Exp*, (2010).
97. Wong SL, *et al.* Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med* **21**, 815-819 (2015).
98. Tiruppathi C, Malik AB, Del Vecchio PJ, Keese CR, Giaever I. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 7919-7923 (1992).
99. Peitsch WK, *et al.* Desmoglein 2 depletion leads to increased migration and upregulation of the chemoattractant secretoneurin in melanoma cells. *PloS one* **9**, e89491 (2014).
100. Wehling C, *et al.* Monitoring of complement activation biomarkers and eculizumab in complement-mediated renal disorders. *Clinical and experimental immunology* **187**, 304-315 (2017).
101. Joiner KA, Hawiger A, Gelfand JA. A study of optimal reaction conditions for an assay of the human alternative complement pathway. *Am J Clin Pathol* **79**, 65-72 (1983).

102. Talsma DT, *et al.* Endothelial heparan sulfate deficiency reduces inflammation and fibrosis in murine diabetic nephropathy. *Laboratory investigation; a journal of technical methods and pathology* **98**, 427-438 (2018).
103. Denk S, *et al.* Complement C5a-Induced Changes in Neutrophil Morphology During Inflammation. *Scandinavian journal of immunology* **86**, 143-155 (2017).
104. Khameneh HJ, *et al.* C5a Regulates IL-1beta Production and Leukocyte Recruitment in a Murine Model of Monosodium Urate Crystal-Induced Peritonitis. *Frontiers in pharmacology* **8**, 10 (2017).
105. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* **16**, 1438-1444 (2009).
106. Ling GS, *et al.* C1q restrains autoimmunity and viral infection by regulating CD8⁺ T cell metabolism. *Science (New York, NY)* **360**, 558 (2018).
107. Zhang Y, Zhang Z, Cao L, Lin J, Yang Z, Zhang X. A common CD55 rs2564978 variant is associated with the susceptibility of non-small cell lung cancer. *Oncotarget* **8**, 6216-6221 (2017).
108. Fust G, *et al.* C1 and C4 abnormalities in chronic lymphocytic leukaemia and their significance. *Immunol Lett* **14**, 255-259 (1987).
109. Sample A, He YY. Mechanisms and prevention of UV-induced melanoma. **34**, 13-24 (2018).
110. Bald T, *et al.* Ultraviolet-radiation-induced inflammation promotes angiogenesis and metastasis in melanoma. *Nature* **507**, 109-113 (2014).
111. Hammerberg C, Katiyar SK, Carroll MC, Cooper KD. Activated complement component 3 (C3) is required for ultraviolet induction of immunosuppression and antigenic tolerance. *The Journal of experimental medicine* **187**, 1133-1138 (1998).
112. Stapelberg MP, Williams RB, Byrne SN, Halliday GM. The alternative complement pathway seems to be a UVA sensor that leads to systemic immunosuppression. *The Journal of investigative dermatology* **129**, 2694-2701 (2009).
113. Augusto JF, *et al.* Low Serum Complement C3 Levels at Diagnosis of Renal ANCA-Associated Vasculitis Is Associated with Poor Prognosis. *PloS one* **11**, e0158871 (2016).
114. Ajona D, Ortiz-Espinosa S, Pio R. Complement anaphylatoxins C3a and C5a: Emerging roles in cancer progression and treatment. *Seminars in cell & developmental biology* **85**, 153-163 (2019).
115. Kerk N, Strozyk EA, Poppelmann B, Schneider SW. The mechanism of melanoma-associated thrombin activity and von Willebrand factor release from endothelial cells. *The Journal of investigative dermatology* **130**, 2259-2268 (2010).
116. Desch A, *et al.* Highly invasive melanoma cells activate the vascular endothelium via an MMP-2/integrin α v β 5-induced secretion of VEGF-A. *The American journal of pathology* **181**, 693-705 (2012).

117. Ytting H, Christensen IJ, Jensenius JC, Thiel S, Nielsen HJ. Preoperative mannan-binding lectin pathway and prognosis in colorectal cancer. *Cancer immunology, immunotherapy : CII* **54**, 265-272 (2005).
118. Babu D, Runions J, Kadhim M, Brooks SA. N-acetylgalactosamine glycans function in cancer cell adhesion to endothelial cells: A role for truncated O-glycans in metastatic mechanisms. *Cancer Lett* **375**, 367-374 (2016).
119. Srinivasan N, Bane SM, Ahire SD, Ingle AD, Kalraiya RD. Poly N-acetyllactosamine substitutions on N- and not O-oligosaccharides or Thomsen-Friedenreich antigen facilitate lung specific metastasis of melanoma cells via galectin-3. *Glycoconj J* **26**, 445-456 (2009).
120. Kieda C, Dus D. Endothelial cell glycosylation: regulation and modulation of biological processes. *Advances in experimental medicine and biology* **535**, 79-94 (2003).
121. Weston BW, *et al.* Expression of human alpha(1,3)fucosyltransferase antisense sequences inhibits selectin-mediated adhesion and liver metastasis of colon carcinoma cells. *Cancer Res* **59**, 2127-2135 (1999).
122. Beauvais DM, Ell BJ, McWhorter AR, Rapraeger AC. Syndecan-1 regulates alphavbeta3 and alphavbeta5 integrin activation during angiogenesis and is blocked by synstatin, a novel peptide inhibitor. *The Journal of experimental medicine* **206**, 691-705 (2009).
123. Torres Filho IP, Torres LN, Salgado C, Dubick MA. Plasma syndecan-1 and heparan sulfate correlate with microvascular glycocalyx degradation in hemorrhaged rats after different resuscitation fluids. *Am J Physiol Heart Circ Physiol* **310**, H1468-1478 (2016).
124. Johansson PI, Stensballe J, Rasmussen LS, Ostrowski SR. A high admission syndecan-1 level, a marker of endothelial glycocalyx degradation, is associated with inflammation, protein C depletion, fibrinolysis, and increased mortality in trauma patients. *Ann Surg* **254**, 194-200 (2011).
125. Jane-wit D, *et al.* Complement membrane attack complexes activate noncanonical NF-kappaB by forming an Akt+ NIK+ signalosome on Rab5+ endosomes. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 9686-9691 (2015).
126. Morgan BP, Dankert JR, Esser AF. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *Journal of immunology (Baltimore, Md : 1950)* **138**, 246-253 (1987).
127. Shingu M, Nonaka S, Nishimukai H, Nobunaga M, Kitamura H, Tomo-Oka K. Activation of complement in normal serum by hydrogen peroxide and hydrogen peroxide-related oxygen radicals produced by activated neutrophils. *Clinical and experimental immunology* **90**, 72-78 (1992).
128. Douda DN, Yip L, Khan MA, Grasemann H, Palaniyar N. Akt is essential to induce NADPH-dependent NETosis and to switch the neutrophil death to apoptosis. *Blood* **123**, 597-600 (2014).
129. Schreiber A, Rousselle A, Becker JU, von Massenhausen A, Linkermann A, Kettritz R. Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E9618-e9625 (2017).

130. Fuchs TA, *et al.* Extracellular DNA traps promote thrombosis. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 15880-15885 (2010).
131. Gloude NJ, *et al.* Circulating dsDNA, endothelial injury, and complement activation in thrombotic microangiopathy and GVHD. *Blood* **130**, 1259-1266 (2017).
132. Kazatchkine MD, Fearon DT, Metcalfe DD, Rosenberg RD, Austen KF. Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. *The Journal of clinical investigation* **67**, 223-228 (1981).
133. Neely JL, Carlson SS, Lenhart SE. Tinzaparin sodium: a low-molecular-weight heparin. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* **59**, 1426-1436 (2002).
134. Cheer SM, Dunn CJ, Foster R. Tinzaparin sodium: a review of its pharmacology and clinical use in the prophylaxis and treatment of thromboembolic disease. *Drugs* **64**, 1479-1502 (2004).
135. Park JL, Kilgore KS, Naylor KB, Booth EA, Murphy KL, Lucchesi BR. N-Acetylheparin pretreatment reduces infarct size in the rabbit. *Pharmacology* **58**, 120-131 (1999).
136. Chen M, Jayne DRW, Zhao M-H. Complement in ANCA-associated vasculitis: mechanisms and implications for management. *Nature Reviews Nephrology* **13**, 359 (2017).
137. Keragala CB, Draxler DF, McQuilten ZK, Medcalf RL. Haemostasis and innate immunity – a complementary relationship. *British journal of haematology* **180**, 782-798 (2018).

Abbreviations

AAV	Anti-neutrophil cytoplasmic antibody associated vasculitis
APH50	Haemolytic activity of the alternative pathway assay
BCC	Basal cell carcinoma
C1INH	C1 inhibitor
CR1	Complement receptor 1
C4bp	C4-binding protein
C3aR	C3a receptor
C5aR	C5a receptor
sC5b-7	Soluble C5b-7
CitH3	Citrullination of the histone 3
CH50	Haemolytic activity of the classical pathway assay
DAF	Decay-accelerating factor
DAMPs	Damage associated molecular patterns
DCF	2'-7'-dichlorofluorescein
DAPI	4',6-diamidino-2-phenylindole
ECIS	Electric Cell-substrate Impedance Sensing
ECs	Endothelial cells
FB	Factor B
GAG	Glycosaminoglycan
HUVEC	Human Umbilical Vein Endothelial Cells
HIS	Heat inactive serum
ICAM-1	Intercellular cell adhesion molecule 1
KA	Keratoacanthoma
LMWHs	Low molecular weight heparins
MBL	Mannose binding lectin
MAC	Membrane attack complex
MASP	MBL-associated serine proteases
MDSCs	Myeloid derived suppressor cells
MPO	Myeloperoxidase
MMP9	Matrix metalloproteinase 9
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor-κB
NETs	Neutrophil extracellular traps
NE	Neutrophil elastase
NHS	Normal human serum
NCN	Nevocytic nevi
NAH	N-acetylheparin

PNH	Paroxysmal nocturnal hemoglobinuria
PAD4	Protein arginine deiminase 4
ROS	Reactive oxygen species
SDC1	Syndecan 1
TEER	Trans-endothelial electrical resistance
UV	Ultra violet
VWF	Von Willebrand factor
VEGF	Vascular endothelial growth factor
VCAM-1	Vascular cell adhesion molecule 1
WGA	Wheat germ agglutinin

Acknowledgments

This study can't be well performed without the funding and support from Heike und Wolfgang Mühlbauer Stiftung, the European Union's Seventh Framework Program for Research, Technological Development and Demonstration under grant agreement 613931 and the Deutsche Forschungsgemeinschaft within the RTG 2099, the IRTG 1549, SFB/Transregio 23 and SHENC-Unit FOR 1543. My sincere thanks also goes to the China Scholarship Council for providing me the Chinese government scholarship.

In particular, I gratefully acknowledge my supervisor Prof. Stefan W. Schneider for offering me the opportunity to work in their research group in the Department of Experimental Dermatology at the Medical Faculty Mannheim of the University of Heidelberg. I still remember during the first time we met he told me the motivational spirit is more important than hardworking. Thank you all the support and guidance throughout the whole Ph.D. time. Besides, I would like to thank Prof. Viktor Umansky, who enabled this thesis and gave me the chance to study in University of Heidelberg. A great thanks goes to Dr. Christian Gorzelanny for supporting me throughout the years with helpful discussions, valuable guidance and creative suggestions. Thank you for your understanding and encouragement. In addition, I would like to thank Dr. Alexander T. Bauer for his scientific support and helpful suggestions.

Thanks to all the lab members at Mannheim and Hamburg for their great help and technical support: Yuanyuan Wang; José Robador; Dr. Gustavo Ramos; Dr. Volker Huck; Christian Mess; Dr. Yi Yang; Lydia von Palubitzki; Antonia Burmeister; Dr. Anna Desch; Dr. Lukas Goertz; Natalia Halter; Sabine Vidal-Sy; Pia Houdek and Ewa Wladikoski. They are more than my colleagues. I would like to thank our cooperation partners Prof. Weiguo Hu; Dr. Peipei Ding and Prof. Michael Kirschfink for their scientific support and positive encouragement. Moreover, I want to thank Prof. Ralf Bartenschlager and Prof. Peter Angel for being members of my defense committee.

Finally, I would like to thank my family and friends for their unconditional support and encouragement. Particularly, I received enormous love and endless support from my wife Yuanyuan Wang. She always encourages me and makes me smile. Thank you all!