Mechanisms of complement activation under hemolytic conditions

Nicolas Merle

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Mechanisms of complement activation under hemolytic conditions

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RESUME

Le système du complément est une cascade de défense immunitaire complexe et étroitement régulée, conduisant à des dommages tissulaires lorsqu'il est suractivé. L'hème, un motif moléculaire de danger dérivant de l'hémolyse, est capable d'activer le complément dans le sérum et à la surface des cellules endothéliales (CE) *in vitro*, suggérant un rationel pour examiner l'impact de l'activation du complément dans les maladies hémolytiques.

L'objectif de ce projet était d'étudier si et comment l'hémolyse intravasculaire active le complément *in vivo,* et de comprendre les mécanismes sous-jacent conduisant à l'acquisition d'un phénotype activateur du complément par les CE afin d'identifier de nouvelles cibles thérapeutiques.

Nous avons détecté des dépôts de complément, de C3 et de C5b-9, dans des reins de patients souffrants de nephropathie drépanocytaire ainsi que dans un modèle murin de drépanocytose. Nous avons mis en place un modèle murin d'hémolyse intravasculaire massive, déclenchée par la phénylhydrazine (PHZ), et caractérisé l'atteinte rénale. Nous avons détecté des dépôts de C3 au niveau des reins de ces souris. Cet effet a été inhibé par l'administration préventive du scavenger naturel de l'hème, l'hémopexine (Hx), et reproduit par des injections d'hème libre, démontrant une activation hème-dépendante *in vivo*. Les microvésicules d'érythrocytes (MVs) drépanocytaires représentent une source naturelle d'hème, de part leur concentration en hème trois fois supérieure à celle observée chez les donneurs sains. Nous avons démontré que les MVs drépanocytaires activent le complément dans le sérum et sur les CE, de manière en partie hème-dépendante. Ces résultats révèlent le rôle activateur de l'hème sur le complément dans les maladies hémolytiques. De plus, nous avons démontré que l'interaction de l'hème avec TLR4 peut en partie expliquer les dépôts de C3 sur l'endothélium *in vivo* et les CE *in vitro*. Lutilisation d'un inhibiteur de TLR4, le TAK-242, a réduit de 50% les dépôts de complément sur les CE, confirmé par une réduction des dépôts sur l'endothélium vasculaire chez des souris $TLR4^{-/-}$ traitées par PHZ ou hème. De plus, nous avons montré que ces dépôts hème/TLR4 dépendants sont liés à l'expression rapide de P-sélectine, qui recrute C3b et C3(H_2O) à la membrane des CE, révélé par l'analyse des interactions protéiques en temps réel et l'utilisation d'un anticorps bloquant anti-P-sélectine.

Ensemble, ce projet démontre que l'hème et les MVs sont les produits dérivés de l'hémolyse responsables de l'activation du complément. Au niveau cellulaire, l'induction par l'hème d'un phénotype activateur du complément des CE dépend de l'axe TLR4/P-sélectine, induisant des dépôts de C3 à la surface cellulaire. Ainsi, ces études soulignent les bénéfices potentiels de l'Hx et du TAK-242 contre l'activation du complément dans des pathologies associées à une hémolyse.

Mots clés : Maladies hémolytiques, hème, système du complément, TLR4, cellule endothéliale, néphropathie.

ABSTRACT

Complement system is a complex and tightly regulated innate immune defensive cascade, which can promote tissue damage, when overactivated. Hemolysis-derived danger associated molecular pattern heme is able to activate complement in serum and on endothelial cells (EC) in vitro, providing a rational for scrutinizing the impact of complement activation in hemolytic diseases.

The objectives of this work were to study whether and how intravascular hemolysis induces complement activation *in vivo*, and to understand the underlying mechanism that leads to the acquisition of a complement activating phenotype of the endothelium in order to identify novel therapeutic strategies

We found complement deposits, including C3 activation fragments and C5b-9, within kidneys of patients with sickle cell disease (SCD) nephropathy (a prototypical hemolytic disease) as well as in a mouse model of SCD. We set up and characterized the renal injury of a mouse model of massive intravascular hemolysis, triggered by injection of phenylhydrazine (PHZ). We revealed C3 deposition within kidneys of the PHZ-treated animals. It was prevented by heme scavenging with hemopexin (Hx) and reproduced by injections of free heme, thus demonstrating the importance of heme for the complement activation *in vivo*. SCD erythrocytes microvesicles (MVs), are a pathologically relevant source of labile heme, since they carry three times more heme on their surface compare to MVs from healthy donors. We demonstrated that MVs, generated from SCD erythrocytes, activate complement in human serum and on EC surface, in part on a heme-dependent manner. These data highlight the importance of heme as a complement activator in hemolytic diseases. Further, we found that the C3 activation fragments deposits on endothelium *in vivo* and on EC *in vitro* can be in part explained by interaction of heme with TLR4. Indeed, the use of a specific inhibitor of TLR4, TAK-242, reduced about 50% the complement deposits on EC surface and such deposits on vascular endothelium in PHZ- or heme-injected mice were attenuated $TLR4^{-/-}$ mice. Moreover, we found that heme/TLR4-dependent complement deposition was mediated by the rapid expression of P-selectin, which in turn, recruited C3b and C3(H_2O) on the EC surface, as evidenced by real time protein interaction analyses and using of blocking antibodies.

Together our results demonstrated that heme and erythrocytes MVs are the hemolysis-derived products which promoted complement activation. At cellular level, heme induced complement-activating phenotype of EC by triggering TLR4/P-selectin axis and resulting in C3 activation fragments on cell surface. Together, these studies underline the potential benefits of Hx and TAK-242 against complement activation in pathologies related to hemolysis.

Key words: Hemolytic diseases, heme, complement system, TLR4, endothelial cells, nephropathy.

Glossary

Ab:	Antibody
Ag:	Antigen
aHUS:	atypical Hemolytic Uremic Syndrome
AIHA:	Auto-Immune Hemolytic Anemia
AKI:	Acute Kidney Injury
AP:	Alternative Pathway
C1 Inh:	C1 Inhibitor
C3a desArg:	C3a desArginine
C3aR:	C3a Receptor
C3G:	C3 Glomerulopathy
C4BP:	C4 Binding Protein
C5a desArg:	C5a desArginine
C5aR:	C5a Receptor
Casp3:	Caspase3
CCP:	Complement Control Protein
CD:	Cluster of Differentiation
CFHR:	Complement Factor H-Related
CKD:	Chronic Kidney Disease
CP:	Classical Pathway
CR:	Complement Receptor
CRP:	C reactive Protein
DAF:	Decay Accelerating Factor
DC:	Dentritic Cells
deoxyHb:	Deoxyhemoglobin
EC:	Endothelial cells
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic
FB:	Factor B
FD:	Factor D
FH:	Factor H
FI:	Factor I
FP:	Properdin
FSGS:	Focal Segmental GlomeruloSclerosis
GAG:	GlycosAminoGlycan
GFR:	Glomerular Filtration Rate
GPCR:	G Protein-Coupled Receptor
GPI:	Glycosylphosphatidylinositol
Hb:	Hemoglobin

HO:	Heme Oxygenase
Hp :	Haptoglobin
HUVEC:	Human Umbilical Vein Endothelial Cells
Hx:	Hemopexin
ICAM:	InterCellular Adhesion Molecule
IF:	ImmunoFluorescence
IFN:	Interferon
Ig:	Immunoglobulin
IHC:	ImmunoHistoChemistry
IL:	Interleukin
IRAK:	Interleukin-1 Receptor-Associated Kinase
ISC:	Irreversible Sickle Cell
KIM-1:	Kidney Injury Molecule-1
LBP:	Lipopolysaccharide Binding Protein
LP:	Lectin Pathway
LPS:	Lipopolysaccharide
LTB:	Leukotrien B
MAC:	Membrane Attack Complex
MAPK:	Mitogen-activated protein kinases
MASP:	Mannan Binding Lectin-Associated Serin Protease
MBL:	Mannan-Binding Lectin
MCP:	Membrane Cofactor Protein
MCP1:	Monocyte Chemoattractant Protein 1
MD2:	Myeloid Differentiation factor 2
MDSC:	myeloid-derived suppressor cells
MetHb:	Methemoglobin
MPGN:	MembranoProliferative GlomeruloNephritis
MV:	Microvesicles
MyD88:	Myeloid Differentiation primary response 88
NFkB:	nuclear factor-kappa B
NGAL:	Neutrophil Gelatine-Associated Lipocalin
NO:	Nitric Oxide
OxyHb:	Oxyhemoglobin
PAR	Protease-Activated Receptor
PGE2:	Prostaglandine E2
PHZ:	Phenylhydrazine
PI3K:	Phosphoinositide 3-kinase
PNH:	Paroxysmal Noctural Hemoglobinuria
PRR:	Pattern-Recognition Receptors
PT:	Pertussis Toxin
PTX:	Pentraxin

RBC:	Red Blood Cell
ROS:	Reactive Oxygen Species
SCD:	Sickle Cell Disease
SCDG:	Sickle Cell Disease Glomerulonephritis
SP:	Serine protease
SPR:	Surface Plasmon Resonnance
TED:	Thio-Ester containing Domain
TF:	Tissu Factor
TIR:	Toll-Interleukin 1 Receptor
TIRAP:	TIR domain contraining receptor
TLR:	Toll-Like Receptor
TMA:	Thrombotic Microangiopathy
TNF:	Tumor Necrosis Factor
TRAM:	TRIF-related adaptor molecule
TRIF:	TIR-domain-containing adapter-inducing interferon-b
TTP :	Thrombotic Thrompocytopenic Purpura
UL-vWF:	Ultra Large-von Willebrand Factor
VCAM:	Vascular Cell Adhesion Molecule
VEGF:	Vascular Endothelial Growth Factor
VOC:	Vaso-Occlusion Crisis
vWF:	von Willebrand Factor
WB:	Western Blot
WPB:	Weibel Palade Bodies

TABLE OF CONTENT

ACKNOWLEDGEMENT **1**
RESUME **5**
ABSTRACT **6**
GLOSSARY **7**
INTRODUCTION **12**
I. Complement system **13**
1. Mechanisms of activation 13
1.1. Pathways of initiation 13
1.1.1. Classical pathway (CP) 15
1.1.2. Lectin pathway (LP) 15
1.1.3. Alternative pathway (AP) 16
1.1.4. Extrinsic complement pathway activation 16
1.2. Focus on the AP and its amplification loop 17
1.2.1. Tick-over of C3 17
1.2.2. The amplification loop of the complement pathways 18
1.3. Common terminal pathway 19
1.3.1. C5 convertases formation 19
1.3.2. Membrane attack complex 20
1.3.2.1. Lytic activity 20
1.3.2.2. Sub-lytic activity 21
1.3.2.3. Soluble C5b-9 22
2. Role in host homeostasis 22
2.1. Opsonization and phagocytosis 22
2.1.1. Dead cells clearance and role of complement recognition proteins 22
2.1.2. Complement receptors 23
2.2. Anaphylatoxins: powerful mediators of inflammation 24
2.2.1. C3a, C5a and their receptors 24
2.2.1.1. Innate immune cells 25
2.2.1.2. Adaptive immune cells 27
2.2.2. Interaction with endothelial cells 27
3. Regulation of the complement system 28
3.1. Positive regulation of complement 28
3.1.1. Stabilization of the AP C3/C5 by properdin 28
3.1.2. Competition with FH 29
3.2. Negative regulation of the C3 convertases 30
3.2.1. Plasmatic regulators 30
3.2.1.1. Factor I 30
3.2.1.2. Factor H 31
3.2.1.3. C4 Binding Protein 32
3.2.1.4. C1 Inhibitor 33
3.2.2. Membrane regulation 33
3.2.2.1. Membrane cofactor protein (MCP) 33
3.2.2.2. Decay accelerating factor (DAF) 34
3.2.2.3. Complement Receptor 1 (CR1) 35
3.3. Regulation of the Membrane Attack Complex 35
3.4. Platforms for alternative pathway activation 36
II. Hemolysis: a pathological event **39**
1. Cascade and Regulation 39
1.1. Activation 39
1.1.1. Oxydation steps 39
1.1.2. Extravasacular hemolysis 40
1.1.3. Intravascular hemolysis 40
1.2. Inhibition 41
1.2.1. Hemoglobin scavenging 41
1.2.2. Heme scavenging 42

1.2.3. Heme oxygenase-1 ... 43
2. Toxicity ... 44
2.1. Depletion of nitrogen oxide ... 44
2.2. Oxidative stress ... 45
3. Heme: a potent Danger-Associated Molecular Pattern ... 46
3.1. Activation of innate immune cells ... 46
3.1.1. Targeting neutrophils ... 46
3.1.1.1. Neutrophils ... 46
3.1.1.2. Involvement of GPCR in heme sensing ... 47
3.1.2. Activation of monocytes/macrophages ... 48
3.1.2.1. Monocytes ... 48
3.1.2.2. Toll-like receptors – history of these "great" receptors ... 49
3.1.2.3. Functions of TLR4 ... 51
3.1.2.4. Emergence of therapeutics against TLR4 ... 54
3.1.2.5. Heme as a potent trigger of TLR4 signaling pathways ... 55
3.2. Interaction of heme with endothelium ... 57
3.2.1. Endothelium ... 57
3.2.2. The macro- and the micro- vasculature ... 57
3.2.3. Endothelial cells ... 58
3.2.3.1. Activation of endothelial cells ... 59
3.2.3.2. Endothelial cells in hemolytic conditions ... 59
3.3. Interaction of heme with complement system ... 60
4. Experimental models for studying hemolysis ... 61
4.1. Injection of free heme ... 61
4.2. Injection of glycerol ... 62
4.3. Old blood transfusion ... 62
4.4. Autoimmune hemolytic anemia models of hemolysis ... 63
4.5. Phenylhydrazine-induced hemolysis ... 65

III. Hemolytic diseases ... 66

1. Overview ... 66
2. Atypical Hemolytic Uremic Syndrome ... 66
2.1. Complement implication ... 67
3. SCD – a model of chronic hemolytic disease ... 70
3.1. A worldwide genetic disease ... 70
3.2. Vaso-occlusion crisis ... 72
3.3. Clinical manifestations ... 73
3.4. P-selectin: a key player in SCD pathogenesis ... 75
3.5. Therapeutics in SCD ... 76
3.6. Complement activation in SCD ... 78

OBJECTIVES ... 81

RESULTS ... 83

Manuscript 1: Characterization of the renal injury in an experimental model of intravascular hemolysis ... **84**
Manuscript 2: Intravascular hemolysis induces complement system activation mediated by heme and heme-loaded erythrocyte microvesicles ... **113**
Manuscript 3: Intravascular hemolysis induces complement attack on endothelium in a TLR4-dependent manner ... **150**

ANNEXES ... 180

Annex 1: Study of complement deposition in organs ... **181**
Annex 2: Study of the heme binding sites on TLR4 ... **184**
Annex 3: Study of GPCR implication in the heme-induced complement activation on EC surface ... **191**
Annex 4: Study of the heme-induced complement activation in aHUS *in vitro* model ... **196**

DISCUSSION ... 199

PERSPECTIVES ... 212

BIBLIOGRAPHY ... 217

ATTACHMENTS ... 265

INTRODUCTION

I. Complement system

The discovery, in 1888 by Jules Bordet, of anti-bacterial properties of plasma components highlighted a new line of host defense, for which Brodet obtained a Nobel Prize in 1919. Paul Ehrlich calls this substance “Complement” in 1899, defining it as the “activity of the serum, which complement (support) the action of antibodies”. Firstly considered as a side mechanism of the immune system, numerous studies demonstrate over the past decades a critical role of the complement system in immunity (Merle et al., 2015a).

From genetic studies we know that first traces of complement genes appear 1 billion years ago, making one of the oldest homeostasis and immune defense system (Nonaka and Kimura, 2006). This primitive innate immune system is based on chemical interactions without any specific epitope recognition. However, throughout the evolution, complement system became more and more sophisticated and evidences of its implication in inflammatory responses, regulation of adaptive immune system, dead cell clearance as well as coagulation pathway are numerous (Merle et al., 2015a, 2015b).

Thus, complement system constitutes a powerful first line of defense against foreign and altered host cells, operating in plasma, in tissues or within cells (Merle et al., 2015b). Over the last 50 years, mechanisms of complement cascade have been deciphered, highlighting its role of a guardian of the body by discriminating host cells from pathogens. During an infection, complete activation of complement system leads to inflammation, opsonization, phagocytosis, and destruction of the pathogen and ultimately results in activation of the adaptive immune response. However, imbalance of its regulation, resulting in inefficient or over stimultation can be detrimental for the host and is associated with increased susceptibility to infectious or non-infectious diseases, including autoimmunity, chronic inflammation, thrombotic microangiopathy, graft rejection, and cancer.

1. Mechanisms of activation

1.1. Pathways of initiation

Complement system is a cascade, progressing through conformational modifications of proteins, allowing rapid and local reaction but necessitating a tight regulation (Merle et al., 2015b). Complement is composed of about 50 proteins expressed on host cells surfaces, or produced by the liver and secreted in the circulation. These proteins, associated with

activating or regulatory functions, interact with each other in different level to mediate different biological activity, from regulatory processes to pro-inflammatory activity.

Complement activation can be initiated by three distinct pathways: the classical, the lectin and the alternative pathway (Figure 1). All of these three initiation pathways converge to the C3 protein, the central component of the complement system, thus leading to an amplification phase followed by activation of the common terminal pathway.

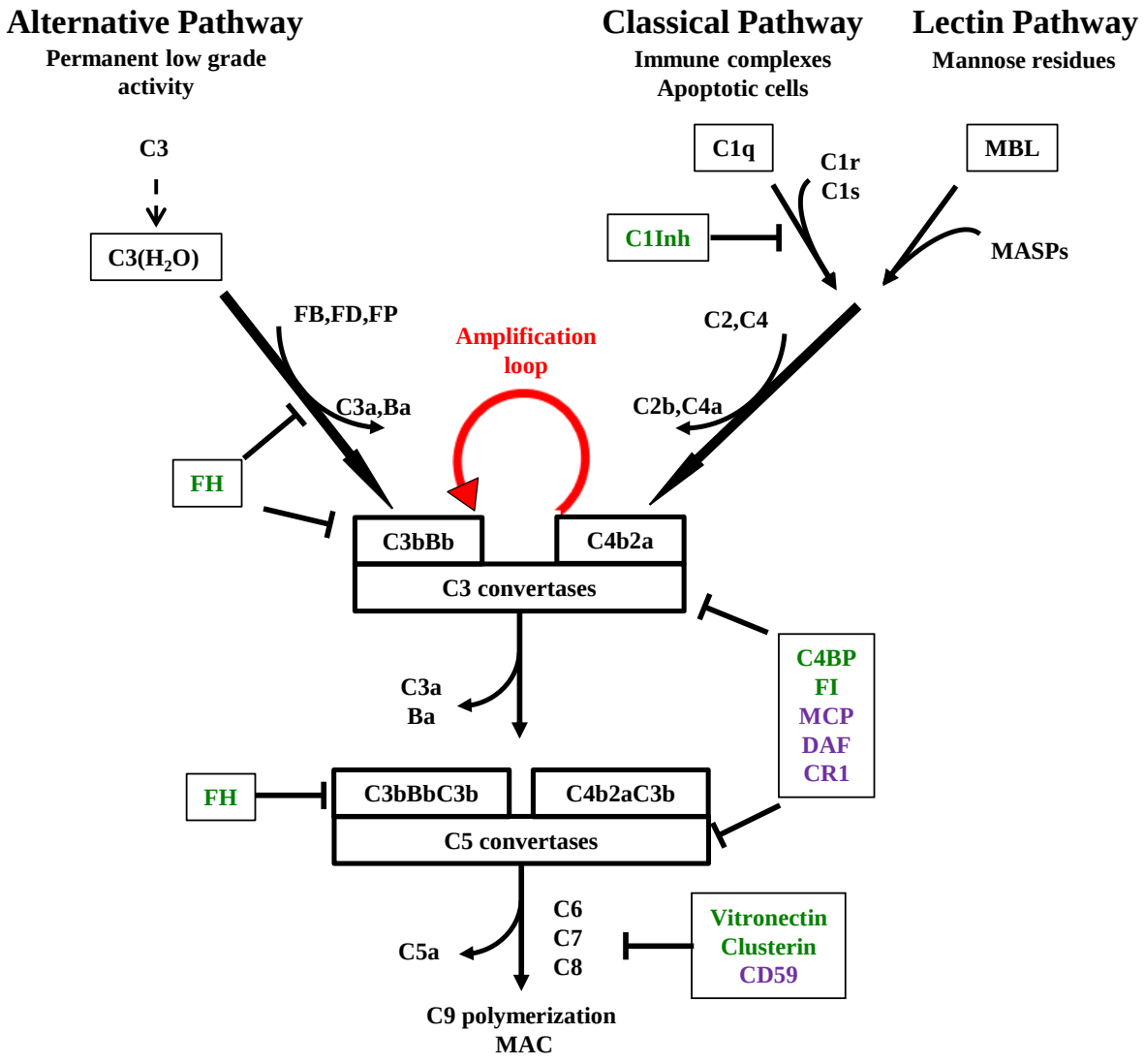

Figure 1: Complement cascade. FB: Factor B; FD: Factor D; FP: Properdin; MBL: Mannan-Binding Lectin; MASPs: Mannan-Aassociated Serine Protease; MAC: Membrane Attack Complex. Membrane regulators: MCP: Membrane Cofactor Protein; DAF: Decay Accelerating Factor; CR1: Complement Receptor 1. Serum regulators: FH: Factor H; FI: Factor I; C4BP: C4 Binding Protein (adapted from Merle et al., 2015a).

1.1.1. Classical pathway (CP)

It has long been established that CP is initiated by the recognition of the Fc part of surface-bound immunoglobulin, such as pentamer or hexamer of IgM or IgG monomer in close proximity, by C1q. Recent data demonstrate the IgG arrange in a specific hexameric ring with for each IgG one Fab in the plane and one Fab bound to the surface to efficiently accommodate the globular domains of C1q (Diebolder et al., 2014). Actually, C1q recognizes more than 100 different target molecules, including pathogen-associated molecular pattern (Roumenina et al., 2008), bacterial porins (Albertí et al., 1996), C-reactive protein (CRP) and pentraxin-3 (PTX3) (Kishore et al., 2004; Nauta et al., 2002), allowing to discriminate pathogen surfaces and apoptotic cells from resting host cells. Upon recognition, a conformational change occurring in C1q allows successive activation of two serine proteases, C1r and C1s, forming the C1 complex in calcium-dependent manner. Activated C1s is then capable of generating classical C3 convertase in a two step process. Firstly, C1s cleaves C4 protein into C4a, an anaphylatoxin domain released in the circulation, and C4b that covalently binds to targeted-cell membrane via a thioesther bond. C4a is an orfan anaphylatoxin, because its receptor is unknown, but very recent studies suggest PAR1/4 (Protease-Associated Receptor 1/4) as potential C4a receptors (Ricklin et al., 2017). Secondly, C2 binds to C4b and C1s cleaves this C2 into C2b, released in the circulation, and C2a, which remains bound to C4b. The complex C4b2a forms a C3 convertase, acquiring a proteolytic activity against C3 exposed by C2a.

1.1.2. Lectin pathway (LP)

The LP shares high similitudes with the CP (Ikeda et al., 1987). Indeed, this pathway is activated by the binding of the Mannan-Binding Lectin (MBL) or ficolins on carbohydrates expressed on pathogen surfaces (Chen and Wallis, 2001; Garred et al., 2016). Both MBL and ficolins have similar structures compare to C1q and associate with MASP-1, -2 and -3 (MBL-Associated Serine Protease), which also share high homology with C1r and C1s (Wallis et al., 2010). MASP-2 binding on activated MBL, or ficolin, leads to the same proteolytic activity observed in C1 complex and induces C4 and C2 cleavages, ending up to C4b2a complex.

1.1.3. Alternative pathway (AP)

Contrary to CP and LP, the AP is not activated upon recognition of a specific ligand. The AP remains permanently activated at low grade, due to a spontaneous hydrolysis of the C3 into C3(H_2O) (Lachmann and Halbwachs, 1975), which has a C3b-like structure (Chen et al., 2016; Pangburn et al., 1981). Without regulation, a fluid phase C3 convertase is formed with FB and FD, cleaving C3 into C3a and C3b (Fishelson et al., 1984; Ricklin et al., 2016). In normal conditions, this C3b is deactivated by the presence of serum and membrane inhibitors. However, surfaces lacking membrane inhibitors, such as pathogens, are unable to regulate C3b deposition, leading to the formation of an alternative C3 convertase (Pangburn et al., 1983).

Formation of the alternative C3 convertase starts with a magnesium-dependent binding of factor B (FB) to a C3(H_2O) or a C3b. Thereafter, conformational modification of FB exposes a binding site for a serine protease, factor D (FD), found in an active form in plasma. FD is generated after cleavage of pro-FD by MASP-3, showing an intricate link between lectin and alternative pathways (Dobó et al., 2016; Pihl et al., 2017). FD cleaves FB in Ba, a fragment released in the circulation, and Bb, which remains bound to C3b and forms the alternative C3 convertase (C3bBb). The half life of the AP C3 convertase is very short and is stabilized by properdin, the only positive regulator of complement (Blatt et al., 2016; Pangburn and Müller-Eberhard, 1986).

1.1.4. Extrinsic complement pathway activation

Accumulating evidence suggests the existence of additional complement activation pathways in the plasma (Huber-Lang et al., 2006) aside from the three established pathways. Thrombin, human coagulation factors IXa, XIa, Xa, and plasmin were all found to effectively cleave C3 and C5 (Amara et al., 2010). C5a and C5b can be generated from C5 via thrombin, independently of the plasma complement system (Huber-Lang et al., 2006; Oppermann et al., 2006). Thrombin cleaves C5 at an alternate site of the C5 convertase, generating intermediates C5T and C5bT. These activation fragments, generated at sites of activation of the coagulation cascade, form a C5bT-9 MAC with signicantly more lytic activity than with C5b-9.

Complement-independent cleavage of C3 by plasmin has also been suggested in the literature (Amara et al., 2010; Barthel et al., 2012; Foley et al., 2014). The fragments generated by plasmin-mediated cleavage (C3a-like, C3b-like, iC3b-like, C3c-like, and C3dg-like) are similar, but not identical to fragments generated by the complement cascade and are biologically active.

1.2. Focus on the AP and its amplification loop

1.2.1. Tick-over of C3

In the plasma, during normal physiological conditions, the dominant active complement pathway is the AP (**Figure 1A**). Tick-over is the spontaneous hydrolysis of a labile thioester bond, which converts C3 to a bioactive form C3(H_2O) in the fluid phase (Lachmann and Halbwachs, 1975). Upon hydrolysis, the TED domain of C3 undergoes a dramatic structural change that exposes a binding site for FB. The C3(H_2O)-bound FB is then cleaved by FD allowing formation of a fluid phase C3 convertase complex C3(H_2O)Bb. C3(H_2O)Bb is able to interact and cleave native C3 molecules to C3a and C3b (Isenman et al., 1981; Li et al., 2010; Nishida et al., 2006; Pangburn et al., 1981; Rodriguez et al., 2014; Winters et al., 2005). Thereafter, C3b can bind covalently via its TED domain to any adjacent surface containing hydroxyl or amine groups that is located within about 60 nm from the convertase, due to the short half-life of the C3b thioester, around 60 μs, with a poor attachment efficiency of 10% (Law and Dodds, 1997) (Figure 4).

Of note, FB binds in a similar manner to C3b on membranes and C3(H_2O) in the fluid phase (Bexborn et al., 2008). Even if FB has a higher affinity to C3(H_2O) than to C3b, the convertase activity remains lower than the C3bBb complex, as measured by C3a released. The fluid phase convertase C3(H_2O)Bb also has higher resistance to decay by complement inhibitors (Bexborn et al., 2008).

On host cells, bound C3b molecules are rapidly inactivated by an armamentarium of membrane-expressed or fluid phase-recruited complement regulators (described in detail below).

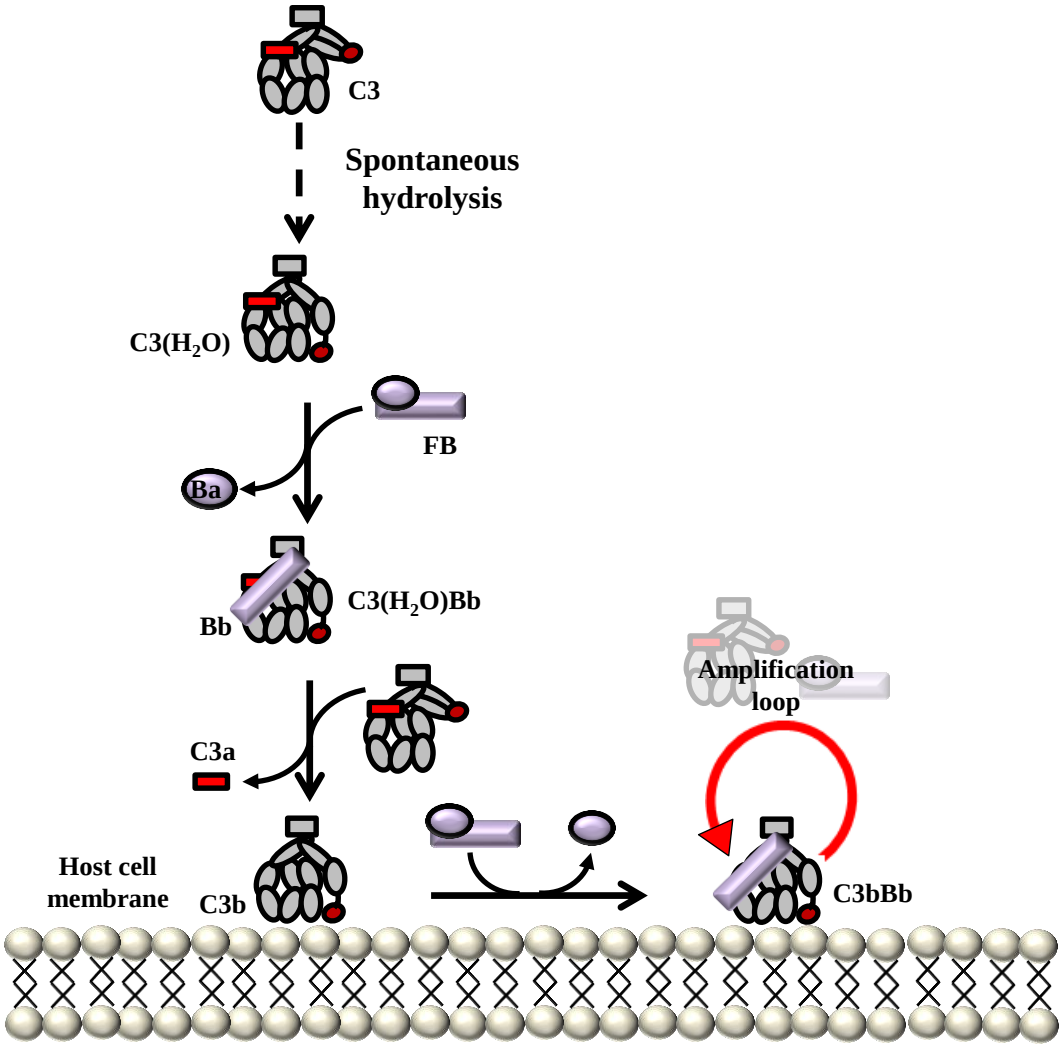

Figure 2: Complement tick-over and amplification loop. Spontaneous hydrolysis of the C3 component allows formation of a fluid phase C3convertase $C3(H_2O)Bb$ able to cleave C3 into C3a and C3b. Thereafter, C3b binds to a cell membrane and generates a membranous C3bBb that promotes auto-amplification of the alternative pathway.

1.2.2. The amplification loop of the complement pathways

Cleavage of C3 constitutes the focal point of the three pathways. Both convertases C4b2a and C3bBb identically cleave C3 into C3a, released in the circulation, and C3b. C3b covalently binds to activated surfaces, and can in turn interacts with FB that will be cleaved by FD in order to form a new C3 convertase (Figure 2). Thereafter, formation of new C3 convertases works as an amplification loop that augments the effect of all pathways, which is crucial for complement cascade (Lachmann, 2009).

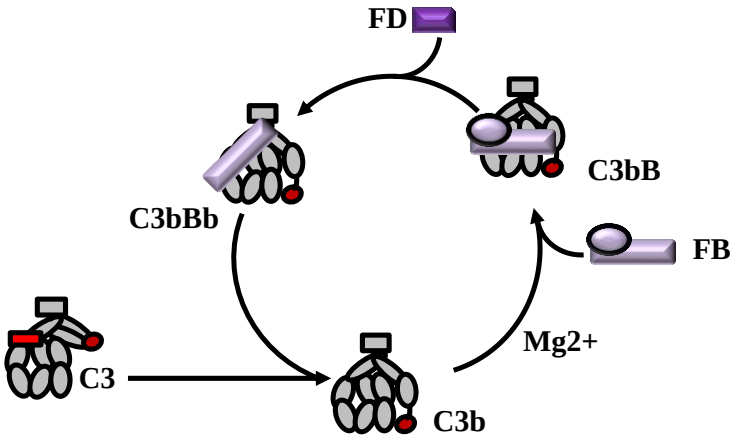

Figure2: Amplification of alternative pathway. Covalently bound C3b binds factor B (FB) in a Mg^{2+} dependent manner to form C3bB complex. Factor D (FD) cleaves bound FB, leading to C3bBb complex, capable of cleaving plasma C3 and form neo-C3b.

1.3. Common terminal pathway

1.3.1. C5 convertases formation

Increasing concentration of C3 cleavage products on membranes provokes binding of an additional C3b on the classical (C4b2a) or alternative (C3bBb) C3 convertase, thus leading to formation of a C5 convertase and modifies the specificity of the enzyme (Kinoshita et al., 1988). This C5 convertase cleaves C5 into bioactive fragments C5a, a powerful anaphylatoxin, and C5b that covalently binds to the membrane. The structures of the C5 convertases and the switch of specificity from C3 to C5 convertase are a matter of debate and intensive research. Indeed, previous data suggested that C3b generated from a C3 convertase can bind in turn to C3 convertase itself and form a new convertase with 1000 fold higher affinity toward C5 cleavage (Pangburn and Rawal, 2002). However, recent studies have highlighted the complexity of the switch from C3 to C5 convertase. Indeed, in vitro study using streptavidin beads highlighted the necessity of high density covalent binding of a second C3b (C3b') via the thioesther domain to form C5 convertases (Berends et al., 2015). Cumulating with structural data, new insights of C5 cleavage arise, suggesting a first binding of C5 to C3b', thus allowing C5 cleavage by a nearly-bound C3 convertase (Jore et al., 2016), echoing an earlier model (Vogt et al., 1978). This model actually fits with the recent clinical observation, revealing that properdin-bound C3 convertase is required for C5 convertase assembly (Marinozzi et al., 2017). This could be explained by the necessity to stabilize C3 convertase to generate high density of C3b' on the cell surface.

1.3.2. Membrane attack complex

Then, C5b undergoes a striking conformational change, similar to C3b (Hadders et al., 2012). C5b is capable of interacting with C6, C7, C8, and multiple copies of C9 that polymerizes, forming a C5b-9 complex, also called membrane attack complex (MAC) (Morgan et al., 2016). Until C5b-7 formation, the complex is lipophilic and anchors to the cell membrane (Preissner et al., 1985). C8 component is the first to penetrate the lipid bilayer thanks to a perforin-like domain (Hadders et al., 2007), forming a first transmembrane pore unable to lyse the cell. The C5b-8 complex allows recruiting between 2 and 18 C9 molecules, forming a tubular channel of 10nm wide, pores in the target membrane (Bubeck, 2014; Podack, 1984; Sharp et al., 2016).

CP, LP and AP generate C5b-9. Thanks to the amplification loop, AP assures more than 80% of the terminal complement activity (Harboe and Mollnes, 2008). Therefore, the AP has a particular importance in health and disease. Tight regulation is needed in order to keep the delicate balance between activation and regulation.

1.3.2.1. Lytic activity

Historically, cell lysis is the first attributed function of the complement system (Morgan et al., 2016). In fact, inert cells, like erythrocytes or liposomes are particularly susceptible to lysis from MAC by colloid osmosis (Koski et al., 1983). Gram-negative bacteria are also particularly sensitive due to a very thin cell wall, especially meningitis causing Neisseria species (Bhakdi et al., 1987; Lewis and Ram, 2014). However, extremely thick cell wall of gram-positive bacteria gives them a resistance against lysis from MAC, similarly to the metabolically active nucleated cells (Cole and Morgan, 2003; Morgan, 1989). To induce lysis in these cells, multiple MACs must be inserted in the cell membrane to induce calcium flux from the extracellular space or released from the intracellular stores (Campbell et al., 1981). Increased calcium concentration induces loss of mitochondrial membrane polarity, a total loss of ATP, ADP and AMP and finally cell death (Papadimitriou et al., 1991; Rus et al., 2001).

1.3.2.2. Sub-lytic activity

In physiology, amount of C5b-9 complexes is limited on the cell surface. Nucleated cells eliminate rapidly membrane-inserted C5b-9 by endocytosis (Carney et al., 1986) or membrane shedding (Scolding et al., 1989), escaping thus complement-mediated killing.

Although C5b-8 is able to induce signal transduction, C5b-9 is the most efficient for inducing calcium flux and activation of PKC, phospholipases and PI3K intermediates through a Gi protein (Carney et al., 1986; Papadimitriou et al., 1991; Wiedmer et al., 1987). Sub-lytic C5b-9 generates signal messengers participating to cell proliferation (Rus et al., 1996), anti-apoptosis pathway (Soane et al., 1999), and pro-inflammatory pathways (MAPK, ERK, NFkB) (Niculescu and Rus, 2001) in HUVEC. Moreover, it induces synthesis of pro-inflammatory cytokines, such as IL-8 and MCP-1 in HUVEC (Kilgore et al., 1997). Complete C5b-9 complex is necessary for inducing adhesion expression markers on cell surface like ICAM-1, VCAM-1 and E-selectin (Tedesco et al., 1997), thus participating to trans-endothelial migration of neutrophils (Dobrina et al., 2002).

Sub-lytic C5b-9 participates to a pro-thrombotic phenotype acquisition by activating platelets and endothelial cells (EC). Indeed, secretion of high molecular weight multimers of vWF and increased expression of P-selectin by EC require complement activation through C5b-9 deposition (Hattori et al., 1989; Ruiz-Torres et al., 2005; Sims and Wiedmer, 1995).

Moreover, it induces tissue factor (TF) synthesis by EC, with an increase of activated factor X (Saadi et al., 1995), reorganizes membrane phospholipids, favoring a coagulation environment. Sub-lytic C5b-9 participates to endothelial and platelet membranes vesiculation, promoting microparticles enriched in factor Va and VIIIa (Sims et al., 1988). It catalyzes pro-thombin into thrombin in absence of factor V (Wiedmer et al., 1986). Finally, sub-lytic induces cellular retractation, exposing sub-endothelial matrix to pro-thrombotic proteins (Saadi et al., 1995).

Regarding adaptive immune cells, a single study has shown the importance of sub-lytic C5b-9 in T cell activation (Chauhan and Moore, 2011).

1.3.2.3. Soluble C5b-9

Soluble C5b-9, derived from fluid phase activation of the AP, is incapable of creating a pore in cell membrane, but represents a witness of AP activation in diseases (Bu et al., 2015). Study of sC5b-9 functions revealed its implication in vascular leakage by opening inter-endothelial junctions, thus promoting extravasation (Bossi et al., 2004), as well as EC activation by inducing synthesis and release of a soluble member of the TNF receptor-family (Corallini et al., 2009). Soluble C5b-9 in plasma is now used as a biomarker for complement activation in pathological conditions (Chapin et al., 2016; Cofiell et al., 2015; Marinozzi et al., 2017).

2. Role in host homeostasis

2.1. Opsonization and phagocytosis

2.1.1. Dead cells clearance and role of complement recognition proteins

Every day, billions of cells die by programed cellular death. During this homeostatic process, critical structural and molecular modifications of the cell membrane lead to “eat-me” signals expression, marking the apoptotic cells for recognition by phagocytes. This process is accompanied by reduction of the expression level of complement regulators. Complement system plays a major role in the tolerogenic perception of apoptosis (Martin and Blom, 2016). This is in part due to the binding of C1q to PS phosphatidylserine (PS), double strand DNA, GAPDH or calreticulin (Païdassi et al., 2008; Terrasse et al., 2012; Verneret et al., 2014). Defect of C1q to opsonize apoptotic cells modifies phagocytosis by monocytes (Fraser et al., 2009) and results in activation of DCs (Castellano et al., 2007), leading to inflammation and auto-immunity (Pickering et al., 2000; Roumenina et al., 2011a). Similar functions have been observed for the LP recognition molecules MBL and the ficolins (Ogden et al., 2001). For instance, binding of C1q to apoptotic cells suppresses macrophage inflammation and inhibits inflammasome activation (Benoit et al., 2012). Moreover, self-antigen presentation by DCs in presence of C1q polarizes T cell subpopulations by decreasing Th1 and Th17 to the benefit of Treg, associated with production of anti-inflammatory cytokines like IL-10 and TGF-β (Clarke et al., 2015).

Apart from the complement activation-independent functions of C1q and MBL, these proteins, as well as the ficolins and properdin, also activate complement on the surface of apoptotic and necrotic cells. This results in opsonization of cells with C3 activation fragments, enhancing phagocytosis and immunologically silent removal of the dead cells (Bohlson et al., 2014; Galvan et al., 2012; Kemper et al., 2008; Nauta et al., 2003; Rajagopalan et al., 2010). The process is controlled by complement regulatory proteins, such as C4b-binding protein and FH, which control the level of activation and inflammation (Leffler et al., 2010).

2.1.2. Complement receptors

Complement opsonization, resulting from the direct activation of the AP on pathogens surface, allows their elimination by phagocytes, avoiding the mounting of an adaptive immune response. Phagocytes express specific receptors for C3 fragments – CR1, CR2, CR3, CR4 and CRIg – that will interact with C3b derived products to promote an immunologically silent clearance (Erdei et al., 2016). Behavior of phagocytes depends on the nature of the opsonin and the receptor engaged, leading to pro-inflammatory response or tolerogenic suppression.

CR1 (CD35) is expressed on monocytes, macrophages, neutrophils, erythrocytes and renal podocytes (Merle et al., 2015b). CR1 contains Complement Control Protein (CCP) domains, allowing binding to the C3b and C4b activation fragments. If CR1 is known to inhibit complement activation, (see below), interaction between C3b-coated targets with CR1 enhances the FcγR-mediated phagocytosis of targets bearing both IgG and C3b.

CR2 (CD21), expressed on B-cells, forms a co-receptor complex with CD19 and CD81. It interacts with iC3b- and C3d-coates surfaces (Vogt et al., 2006). The specificity for these two ligands is explained by the recognition of a common epitope on the TED domain exposed on these two fragments upon conformational modification of the native C3 (Carroll and Isenman, 2012; Szakonyi et al., 2001). C3d recognition plays a role of adjuvant by lowering the B-cell activation threshold by 1000-10000-fold (Dempsey et al., 1996).

CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are heterodimeric transmembrane receptors, composed of a common β-chain and a unique α-chain. They are expressed by macrophages, monocytes, dendritic cells, neutrophils and NK (Min et al., 2014; Ross and

Větvicka, 1993) and are well known as lineage markers for myeloid cells. Upon ligand binding, an outside-in transmitted signal is generated, leading to cellular response, including actin remodeling, phagocytosis, degranulation, or slow responses involving protein neosynthesis. CR3 and to lesser extent CR4 are essential for phagocytosis of C3 fragments, opsonized immune complexes, and pathogens (Springer and Dustin, 2012). CR3 and CR4 both recognize iC3b fragment but differs in their profile of recognition (Bajic et al., 2013a). Indeed, C3d part of iC3b is recognized by CR3 (Diamond et al., 1993), while CR4 binds to C3c (Chen et al., 2012). Both CR3 and CR4 are expressed at low level on the cell surface in an inactivated conformation. Stimuli of phagocytic cell, such as pro-inflammatory cytokines or TLRs activation increase expression of these receptors and are necessary for inducing phagocytosis (Aderem and Underhill, 1999). Moreover, the inside-out signaling generated by these stimuli switches integrins to an active conformation that increases affinity for iC3b (Dupuy and Caron, 2008).

CRIg is a CR expressed on macrophages and kupffer cells in the liver that binds to C3b and iC3b, mediating phagocytosis of opsonized particles and pathogens (Gorgani et al., 2008; Helmy et al., 2006). Critic conformational changes of the native C3 upon activation expose new epitopes on the C3c part of C3b and iC3b, recognized by CRIg. Then, by directly binding to C3b, CRIg acts as an inhibitor of the AP, preventing C3 and C5 convertase formation (Wiesmann et al., 2006).

2.2. Anaphylatoxins: powerful mediators of inflammation

2.2.1. C3a, C5a and their receptors

The anaphylatoxins, C3a, C4a and C5a, are released during complement activation. These small (10–14 kDa) peptides play a critical role in supporting inflammation (Klos et al., 2009). Among these mediators, C3a and C5a are now well-characterized (Merle et al., 2015a). Although C4a has been reported to activate macrophage and monocytes (Tsuruta et al., 1993; Zhao et al., 2014), a lack of cognate C4a receptor slows down identification of the physiological role of C4a, although PAR1/4 may serve as such a receptor (Ricklin et al., 2017).

C3aR, C5aR, and C5aL2 belong to the G protein-coupled receptor (GPCR) family that contains seven transmembrane domains that are able to interact with C3a and C5a. Despite the homologies between C3a, C5a and their distinct receptors, differences in binding and induced-cell signaling pathways have been highlighted (Merle et al., 2015b). In fact, C3a binds in only one specific site on C3aR (Gao et al., 2003) that leads to phosphorylation of PI3K, Akt, and MAPK intermediates (Venkatesha et al., 2005). In contrast, two binding sites have been described for C5a on C5aR (Mery and Boulay, 1993; Siciliano et al., 1994), inducing activation of PI3K-γ (Perianayagam et al., 2002; la Sala et al., 2005), phospholipase Cβ2 (Jiang et al., 1997), phospholipase D (Mullmann et al., 1990), and Raf-1/B-Raf-mediated activation of MEK-1 (Buhl et al., 1994).

In human plasma, regulation of anaphylatoxin effects is provided by carboxypeptidase N, converting C3a and C5a fragments into C3a desArg and C5a desArg by cleavage at the C-terminal arginine (Matthews et al., 2004; Mueller-Ortiz et al., 2009). If C3a desArg is incapable of binding to C3aR (Bajic et al., 2013b), C5a desArg still have 10% pro-inflammatory activity compared to C5a in human (Sayah et al., 2003). By contrast, in mouse, C5a desArg is as efficient as C5a upon binding to C5aR (Schatz-Jakobsen et al., 2014).

Complement anaphylatoxins C3a and C5a play a critical role in the modulation of immune system activity by complement. To enhance inflammation, anaphylatoxins act as chemokine to recruit and activate immune cells to the site of complement activation (Merle et al., 2015a). Both anaphylatoxins impact on innate and adaptive immune cells as well as on EC (Figure 3).

2.2.1.1. Innate immune cells

C3a acts as a chemoattractant molecule for human mast cells, favoring hypersensitivity and inflammatory process (Hartmann et al., 1997). Co-stimulation of C3aR with TLR4 on humans' monocytes and monocytes-derived macrophages promotes synthesis of pro-inflammatory mediators such as TNFα, IL6, IL1β and PGE2 (Asgari et al., 2013; Takabayashi et al., 1996). However, some studies challenge the unique pro-inflammatory role of C3a, revealing a more complex function, since C3a prevents degranulation of neutrophils in ischemia/reperfusion model (Coulthard and Woodruff, 2015; Daffern et al., 1995; Wu et al., 2013). This anti-inflammatory effect has recently been confirmed in a model of melanoma, where tumorigenesis was retarded in mice lacking C3aR (Nabizadeh et al., 2016).

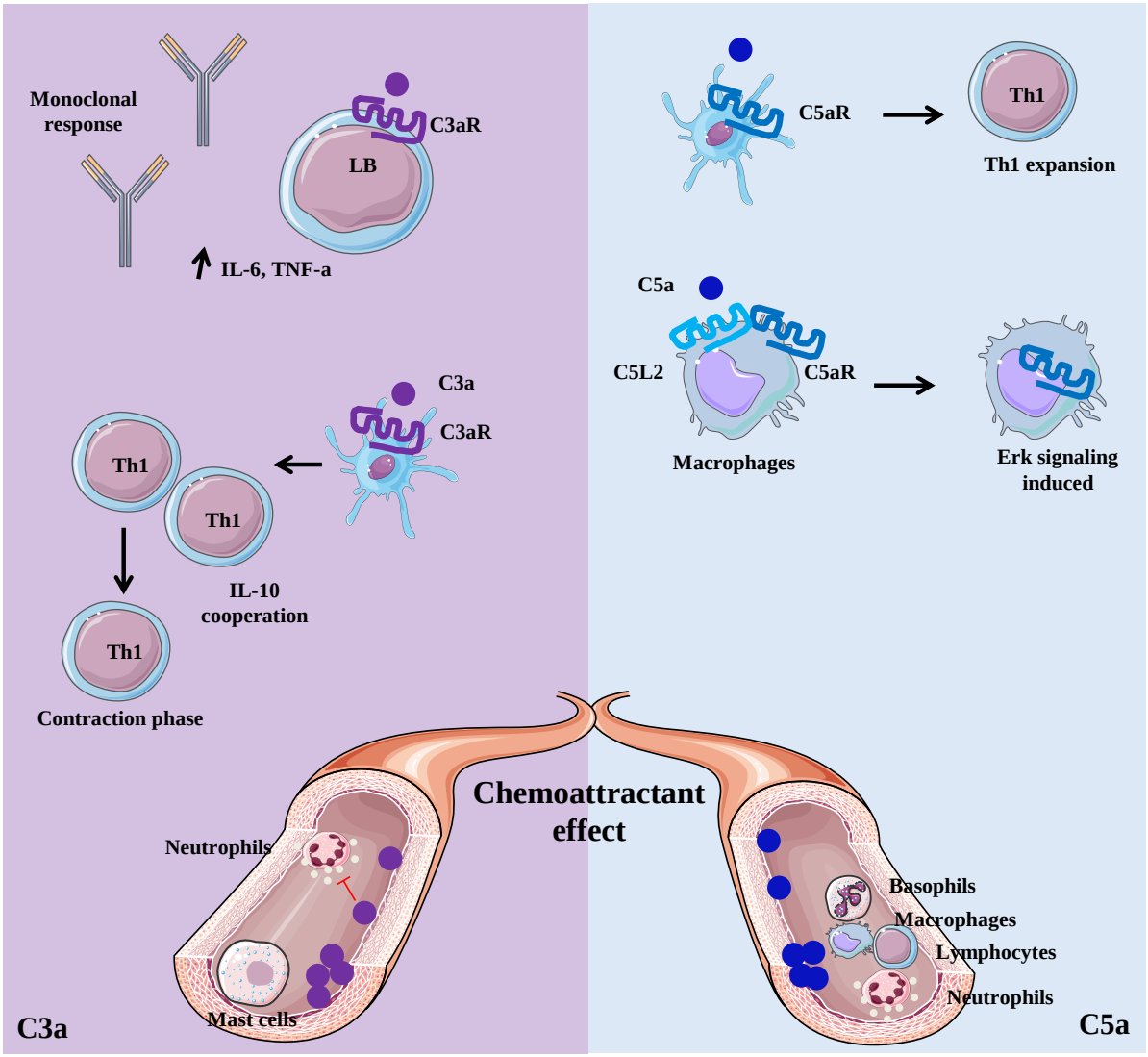

Figure 3: Role of anaphylatoxins C3a and C5a Anaphylatoxins C3a and C5a participate in inflammation by interacting and activating immune cells via C3aR and C5aR, respectively. C3a is implicated in the adaptive immunity by inducing monoclonal response from B cells and up-regulation of pro-inflammatory cytokines. Moreover, C3a facilitates the contraction phase of T cells by increasing IL-10 synthesis. C5a is implicated in Th1 expansion to improve adaptive immunity response, and allows C5aR internalization in presence of C5L2 to induce ERK signalization and pro-inflammatory effect of macrophages. Both are chemoattractant molecule, and allow mast cells migration for C3a, basophils, macrophages, neutrophils, and lymphocytes recruitment for C5a at the inflammatory site. Nevertheless, C3a has an anti-inflammatory effect on neutrophils by inhibiting their degranulation and recruitment (Merle et al., 2015a).

C5a is known to be a powerful chemoattractant molecule by recruiting macrophages (Aksamit et al., 1981), neutrophils (Ehrengruber et al., 1994), basophils (Lett-Brown and Leonard, 1977), eosinophils (DiScipio et al., 1999) and Myeloid Derived Suppressor Cells (MDSC) (Markiewski et al., 2008). In addition, C5a promotes plasminogen activator inhibitor-1 in mast cells and basophils (Wojta et al., 2002).

2.2.1.2. Adaptive immune cells

Adaptive immune cells are also impacted by release of C3a. Indeed, it has a direct effect on B cells by suppressing the polyclonal response and promotes IL-6 and TNFα secretion (86). In addition, C3a has an indirect effect on T cells by polarizing a Th1 phenotype through C3aR activation on DCs (Peng et al., 2006, 2008), and participates to the contraction phase of Th1 by regulating IL-10 expression (Ghannam et al., 2014; Liszewski et al., 2013). Furthermore, recent study has demonstrated the uptake of soluble C3(H_2O) by T cells, thus representing a source of C3a that sustains expression of IL-6 by activated $CD4^+$ T cells (Elvington et al., 2017).

C5a exerts a chemoattractant effect on T cells that constitutively express C5aR on their surface (Nataf et al., 1999), and is essential for induction of Th1 phenotype (Lalli et al., 2007). Moreover, C5a has been described to be crucial for T cells by restoring T cell activation in mouse models of depleted-APC in $CD80^{-/-}$ and $CD86^{-/-}$ APC (Strainic et al., 2008). C5a also impact B lymphocytes, by directing traffick of activated B cells in secondary lymphoid organs (Ottonello et al., 1999).

By contrast, autocrine signaling through C3aR and C5aR have recently been suggested to suppress CD8+ T cell anti-tumor effect by inhibiting IL-10 production (Wang et al., 2016)

2.2.2. Interaction with endothelial cells

Both C3a and C5a have been described to induce signals transduction in EC upon binding to their own receptor (Schraufstatter et al., 2002). However, different functions have been attributed, due to the use of different Gα protein.

Stimulation of C3aR on EC induces a rapid mobilization of intracellular granules, Weibel–Palade bodies (WPB), containing von Willebrand factor (vWF) and P-selectin (Morigi et al., 2011). Hence, the cells acquire a pro-inflammatory and pro-thrombotic phenotype, since P-selectin helps the recruitment of leukocytes via binding to PSGL-1 (Goligorsky et al., 2009) and vWF mediates platelet adhesion (Sadler, 1998). Furthermore, a recent study has provided evidence that brain vasculature of $C3^{-/-}$ mice, but not $C6^{-/-}$, have decreased expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 in response to LPS

administration, suggesting the critical role of C3a/C3aR axis in endothelial activation (Wu et al., 2016)

C5aR is expressed by macrovascular EC (Gasque et al., 1997). C5a induces increase of intracellular Ca^{2+} followed by rapid expression of P-selectin and secretion of vWF (Foreman et al., 1994), and induces TF activity (Ikeda et al., 1997). Moreover, accompanied with additional agonist, such as LPS, IFNγ or IL-6, C5a enhances C5aR mRNA and synthesis of CXCL2 (MIP-2) and CCL2 (MCP-1), two potent chemokines attracting monocytes and macrophages (Laudes et al., 2002).

3. Regulation of the complement system

Complement system has to be tightly regulated to avoid accidental damage of the host tissue (Schmidt et al., 2016). A multitude of inhibitors of complement, primarily against AP, exist in the fluid phase and on host cells. Binding of these inhibitors to C3b and/or C4b avoids C3 convertase formation and works as co-factors for factor I (FI), a serine protease, to induce C3b and C4b cleavage and generation of inactivated fragments. Moreover, different regulators can bind to the C3 convertase, promoting the decay of C3bBb complexes. Complement FH is the master inhibitor of the AP, combining in it three regulatory functions: dissociation of the AP C3 convertase, prevention of its formation and cofactor activity towards FI.

Apart from the negative regulation, positive regulators of the AP also exist, allowing its strong activation on pathogen surface.

3.1. Positive regulation of complement

The positive regulation of the complement cascade occurs at two steps – stabilization of the AP C3/C5 convertase and deregulation of FH binding to surfaces.

3.1.1. Stabilization of the AP C3/C5 by properdin

The C3bBb is a short-lived complex with a half-life of about 90 sec (Pangburn and Müller-Eberhard, 1986) and, therefore a stabilization of this complex is required to assure

efficient complement activation (Kemper et al., 2010). The AP C3/C5 convertase is stabilized 5- to 10-fold by association with properdin (Fearon and Austen, 1975). Properdin is synthesized and secreted by monocytes/macrophages and T lymphocytes (Schwaeble et al., 1994; Wirthmueller et al., 1997) and is stored in secondary granules of neutrophils and rapidly released upon stimulation (Camous et al., 2011; Wirthmueller et al., 1997).

Properdin is constituted of identical protein subunits (Sun et al., 2005) that are associated head-to-tail to form cyclic dimers, trimers, and tetramers that resemble rods, triangles, and squares, respectively. The capacity of the properdin to stabilize the C3 convertase positively correlates with its polymerization level (Pedersen et al., 2017). For instance, the tetramer has been described to be 10-fold more efficient than the dimer (Pangburn, 1989). Properdin binds C3bBb, as well as, the pro-convertase C3bB and C3b (Hourcade, 2006). Structural studies brought explanation of the capacity of properdin to stabilize the C3 convertase. Indeed, upon binding to C3bBb, structural changes occurring in the C3bBb complex, that distort the FH binding site, thus providing a relative resistance to the regulation by FH (Kajander et al., 2011; Morgan et al., 2011; Wu et al., 2009).

3.1.2. Competition with FH

The interaction of FH with different cell surfaces is controlled by CFHR proteins. CFHRs belong to the FH family and comprise five different members (CFHR1–5) (Medjeral-Thomas and Pickering, 2016). They are all composed of four to nine CCP domains, sharing a high sequence homology with FH. They exist in homo or heterodimers (Goicoechea de Jorge et al., 2013; Tortajada et al., 2013). Due to the high sequence homology of their C-termini with FH, it has been proposed that they can compete with FH for the binding to C3b, C3d and GAG, then enhancing complement activation (Fritsche et al., 2010; Goicoechea de Jorge et al., 2013; Tortajada et al., 2013).

CFHR1 has a capacity to bind heparin and C3b via its CCPs3-5, presenting high homology with FH CCPs18-20 and can also compete with FH for its binding to C3b (Fritsche et al., 2010). Isolated reports suggest that it can prevent C5 convertase formation (Heinen et al., 2009). CFHR2 binds to C3b via CCPs3-4 and could also compete with FH to regulate the C3 convertase. CFHR3 and CFHR4 also bind to C3b and C3d. CFHR4 can activate complement since the AP C3 convertase can assemble on its surface (Hebecker and Józsi,

2012). CFHR5 binds to heparin through CCPs5-7 (high sequence homology with CCPS12-14 of FH), and binds also CRP, PTX3 and C3b (Csincsi et al., 2015; Goicoechea de Jorge et al., 2013; Tortajada et al., 2013). At a high concentration, CFHR5 expresses a co-factor activity for FI as well as a capacity to dissociate the C3 convertase (McRae et al., 2005).

The FH gene locus is prone to genetic rearrangements, resulting in different hybrid proteins between FH and the CHFRs or between different CFHRs, which outcompete FH from host cells and are predisposing factors for severe renal diseases (Chen et al., 2014b; Gale et al., 2010; Goicoechea de Jorge et al., 2013; Togarsimalemath et al., 2017; Tortajada et al., 2013).

3.2. Negative regulation of the C3 convertases

3.2.1. Plasmatic regulators

3.2.1.1. Factor I

FI is a serine protease (SP) found in the plasma that regulates AP and CP by cleaving C3b and C4b, respectively. FI is mainly synthesized by the liver, but also EC, monocytes, fibroblasts and keratinocytes have shown a capacity to produce this complement protein (Julen et al., 1992; Nilsson et al., 2011; Timár et al., 2006). FI circulates in a proteolytic form but in inhibited conformation. The proteolytic activity of FI is carried by a light chain, which is allosterically inhibited in the circulation by a non-catalytic heavy-chain (Roversi et al., 2011). To exert its proteolytic activity, FI must bind to a co-factor, such as FH, MCP, CR1 or C4BP. By contrast, DAF is the only surface-bound inhibitor without co-factor activity. The recent structural basis of FI interaction with C3b/co-factor revealed conserved hydrophobic patches in MCP, CR1 and FH, while DAF does not have these hydrophobic patches (Xue et al., 2017). Upon binding to its cofactors, the non-catalytic heavy-chain undergoes reorientation without critical conformational changes, allowing FI to irreversibly degrade C3b into iC3b fragment at an Arg residue, which is unable to bind FB (Davis et al., 1984; Roversi et al., 2011; Xue et al., 2017). C4b is also degraded by FI to C4c and C4d, the latter remaining covalently bound to the surface.

Several proteins have been shown to enhance FI-mediated cleavage in the presence of cofactors. Thrombomodulin binds to C3b and FH and negatively regulates complement by

accelerating FI-mediated inactivation of C3b in the presence of FH and C4BP (Delvaeye et al., 2009). VWF also enhances the efficacy of FH as a cofactor for FI (Rayes et al., 2014). In specific *in vitro* buffer conditions, vWF has been suggested to have direct cofactor activity, but the physiological function of this interaction requires further validation (Feng et al., 2014).

3.2.1.2. Factor H

Discovered in 1965 (Nilsson and Mueller-Eberhard, 1965), FH regulates the AP and the amplification loop of the complement pathways. FH is mainly produced by the liver (around 80%) but can also be locally produced by EC (Brooimans et al., 1990), platelets (Licht et al., 2009), retinal epithelial cells (Chen et al., 2007) and glomerular mesangial cells (van den Dobbelsteen et al., 1994). It is a soluble inhibitor of the C3 convertase competing with FB for binding to C3b (Kazatchkine et al., 1979). FH is composed of 20 CCP domains arranged as beads on a string (Perkins et al., 2012). The fluid phase convertase C3(H_2O)Bb is more resistant than the alternative cell-bound convertase and is less susceptible to regulation by FH (Bexborn et al., 2008). FH has two main ligands, C3b and the GAG, found on host cells surface. Thus, FH has the particularity to regulate the AP in fluid phase (Weiler et al., 1976) as well as on host cell surfaces (Kazatchkine et al., 1979).

FH binds to C3b and C3(H_2O), but not to uncleaved C3. The conformational changes occurring to form C3b and C3(H_2O) expose the FH-binding sites. FH binds to C3b in at least two regions, at the N-terminus and the C-terminus of the protein. The N-terminal four CCPs compete with FB causing its dissociation from C3b and prevent C3 convertase assembly (Wu et al., 2009). It induces the irreversible dissociation of the C3bBb complex by releasing the Bb fragment (Weiler et al., 1976; Whaley and Ruddy, 1976). Moreover, CCP1-4 carries a co-factor activity for FI, increasing the affinity of FI for C3b and promoting the generation of iC3b fragments (Pangburn and Müller-Eberhard, 1983; Pangburn et al., 1977; Wu et al., 2009) (Figure 5).

The C-terminal part of FH, composed of CCPs19–20, carries two distinct binding sites, one for C3d as well as for glycosaminoglycans (GAGs) (Hellwage et al., 2002; Jokiranta et al., 2000; Kajander et al., 2011; Morgan et al., 2011). Moreover, it has been suggested that CCPs19–20 domains may bind both C3d and GAG at the same time (Blaum et al., 2014;

Lehtinen et al., 2009; Morgan et al., 2011, 2012). GAGs are an important constituent of the cell membrane and play a critical role in complement regulation. In addition to the GAG-binding site in CCP20, FH carries another GAG-binding site located in CCP7 (Blackmore et al., 1998; Ormsby et al., 2006; Schmidt et al., 2008). These two regions anchoring FH to the cell membrane are both necessary to assure functional activity of FH on the cell surface (Perkins et al., 2014), making a powerful regulator against complement activation on host cells. FH has been shown to interact with orther ligands, such as platelets through CCPs19-20 (Vaziri-Sani et al., 2005), PTX3 (Deban et al., 2008) , DNA, histones, annexin II (Leffler et al., 2010), as well as oxidative stressed marker or inflammation like CRP (Jarva et al., 1999), malondialdehyde acetaldehyde (Weismann et al., 2011) or oxidized lipids (Shaw et al., 2012).

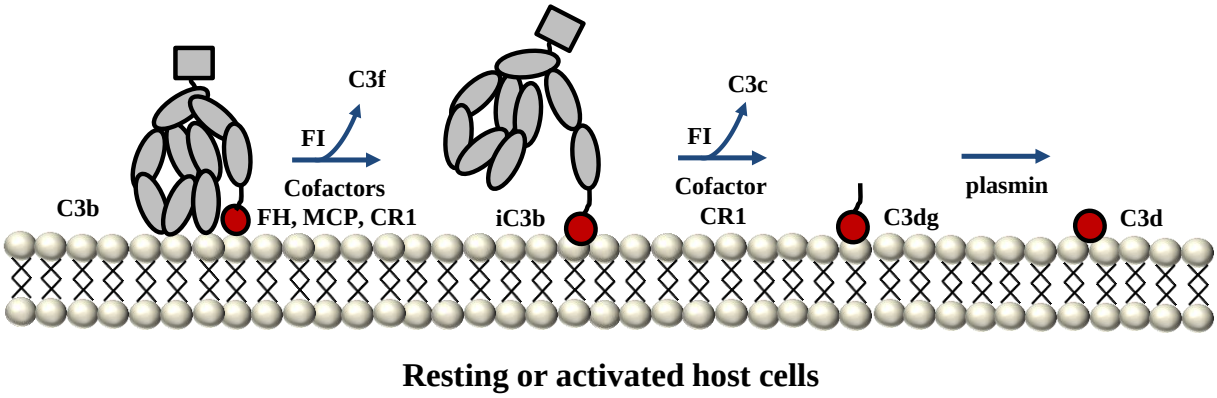

Figure 5: Regulation of the alternative pathway. (A) FH as a master regulator of C3b in the fluid phase and on the cell surface. FH binds to C3b in fluid phase preventing novel convertase formation. FH may bind to C3b and GAGs on the cell surface and the architecture of the complex depends on the level of activation of the cell and the density of deposited C3 fragments. Resting cells have only a few C3b molecules that are deposited and FH binds to them with the regulatory domains CCP1-4. CCP7 and CCP20 interact with GAG on the membrane. Alternately, CCP19 may bind to the thioester domain (TED) of C3b allowing CCP20 to interact with GAGs. If the cell is activated and C3b and C3d (or two C3b molecules) are deposited in close proximity, FH may bind to two of these molecules, allowing GAG binding by CCP20 (adapted from Merle et al, 2015b).

3.2.1.3. C4 Binding Protein

C4BP can act as a plasmatic cofactor for FI to inactivate C4b (Blom, 1999 ; Blom 2001 ; Blom 2003 ; Rawal 2009). C4BP has an octopus-like structure containing CCP domains constituted with α- and β-chains (Hofmeyer 2012, Dahlback, 1983). Cofactor activity and convertase dissociation functions are carried by the first three CCP domains of each α-chain. C4BP can simultaneously bind up to four C4b molecules (Ziccardi, 1984). By contrast, the β-chain interact with the coagulation protein S and participate to coagulation cascade regulation (Dahlback, 1983).

3.2.1.4.C1 Inhibitor

C1 Inh is a serpin molecule, able to control activation of the CP. C1 Inh dissociates C1 complex by binding to C1r and C1s, releasing free C1q and inactive covalent complexes between C1 Inh and C1r or C1s (Beinrohr et al, 2008 ; Davis et al, 2010). Recent study has demonstrated the crucial role of glycosylation C1 Inh on its function (Ghannam et al, 2016).

C1 Inh plays a role outside complement system, related to kinin pathway. Indeed angioedema, a disease caused by hereditary or acquired C1 Inh deficiency, is associated with elevated levels of bradykinin, thus increasing permeability of blood vessels. Recombinant and plasma-derived C1Inh are approved therapeutic agents for hereditary angioedema (Longhurst et al, 2012).

C1 Inh has the capacity to regulate the LP by binding to MASP-1 and MASP-2, thus preventing their activity (Paréj, 2013; Pressanis, 2003).

3.2.2. Membrane regulation

3.2.2.1. Membrane cofactor protein (MCP)

MCP, or CD46, is a transmembrane glycoprotein, ubiquitinously expressed on all cells but erythrocytes, composed of 4 extracellular CCPs. Alternative splice generates 4 isoforms with a molecular weight between 51 and 68 kDa, coexisting in the same cell surface. MCP participates to the regulation of CP, LP and AP via its function of FI co-factor. The four extracellular domains CCPs1–4 are structurally similar to the four N-terminal domains of FH. Thus, MCP binds to C3b and C4b. Moreover, it works as a co-factor for FI and helps to the cleavage of C3b into iC3b (Barilla-LaBarca et al., 2002; Liszewski et al., 1991; Persson et al., 2010; Wu et al., 2009) (Figure 5).

Over the past 10 years, other functions have been attributed to MCP. Indeed, a crucial role has been imputed to MCP in the regulation of adaptive immune system, by controlling proliferation and contraction phase of T $CD4^+$ cells. For instance, CD46 engagement on $CD4^+$ T cells promotes Th1 phenotype (Cardone et al., 2010). CD46 have been identified as a new natural ligand for Notch family member Jagged 1, thus controlling proliferation and contraction phase of T cells (Le Friec et al., 2012). In addition, CD46 inhibits macrophages-

induced IL-12 production and NK cells activity (Cardone et al., 2011). MCP is also implicated in fertility and can constitute a receptor for bacteria and viruses (Kim and Song, 2006).

3.2.2.2. Decay accelerating factor (DAF)

DAF, or CD55, is a GPI-anchored glycoprotein composed of 4 extracellular CCPs. As MCP, DAF is ubiquitinously expressed and participates to the regulation of CP and AP. However, DAF has no co-factor activity for FI, but binds to C3b and accelerates dissociation of C3 and C5 convetases (Forneris et al., 2016; Lublin and Atkinson, 1989). In addition to the lack of hydrophobic pocket, structural analysis of C3b/DAF interaction revealed a particular orientation of C3b CUB domain that is not compatible with the described position of the SP domain of FI for catalysis (Xue et al., 2017).

Contrary to other regulating proteins of the complement system, expression of DAF appears to be stimulation-dependent on cell surface (Tsuji et al., 1994). Indeed, inhibition of protein synthesis rapidly decreases DAF expression on HUVEC membrane, increasing DAF soluble concentration. This obvious membranous cleavage of DAF is not dependent on PIPLC (phosphatidylinositol-specific phospholipase C) since expression GPI-anchored CD59 is not impacted in this model (Tsuji et al., 1994). Moreover, DAF expression is inducible by numerous stimuli on EC, such as IFN-β, TNFα (Mason et al., 1999), β-FGF (β-fibroblast growth factor) (Mason et al., 2002), histamin (Tsuji et al., 1994), VEGF (vascular endothelial growth factor) (Mason et al., 2001) or thrombin (Lidington et al., 2000).

DAF has also been identified as a regulator of the immune response, since direct stimulation of CD55 by CD97 serves as a molecule of co-stimulation for $CD4^+$ T cells (Capasso et al., 2006). However, studies show that DAF expression regulating complement activation on T cell surface is necessary for homeostasis of T cells, otherwise exposing mice to IFNγ and IL-2 hypersecretion, and thus auto-immune reaction (Liu et al., 2005). The importance of this regulator has recently been confirmed, for the first time in a patient lacking DAF expression (Ozen et al., 2017), leading to uncontrolled complement activation on T cells. CD55 deficiency is shown to expose patients to angiopathic thrombosis.

3.2.2.3. Complement Receptor 1 (CR1)

CR1 (or CD35) is a glycoprotein containing 30 extracellular CCP domains. The first 28 CCP of CR1 are 4 long homologous repeats (LHR) for 7 CCP domains containing the binding sites for C3b and C4b (Klickstein et al., 1988; Wong et al., 1989). CR1 is expressed on circulating cells (Langford-Smith et al., 2015), more particularly on erythrocytes that express 90% of total CR1 (Hourcade et al., 2000). Within tissues, CR1 is found on glomerular podocytes (Chauvet et al., 2016; Gelfand et al., 1975), astrocytes and dendritic follicular cells (Hourcade et al., 2000). It is not expressed on EC (Roumenina et al., 2009), making MCP the only inhibitor with co-factor activity for FI on these cells. CR1 has 3 binding sites for C4b and 2 for C3b, and cumulates functions of both DAF and MCP. Indeed, CR1 accelerates dissociation of C3 and C5 convertases from CP and AP (Iida and Nussenzweig, 1981), and induces cleavage of C4b and C3b by working as a co-factor for FI (Forneris et al., 2016; Ross et al., 1982) (Figure 5). Moreover, CR1 is a unique cofactor of FI due to its ability to induce further cleavage of iC3b, generating C3c and C3dg degradation fragments (Lambris et al., 1996), ligands for CR2 expressed on B cells.

3.3. Regulation of the Membrane Attack Complex

MAC formation is strictly controlled to avoid accidental attack on host cell damage and subsequent activation (Schmidt et al., 2016). The vitronectin (S protein), and the clusterin (SP-40) inhibit C5b-9 formation by binding to C5b-7, C5b-8 and C5b-9, rendering it water-soluble and preventing membrane binding (Choi et al., 1989; Hadders et al., 2012; Preissner et al., 1989; Tschopp et al., 1993). C8 was suggested to play a dual role in C5b-9 formation and regulation. In the absence of a cell membrane, the binding of C8 to C5b-7 induces conformational changes that results in a loss of ability to form pores, thus playing a role of C5b-9 inhibitor (Nemerow et al., 1979).

The GPI anchored protein CD59 is expressed on most tissues and on all circulating cells (Fletcher et al., 1994; Leath et al., 2007). This inhibitor blocks membrane perforation by C5b-8 and C5b-9 (Farkas et al., 2002; Meri et al., 1990). CD59 does not bind free C8 and C9, but does interact with C5b-8 upon conformational changes into C8 and then blocks recruitment and polymerization of C9 (Huang et al., 2006; Lovelace et al., 2011; Wickham et al., 2011).

Furthermore, evidences of signaling capacity of CD59, leading to calcium release, have suggested its implication in cell homeostasis (Kimberley et al., 2007). Indeed, CD59 expression on NK-cells has been linked to enhanced killing capacity through trans-interaction with NKp46 and NKp30 (Marcenaro et al., 2003). Moreover, CD59 activation has been related to Lck signal transmission in T cells *in vitro* that enhances time of CD3 internalisation upon TCR activation (Lipp et al., 2014). However, these non-canonical functions of CD59 have never been studied *in vivo* yet, and thus require further investigations.

3.4. Platforms for alternative pathway activation

Medical interventions necessitate the use of specific materials. Biomaterial surfaces, biological and artificial interfaces, lipid surfaces and complexes as well as gas bubbles are all capable of improving the rate of hydrolysis of C3 to C3(H_2O) (Nilsson and Nilsson Ekdahl, 2012). This characteristic of C3 has to be taken into account during *in vitro* studies.

Hourcade and colleagues demonstrated that properdin is not merely a stabilizer of the C3 convertase, but also a pattern-recognition molecule that binds to microbial surfaces including GAGs, apoptotic, and necrotic cells (Kemper et al., 2010). This study provided evidence of a potential new role of properdin as a platform for C3 convertase assembly. Actually, this non-canonical role of properdin was suggested by Pillemer and colleagues in 1954 (Pillemer et al., 1954). However, a conflicting study published by Robert Nelson claimed an artefact effect of properdin due to immunoglobulin contamination (Nelson, 1958), thus burrying interest about properdin properties for decades.

It has been shown that activated platelets can activate complement system via classical (Peerschke et al., 2006) and alternative pathway (del Conde et al., 2005). Indeed, activation of platelets, by thrombin, C3a, collagen interaction or vWF, induces the release of components contained in their α-granules (Polley and Nachman, 1983; Rendu and Brohard-Bohn, 2001). These granules are enriched in complement components, such as C3, FD, FH and clusterin, as well as P-selectin (Davis and Kenney, 1979; Devine and Rosse, 1987). This protein is an adhesive protein, contained in α-granules of platelets and in weibel-palade bodies (WPB) of EC, rapidly excreted upon activation of these cells, promoting diapedesis of neutrophils. In 2005, Del Conde et al. described that P-selectin can bind to C3b, allowing alternative

complement activation activated platelet surface (del Conde et al., 2005). This phenomenon was confirmed on EC (Morigi et al., 2011) (Figure 6).

VWF, also contained in WPB of EC, has been demonstrated to serve as a platform for complement activation. First evidence came from *in vitro* study of histamine-induced EC degranulation (Turner and Moake, 2013). Indeed, ultra-large vWF (UL-vWF) strings released was shown to bind C3, FB, FD, FP and C5, as well as FH and FI, thus providing a link between thrombotic phenotype and complement activation. This has been recently confirmed, showing AP activation due to anchored-UL-vWF on EC with *ex vivo* serum from ADAMTS13 deficient patients (Bettoni et al., 2017).

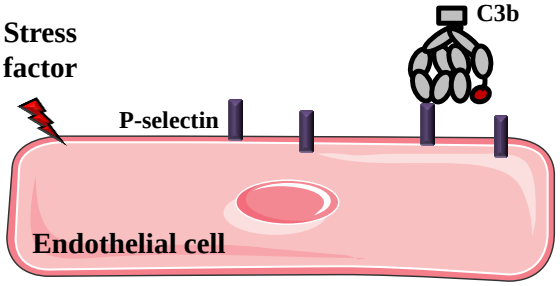

Figure 6: P-selectin expression on EC surface promotes complement deposition.

Same capacity of activation has been reported to platelet microparticles. These tiny vesicles from platelets membranes are implicated in inflammation (Barry et al., 1998) and hemostasis via their 50 to 100 times more procoagulant property compare to activated platelets (Sinauridze et al., 2007). They express P-selectin (George et al., 1986) and gC1qR/p33, allowing activation of AP (del Conde et al., 2005) and CP (Yin et al., 2008a), respectively.

Heme, the end-stage of hemolysis derived-product, has been described to be a potent inducer of C3 hydrolysis, inducing an effective C3(H_2O) able to bind FB and to form a C3 convertase in the fluid phase (Pawluczkowycz et al., 2007). Molecular docking of the heme to C3 revealed at least 3 potential determinant areas (Frimat et al., 2013). Because of its location, close to the TED domain, one model (binding site < 10Å) seems to be particularly relevant for explaining the potential role of heme as a new complement activator (Figure 7). Moreover, heme interacts with lipid structures (Roumenina et al., 2016a) and can bind to the cell membrane. It was suggested that this cell-bound heme can recruit C3(H_2O) and C3b to the cell surface (Frimat et al., 2013).

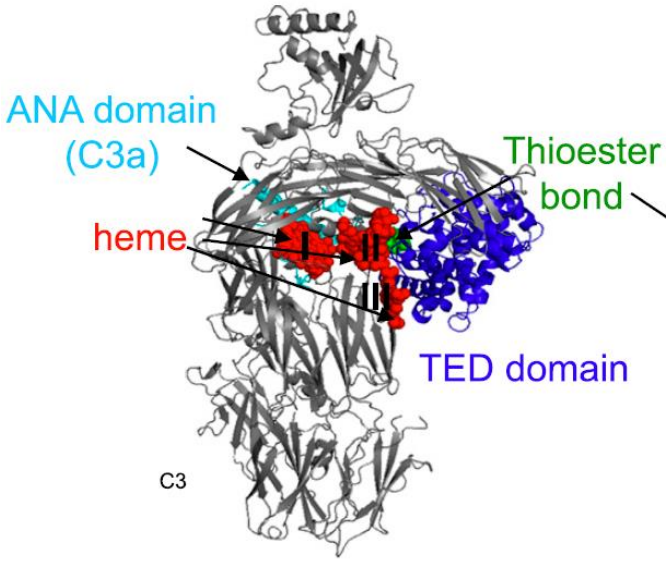

Figure 7: Heme-binding sites on C3 component (Frimat et al., 2013). Predictive software for binding affinity of free heme to C3 detected three potential zones. I: near the anaphylatoxin (ANA) domain; II: less than 10 Å from the thioester bond; III: located at the interface between Thioester (TED) and MG2 domains.

Thereby, complement system is a complex cascade of proteins, interacting with immune cells, erythrocytes, coagulation cascade, and blood barrier. The balance between activation and inhibition must be well orchestrated to avoid auto-reaction as well as pathogen invasion. Hemolysis is now considered as a new activator of alternative pathway, highlighting the need to gain insight into the mechanisms of this process to understand pathogenesis of hemolytic diseases.

II. Hemolysis: a pathological event

1. Cascade and Regulation

1.1. Activation

1.1.1. Oxydation steps

The red blood cells contain hemoglobin (Hb), which plays an essential role of gas carrier in the organism. This 64 kDa protein is made of four globin chains (two α and two β), and each of them is bound to a prosthetic group (a cofactor): heme. Heme is an ubiquitous iron-containing compound, present in large amount in many cells and organisms. It is a tetrapyrrole ring – also called protoporphyrin IX – with an iron (Fe^{2+}) atom bound in the center, considered as a versatile biological catalyst in many reactions such as oxidation, electron transfer processes and molecular oxygen (O_2) delivery to tissues, due to the capacity of its Fe (which is the site of oxygen binding) to be chelated. For these reasons, heme is contained in a hydrophobic pocket in order to maintain it in a Fe^{2+} state during oxygenation/deoxygenation steps (Figure 8). Heme is attached in the hydrophobic pocket on one side to an amino acid residue histidine (His 63), and a second histidine (His 92) on the other side of the pocket (Marengo-Rowe, 2006). Thus, heme plays major functions in the metabolism and in the cellular physiology.

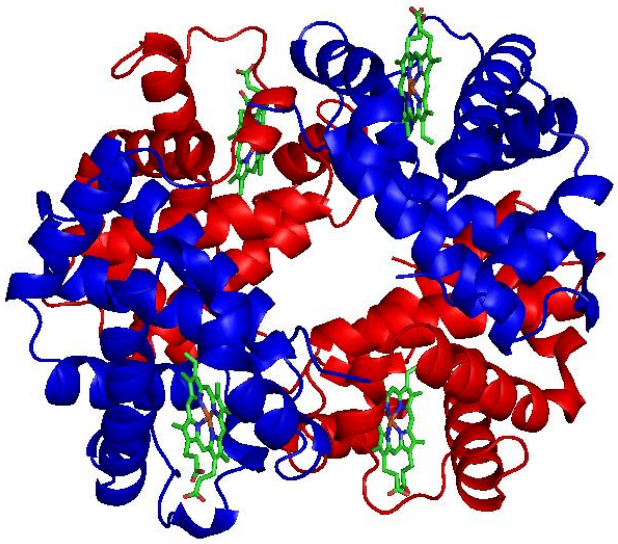

Figure 8: DeoxyHemoglobin structure. Structure obtained from PDB (code: 1A3N), cartoon representation of α and β chains, heme is represented as sticks with PyMol. Red: α-chain; Blue: β-chain (Tame and Vallone, 2000)

1.1.2. Extravasacular hemolysis

Physiological hemolysis happens in extravascular compartment. After 120 days in the circulation, senescent erythrocytes are phagocytized by macrophages in bone marrow, spleen and liver (Bratosin et al., 1998). Hb is degraded, globin's amino acids are recycled, iron is stored in macrophages by binding to the cytosolic ferritin and the protoporphyrin IX is catabolized into biliverdin and free bilirubin. Free bilirubin is then delivered to the liver by albumin (Alb) to be eliminated in faeces.

1.1.3. Intravascular hemolysis

Intravascular hemolysis represents 10-20% of the total physiological hemolysis (Garby and Noyes, 1959), exposing host cells to free Hb harmful effect. During massive intravascular hemolysis, red blood cells destruction leads to release of Hb in the circulation. Once released, Hb goes through different oxidation stages that finally induce the release of free heme. This results in oxidation of lipoprotein which leads to toxic effects on cells, vascular dysfunction, oxidative tissue damage and altered inflammatory response. The first form of Hb released is oxyHb(Fe^{2+}), also called ferrousHb(Fe^{2+}), due to its oxygenation state (the Fe of heme is still bound to oxygen) (Figure 9).

The presence in the vessels of oxyHb(Fe^{2+}) provokes nitric oxide (NO) depletion. Indeed, the high affinity of oxyHb(Fe^{2+}) for NO leads to an irreversible reaction of deoxygenized NO, generating ferricHb(Fe^{3+}) (also called MetHb) and NO_3^-. OxyHb(Fe^{2+}) can also spontaneously auto-oxidized, releasing ferricHb(Fe^{3+}) (MetHb) and a superoxide radical O_2^- (Deuel et al., 2015). Free heme is then released from ferricHb(Fe^{3+}), leading to heme-driven oxidation of unsaturated fatty acids and so to endothelial damage.

A last oxidation can produce ferrylHb(Fe^{4+}), which is unstable and may return to the Fe^{3+} state by reacting with specific amino acids of the globin chains. It has been demonstrated that the accumulation of toxic lipid peroxidation products (oxidative degradation of lipids) ultimately leads to endothelial damage, with specific gene expression pattern, reduced cellular ATP and monolayer disintegration (Deuel et al., 2015). Monocytes, macrophages and neutrophils are also affected by the pro-oxidative and pro-inflammatory effects of heme, resulting in their activation and recruitment.

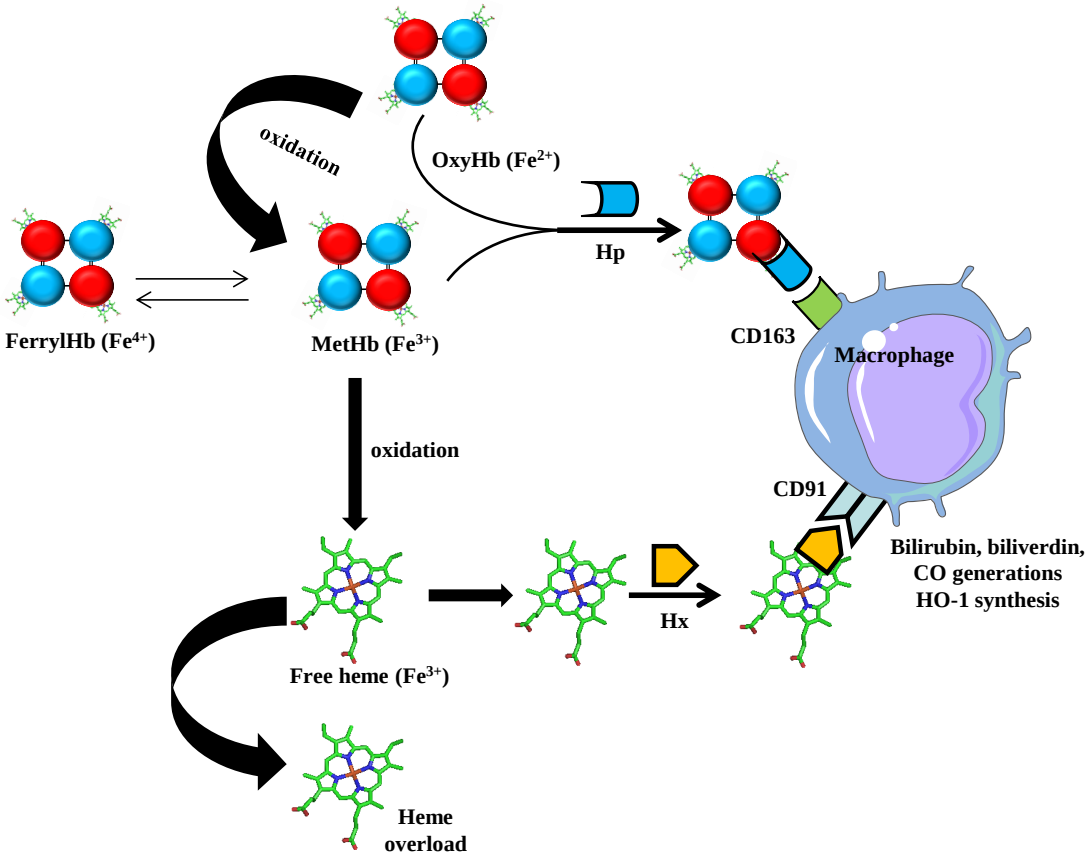

Figure 9: Oxidation steps and scavenging of hemolysis-derived products. Circulating OxyHb(Fe^{2+}) is oxidized into MetHb(Fe^{3+}), which both can be scavenged by haptoglobin (Hp). The complex will be internalized upon recognition by CD163 expressed on macrophages. Overload of MetHb leads to the release of free heme in the circulation, which can be scavenged by hemopexin (Hx). The complex is recognized by CD91 expressed on macrophages to degrade heme. Phagocytosis through Hp and Hx is needed to generate bilirubin, biliverdin and HO-1 synthesis. Upon internalization, both Hp and Hx are not recycling upon degradation of RBC breakdown products, leading to heme overload in the circulation.

1.2. Inhibition

1.2.1. Hemoglobin scavenging

To avoid tissue damage caused by hemolysis, different scavengers are present in the blood and are specific to certain hemolysis products. Haptoglobin (Hp) is the first line of defense against free Hb. Hp is a plasmatic glycoprotein (0.3-3 g/l) of about 64 kDa that belongs to acute inflammation proteins. Its expression is inducible under pro-inflammatory cytokines (Raynes et al., 1991). Hp is mainly produced by the liver, but can be also locally produced by the kidney (Kalmovarin et al., 1991). It is composed of 4 subunits α and β, organized in dimers αβ, linked by a disulfide bond on the subunit. Hp is a scavenger that

binds free Hb with a very high affinity, though non-covalent, with a dissociation constant (Kd) of 10^{-15}M (Lim et al., 2001). This binding provides a protection by preventing free heme release. The mechanism is likely related to the structural stabilisation of Hb within the Hp:Hb complex. These complexes are recognized by the cluster of differentiation receptor 163 (CD163) expressed on mature tissue macrophages present in the spleen, liver, lymph nodes, bone marrow, lung, placenta, peritoneum, thymus, and on Kupffer cells in the liver. This recognition promotes the complex endocytosis by macrophages, thus leading to globin degradation in lysosomes and heme catabolism by heme-oxygenase 1 (HO-1) in cytosol (Figure 9). It has been established that at least iso-stoichiometric quantities of Hp in relation to oxyHb(Fe^{2+}) are necessary to provide a protection (Deuel et al., 2015). *In vitro*, Hp could not block lipid peroxidation beyond Hb concentration exceeding the binding capacity of Hp. In this case, Hb oxidation can occur and leads to ferricHb(Fe^{3+}), and so to free heme release.

1.2.2. Heme scavenging

Once Hp is saturated, free Hb is oxidized and release free heme that is rapidly scavenged by a second protein implicated in the detoxification process: the hemopexin (Hx). Hx is a plasmatic glycoprotein (0.5-1.15 g/l) (Tolosano and Altruda, 2002), of about 60 kDa. Hx is mainly produced by the liver, but also by central nervous system cells (Morris et al., 1993), retinal photoreceptor cells and renal mesangial cells (Tolosano et al., 2010). Hx production is induced by inflammation.

Hx is a scavenger that binds free heme with a very high affinity (Kd< 10^{-13}M) (Lin et al., 2015; Schaer et al., 2014), which makes it virtually irreversible. Upon heme:Hx complex formation, Hx undergoes a conformational change allowing to bind CD91, a receptor that belongs to LDL receptor superfamily (Hvidberg et al., 2005). Around 40 different ligands of CD91 have been identified, and so several functions (Lillis et al., 2005). This receptor is expressed on macrophages and hepatocytes, but also on fibroblasts, adipocytes and neurons (Moestrup et al., 1992). Binding of the heme:Hx complex to CD91 results to endocytosis where a part of Hx may be recycled (majority is degraded in the lysosome as Hp), heme is degraded and its iron re-claimed. These cells express HO-1 that leads to the degradation of free heme and the release of anti-inflammatory components (biliverdin is then transformed

into bilirubin, and carbon monoxide, CO). Free iron is released in the cell and complexes to intracellular ferritin (Figure 9).

Furthermore, the protection provided by Hx has a specific biochemical function. Indeed, other proteins are able to bind heme with lower affinity. For instance, Alb, a main circulating protein in the blood, has also a high affinity for heme ($Kd < 10^{-9}M$). However, it has been shown that binding of Alb to heme is reversible and can transfer heme to Hx (Ascenzi et al., 2015). Sub-stoichiometric quantities of Hx are sufficient to significantly attenuate Hb triggered lipid peroxidation and endothelial damage (Deuel et al., 2015). *In vitro*, Hx exhibited a dose-dependent protection of endothelial monolayer integrity. In this cascade, Hp acts as a primary protection system which handles all negative activities of free Hb. Once all the Hp is saturated, Hx operates as a backup protection system, providing sustained control of heme-driven oxidative damage. Hx is unused until the capacity of the primary protection system is depleted.

1.2.3. Heme oxygenase-1

Heme-oxygenase is the principal physiological mechanism of heme degradation, whose catabolism constitutes a defense mechanism against oxidative stress induced by heme. Indeed, carbon monoxide and bilirubin have powerful anti-oxidant properties (Olsson et al., 2012).

Two isoforms of HO have been described – HO-1 and HO-2. HO-1 and HO-2 are differentially expressed and distributed within tissues and have a catalytic activity towards heme.

HO-2 expression is constitutive, particularly in central nervous system cells (Ryter et al., 2002). On the contrary, HO-1 is inducible. It has an ubiquitously intracellular expression, on the endoplasmic reticulum membrane. HO-1 expression is induced by oxidative stress, and by recognition of Hb:Hp and heme:Hx complexes by their own receptors (Gozzelino et al., 2010). Rapid increase of HO-1 expression upon cellular stimulation is regulated by several transcriptional factors, such as Nrf-2 (Tong and Zhou, 2017).

In case of intense hemolysis, large amount of free heme is released in the circulation, leading to cell damages. In cases of extensive and chronic hemolysis, levels of Hp and Hx in

plasma of patients have been demonstrated to markedly decrease. Accordingly, high concentrations of free heme (20-50 µM) in plasma of patients with severe hemolytic events may be achieved (Muller-Eberhard et al., 1968; Reiter et al., 2002). In mouse models of hemolytic diseases this concentration reaches 75 -100 µM (Vinchi et al., 2013).

2. Toxicity

Free Hb and free heme have multiple effects on coagulation system, endothelium, as well as on tissues, particularly on the kidney. Their effects are time and concentration dependent, which underlines different phenotypes regarding a massive acute hemolysis against a chronic background hemolysis. Release of Hb and heme drives pro-thrombotic and pro-inflammatory vascular phenotypes.

2.1. Depletion of nitrogen oxide

Nitrogen oxide (NO) is a key actor of vascular homeostasis. Indeed, NO is a powerful inducer of endothelium vasodilation (Furchgott and Vanhoutte, 1989; Palmer et al., 1987). Moreover, NO is implicated in smooth muscular cell migration and proliferation, leucocyte adherence and hemostasis. Endothelium NO synthesis is controlled by endothelial NO Synthase (eNOS), a constitutive enzyme that catalyzes oxidation of L-Arginine to NO and L-citrullin in presence of NADPH. eNOS can be activated via different mechanisms. The first one is a calcium-dependent pathway, where increase of intracellular Ca^{2+} induced by mediators (acetylcholine, bradykinin), releases eNOS from caveoline. The second one is regulated by shear stress and VEGF receptors (Dimmeler et al., 1999; Jin et al., 2003). Afterwards, NO diffuses through cells membranes into smooth muscle cells to induce cell relaxation by increasing intracellular rate of cGMPc (cyclic Guanosine MonoPhosphate) (Furchgott and Vanhoutte, 1989).

Hypoxic environment, subsequent to NO depletion by oxyHb has been described to induce vasoconstriction (Doherty et al., 1998; Minneci, 2005; Olson et al., 2004) (Figure 10). Thus, from this alteration of vascular tonus results systemic arterial or pulmonary hypertension as well as a modification of organ perfusion (Deem et al., 2002; Minneci, 2005; Vogel et al., 1986). Inhibition of NO/Hb interaction, using recombinant Hb in rat, has shown a decrease vasoconstriction and arterial hypertension (Doherty et al., 1998).

The dimeric form of Hb, due to its low molecular weight (32 kDa), induces toxicity by diffusing through the endothelium via sub-endothelial and peri-vascular space (Nakai et al., 1998). Several studies have shown a decrease of the vasoactive effect of free Hb upon polymerization due to an increased molecular weight (Doyle et al., 1999). For instance, one of the inhibitory effect mediated by Hp is the high molecular weight complex formation (>150 kDa), which remains in blood vessels (Schaer et al., 2013).

2.2. Oxidative stress

Oxidation of Hb generates degradation-product carrying Fe^{3+} or Fe^{4+} (ferricHb) and then free heme that generates ROS. These products have pro-oxidant properties and affect plasmatic, membrane or intracellular components. For instance, free heme has been described to induce DNA damages through oxidative stress. Free heme catalyzes also oxidation and aggregation of proteins, thus leading to protein degradation into small peptides (Kumar and Bandyopadhyay, 2005). Moreover, it can induce lipid peroxidation. For instance, heme has a high affinity for lipoproteins, like LDL, that recruits macrophages upon oxidation, leading to endothelium injury and participates to development of atherosclerosis (Balla et al., 2000; Jeney et al., 2002).

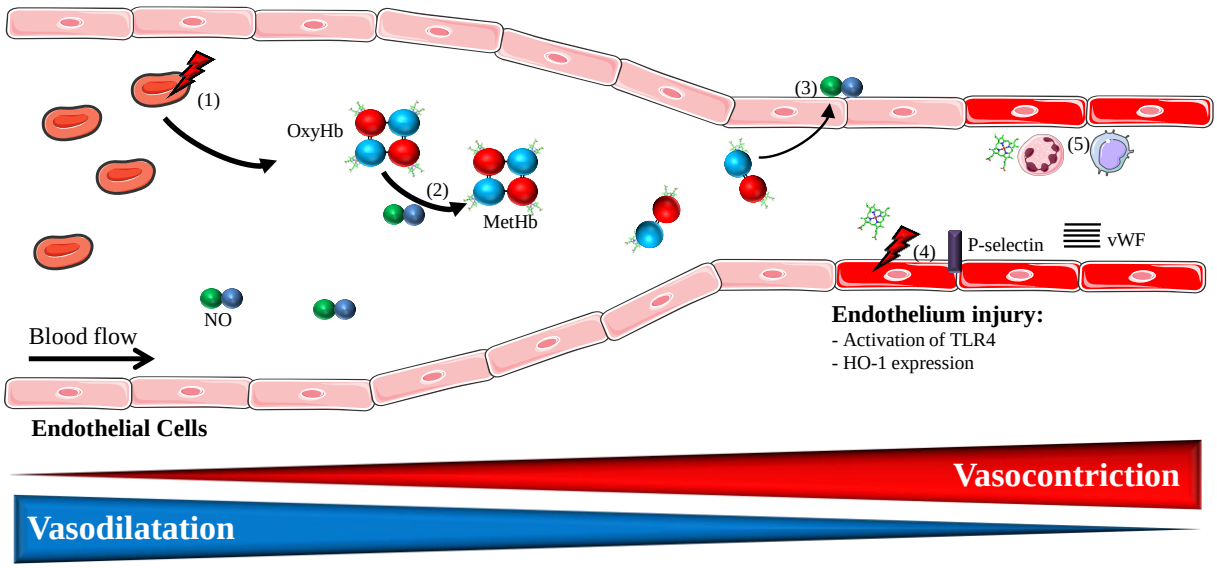

Figure 10: Harmful effects of hemolysis *in vivo*. (1) Hemolysis induces release of RBC content like OxyHb; (2) OxyHb is converted into MetHb by binding to NO, inducing its depletion; (3) dimers of Hb diffuse through endothelium barrier and deplete NO; (4) Free heme is released and mediates endothelial injury, P-selectin and vWF exocytosis from WPB; (5) Free heme promotes recruitment and activation of neutrophils and macrophages activation through TLR4.

3. Heme: a potent Danger-Associated Molecular Pattern

Heme acts as a prototypic Damage-Associated Molecular Pattern (DAMP) that is recognized by Pattern Recognition Receptors (PRR), mediating inflammatory processes by targeting innate immune cells and EC.

3.1. Activation of innate immune cells

3.1.1. Targeting neutrophils

3.1.1.1. Neutrophils

In humans, polymorphonuclear neutrophils (PMN) are continuously generated in the bone marrow and represent 50-70% of circulating leukocytes, making neutrophils major effectors of acute inflammation (Kolaczkowska and Kubes, 2013). Neutrophils contain three types of granules: azurophilic (primary), specific (secondary) and gelatinase (tertiary) granules, containing myeloperoxidase (MPO), lactoferrin, and metalloproteinase 9 (MMP9), respectively.

During inflammation, neutrophils eliminate pathogens by three different mechanisms. They can internalize microorganisms in phagosomes to kill them in a NADPH oxygenase-dependent manner, generating reactive oxygen species (ROS), or using antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme) (Borregaard, 2010; Häger et al., 2010). In addition, antibacterial proteins can be degranulated and act on extracellular pathogens. Finally, a terminal activation state of neutrophils has been described, called neutrophil extracellular trap (NET). This strong activation of neutrophils leads to the release of intracellular structures such as chromatin DNA, histones and granular proteins. Neutrophils can be recuited into tissues, following 4 major steps: rolling, by recognition of adhesion marker such as P-selectin on endothelium; adhesion, by interaction with chemokines present on endothelium; crawling, and finally transmigration (Kolaczkowska and Kubes, 2013).

Recently, heme overload has been demonstrated to induce NETs formation *in vitro* and *in vivo*, which was inhibited by the use of Hx (Chen et al., 2014a; Kono et al., 2014; Ohbuchi et al., 2017). Interestingly, this terminal-activated state of neutrophil was ROS dependent, since anti-oxidant N-acetyl-L-cysteine (NAC) abrogated heme-induced NET

formation. Interestingly, the implication of G protein-coupled receptors (GPCRs) in heme-mediated neutrophil activation process has been suggested.

3.1.1.2. Involvement of GPCR in heme sensing

GPCRs history closely evolved with the emergence of the concept of receptors (Lefkowitz, 2013). Existence of "receptors" has been largely accepted only 30 years ago. However, the notion of cellular receptor appeared at the beginning of the 20th century, when the British pharmacologist, J. N. Langley wrote in 1905: "*So we may suppose that in all cells **two constituents** at least are to be distinguished. The **chief substance** which is concerned with the chief function of the cell as contraction and secretion and **receptive substances** which are acted upon by chemical bodies and in certain cases by nervous stimuli. The receptive substance affects or is capable of affecting the metabolism of the chief substance*". Skepticism of the scientific world slowed down the progression of receptor research. Ironically, J. N. Langley's student, Henry Dale, Nobel Prize in medicine in 1936 for his research on cholinergic transmission; and later Raymond Ahlquist, awarded with Lasker Prize in 1948 for his work on adrenergic receptors, were both eminent skeptic researcher about receptors existence. Even 100 years later, GPCRs, constituting the largest protein receptor family (around 800), still counts 150 orphan receptors. In 2012, R. Lefkowitz and B. Kobilka won the Nobel Prize in chemistry for the crystallization of a bovine G-protein-coupled Receptor Kinase (GRK) (Lodowski et al., 2003) and human β2 Adrenergic Receptor (Rasmussen et al., 2007), respectively, opening a new field for investigating and understanding GPCRs.

GPCRs are seven transmembrane domain receptors ubiquitously expressed, linked to three G proteins subunits – G_{α}, G_{β} and G_{γ} – in the intracellular part of the receptor (Rosenbaum et al., 2009). Commonly, upon recognition of a specific ligand outside the cell, GDP bound to Gα protein is exchanged into GTP, allowing dissociation of β and γ subunits and initiation of signaling pathways dependent on intermediates like cAMP and phosphatidylinositol, for example. $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ are 4 different Gα-subunits that have been identified, initiating different signaling pathways. GPCRs ligands are mainly small molecules, implicated, for instance, in metabolic system, sensory signals, and inflammatory pathways. These receptors are expressed in a large number of cell types implicated in sensory signals, metabolic system, homeostasis, and regulation of the immune

system, by inducing anti- or pro-inflammatory pathways. For instance, activation of GRKs, a subfamily of GPCRs signaling without Gα proteins, has been shown to induce NFκB and MAPK pathways (Packiriswamy and Parameswaran, 2015).

In hemolytic models, it has been demonstrated that heme acts as a chemokine, by inducing neutrophils recruitment in GPCRs-dependent manner *in vitro* and *in vivo* (Graça-Souza et al., 2002; Porto et al., 2007). Indeed, using pertussis toxin (PT), a powerful inhibitor of both $G_{\alpha s}$ and $G_{\alpha i/o}$ GPCRs, authors observed a lack of neutrophil migration (Porto et al., 2007). In addition, ROS production by neutrophils was determined to be $G_{\alpha i}$ and PKC dependent (Porto et al., 2007).

3.1.2. Activation of monocytes/macrophages

3.1.2.1. Monocytes

Monocytes are circulating innate immune cells, representing 10% of nucleated cells in the blood (Ginhoux and Jung, 2014). Moreover, marginal pools are found in the spleen and lungs that can be rapidly mobilized. These cells play a key role during inflammation, and constitute a precursor reservoir for tissue-resident macrophages, which have a high phagocytic activity (van Furth and Cohn, 1968; Yang et al., 2014).

Monocytes are present in the blood, defined as $CD14^{+}$ cells, subdivided into two distinct populations ($CD14^{hi}$ and $CD14^{low}$) differing from capacities to secrete inflammatory cytokines (Ginhoux and Jung, 2014). Indeed, $CD14^{hi}$ represent classical monocytes that are essential for response against inflammation and are precursors of peripheral mononuclear phagocytes (Ziegler-Heitbrock and Hofer, 2013), while evidences suggest a capacity to protect endothelial integrity for $CD14^{low}$ subpopulation (Auffray et al., 2007; Carlin et al., 2013). By opposition, tissue-resident macrophages are found in all tissues and are uncapable of circulate in the blood, but insure development, homeostasis, tissue repair and local immunity (Wynn et al., 2013).

Both monocytes and macrophages have the crucial role in T cell response (Randolph et al., 2008). These cells can phagocyte pathogens, which are degraded in phagosomes in order to promote Ag presentation on HLA II, thus orchestrating the mounting of an adaptive

immune response. Moreover, both monocytes (Rutkowski et al., 2003) and macrophages (Kalupahana et al., 2005) can promote inflammation by recognition of danger-associated molecular patterns (DAMP) via their pattern recognition receptor (PRR) – including recognition of LPS, the natural ligand of Toll-like receptor 4 (TLR4). Indeed, recognition of DAMP leads to induction of intracellular pathways that promotes expression of activation markers, such as CD80 and CD86, and synthesis of pro-inflammatory cytokines. However, evidences of monocytes and macrophages activation have been observed in response to self antigen. For instance, an activation profile of monocytes from SCD patients during vaso-occlusion crisis (VOC) was detected, including increased IL1-β and TNFα production, suggesting a potential role of hemolysis as a trigger of monocyte activation (Belcher et al., 2000). Since, several studies have demonstrated the importance of TLR4 in the heme-induced monocytes/macrophages activation.

3.1.2.2. Toll-like receptors – history of these "great" receptors

The discovery of TLR4 is attributed to the work of J. Hoffmann and B. Beutler, awarded with Nobel Prize in medicine in 2011. Indeed, TLR4 was already identified as a key receptor in embryonal development of drosophila in 1985 by Christiane Nüsslein-Volhard, for which she received Nobel Prize in physiology in 1995 (Anderson et al., 1985). Upon her discovery, she named this receptor "Toll-like receptor", litteraly "Great-like receptor". Implication of TLR4 in immune response was characterized in 1996, when B. Lemaître, from the team of J. Hoffmann, fortuitously found a parent tree of drosophila incapable of reacting against infection (Lemaitre et al., 1997). By genetic analysis, they detected a mutation located in Toll receptor gene. In 1998, the team of B. Beutler determined the specific ligand of TLR4 in mice carrying a mutation in this Toll-receptor, conferring a resistance to lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (Poltorak et al., 1998). In the following years, from 1999 to 2003, TLRs 1, 2, 3, 5, 6, 7 and 9 ligands were discovered (Akira and Takeda, 2004) (see Table 1).

Receptor	Localisation	Natural ligand	Origin of ligand
TLR1	Plasma membrane	Triacyl lopopetides Soluble factors	Bacteria and mycobacteria Neisseria Meningitidis
TLR2	Plasma membrane	Lipoproteins Peptidoglycans	Various Pathogens Gram-positive bacteria
TLR3	Endosome	dsRNA	Viruses
TLR4	Plasma membrane & endosome	LPS Taxol Fusion Protein Heme HMGB1	Gram-negative bacteria Plants Respiratory syncytial virus Host Host
TLR5	Plasma membrane	Flagellin	
TLR6	Plasma membrane	Diacyl lipopeptides Zymosan	Mycoplasma Fungi
TLR7	Endosome	ssRNA	Viruses
TLR8	Endosome	ssRNA	Viruses
TLR9	Endosome	CpG DNA	Bacteria and viruses
TLR10	Plasma membrane	N.D. Lipopeptides	Influenza virus Bacteria

Table 1: Toll-like receptors and their natural ligands - (adapted from Akira & Takeda, 2006)

Nowadays, 10 different TLRs have been identified in humans and belong to the family of Pattern-Recognition Receptors (PRRs) (Akira et al., 2006). TLRs are expressed on innate immune cells, such as monocytes, macrophages and dendritic cells (Akira et al., 2006), as well as on EC (Fitzner et al., 2008). We can separate TLRs in two categories, the ones expressed on the cell surface and the ones - on endosomes membranes, having different properties. All TLRs recognize molecules, commonly named Pathogens-Associated Molecular Patterns (PAMP), and induce different signaling pathways dependent on their location. For instance, cell surface TLRs recognize extracellular particles, such as pieces of bacteria membranes, and induce MyD88/IRAK (Myeloid differentiation primary response 88/Interleukin-1 Receptor-Associated Kinase) dependent pathways leading to phosphorylation of MAPK, p38 and NFκB (Fitzgerald et al., 2001). These pathways are associated with synthesis of pro-inflammatory cytokines, such as IL-6, IL-1α and IL-1β. In contrast, endosomal TLRs recognize intracellular motifs like single and double strands of DNA and RNA, inducing TRAM/TRIF dependent pathways leading to translocation of IRF-3 and induction of type I interferon (O'Neill et al., 2013).

3.1.2.3. Functions of TLR4

TLR4 is expressed at basal level or is inducible on DC, monocytes, macrophages, neutrophils, EC, epithelial cells and keratinocytes, reacting against LPS. LPS is composed of an amphiphatic lipid A component, a core polysaccharide of six sugars and a O antigen (Raetz and Whitfield, 2002). Lipid A represents the conserved molecular pattern of LPS and is the main inducer of immunological responses to LPS (Figure 11).

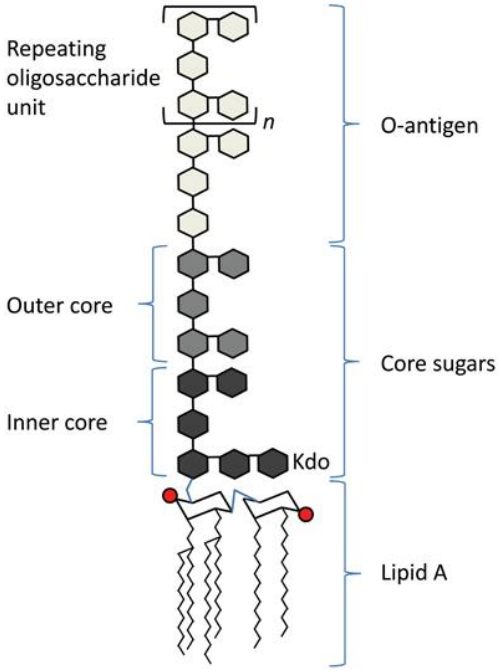

Figure 11: Schematic representation of LPS. LPS is composed of an amphipathic lipid A component, a core polysaccharide of six sugars and a O antigen.

TLR4 activation necessitates the intervention of many actors. In the circulation, Lipopolysaccharide Binding Protein (LBP) binds to LPS with a high affinity (Mathison et al., 1993; Schumann et al., 1990). LBP can extract LPS from bacterial membranes (Vesy et al., 2000), or catches LPS to address it to CD14 (Wright et al., 1990). CD14 is a GPI-anchored co-receptor of the TLR4, mainly expressed on myeloid cells (Figure 12). Although CD14 expression was firstly thought to be exclusive of myeloid cells, studies have documented that CD14 is expressed on HUVEC (Jersmann et al., 2001), corneal (Song et al., 2001) and liver sinusoidal EC (Gong et al., 2002) as well as on epithelial cells (Song et al., 2002). CD14 is also found in soluble form in serum upon activation of monocytes (Shive et al., 2015) and epithelial cells (Funda et al., 2001). If sCD14 function was described to promote epithelial cells activation in presence of LPS (Landmann et al., 1996), it is now more accepted that it is generated by cleavage from the cell surface or released from intracellular pools, and is used as

a marker of monocytes activation (Shive et al., 2015). Without LBP, LPS binding to sCD14 is poorly efficient (Fujihara et al., 2003; Viriyakosol et al., 2001). Membranous CD14 is essential for presentation of LPS to TLR4:MD2 complex and to induce inflammatory response (Gioannini et al., 2004). Moreover, it has been shown that CD14 controls endocytosis of TLR4 induced by LPS (Zanoni et al., 2011), responsible for TRAM signaling pathways.

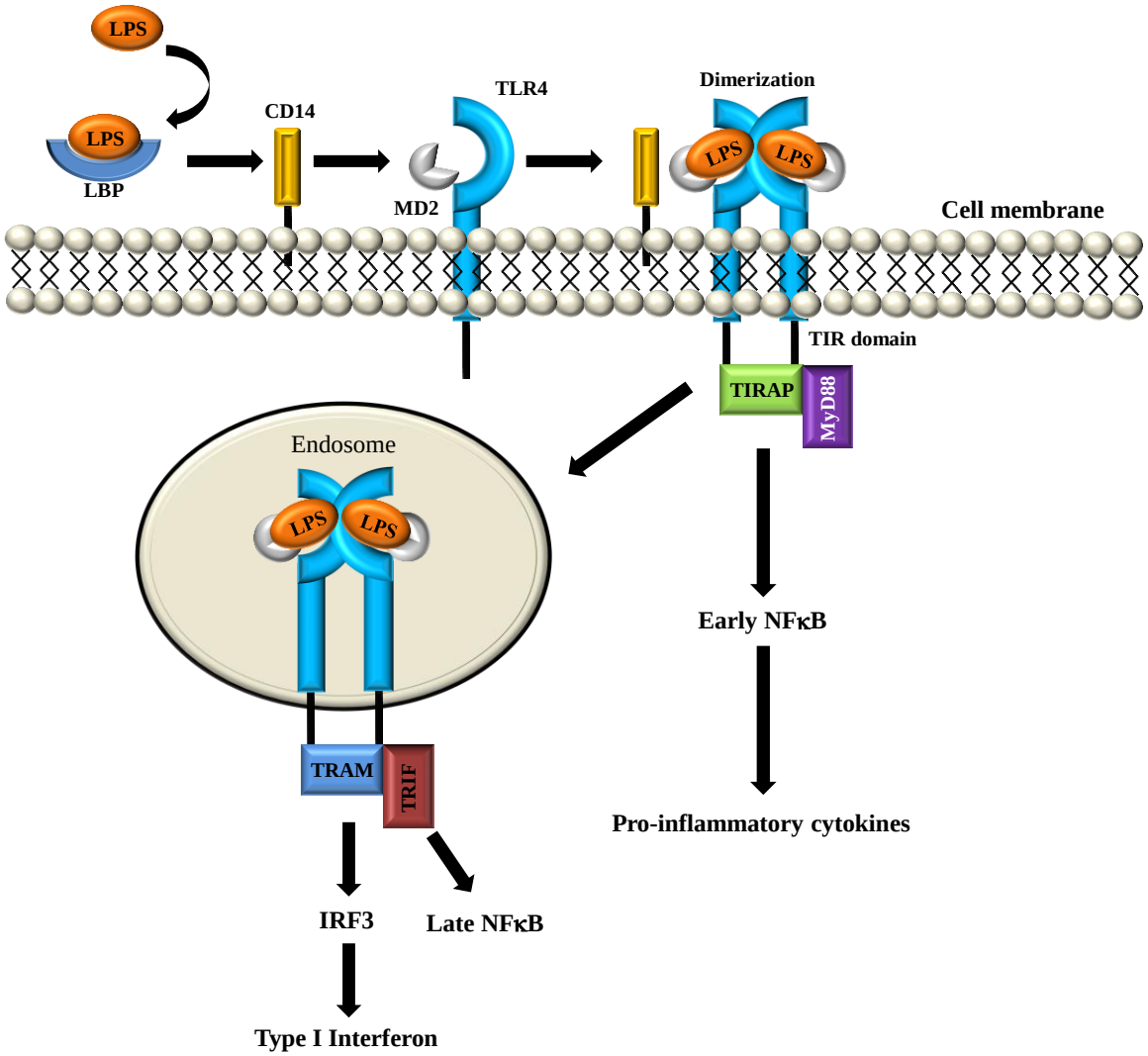

Figure 12: Toll-like receptors 4 signaling pathways. LBP can extract LPS from bacterial membranes or catches LPS to address it to CD14. Membranous CD14 is essential for presentation of LPS to TLR4:MD2 complex and to induce inflammatory response through TIRAP/MyD88 adaptive molecules. Moreover, CD14 controls endocytosis of TLR4 induced by LPS responsible for TRAM signaling pathways.

MD2 is a TLR4-associated glycoprotein that provides a hydrophobic pocket able to bind fatty acids. Thus, without MD2, TLR4 is not capable of inducing signaling pathway in response to LPS (Kawasaki et al., 2000; Schromm et al., 2001; Shimazu et al., 1999). Only

monomers of LPS – containing one lipid A and one polysaccharide chain – can bind to MD2 (Teghanemt et al., 2005). Five of the six lipid chains of LPS bind to the hydrophobic pocket MD2, the sixth chain remaining exposed to the surface to form a hydrophobic interaction with the two conserved phenylalanines (F440 and F463) of TLR4, forming a core hydrophobic interface (Park et al., 2009). Thereafter, the close proximity between TLR4 and MD2 allows formation of interactions between hydrophilic residues, forming hydrogen bonds and ionic interactions to support the hydrophobic core. These interactions lead to dimerization of two TLR4:MD2 complexes, linking two intra-cytoplasmic TIR domains (Toll-Interleukin-1 Receptor). On cell surface, TIR linkage recruits adaptive molecules, such as MyD88 to initiate intracellular signaling pathways, inducing the production of pro-inflammatory mediators such as TNF-α, IL-6, IL-12, and IL1-β (Figure 12).

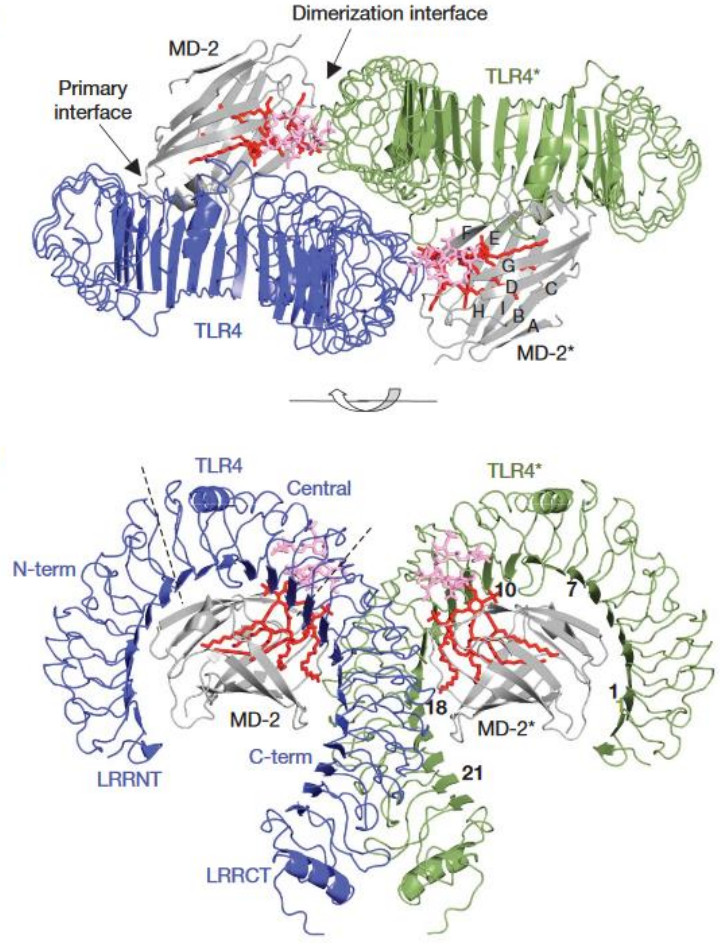

Figure 13: Overall structure of the TLR4:MD2 complex with LPS (Park et al., 2009). LPS binds to the hydrophobic pocket of MD2 in the TLR4:MD2 heterodimer. The sixth chain of LPS forms a hydrophobic interaction with the two conserved phenylalanines (F440 and F463) of TLR4 (dimerization interface) and allows dimerization of a quaternary complex TLR4:MD2/TLR4*:MD2*.

3.1.2.4. Emergence of therapeutics against TLR4

Studies about LPS and TLR4 have highlighted the crucial role of TLR4 in the mounting of immune response against pathogens. However, evidence emerges also about the implication of TLR4 on systemic inflammatory responses, such as septic shock (Cristofaro and Opal, 2003), encouraging public and private laboratories to search for ways to inhibit TLR4 activation by LPS (Figure 14).

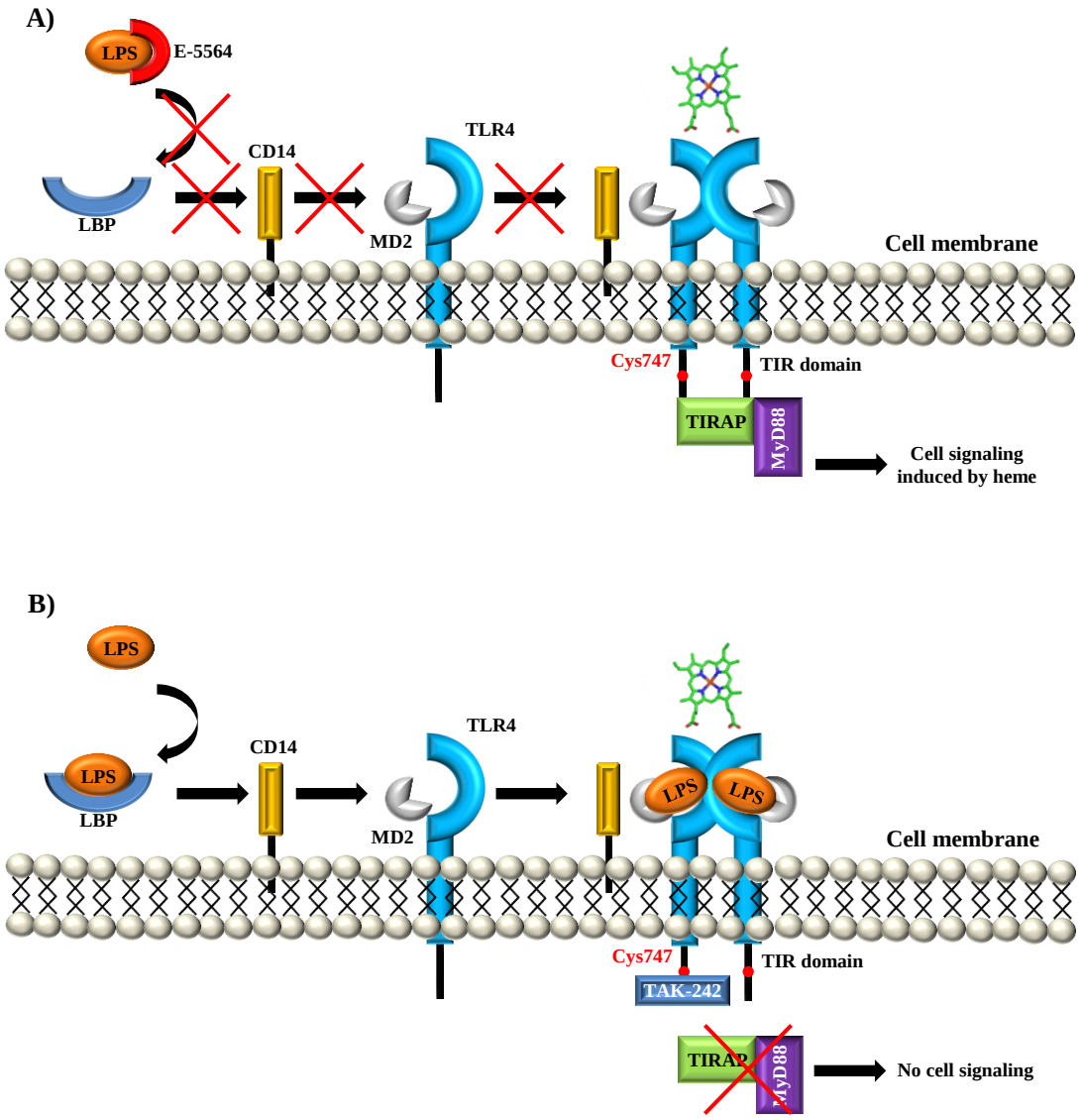

Figure 14: Inhibitory effects of E-5564 and TAK-242. A) Synthetic inhibitor E-5564 binds to LPS and prevents its binding to TLR4. However, free heme would still be able to induce TLR4 activation. B) By binding to the specific amino acid of TLR4 intracellular tail, Cys747, TAK-242 prevents all possibilities of recruitment of adaptive molecules.

Nowadays, several molecules have enriched the panel of possibilities to prevent TLR4 activation (Savva and Roger, 2013). Chemical industry provides synthetic molecules able to specifically block TLR4. Eritoran-E5564 is the second generation of a lipid A antagonist molecule, blocking pro-inflammatory cytokines production and reducing clinical symptoms in a phase 1 clinical trial (Liang et al., 2003; Lynn et al., 2004; Rossignol et al., 2004, 2008) (Figure 14A).

TAK-242 – also known as CLI-095 – exhibited promising therapeutic properties improving septic shock symptoms in guinea pigs (Kuno et al., 2009). This small molecule binds to a specific cysteine (Cys747) in the TIR domain of TLR4, preventing both MyD88 – dependent and –independent pathways activated by LPS (Matsunaga et al., 2011; Takashima et al., 2009). However, both molecules failed clinical trials due to inefficacy of the treatment in septic patients, pointing out the aggressiveness of the disease (Rice et al., 2010; Tidswell et al., 2010) (Figure 14B).

If Eritoran-E5564 has a preventing effect on the canonical LPS-induced TLR4 activation, we can expect an inefficiency of this synthetic inhibitor on heme-induced TLR4 signaling. However, TAK-242 opens a new field of investigation for studying the implication of TLR4 as a receptor sensing new ligands.

3.1.2.5. Heme as a potent trigger of TLR4 signaling pathways

First evidence of heme as a new ligand for TLR4 was described in 2007 *in vitro* (Figueiredo et al., 2007). Authors found that free heme can promote synthesis of TNF-α by macrophages through a specific binding to TLR4. Contrary to LPS, TLR4 activation by heme was shown to be MyD88-dependent but independent on MD2, since the use of an anti-TLR4/MD2 antibody (Ab) only prevented LPS-induced TNF-α secretion (Figure 15A). By contrast, TLR4 activation by heme was abrogated in $CD14^{-/-}$ macrophages, while a weak inflammatory response was still detected in presence of LPS (Figueiredo et al., 2007). *Ex vivo*, heme is capable of polarizing macrophages by promoting phenotype switch to type 1 (M1) and even switching from type 2 (M2) to type 1 (M1) macrophages through TLR4 (Vinchi et al., 2015). Indeed, the use of the TAK-242 prevents the switch of the phenotype (Figure 15).

Even if ferricHb seems to have a weak oxidative potential, some recent studies have shown that ferricHb can induce signaling pathway through TLR4 receptor in microglia (Kwon et al., 2015). Also in astrocytes, heme was suggested to activate TLR2 (Min et al., 2017).

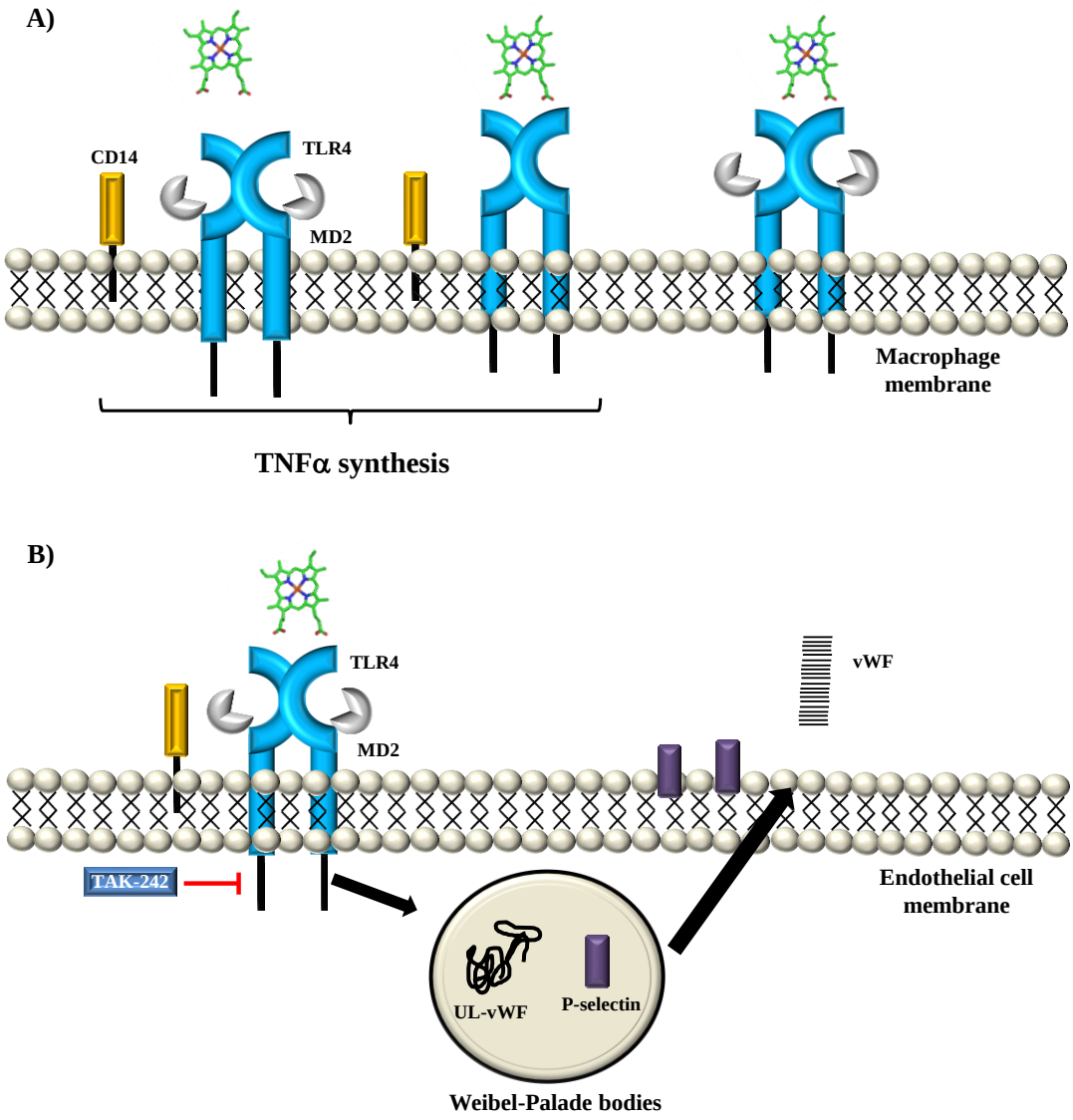

Figure 15: Free heme induces TLR4 activation on macrophages and EC. A) Free heme promotes TLR4 activation in MD2-independent manner. B) Free heme provokes P-selectin and vWF excretion in TLR4-dependent manner.

3.2. Interaction of heme with endothelium

3.2.1. Endothelium

Endothelium lines the vasculature, acting as an active regulatory organ that maintains the vascular homeostasis (Roumenina et al., 2016b). EC, which constitute the endothelium, are in dynamic equilibrium with plasma proteins and the sub-endothelium. EC work as a barrier, controlling vascular permeability, smooth tone, inflammatory and immune responses, angiogenesis and thromboresistance (Roumenina et al., 2016b). Despite the shared common characteristics of the EC, they express structural and functional heterogeneity depending on the vascular area: arteries, arterioles, capillaries, veinlets post-capillaries, veins, as confirmed by gene expression (Chi et al., 2003). This heterogeneity confers multiple functions to endothelium and different behavior in reaction to stimuli. For instance, specific intercellular junctions are found among the endothelium (Dejana, 2004). The macrovascular endothelium is continuous and non-fenestrated, providing a low permeability to this vascular wall. Capillaries supplying organs necessitating a barrier-maintenance, such as the brain, heart and lung, are also continuous and non-fenestrated. However, other capillaries are discontinuous with open pores, lacking basal lamina, allowing cellular trafficking, such as in liver, spleen and bone marrow. In kidney glomeruli, endothelium is fenestrated supported by a basal lamina, conferring efficient secretion or filtration and selective permeability. As a consequence of the constant interaction with the environment, EC express a very high plasticity in order to promote phenotypic adaptations. Depending on the organ and on the stimuli, including shear stress, hypoxia and chemical mediators, EC undergo phenotypical changes, leading to expression of pro- or anti-inflammatory, pro- or anti-thrombotic and homeostasis-related proteins (Roumenina et al., 2016b).

3.2.2. The macro- and the micro- vasculature

Microvascular endothelium includes small blood vessels (diameter < 200-300 µm) such as capillaries, arterioles and veinlets, and represents the most important endothelial surface (50 fold more than macrocirculation) (Wolinsky, 1980). It is responsible for distribution of oxygen and nutriments to the tissues. Macrovessels are characterized by a complex structure with superposition of three layers: (the intima, the media and the adventice). In contrast, capillaries structure is composed of endothelium monolayer, a basal

lamina and a thin layer of sub-endothelium connective tissue, surrounded by some smooth muscle cells (pericytes). By contrast, arterioles are surrounded by a monolayer of smooth muscle cells.

Arterioles are responsible for vascular tonus and regulate blood flow upstream capillaries. Capillary nets are characterized by a low pressure and low flow velocity to allow plasma/tissue exchange.

3.2.3. Endothelial cells

All along the blood circulation, a structural heterogeneity of endothelium has been characterized following electron microscopy observations, and showed differences in intercellular junctions (Bennett et al., 1959). From this, a classification emerged, leading to three subtypes of endothelium: continuous, fenestrated and discontinuous endothelium. Each subtype has its own behavior, morphology and different properties. For instance, continuous endothelium is composed of thick EC, promoting a high resistance to shear stress, whereas very thin (around 2 µm) EC form the fenestrated endothelium, favoring molecules exchange with tissues through the blood barrier. EC contains specific granules in their cytoplasm called WPB, providing an intracellular storage of bioactive substances that participates to inflammation and hemostasis (Goligorsky et al., 2009). Degranulation of WPB is induced in response to acute stress and promotes releasing of several proteins. Among them, WPB has been described to contain IL8, endothelin 1, P-selectin and vWF. Thus, upon activation of EC, WPB mobilization provides EC a capacity to mediate leukocyte rolling, platelet recruitment and vasoconstriction.

EC are linked by intercellular junctions (tight, adherens, gap) and form a barrier between bloodstream and sub-endothelium. If presence of tight junctions is predominant in arteries, adherens junctions are more represented in capillaries in order to promote paracellular diffusion (Dejana, 2004). Endothelium permeability can be increased by histamine- or thrombin-induced cellular retraction (Majno and Palade, 1961; McDonald et al., 1999). Particularity of microvascular endothelium is to rapidly induce expression of adhesion molecule like E-selectin, P-selectin, ICAM-1 and VCAM-1 (Aird, 2007; McEver et al., 1989; Petzelbauer et al., 1993). Moreover, the endothelial inhibitor of TF (TFPI) is highly expressed on microvascular EC surface (Bajaj et al., 1999; Osterud et al., 1995).

3.2.3.1. Activation of endothelial cells

Under stimulation, such as TNF-α, IL-1, bacteria endotoxins, hypoxia, EC acquire a pro-inflammatory phenotype by inducing expression of adhesion molecules, synthesis of pro-inflammatory cytokines, release of vasoactive mediators and of proteases, thus promoting platelets and leukocytes activation, and modulate vasomotricity and permeability (Cines et al., 1998; Danese et al., 2007; van Hinsbergh, 2012; Pober and Sessa, 2007). Moreover, endothelium constitutes an extra-hepatic site of production of complement proteins, thus participating to local complement activation. Indeed, under stimulation of pro-inflammatory cytokine, EC are capable of expressing properdin (Bongrazio et al., 2003), C1q (Bulla et al., 2008; Dauchel et al., 1990), C3 (Warren et al., 1987), C5, C6, C8 and C9 (Johnson and Hetland, 1991) proteins, as well as FH (Brooimans et al., 1990) and FI (Ripoche et al., 1988).

In case of endothelium activation, EC, which are anti-thrombotic in resting conditions, undergo a profound phenotype modification by acquiring a pro-thrombotic phenotype. Indeed, activation of EC induces a rapid excretion of vWF from WPB, and expression of TF.

3.2.3.2. Endothelial cells in hemolytic conditions

EC are directly exposing to RBC-degradation products in case of hemolysis. Thus, several studies have investigated the impact of hemolysis on EC functions. *In vitro*, heme promotes cytokine production and HO-1 synthesis by EC. *In vitro* and *in vivo*, heme promotes degranulation of WPB, thus inducing release of vWF and a rapid expression of P-selectin in TLR4 dependent manner (Belcher et al., 2014). The use of the TAK-242 against TLR4 revealed the crucial role of this receptor in EC activation by heme. Furthermore, our team has shown that heme promotes complement deposition on EC, suggesting the acquisition of a complement activation phenotype by EC (Frimat et al., 2013). Moreover, heme has been linked to the expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin, as well as increased expression of HO-1 (Wagener et al., 1997).

TLR4 is also expressed on EC (Fitzner et al., 2008). In 2014, Belcher et al have demonstrated the crucial role of the activation of TLR4 by heme on EC (Belcher et al., 2014). Indeed, blocking the TLR4 signaling pathway with TAK-242 prevents mobilization of WPB, then preventing release of vWF as well as rapid expression of P-selectin by these cells (Figure

15B). In a sickle cell anemia disease mouse model, the blocking of TLR4 signaling pathway with TAK-242 reduced heme-induced stasis. Moreover, the use of a blocking Ab against P-selectin, or vWF provided the same inhibitory effect on stasis, showing the crucial role of TLR-4 activation on EC *in vivo*.

3.3. Interaction of heme with complement system

Heme has been shown to interact with both classical and alternative complement pathways (Roumenina et al., 2016a). Heme directly binds to C1q, causing a change in the mechanism of recognition of CRP and IgG (Roumenina et al., 2011a). This interaction induces inhibition of complement activation by significantly reducing CP initiation.

First evidence of the interaction between heme and AP has been provided in 2007, in a model of malaria (Pawluczkowycz et al., 2007). In this study, the authors demonstrated that heme activates complement AP by inducing C3b deposition on normal erythrocytes. CR1 erythrocytes expression correlated with C3dg deposits and even CR1 was shown to be the platform for C3b deposition. The opsonization of the RBC with C3d could promote the clearance of these erythrocytes by CR2 expressing B cells in the spleen. More recently, our team has shown that heme induces alternative complement pathway activation directly in serum and on EC (Frimat et al., 2013), by promoting hydrolysis of C3 and formation of a fluid phase C3 convertase. C3a, C5a and sC5b9 are detected in sera in a dose-dependent manner of heme concentration. Moreover, heme promotes complement deposition on stressed-EC expressing P-selectin.

Taken together, these results point towards a hypothesis of a link between hemolysis and complement activation in the context of hemolytic conditions and diseases. To find out to what extend the complement activation can occur during hemolysis and what is the underlying mechanism, we need to define suitable *in vivo* models and to select appropriate pathological conditions.

4. Experimental models for studying hemolysis

4.1. Injection of free heme

Several animal models have been set up to study hemolysis. One of them consists in intraperitoneal or intravenous injections of soluble heme in mice, leading to systemic presence of free heme. This setting has been extensively studied in the context of SCD mouse models (Belcher et al., 2014; Vinchi et al., 2013, 2015), showing that heme promotes vaso-occlusion and contribute to the severity of the crisis.

This model, however, has multiple limitations, the main being the administration of an exogenous solubilized heme. This commercially available free heme is either purified from porcine or bovine hemoglobin (Sigma, Fluka) or chemically synthesized (Frontier Scientific). This crystalline heme has poor solubility in water at neutral pH. It is well solubilized in high pH buffers (hematin) or DMSO (hemin). Nevertheless, when it is further diluted in aqueous interaction buffer, with physiologically relevant pH, there is a high risk of generation of heme dimers and higher order heme-heme complexes and aggregates (Roumenina et al., 2016a). This can decrease the active concentration of heme or confer to the preparation properties, not necessarily shared by the heme *in vivo*, which is loosely bound heme to different proteins.

The levels of free heme in the circulation are debated, since heme is a hydrophobic molecule that binds to a large number of proteins in the circulation (Gouveia et al., 2017). Measurement of the exact concentration of protein free heme in plasma of patients with hemolytic diseases is technically very difficult and contradictory results have been published depending on the used method, ranging from 5 µM (Gouveia et al., 2017) to 110 µM (Vinchi et al., 2013) in mice, and between 20 and 50 µM in human (Reiter et al., 2002). The existence of Hb-free heme, though, is not a matter of debate due to the protective effect of Hx (interacting with heme but not hemoglobin), which is efficient in hemolytic conditions *in vivo* (Belcher et al., 2014; Ingoglia et al., 2017; Schaer et al., 2014; Vinchi et al., 2013, 2015).

A French team has demonstrated the impact of human erythrocytes microvesicles as a trigger of vaso-occlusion in a murine model of SCD (Camus et al., 2012, 2015). Indeed, authors described that 1000 RBC microvesicles per microliter have the capacity to carry around 5 µM of heme, making these microvesicles a platform able to disseminate heme in the

circulation. The use of such microvesicles can overcome the limitations of the injection of free heme.

Another difficulty of this method is the fact that the injection of free heme results in induction of HO-1 expression and anti-inflammatory response (Balla et al., 2007). This property is used in multiple models of organ transplantation, malaria, etc as a protective, therapeutic effect of free heme (Pamplona et al., 2007; Soares et al., 1998). This makes difficult to distinguish the dualistic inflammatory and anti-inflammatory properties of this molecule.

4.2. Injection of glycerol

The capacity of glycerol to induce RBC lysis has been frist described in 1897 (Parpart and Shull, 1935). Since, study of the mechanism revealed that glycerol releases lipids from the red cell membrane into the medium, dehydrating the membrane and thereby promoting hemolysis (Arakawa et al., 1977). This mechanism can be counteracted by addition of fructose and was revealed to be pH dependent (Arakawa et al., 1977; Zou et al., 2000). This model has been used in rats for studying brain edema, and acute renal failure in rhabdomyolysis (Bralet et al., 1983; Lochhead et al., 1998; Singh et al., 2012). However, glycerol also exerts adverse effects on many types of cells, limiting its use in our conditions (Héliès-Toussaint et al., 2006; Wiebe and Dinsdale, 1991).

4.3. Old blood transfusion

Hemorrhagic shock is a common complication occurring in patients suffering from polytrauma, gastrointestinal bleeds, obstetric hemorrhage, or after major surgery (Pham and Shaz, 2013). Massive blood transfusion is required to maintain circulation and hemostasis. However, resorting to blood transfusion pointed out a unique set of complications, such as hemolytic transfusion reaction (HTR). The implication of both IgM and IgG to promote complement-mediated hemolytic transfusion reaction have been well documented, supporting the role of complement system in RBC lysis (Hod et al., 2008a). Emergence of animal models has provided new insights on intravascular hemolysis-mediated organ injury. Indeed, study of

HTR in mouse provided evidence that IgG-mediated RBC clearance is responsible for cytokine storm, which supports HTR pathogenesis, while complement system was only moderately implicated in this process (Hod et al., 2008b). Furthermore, depletion of NO observed during HTR has been associated to pulmonary vascular endothelial dysfunction and increased vascular tone (Hsu et al., 2007). Study of this HTR complication highlighted the time of storage-dependent effect on RBC (Baek et al., 2012). Indeed, this study clearly demonstrated a harmful effect on RBC due to long-term storage of blood, resulting intravascular hemolysis leading to acute hypertension, vascular injury and kidney dysfunction in guinea pigs. This study echoed to an earlier study demonstrating a harmful effect of long-term storage of erythrocytes, producing acute tissue iron deposition and initiating inflammation in mouse (Hod et al., 2010). Moreover, recent study has shown evidence of the beneficial therapeutic effects provided by treatment with Hp or Hx on long-term storage RBC-induced hemoglobinuria and kidney injury (Graw et al., 2016). Actually, long-term storage RBC release microvesicles that aggravate pulmonary leukocyte sequestration and peripheral blood leukopenia induced by LPS (Zecher et al., 2014). In this study, authors have shown a complement dependent effect, since these effects were not seen in C5aR-deficient mice.

Therefore another method to study hemolysis consists in injecting RBC, stored for prolonged intervals (stored for 35–40 days in humans or 14 days in mice). Part of these RBC will lyse in circulation due to progressive deleterious morphological and biochemical changes which occur during storage. This method has the advantage that intravascular heme is generated *in vivo* by the hemolysis, but is difficult to standardize since the stored RBC may vary from one lot to another.

4.4. Autoimmune hemolytic anemia models of hemolysis

Autoimmune hemolytic anemia (AIHA) is a rare disease associated to autoantibodies directed against self erythrocytes (Zanella and Barcellini, 2014). AIHA is divided in two subgroups, depending on the direct antiglobulin test (DAT): warm AIHA, which is defined as anti-IgG antisera associated, in some cases, with C3d on RBC; and cold AIHA, which is defined as anti-IgM antisera associated with C3d on RBC. In rare cases, IgA autoantibodies are associated with warm AIHA (Chadebech et al., 2010).

Using murine model of AIHA, Baudino et al have described a subclass-dependent pathogenicity of immunoglobulins, related to different mechanisms (Baudino et al., 2006). Indeed, IgG2a and IgG2b exhibit the highest pathogenicity due to the activation of both FcγR and complement system, while IgG1 and IgG3 exhibit less pathogenicity by activating only FcγR or complement, respectively. Apart from IgG, IgM are associated to complement activation and weak pathogenicity. Pathogenicity of these immunoglobulins operates mainly through phagocytosis of erythrocytes for IgG subgroup, while it is more related to RBC agglutination with IgM subgroup. Interestingly, experiments performed on C3 deficient mice did not improve AIHA outcome, suggesting that complement system is not involved in the disease (Shibata et al., 1990; Sylvestre et al., 1996). However, results suggest that complement activation significantly contribute to severe anemia through activation of complement receptor (Baudino et al., 2006).

In warm AIHA, erythrophagocytosis by reticulo-endothelial cells in the liver is the principal mechanism, thus leading to extravascular hemolysis. The lack of complement-mediated intravascular hemolysis can be explained by the expression of complement inhibitory proteins on RBC surface, such as CD55, CD59 and CR1 (Berentsen, 2015).

However, in cold AIHA, antigen-bound oligomeric IgM promotes classical pathway activation through binding of C1q (Berentsen and Sundic, 2015; Jaffe et al., 1976; Jonsen et al., 1961; Ulvestad et al., 2001). Indeed, recent studies have shown a complete inhibition of erythrophagocytosis with anti-C1s Ab *in vitro* (Berentsen and Sundic, 2015), and improve parameters in cold AIHA patients upon administration of C1 Inh (Desai and Broome, 2016). If common terminal complement activation does not occur in stable disease due to surface-bound regulatory proteins, intravascular hemolysis can be triggered by C5b-9, as shown by the improved clinical features in a case report of a patient with cold AIHA using anti-C5 strategy (Röth et al., 2009). Thus, inhibition of complement activation using anti-C3 strategy could provide a beneficial effect by inhibiting complement-induced both extravascular and intravascular hemolysis (Wouters and Zeerleder, 2015).

Therefore, AIHA is an interesting model to study extravascular hemolysis mediated by immunoglobulins and/or complement system. However, the low generation of RBC-breakdown products into the bloodstream discards the use of this model in our study.

4.5. Phenylhydrazine-induced hemolysis

Disrupting erythrocyte membranes leads to release of RBC components. Phenylhydrazine (PHZ), by its powerful pro-oxidative properties, leads to the destruction of RBC, resulting in systemic and massive intravascular hemolysis. Lytic effect of PHZ has been observed in 1885, when blood from rabbits treated with PHZ was found to be brown in color (Magnani et al., 1988). PHZ and its derivatives were used in medicine in 19th and early 20th century firstly as antipyretics (Ranvers, 1891) and later as a drug for polycythemia vera (Falconer, 1933), a clonal disorder (Spivak, 2002) characterized by a very high number of erythrocytes in the blood. The strong adverse effects limited the clinical application of PHZ but it remained reagent of choise when intravascular hemolysis is to be studied.

The cellular and chemical responses of the RBC to PHZ have been thoroughly scrutinized. PHZ induces lipid peroxidation within cellular membranes of erythrocytes (Goldstein et al., 1980), destabilizes the globin portion of hemoglobin, leading to its denaturation and precipitation and release of heme (Augusto et al., 1982). Several studies have employed PHZ *in vivo* in order to study erythropoietin (EPO) synthesis, responsible for erythrocytes production, in response to anemia (Paulson et al., 2011). However, mice recovery after acute anemia stress induced by PHZ was shown to be EPO independent (Zeigler et al., 2010). Particular attention was given to PHZ because of the increase of fetal Hb containing erythrocytes production during the recovering process of RBC upon PHZ treatment in nonhuman primates, underlining a potential role of PHZ in sickle cell disease (DeSimone et al., 1978, 1979, 1982). PHZ constitutes a good model for studying acute phase or chronic background intravascular hemolysis, by injection of a single high concentration (Tolosano et al., 1999) or by repetitive injections of low PHZ concentration (Ingoglia et al., 2017), respectively. In these models, authors demonstrate the safeguardian role of Hx on kidney function under hemolysis (Tolosano et al., 1999), and that Hx counteracts ROS production and HO-1 synthesis in heart as a consequence of impairment of calcium homeostasis induced by heme in cardiomyocytes (Ingoglia et al., 2017).

III. Hemolytic diseases

1. Overview

The group of hemolytic diseases gathers a large spectrum of pathologies with heterogeneous etiologic factors and clinical features. Indeed, hemolytic event can be observed in autoimmune disorders, cancer, infectious and genetic diseases. Sickle cell disease (SCD), β-Thalassemia, Thrombocytopenia Thrombotic Purpura (TTP), Paroxysmal Nocturnal Hemoglobinuria (PNH) and atypical Hemolytic Uremic Syndrome (aHUS) are all hemolytic diseases associated with mutations on various genes. For instance, mutation in Hb gene has been described in SCD and β-Thalassemia, causing systemic hemolysis upon release of oxygen (Nath and Hebbel, 2015); deficiency in ADAMTS-13 leading to thrombi formation is related to TTP and local mechanical hemolysis (Lian, 2005); lack of CD55 and CD59 on erythrocytes promotes uncontrolled complement activation-driven systemic hemolysis in PNH (Brodsky, 2014); lack of regulatory proteins of complement system leads to fibrin-platelet thrombi and local mechanical hemolysis in aHUS (Fakhouri et al., 2017).

Thereby, premature intravascular erythrocytes destruction – causing Hb and heme release and overwhelming scavenging systems – is a common critical event in the pathogenesis of these diseases, and might lead to a common mechanism of pathogenesis. We will focus on two hemolytic diseases, where hemolysis-derived products and complement contribute to the pathogenesis. In SCD the pathogenic role of hemolysis-derived products, such as heme, Hb and RBC microvesicles is well described, but the contribution of complement is poorly studied. On the contrary, in aHUS the role of complement is well understood but studies on the contribution of the hemolysis-derived products are scarce.

2. Atypical Hemolytic Uremic Syndrome

Atypical HUS is a rare disease allying acute renal injury, thrombocytopenia and mechanical hemolytic anemia (Fakhouri et al., 2017). These are due to acquisition of a pro-thrombotic phenotype of the renal microvessels and complement overactivation. This result is thrombotic microangiopathy (TMA) injury in kidney glomeruli. TMA affects capillaries (vessel diameter < 200 µm), characterized by a swelling of sub-endothelial space as a result of

flocculent material accumulation, leading to EC detachment in the lumen of microvessels. Fibrin-platelet thrombi formation, subsequent to exposure of glomerular basement membrane leads to occlusion of glomerular capillaries. This occlusion promotes RBC destruction on the platelet-forming clot, thus leading to a local hemolysis, enhancing the stress of endothelium.

2.1. Complement implication

In more than 50% of the cases, aHUS is due to genetic variants of complement-related proteins, such as FH, FI, MCP, FB, C3 as well as in Diacylglycerol Kinase epsilon (DGKE) and thrombomodulin. On the one hand, loss-of-function mutations in FH, FI and MCP induce an inefficient complement regulation at the level of the AP C3 convertase on EC (Fakhouri et al., 2017). Most of the FH mutations have been reported on CCPs19-20, inducing a loss of capacity of FH to bind GAGs and heparin and so reducing regulatory activity of complement activation by FH glomerular endothelium (Loeven et al., 2016; Manuelian et al., 2003). Mutations in the CCPs1-4 have also been reported, leading to a defective ability of FH to control AP in both fluid phase and on cell surfaces (Pechtl et al., 2011). Several mutants of FI have been characterized, leading to a deficiency or loss of regulatory activity in the fluid phase and on cell surfaces (Bienaime et al., 2010; Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2008; Nilsson et al., 2010). Mutations of MCP have been found on the four extracellular CCP domains, resulting primarily in protein deficiency and more rarely in reducing its ability to bind C3b and C4b (Fremeaux-Bacchi et al., 2006; Richards et al., 2003). On the other hand, gain-of-function mutations of FB and C3 have been reported, mostly located in the binding area between C3b and FB or in the regulators binding sites on C3b, enhancing C3 convertase formation and resistance to FH, MCP and DAF regulation (Frémeaux-Bacchi et al., 2008; Goicoechea de Jorge et al., 2007; Marinozzi et al., 2014; Roumenina et al., 2009; Sartz et al., 2012; Schramm et al., 2015).

Clinical studies have shown about 85% of sporadic forms in spite of familial inheritance of the mutation, suggesting that genetic background is not a cause but predisposes to the disease (Fremeaux-Bacchi et al., 2013). In fact, a variety of events have been associated with aHUS including infections, pregnancy, transplants, drugs, autoimmune conditions and metabolic conditions (Kavanagh et al., 2013).

In acute phase, patients suffering from aHUS have a variable renal presentation that might include proteinuria as a result of glomerular basement membrane damage (Fakhouri et al., 2014), associated with C3 deposits (Noris and Remuzzi, 2015). Without specific treatment, more than 50%, aHUS cases evolve towards renal failure, requiring dialysis and even renal transplantation. Extra-renal complication occurs in 8% to 25% in adult and in 16% to 29% in children, with variable manifestations. Predominantly, it includes neurological symptoms, pancreato-intestinal involvement, gangrene of fingers and toes, ulcerative-necrotic skin lesions, myocardial infarction or ischemic cardiomyopathy (Fakhouri et al., 2017).

The implication of complement system as a trigger of kidney pathogenesis in aHUS is now well established thanks to the use of eculizumab. Eculizumab is a recombinant humanized monoclonal Ab that blocks the common terminal pathway by binding the native C5 component, creating a steric hindrance, thus avoiding the cleavage of C5 into C5a and C5b by the C5 convertase (Jore et al., 2016; Rother et al., 2007; Schatz-Jakobsen et al., 2016). Actually, recent structural analysis of the C5 convertase function support the hypothesis that C5 firstly binds to C3bBb3b complex and is secondary cleaved by a close C3bBb complex (Jore et al., 2016). In this context, eculizumab should prevent binding of the C5 component to the first C3bBb3b complex. This therapeutic approach pointed up the harmful effect of the common terminal complement activation, at least on kidneys (Zuber et al., 2012), revolutionizing the management of the disease with approximately 85% of patients becoming disease-free (Wong et al., 2013) (Figure 16). Moreover, it drastically increased survival of patients upon kidney transplantation. Indeed, since eculizumab is available for aHUS patients, this therapeutic Ab improves safely and successfully the renal transplantation, reducing from 56% to around 10% of aHUS patients progressing to end-stage renal disease or died after 1 year (Fakhouri et al., 2017).

During the acute phase, following the fibrin-platelet thrombi formation, erythrocytes are mechanically lysed, releasing Hb and thus heme in the circulation (Jokiranta, 2017). Our team suggested that massive hemolysis promotes C3b deposition and C5b-9 formation on EC, thus participating to platelet aggregation via a rapid increase of P-selectin expression on EC surface and release of vWF in the bloodstream (Frimat et al., 2013). Moreover, C3a can autoamplify this phenomenon, since by binding to C3aR, C3a promotes WPB mobilization (Morigi et al., 2011).

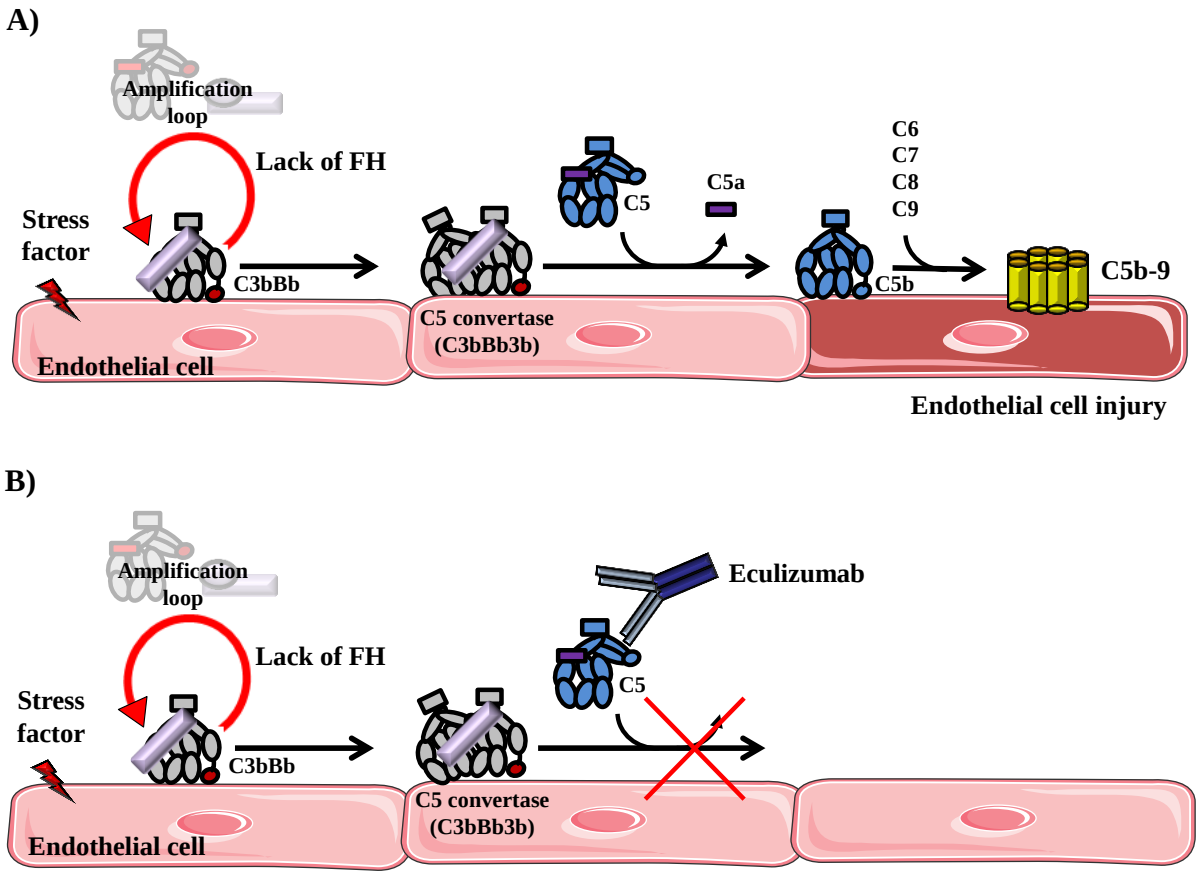

Figure 16: Impact of the common terminal complex of complement activation on EC. A) Upon a first trigger leading to EC activation, overactivation of alternative complement pathway leads to C5b-9 formation and EC injury leading to kidney dysfunction. B) Treatment with the anti-C5 Ab, Eculizumab, C5 cleavage is prevented, thus inhibiting endothelium injury and renal disorders.

Thereby, studies about aHUS provide a critical role of complement system (and more importantly about C5b-9 deposition on EC), as a trigger of kidney failure. The recent discovery underlining the relationship between hemolysis and complement system raises the question about the implication of complement activation in other diseases characterized by hemolytic event, such as SCD.

3. SCD – a model of chronic hemolytic disease

SCD is a widespread inherited disease, affecting over 6 million people worldwide, mostly in sub-Saharan Africa (Serjeant, 2013). In France there are between 400 and 500 births of children with SCD per year, making it the most frequent genetic disease in France. A mutation in the Hb gene leads to sickle form of RBC upon releasing oxygen to organs and muscles. Vaso-occlusion emerging from this phenotypical modification of erythrocytes induces hemolysis and powerful inflammation, making it a perfect disease to study the mechanisms and the impact of hemolysis on tissues.

3.1. A worldwide genetic disease

This disease is characterized by the presence of sickle Hb (HbS), by opposition to two normal forms of Hb, HbA, and HbA2, which represent around 95-98% and 2-3% of Hb found in adults, respectively. This HbS phenotype is due to a mutation on the β-globin chain of Hb, described as a substitution of valine for glutamic acid at the sixth amino acid of the HbA (Hebbel, 1991, 1997).

SCD exists in two different forms, the most severe form Sickle Cell Anemia (SCA) and a less severe form called Sickle Cell Trait (SCT), due to inheritance of homozygous (HbSS) or heterozygous (HbS) mutation, respectively (Nath and Hebbel, 2015). In 2010, SCT patients have been estimated of approximately 5,5 million neonates, and 312000 born with SCA (Piel et al., 2013a). This number is expected to critically increase in the next decades, highlighting a major societal and health issue (Piel et al., 2013b). SCT conferts adaptive advantage since these individuals are resistant to malaria, explaining thus the disease prevalence, especially in the population of the regions of the world, affected by malaria (Allison, 1954; Elguero et al., 2015). Functionally, HbS mutation leads to Hb polymerization upon exceeding a critical threshold of oxygen-free Hb (deoxyHb) (Nath and Hebbel, 2015). It is now well established that this phenomenon acts as the primary event that triggers sickling crises (Alayash; Vekilov, 2007). The β6Glu→Val was shown to induce hydrophobic patch on the surface and link to other hydrophobic amino acids (e.g. Phe85 and Leu88) close to the heme containing pocket (Figure 17). Thus, these interactions participate to Hb aggregation

into long fibers and deform RBC (Carragher et al., 1988; Dykes et al., 1979; Fronticelli and Gold, 1976; Mickols et al., 1985; Wishner et al., 1975).

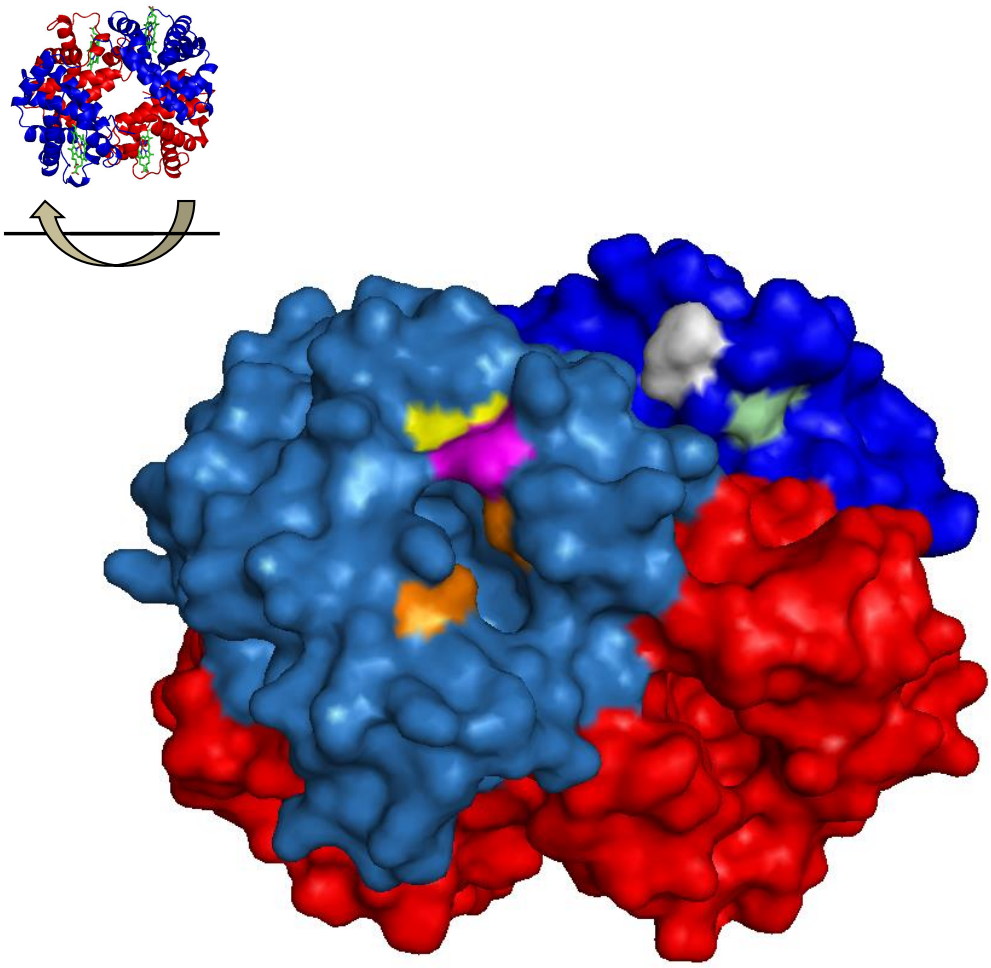

Figure 17: Representation of the β6Glu→Val mutation and its binding site on the opposite β-chain in SCD. Red: α-chains; blue and lightblue: β-chains; grey: N-terminal histidine; palegreen: glutamate amino-acid; orange: His 63 and His 92 responsible for binding of heme on the hydrophobic pocket of β-chain; yellow: Phe 85; magenta: Leu 88.

Studies of transgenic mice expressing human HbS and expressing SCD phenotype showed that switching of Thr87 residue into Gln delayed fiber formation and corrected hematological parameters and splenomegaly as well as inhibition of sickled RBC formation (Pawliuk, 2001).

Moreover, it has been shown that acidosis, subsequent to hypoxia, decreased HbS affinity for oxygen. In addition, hyperosmolarity increases RBC HbS concentration and participates to polymerization of Hb. Cumulating these phenomena, it becomes evident that sickle RBC promote VOC, leading to organ failure in patients.

3.2. Vaso-occlusion crisis

VOC are a result of multifactorial events. Reduction of the lumen of the vessels results in painful crisis. After several sickling/unsickling cycles, a RBC becomes irreversible sickle cell (ISC). During VOC, increased adhesion of sickled RBC on endothelium plays a major role in this mechanism (Cartron and Elion, 2008). Indeed, SS RBC exhibit increased adhesion property on EC (Hoover et al., 1979). This was shown to be exacerbated by a lack of repulsive effects between endothelium and erythrocytes due to the loss of sialic acid expression on sickle RBC (Hebbel et al., 1980a, 1980b). Moreover, intravital microscopy has demonstrated that ICAM-4 expressing SS RBC participates to erythrocytes adhesion to endothelium (Kaul et al., 2006).

Repetition of sickling/unsickling cycles results in the destruction of RBC, leading to the release of hemoglobin. However, deciphering the vaso-occlusive crisis revealed that not only erythrocytes membrane, but also EC could be altered (Nath and Hebbel, 2015). Endothelium, constituting the blood barrier, works as a guardian of tissue integrity against hemolysis. Thus, several studies have been carried out in order to decrypt the EC behavior during VOC. Indeed, over the past decades, *in vitro* studies have shown that pro-inflammatory environment induces EC activation and promotes adhesion expression markers, leading to SS RBC adhesion. First evidences were provided in 1993, when TNFα-activated EC expressed VCAM1 was shown to promote binding of SS RBC-expressing α4β1 integrin in a direct interaction (Joneckis et al., 1993; Swerlick et al., 1993). Stressed EC have been reported to express αvβ3 integrin on their surface, acting as a receptor for ICAM4 expressed on sickle RBC, determined as a key factor for initiating vaso-occlusive episodes (Zennadi et al., 2008).

However, other studies tend to demonstrate that SS-RBC/EC interaction required plasmatic proteins. For instance, thrombospondin-1 (TSP1) and fibronectin are two major plasmatic factors promoting vaso-occlusion. Normally implicated in coagulation cascade, TSP1 is highly secreted by activated platelets in SCD patients. In the context of SCD, TSP1 was shown to interact with CD36, a stress marker expressed on both sickle RBC and microvascular EC and promotes sickle erythrocytes adherence to microvascular endothelium (Brittain et al., 1993).

In association with these phenomena, depletion of NO participates to the vascular instability in SCD. Studies on murine models and in human with SCD indicate impairment in

NO-dependent relaxation in the vasculature (Nath et al., 2004). In fact, whether eNOS and iNOS expression remain unaltered, NO can be scavenged by superoxide anion generated by the vasculature (Aslan et al., 2001) and by released Hb (Reiter et al., 2002). This inhibition of NO impairs vasodilation, platelet inhibition and dowregulation of VCAM and E-selectin (Reiter and Gladwin, 2003; Reiter et al., 2002). Moreover, by depleting NO, release Hb during VOC favors endothelin-1, the most potent circulating vasoconstrictor in humans (Graido-Gonzalez et al., 1998; Phelan et al., 1995).

3.3. Clinical manifestations

SCD shows considerable phenotypic heterogeneity resulting from both genetic and environmental factors (Kanter and Kruse-Jarres, 2013). As a consequence of painful crisis, complications can occur within the central nervous system, lung, heart, spleen, liver, skin, bones, genitals and kidneys. In children, acute chest syndrome (ACS), subsequent to vaso-occlusion episode in the pulmonary vasculature, is described as a combination of hypoxia and fever, similar to pneumonia, making the greatest cause of mortality after 2 years of age in developed countries. Hemorrhagic stroke is a major morbidity and mortality complication of SCD (Strouse et al., 2009). The incidence of overt stroke is 11% before 20 years old (Wang, 2007), but silent cerebral infarcts are more frequent (up to 30%) (DeBaun et al., 2012) that may lead to irreversible cerebral consequences. Asthma, with prevalence between 8 and 53%, is a common pulmonary complication in children, associated with ACS (Anim et al., 2011). Adults have the same complications, but additional disease manifestations may occur. For instance, leg ulcers, sickle retinopathy, thromboembolic complications, cardiac failure, priapism or avascular necrosis are associated with SCD adult complications (Schnog et al., 2004).

Since a decade, a particular attention has been granted to nephropathy complications. Actually, first studies of nephropathy in SCD described nephrotic syndrome, hypertension, hematuria and renal insufficiency in more than half of the 7 patients presenting glomerular disease (Pardo et al., 1975). Complement deposition was associated to this phenotype on glomerular basement membrane in all patients in a parallel study (Strauss et al., 1975). Renal complications can occur throughout the life of a patient and are a major cause of death in SCD, imputing 16-18% of overall mortality in these patients (Hamideh and Alvarez, 2013;

Platt et al., 1994). SCD nephropathy is associated with urinary excretion of early markers of tubular injury like KIM-1, NGAL and NAG proteins (Sundaram et al., 2011).

Pathogenesis of sickle cell nephropathy (SCN) can be the consequence of different etiology. Acute kidney injury (AKI) occurs in 4-10% of hospitalized patients with SCD in adulthood, associated in 13.6% of the case to ACS (Audard et al., 2010), while it represents a complication in 17% of the cases in childhood (Baddam et al., 2017). However, a persistent urinary excretion of NGAL, underlining an acute subclinical tubular injury in patients with painful crisis but a normal serum creatinine level (Audard et al., 2014). Multi-organ failure is observed in 75% of AKI episodes, requiring hemodialysis in 18% of the cases (Hassell et al., 1994). Whether haematuria, hyperfiltration, hypertrophy, impaired urinary concentration and AKI can occur at any age, some manifestations are more restrictive to certain age category.

The perfusion paradox of SCN results from the co-existence of hyperperfusion in the whole organ, increasing renal plasma and blood flow rate as well as glomerular filtration rate (GFR). On the contrary, reduced perfusion is endemic in microcirculation, such as in the medulla, due to HbS polymerization in vasa recta (Nath et al., 2004). Indeed, relative hypoxia and reduced pH in the vasa recta are both likely to promote sickling of erythrocytes. This perfusion paradox predisposes to tubular functional defects, including disorders of renal concentrating capacity and tubulointerstitial disease (De Jong and Statius van Eps, 1985).

Hyperperfusion can lead to cortical defect, which is usually accompanied by hyperfiltration and proteinuria. With recurrent AKI, both can lead to the emergence of chronic kidney disease (CKD). CKD and macroalbuminuria tend to appears in later stages, and can be associated to regression of the glomerular filtration rate (GFR) (Nath and Hebbel, 2015). Both proteinuria and reduced GFR are associated with increased mortality among SCD patients (Darbari et al., 2013; Elmariah et al., 2014; Gladwin et al., 2014). In later stages, renal biopsies of patients with proteinuria associated with acute or progressive impairment of renal function showed glomerular injury: focal segmental glomerulosclerosis (FSGS) in 39%, membranoproliferative glomerulonephritis (MPGN) in 28%, TMA in 17% and sickle cell disease glomerulopathy (SCDG) in 17% of the cases (Maigne et al., 2010). Moreover, using magnetic resonance imaging, a recent study has confirmed defective renal perfusion during VOC by alteration of cellular integrity and local perfusion in medullary and cortical areas compared to resting state's patients (Deux et al., 2016).

Hemolysis and proteinuria are both associated with CKD (Hamideh et al., 2014). In fact, prospective study has clearly correlated hemoglobinuria and progression of CKD (Saraf et al., 2014). The main mechanism could be related to dimers of Hb, released during hemolysis, that are small enough to traffic through glomerular filtration barrier and promote stress of tubular cells. To counteract hemoglobinuria, tubular cells incorporate HbS, thus explaining the high HO-1 expression by these cells in case of hemolysis (Nath et al., 2001). Moreover, TLR4 is expressed on endothelial, mesangial, tubular epithelial cells and on podocytes, thus rendering possible the trigger of pro-inflammatory responses by heme (Tracz et al., 2007). HO-1 activity is required to protect kidney from heme deleterious effects, as shown in HO-$1^{-/-}$ mice (Nath et al., 2000a). The protective role of HO-1 is mediated by generation of CO that counteracts vasoconstriction, releasing of iron that stimulates ferritin synthesis – essential for RBC production – and generation of anti-oxidant and anti-inflammatory mediators by converting heme into biliverdin and bilirubin.

3.4. P-selectin: a key player in SCD pathogenesis

Rapid translocation of pre-synthesized P-selectin to the cell surface plays a key role in leukocytes recruitment on stressed endothelium (Goligorsky et al., 2009). First evidences came at the beginning of the 90's, when P-selectin deficient mice had defective leukocyte rolling and a delay of neutrophil recruitment (Mayadas et al., 1993) (Figure 18). Since, study of adherent leukocytes revealed a major role of these cells in vaso-occlusion (Turhan et al., 2002). Indeed, by cross-linking SS phenotype with P-selectin deficiency in mouse, authors demonstrated that defect in leukocytes recruitment protects mice from vaso-occlusion. More recently, using a SCD mouse model, it was shown that injection of heme provokes SCD phenotype, caused by expression of endothelial-stressed marker, promoting VOC (Belcher et al., 2014). Rapid expression of P-selectin and release of vWF were strikingly correlated with vaso-occlusion, since the use of specific Ab directed against P-selectin or vWF prevents the percentage of stasis promoted by heme injection (Belcher et al., 2014) (Figure 18). Interaction between platelets and neutrophils has been identified as a process leading to both platelets and neutrophils activation in mouse and human (Polanowska-Grabowska et al., 2010). The use of an anti-P-selectin in mouse prevented platelet-neutrophil aggregation and neutrophils activation, underlining the role of P-selectin in this process.

The critical role of P-selectin in the pathological process is now deeply investigated. Indeed, the use of an anti-P-selectin in SCD mouse model has highlighted the therapeutic potential benefit of such alternative to prevent vaso-occlusion (Gutsaeva et al., 2011). Furthermore, administration of Crizanlizumab, a monoclonal Ab directed against P-selectin, resulted in a significantly lower rate of sickle cell-related pain crisis and was associated with a low incidence of adverse events (Ataga et al., 2017). GMI-1070, an anti-pan-selectin Ab has also been tested in patients, improving time to resolution of VOC (Telen et al., 2015).

3.5. Therapeutics in SCD

Nowadays, hydroxyurea is the only therapeutic alternative commercially available for SCD patients. By promoting fetal Hb level and increasing NO production, hydroxyurea exerts a double benefit for patients and then reduces the incidence of VOC. It is surprising that the mechanism leading to foetal Hb induction remains still not completely understood. One of the possible mechanisms would be provided by the inhibitory property of hydroxyurea on ribonucleotide reductase. Inhibition of this protein, which promotes DNA synthesis, causes cytotoxic depletion of erythroid progenitors and cell stress signaling. Thus, new erythroid progenitors are recruited with increased foetal Hb level (Agrawal et al., 2014). Increase of NO has been determined to be released from hydroxyurea itself by a still unknown mechanism (Gladwin et al., 2002). In addition to these beneficial effects, recent prospective study underlined the interest of long-term hydroxyurea therapy on renal function (Bartolucci et al., 2016). Indeed, administration of hydroxyurea counteracted microalbuminuria in patients presenting increased hemolysis markers, highlighting the relationship between renal function and hemolysis.

Hx, the natural heme-scavenger, is also tested as a new therapeutic approach in SCD. Over the past decades, studies have demonstrated the implication of hemolysis in the pathogenesis of the disease (Belcher et al., 2014; Camus et al., 2012; Vinchi et al., 2013). Indeed, Hx could be another symptomatical therapeutic approach, even better than anti-P-selectin treatment, by counteracting the pro-inflammatory effects of heme release. In fact, by binding to heme, Hx would prevent heme-induced EC injury, and so P-selectin expression on these cells. In the past few years, *in vitro* and *in vivo* studies have shown the efficacy of Hp and Hx to counteract hemolysis and represent now a burning issue for the next decade.

Indeed, Hp, but not Hx, is able to prevent NO depletion by Hb *in vitro* and *ex-vivo*, and thus prevents Hb-induced systemic hypertension by compartmentalizing cell-free Hb in plasma (Deuel et al., 2015; Graw et al., 2017). Molecular approaches have shown the benefit of Hp treatment in hemolytic mice models to prevent molecular stress signature of the kidney (Graw et al., 2016). For instance, HO-1, NGAL and KIM-1 increase expression by injection of stored RBC (SRBC) was prevented by addition of Hp, but not by Hx. However, administration of Hx was tested in several studies. First evidence has been claimed in 1999, when Tolosano et al. demonstrated the the key role of Hx as a guardian of kidney integrity in $Hx^{-/-}$ mice upon treatment with PHZ (Tolosano et al., 1999). Since, the same team has highlighted the ability of Hx to protect from cardiovascular injury and cardiomyocytes alteration after heme treatment in SCD mouse model (Ingoglia et al., 2017; Vinchi et al., 2013, 2015). In a more relevant model, erythrocyte microvesicles from SCD patients, injected in a SCD mouse model induced kidney vaso-occlusion and promoted endothelium injury by transferring heme to EC (Camus et al., 2012, 2015). This pro-active effect of erythrocytes microparticles is prevented by Hx injection, underlying the role of heme in this mechanism. Moreover, Hx provides protection against stasis in SCD mice by preventing P-selectin expression and vWF release by EC upon triggering VOC by heme injection (Belcher et al., 2014). By scavenging cell-free heme, Hx promotes Nrf2/HO-1 antioxidant axis and ameliorates inflammation and vasoocclusion (Vercellotti and Zhang, 2016) (Figure 18).

Expression of P-selectin in SCD has never been linked to complement activation, despite the published interaction between P-selectin and the C3 activation fragment (del Conde et al., 2005; Morigi et al., 2011). In fact, complement system implication in SCD has been poorly studied these last years, though old studies clearly demonstrated an increase of activated complement markers in plasma.

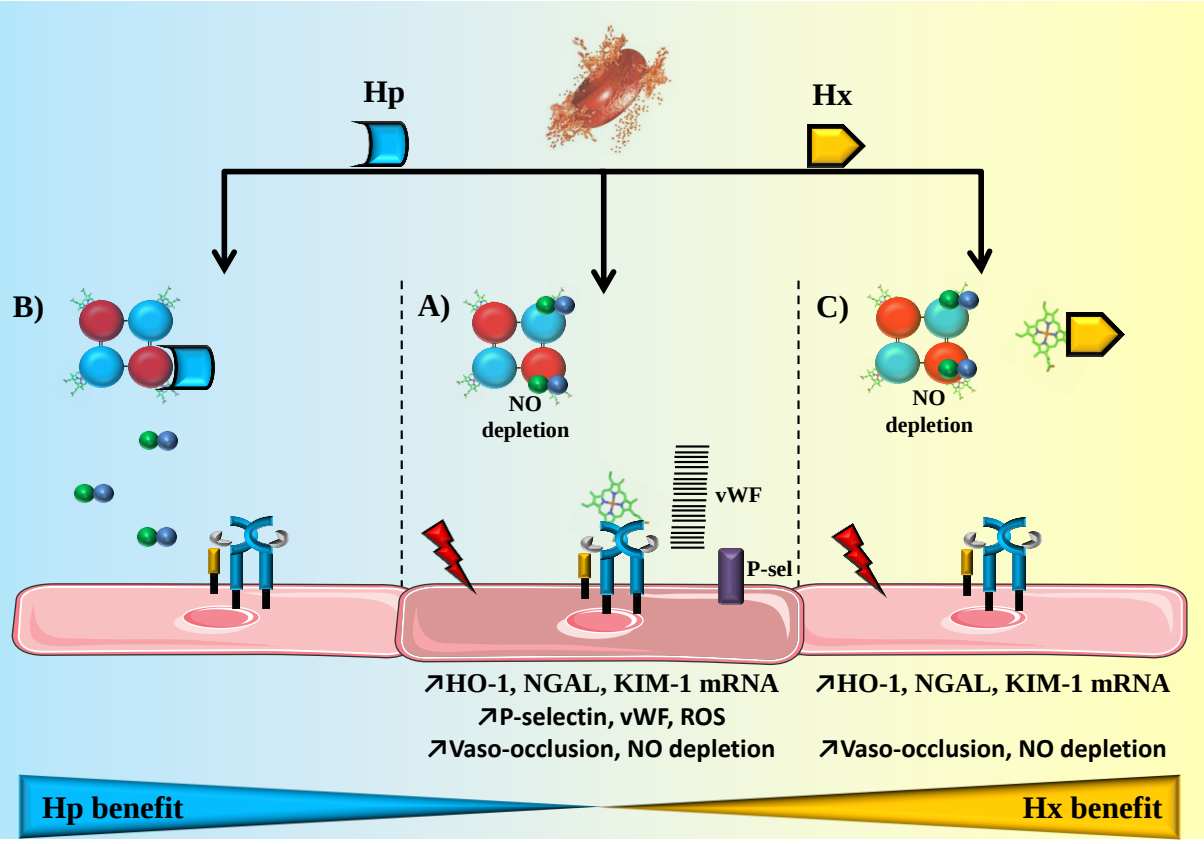

Figure 18: Benefit effects provided by Hp and Hx. A) Hemolysis releases both Hb and heme upon saturation of scavenging system. Hb provokes NO depletion and vasoconstriction while heme promotes P-selectin and vWF release via TLR4. HO-1, NGAL and KIM-1 mRNA expression is increased. B) The use of Hp inhibits NO depletion by Hb and its conversion to MetHb and heme. C) The use of Hx prevents heme-mediated EC injury. However, NO depletion leading to vaso-occlusion, and increase mRNA of HO-1, NGAL and KIM-1 are not averted.

3.6. Complement activation in SCD

If the impact of hemolysis has been well characterized in SCD, little is known about the implication of complement system in organ injury during VOC. First evidence of alternative complement pathway activation in SCD emerge during the 70's, when authors revealed decreased levels of C3 and FB in sera from SCD patients (deCiutiis et al., 1978). Few years later, elevated C3b,P complexes were detected in 89% of SS patients sera (Chudwin et al., 1985). Further, an AP activation dependent on SS RBC has been described, due to PS/PE exposure on SS erythrocytes and promoting anchoring of C3b (Chudwin et al., 1994; Wang et al., 1993). In addition to C3 deposition on SS erythrocytes, Bb, C4d and C3a fragments were detected in SCD patients' plasma, as well as C3 deposition on RBC (Mold et al., 1995; Wang et al., 1993) (Figure 18). Interestingly, detection level of this activated

fragments depended on patient status. For instance, elevated C3a and C4d fragments were correlated with continuous pain, while Bb and C3a fragments were highly increased during painful crisis, suggesting different activating profile of the complement system. Actually, even though DAF and CD59 expression were not altered in SSRBC compared to normal erythrocytes, complement activation was shown to drive much hemolysis on dense ISC (Test and Woolworth, 1994). These data allow considering complement system as a supporting process of hemolysis in patients due to its lytic activity against RBC. In fact, this phenomenon is well characterized in other hemolytic anemia diseases, such as PNH, where complement-driven hemolysis is due to a lack of CD55 and CD59 expressions on RBC (Chapin et al., 2016). Interestingly, increased sC5b-9 have been measured in patients with SCD, correlating with age and HbS percentage (Chapin et al., 2016; Gavriilaki et al., 2017). Moreover, this last study demonstrates the lytic activity of sera from SCD patients against RBC, dependent on the common terminal complement association in 3 tested patients (Gavriilaki et al., 2017). However, if hemolysis has been shown to induce complement-activating phenotype on EC (Frimat et al., 2013), no detailed studies, so far, have been done regarding the impact of complement system on organ injury observed in SCD patients during VOC.

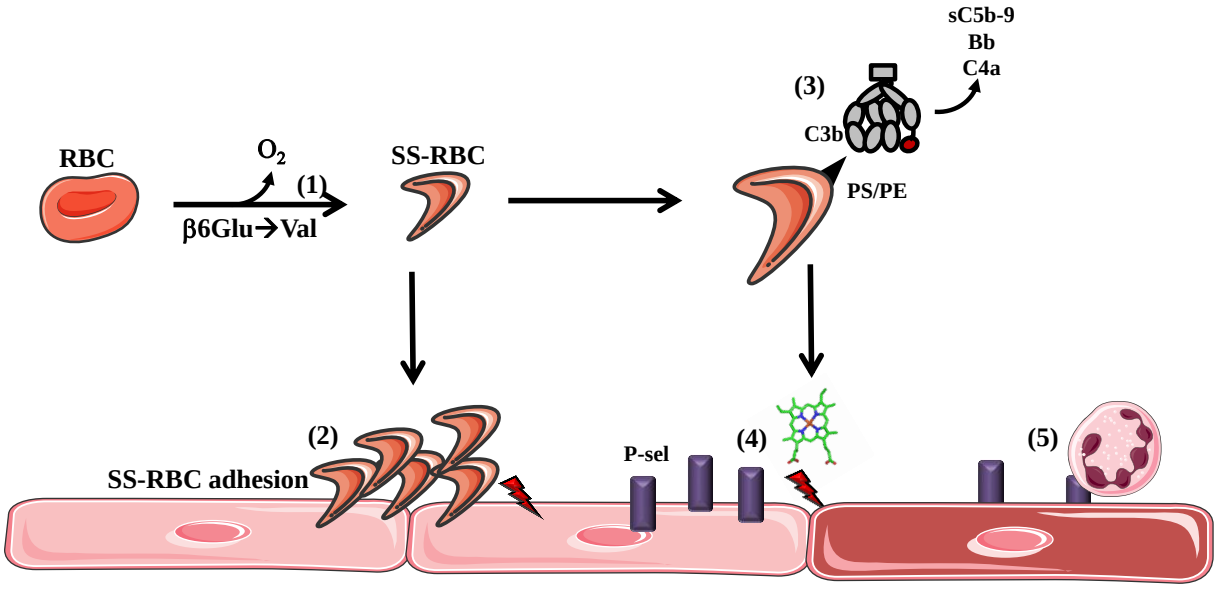

Figure 19: Interelation between hemolysis, EC and complement system activation in SCD. (1) Upon release of oxygen, mutated-Hb carrying the β6Glu→Val polymerizes, leading to sickle form of RBC (SS-RBC). (2) SS-RBC, due to the high expression of adhesion markers and the loss of sialic acid, adhere to EC. (3) High expression of phosphatydilserin (PS) and phosphatydilethanolamine (PE) promotes complement activation on SS-RBC, thus leading to release of sC5b-9, Bb and C4a in the bloodstream. (4) After several sickling/unsickling cycles, SS erythrocytes are lyzed and release Hb and heme in the circulation, inducing EC stress and P-selectin (P-sel) expression. (5) P-sel expression on EC provokes recruitment of neutrophils at the vaso-occlusion site, playing a crucial role in the painful crisis.

Moreover, C3 deposition is commonly accepted as a consequence of inflammation more than a leading cause of organ dysfunction. Scarce data about C3 deposition in kidney biopsies is available in the literature in patients suffering from SCD (Strauss et al., 1975), but systemic study for the types and localization of the deposits in large cohorts of SCD nephropathy are lacking. Furthermore, no study has been performed regarding C5b-9 formation and its implication in renal injury.

Evidence for the pathogenic role of complement comes from cases and models, where direct complement inhibition was tested in hemolytic diseases, including SCD. Targeting C5 with a blocking Ab was shown at a congress abstract to inhibit vaso-occlusions in a mouse model of SCD (Schaid et al., 2016). Complement inhibition at the level of C5 is now possible in the clinic, using Eculizumab, which abrogates terminal pathway activation. Cases of the efficacy of Eculizumab against delayed transfusion reaction and hemolysis in SCD are emerging (Dumas et al., 2016), showing the importance of complement for the disease process.

OBJECTIVES

The analyses of the literature clearly demonstrate that endothelial damage is a major complication of diseases with intravascular hemolysis, including in SCD and aHUS. In addition, heme appears to be a potent inducer of complement activation *in vitro* (Frimat et al., 2013; Lindorfer et al., 2016; Pawluczkowycz et al., 2007), but the relevance of these findings needs *in vivo* validation. Kidney, and especially in the renal microvasculature, is particularly susceptible to complement attack and hemolytic diseases frequently have renal complications. In SCD nephropathy, the exact mechanism and pathophysiological relevance of complement activation is not established. Further studies are needed to establish the exact link between complement activation, endothelial damage, hemolysis and renal injury in hemolytic diseases. Moreover, the exact molecular and cellular mechanisms governing the hemolysis-mediated complement activation need detailed investigation. Such studies are of critical importance to delineate the extent of hemolysis-mediated complement activation in different human diseases and disease-relevant animal models and to develop targeted therapeutic straregies.

To shed light on the mechanisms by which hemolysis promote complement activation in the bloodstream as well as on EC and its relevance *in vivo*, we set up the following objectives:

Thus, the **first objective** of this work was to study whether and how intravascular hemolysis induces complement system activation *in vivo*, in disease-relevant models of SCD. The **second objective** was to understand the underlying mechanism that leads to the acquisition of complement-activating phenotype that promotes complement deposition on EC. Finally, the **third objective** was to test therapeutic strategies, which can be applied to control the hemolysis-mediated complement activation.

In order to respond to these objectives, first we had to set up the appropriate experimental models and to validate the anecdotal reports, stating that complement deposits occur indeed in patients with SCD. Administration of free heme is classically used in *in vitro* and *in vivo* models but the existence of free heme in the circulation remains controversial. Therefore, we had to select other, more pathologically relevant, heme-generating conditions and heme-carrying vehicles to compare and validate the results, obtained by using cell-free heme and hemoglobin.

To answer the first objective, initially we analysed renal biopsies from patients with SCD nephropathy and from a SCD mouse model in order to evaluate complement deposition in a relevant hemolytic context. Further, we set up an acute systemic model of hemolysis on C57Bl/6 WT mice, by injection of PHZ and characterized complement deposits in different organs and particularly the kidneys. To understand which hemolysis-derived products activate complement, we injected the heme scavenger Hx in the PHZ-treated mice or free heme in naïve mice. *In vitro*, complement activation was studied in presence of free heme, Hb or heme-loaded erythrocytes microvesicles from healthy donors and SCD patients (disease relevant vehicles disseminating heme in the body) in serum and on EC, to understand the mechanism by which hemolysis drives complement activation.

To address the second objective, we studied the implication of TLR4 in the heme-mediated complement activation process on EC. Phosphorylation of cell signaling intermediates was assessed in presence of heme or a classical TLR4 ligand - LPS. Complement deposition was evaluated on heme-treated HUVEC with or without the specific inhibitor of TLR4, TAK-242. Following the same protocol, P-selectin expression and vWF release was also stained. P-selectin/C3b interaction was studied using surface plasmon resonnance (SPR) and its importance for the complement deposits was assessed by using a blocking Ab directed against P-selectin on heme-exposed HUVEC. Biological relevance of TLR4-mediated signaling for complement deposition on endothelium was determined *in vivo*, using $TLR4^{-/-}$ mice.

Using the established models in the first two objectives, finally we tested the capacity of the heme scavenger Hx and the TLR4 inhibitor TAK-242 to inhibit hemolysis-mediated complement activation *in vitro* and *in vivo*. With this third objective we evaluated the potential of these two molecules with a therapeutic potential to serve as indirect complement inhibitors under hemolytic conditions.

RESULTS

Manuscript 1: **Characterization of the renal injury in an experimental model of intravascular hemolysis**

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Characterization of the renal injury in an experimental model of intravascular hemolysis

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Conflicts of interest

N.B., T.G. and S.M are employees of CSL Behring. LTR receives research funding from CSL Behring. The remaining authors declare no conflict of interest.

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Abstract

Intravascular erythrocyte destruction, accompanied by the release of hemoglobin and heme, is a common event in the pathogenesis of numerous diseases with heterogeneous etiologic factors and clinical features. A frequent adverse effect related to massive hemolysis is the renal injury. Well-characterized animal models of hemolysis with kidney impairment are needed to investigate how hemolysis drives kidney injury and to test novel therapeutic strategies. Here we characterized the pathological processes leading to acute kidney injury during massive intravascular hemolysis, using a mouse model of phenylhydrazine (PHZ)-triggered erythrocyte destruction. We observed profound changes in gene expression levels for markers of tubular damage (Kim-1, NGAL, Ki67), and tissue and vascular inflammation (IL-6, E-selectin, P-selectin and ICAM-1) in kidneys of PHZ-treated mice, associated with ultrastructural signs of tubular injury. Moreover, mass spectrometry revealed the presence of markers of tubular damage in urine, including meprin-α, cytoskeletal keratins, α-1-antitrypsin and α-1-microglobulin. Signs of renal injury and inflammation rapidly resolved and the renal function was preserved, despite major changes in metabolic parameters of PHZ-injected animals. Mechanistically, renal alterations were largely heme-independent, since injection of free heme could not reproduce them, and scavenging heme with hemopexin in PHZ-administered mice could not prevent them. The reduced fitness of the mice suggested multi-organ involvement. We detected amylasemia and amylasuria, two markers of acute pancreatitis. We also provide detailed characterization of renal manifestations associated with acute intravascular hemolysis, which may be mediated by hemolysis-derived products upstream of heme release. This analysis provides a platform for further investigations of hemolytic diseases and associated renal injury and the evaluation of novel therapeutic strategies that target intravascular hemolysis.

Introduction

Intravascular erythrocyte destruction, accompanied by the release of hemoglobin and heme, is a common event in the pathogenesis of numerous diseases with heterogeneous etiologic factors and clinical features, such as sickle-cell disease, microangiopathic hemolytic anemias, ABO mismatch transfusion reaction, paroxysmal nocturnal hemoglobinuria, autoimmune hemolytic anemia, malaria, cardiopulmonary bypass, mechanical heart valve–induced anemia and chemical-induced anemias and many others[1]. Excessive or chronic intravascular hemolysis may overwhelm the protective, scavenging system made of haptoglobin and hemopexin (Hx) in plasma, and lead, in a fraction of the patients to common lesions, one being renal injury. Adverse clinical effects related to free hemoglobin and heme release can be caused by direct cytotoxicity, nitric oxide scavenging and oxidative reactions[2]. Heme exerts direct cytotoxic effects, drives pro-oxidative, pro-thrombotic and pro-inflammatory effects on endothelium and induces immune cell activation[3]. Free hemoglobin and free heme have multiple effects on plasma systems (complement[4], coagulation[5], antibodies[6]), on endothelium[7], and tissues, particularly on kidneys[8].

Intravascular hemolysis is associated with acute kidney injury (AKI), most likely due cytotoxicity resulting in tubular necrosis, intratubular casts and pro-inflammatory effects[8]. In order to understand to what extend the hemolysis induces kidney injury, what are the underlying mechanisms of these processes and to test novel therapeutic strategies, animal models of hemolysis with well-characterized kidney manifestations are needed. Several animal models have been set up to study hemolysis, but kidney function was not always fully described. One of the most frequently used methods to trigger intravascular hemolysis in experimental animals is the injection of a hemolytic drug phenylhydrazine (PHZ). PHZ constitutes a good model for studying acute phase or chronic intravascular hemolysis[9,10].

The objective of this study is to characterize the pathological processes leading to AKI in acute drug-induced intravascular hemolysis. We aimed to characterize the renal phenotype of a mouse model of intravascular hemolysis triggered by PHZ and to find out to what extend it is dependent on the release of free heme.

Materials and methods

Reagents

Solution of phenylhydrazine (PHZ) of 25 mg/mL (Sigma-Aldrich) was prepared in PBS immediately before use. The Fe^{3+} form of heme (hemin [ferriprotoporphyrin IX], designated as heme, (Frontier Scientific Inc. or Sigma-Aldrich) was dissolved to 20 mM in 50 mM NaOH and 145 mM NaCl, and further diluted in PBS just before use. Plasma-purified Hx was provided by CSL Behring.

Mouse treatment

All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with the approval APAFIS#3764-201601121739330v3 of the French Ministry of Agriculture. C57Bl/6 mice were from Charles River Laboratories, (L'Arbresle, France). Eight week old female C57Bl/6 mice were injected i.p. with 200 µL of PBS (Gibco), or freshly prepared heme (40 µmol/kg, corresponding to 28 µg/g body weight[11]) repeated 24 hours later (day 0 and day 1). PHZ (900 µmol/kg, corresponding to 0.125 mg/g body weight) was injected i.p. in at day 0 and C57Bl/6 mice were sacrificed at day 1, 2 or 4. To test the efficacy of Hx, mice were pre-treated with i.p. injection of 40 µmol/kg of human Hx one hour before i.p. injection of 900 µmol/kg of PHZ. Concentrations and route of administration were chosen as described[10–12]. Mice kidneys were recovered and snap-frozen in liquid nitrogen or fixed and included in paraffin.

Evaluation of the renal function

The follow-up of renal function was performed by placing 6 weeks old C57Bl/6 female mice (Charles Rivers) in individual metabolic cages for acclimation for 5 days, with daily weight measurement and fixed, daily amounts of food. After stabilization of body weight (day -3) metabolic parameters were measured (diuresis, amount of excrements, water and food uptake and body weight). Urine was collected at fixed times for 3 days at baseline, and two days after injections. PHZ, heme or PBS were injected at day 0. Blood was taken from the retro-orbital sinus before and at day 2 after treatment. Blood was taken and blood gas was measured

(Alere system, self-calibrated epoc® BGEM Test Card). Organs were recovered at day 2. In alternative experiments in regular cages organs were recovered at day 4. Organs were snap-frozen in liquid nitrogen in Cryomatrix (Thermoscientific). Urinary protein and creatinin levels, as well as plasma urea concentrations were measured using Konelab equipment. For comparative purposes, similar experiments were performed in regular cages, with 5 animals per group.

Immunohistochemistry on mouse tissues

Three µm-thick, fixed (PFA 4%) or frozen sections of kidneys were cut with Cryostat Leica AS-LMD. HO-1 expression was studied using rabbit anti-mouse HO-1 (Abcam, Ab13243) followed by a polymer anti-rabbit (DAKO, K4003). Staining was revealed with DAB solution. Slides were scanned by Nanozoomer (Hamamatsu). Hematoxylin-Eosin, Perl's Prussian blue and PAS coloration were performed by routine procedures using sections of paraffin-embedded kidneys at day 4. Coloration of slides was scanned by Slide Scanner Axio Scan (Zeiss).

Gene expression analyses

Snap-frozen kidney sections were recovered in RLT buffer (Qiagen)+1% β-mercaptoethanol (Gibco) and used for mRNA extraction with Qiagen RNeasy miniKit. The quality and quantity of mRNA were evaluated with bioanalyser Agilent 2100 using Agilent TNA 6000 NanoKit and if the RNA integrity number was >7 the mRNA was retrotranscribed to cDNA. Gene markers of early tubular and endothelium activation/injury relevant for hemolysis and SCD were analyzed by low density array (LDA, ThermoFisher) including NGAL, Kim-1, HO-1, Il-6, Ki67, ICAM-1, E-selectin and P-selectin, Caspase-3 and CD31[13–17] and validated by RTqPCR for NGAL, Kim-1, HO-1 and Ki-67.

Urinary electrophoresis

Urine of the mice, recovered from the metabolic cages was centrifuged and diluted 1/2 with a sample buffer containing glycerol and bromophenol blue (but not SDS) and 20 µl are deposited on a 10 wells 12% gels and migrated for 40 min. Staining the gel-resolved proteins was performed with Coomassie blue.

Mass spectrometry analyses

After discoloration, bands of interest were excised from the the coomassie blue-stained SDS page gel at the same level for the PBS, heme and PHZ-treated animals. Samples were then reduced with the addition of DTT (4.2 mM, final concentration) for 45 min at 37°C, then alkylated with iodoacetamide (7.6 mM, final concentration) and subjected to in-gel tryptic digestion using porcine trypsin (Promega, France) at 12.5 ng/µL. The dried peptides extracts were dissolved in 12 µL of solvent A (2% acetonitrile, 0.1% formic acid) and analyzed by on-line nanoLC using an Ultimate 3000 System (Dionex) coupled to a LTQ-XL Orbitrap mass spectrometer (Thermo Fisher Scientific). Each peptide extract (5 µL) were loaded on a C18 precolumn (Acclaim PepMap C18, 5 mm length x 300 µm I.D., 5 µm particle size, 100 Å porosity, Dionex) at 20 µL/min in solvent A. After 5 min desalting, the precolumn was switched online with a C18 capillary column (Acclaim PepMap C18, 15cm length x 75 µm ID x 3 µm particle size, 100 Å porosity, Dionex) equilibrated in solvent A. Peptides were eluted using a 0 to 70% gradient of solvent B (80% acetonitrile, 0.1% formic acid) during 50 min at a flow rate of 300 nL/min. The LTQ-XL Orbitrap was operated in data dependent acquisition mode with the XCalibur software. Survey scan MS were acquired in the Orbitrap in the 400–1600 m/z range with the resolution set to a value of 60,000. The five most intense ions per survey scan were selected for collision-induced dissociation (CID) fragmentation and the resulting fragments were analyzed in the linear trap (LTQ). Dynamic exclusion was employed within 45 seconds to prevent repetitive selection of the same peptide.

Peak lists extraction from XCalibur raw files were automatically performed using Proteome Discover software (version 1.4, Thermo Fisher scientific). Database searches were performed using the Mascot server v2.2.07 with the following parameters: database *Mouse*; enzymatic specificity: tryptic with two allowed missed cleavages; fixed modification of cysteine residues (carbamidomethylated (C)); variable oxidation of methionine residues; 5 ppm tolerance on

precursor masses and 0.6 Da tolerance on fragment ions; fragment types taken into account were those specified in the configuration 'ESI-trap'.

Western blotting

The gels with urine proteins, resolved by electrophoresis as above were transferred to nitrocellulose membranes using iBlot technology (Invitrogen) and stained with Rabbit polyclonal anti-pancreatic alpha amylase antibody (Abcam, ab199132) using SNAP technology (Merk Millipore), followed by immunodetection with Goat anti Rabbit IgG-HRP (Santa Cruz, 1/5000). The signal was revealed by chemiluminescence.

Quantification of plasmatic levels of alpha amylase

The levels of α-amylase were detected in mouse plasma, using Colorimetric Amylase Assay Kit (Abcam, ab102523) using the protocol provided by the manufacturer.

Statistics

Results were analyzed using a statistical software package (GraphPad Prism 5) as indicated in figures legends. Briefly, *: $p<0.05$, **: $p<0.005$, ***: $p<0.001$. Multiple groups in the gene expression analyses were compared with Kruskal-Wallis with Dunn's test for multiple pairwise comparisons. When only two groups were compared, Mann–Whitney test was applied. The metabolic parameters were compared for the mice before and after indicated treatments using Two-Way ANOVA with Tukey's test for multiple pairwise comparisons.

Results

Characterization of the kidney function in the PHZ-induced intravascular hemolysis model.

To find out to what extend the acute intravascular hemolysis and heme induce renal injury, we evaluated the parameters of the kidney function, placing the mice in metabolic cages. Kidneys and blood of PHZ-treated mice were dark in color, compared to PBS injected controls, while heme-treated mice showed mild dark coloration. Blood gas analyses revealed that heme and PHZ-treated mice had significantly decreased blood hemoglobin at day 2 compared to PBS controls of about 8% and 46%, respectively (Figure 1A), indicating a red blood cell (RBC) lysis. This RBC lysis was associated with a significant increase in lactate as a surrogate marker of hypoxia and cell damage (Figure 1B) and a decrease in the HCO_3^-, a marker of metabolic acidosis (Figure 1C) in the PHZ group. The heme-treated group presented with slightly decreased blood glucose at day 2, without reaching significance (Figure 1D). Plasma Na^+ was increased in the heme- and PHZ-treated groups (Figure 1E), while K^+ (Figure 1F) and Ca^{2+} (Figure 1G) remained unaltered. Further, we measured urea and creatinine to estimate the changes in glomerular filtration rate. Plasma creatinine concentration (Figure 1H) and the plasma urea (Figure 1I) were significantly increased in PHZ-treated mice compared to the baseline. Moreover, heme treatment also increased the plasma urea in a significant manner compared to baseline (Figure 1I). PHZ and heme-injected mice also presented with an oliguria (24h diuresis) at day 1 (Figure 1J) which persisted in day 2 for PHZ but normalized for heme-injected mice. These were accompanied by reduced water (Figure 1K) and food (Figure 1L) intake, resulting in a decrease of excrements (Figure 1M) and 13% weight loss at day 2 for the PHZ-injected mice (Figure 1N). Proteinuria, normalized by the creatininuria was not significantly different in PHZ and heme-treated mice compare to PBS group (Figure 1O). Alteration of kidney function parameter is most likely functional, due to the anemia and reduced food and water uptake and less likely due to renal tissue damages.

Histological analyses of mouse kidneys do not reveal major structural alterations

To gain a deeper insight whether acute hemolysis induced tissue injury in the kidney, renal sections were analyzed by histology. By light microcopy, paraffin-embedded kidney sections

of the mice treated with PBS or PHZ were similar regarding the analysis of glomeruli, that were normal with no modification of glomeruli size, endocapillary proliferation, glomeruli were not congested and did not show neither TMA lesions nor mesangial proliferation at day 2 (not shown) and 4 (Figure 2A, B). No major modification of renal histology was detected, except minor but significant increase of tubular dilatation at day 4, a sign of acute tubular necrosis at day 4, ($p=0.009$) (Figure 2C). Perl's Prussian blue staining for hemosiderin, a hallmark for hemolysis, was positive in the PHZ-injected mice (Figure 2D).

Hemolysis induced upregulation of tissue injury and inflammation-related genes in the kidney in a heme-independent manner

Further, we analyzed at gene level a number of more sensitive markers for inflammation and tissue injury in the acute hemolysis model. Sustained expression of markers of tubular injury NGAL and Kim-1 till day 4 (Figure 3A, B); increase of markers of inflammation Ki67 and IL-6 (Figure 3C, D) and endothelial activation E-selectin, P-selectin, ICAM-1 (Figure 3E, F, G) were detected at day 1. No difference in CD31 and Caspase 3 was detected (not shown).

To find out whether these changes are heme-dependent or occur due to upstream products (as hemoglobin), we investigated expression of NGAL, Kim-1 and IL6 in heme-treated mice. We detected a weak but significant increase of NGAL expression (Figure 4A), but no modification of mRNA levels of Kim-1 and IL-6 (Figures 4B,C). Furthermore, the i.p. injection of the heme scavenger human plasma-derived hemopexin (Hx) did not modify the expression of these genes (Figure 4D-I), showing a largely heme-independent process.

We analyzed the expression of the same gene panel in liver and spleen and observed similar alterations of a set of genes in the PHZ-treated mice, indicating that this model is associated with multiorgan lesions (data not shown).

Renal tubules upregulate HO-1 after exposure to PHZ

Strong up-regulation of the cytoprotective enzyme heme oxygenase (HO-1) till day 4 at both gene (Figure 5A,B) and protein levels (Figure 5C) was detected in kidneys of PHZ-injected mice. Pre-injection of Hx did not modify expression of HO-1 in PHZ-treated mice (Figure

5B). Treatment with heme also resulted in upregulation of this enzyme (Figure 5C). HO-1 staining was localized in the tubules. Immunohistochemistry revealed no HO-1 expression in PBS-treated mice (Figure 5C).

Markers of tubular injury were found in the urine of PHZ-injected mice

To find out if the tubular necrosis and the oxidative and inflammatory stress in the kidney result in alteration of the profile of the urinary proteins, the profile of the urinary protein content was examined by electrophoresis (Figure 6A). If 24h urine was deposited at equal volume, without normalization to creatinine, it revealed increased total protein content in the PHZ-injected mice (in agreement with the measurement of total protein), which though, was brougth to normal when the urine was diluted to normalize to creatinine (not shown). Alteration of the renal endothelium could affect the glomerular microvasculature and the filtration function of the kidney. A distinction between glomerular and tubular proteinuria could be made in experimental models based on the profile of urine electrophoresis, appearance of low molecular weight proteins (< 60 kDa) suggesting tubular damage, while high molecular weight proteins (> 60 kDa) indicating glomerular one. No apparent imbalance of the higher and lower molecular weight proteins was revealed by the electrophoresis.

Nevertheless, several proteins that were not present in the PBS controls were specifically identified in the PHZ and heme samples. To identify these proteins and find out whether they reflect alteration of the tubular or glomerular function, a mass spectrometry analyses were performed by in-gel trypsin digestion of the proteins. Urine of PHZ treated animals was positive for meprin α, cytoskeletal keratins, α-1-antitrypsine and α-1-microglobulin (protein AMBP), while heme-treated mice urine was positive for meprin α only (Table 1).

Hemolysis and heme injection induced signs of acute pancreatitis

The gels revealed also a presence of a tick ~55 kDa band, characteristic for the heme and to less extend to PHZ-injected mice, which was absent in the PBS controls. Mass spectrometry analyses revealed that the protein is pancreatic α-amylase (Table 1). In depth peptides analyses were performed to distinguish between salivary amylase and pancreatic amylase,

confirming the presence of the pancreatic isoform of the enzyme (data not shown). This result was confirmed by western blot in the PHZ-injected group (Figure 6B).

Presence of pancreatic α-amylase in the urine could suggest an acute pancreatitis, caused by heme toxicity and hemolysis. To test this hypothesis, measurement of plasmatic pancreatic α-amylase was performed and these were found indeed increased in PHZ treated animals, compared to controls (Figure 6C).

Discussion

Here we provide a detailed description of the renal phenotype of a mouse model of intravascular hemolysis, triggered by injection of PHZ. Hemolysis induced rapidly renal inflammation and mild tubular injury, which were largely heme-independent. In addition, we found signs of multi-organ injury, as revealed by major alterations of multiple metabolic parameters in mice, including acute pancreatitis in response to heme and PHZ.

Diverse experimental models of intravascular hemolysis have been described, but the most common one is drug-induced hemolysis with PHZ. PHZ induces massive intravascular hemolysis by lipid peroxidation of erythrocyte membranes [18], destabilizing the globin portion of hemoglobin, leading to its denaturation, precipitation and the release of globin-free heme. Using this model, the protective role of hemoglobin- and heme-scavenging proteins, such as haptoglobin and Hx, as well as heme-degrading enzyme HO-1 function have been demonstrated [9,16,19,20].

Kidneys are the primary route for hemoglobin clearance after saturation of the natural scavenging systems, and they are therefore highly susceptible to organ dysfunction during hemolysis. Renal lesions are described as major complications of hemolysis[21], but not all patients develop overt renal manifestations. Our histological work in the PHZ-injected mice revealed minor but significant tubular dilatation, suggestive of tubular necrosis. We observed an upregulation of sensitive markers for tubular injury, such as NGAL and Kim-1[22], in agreement with other models of hemolysis, including old blood transfusion in mice [16] and guinea pigs[17]. Persistent Kim-1 expression at day 4, when most other markers have returned to normal, indicated that the reparative process was rapid, yet incomplete at this time point. Multiple markers pointed at tubular injury, such as increased proliferation (Ki-67), an indirect

marker of tubular repair. Gel electrophoresis of urinary proteins revealed also presence of (proximal) tubular damage protein markers, such as meprin-α[23], α-1-antitrypsine, renal keratins[24] and α-1-microglobulin[25], indicating tubular alterations. Interestingly, α-1-microglobulin is a heme-binding protein[26], which contributes to the clearance of heme and free radicals, released from Hb in extravascular fluids[27]. Meprin α is involved in the activation and degradation of inflammatory cytokines such as IL-1 and IL-6, respectively[28,29]. All these parameters demonstrate that tubular injury is one of the major lesions of hemolysis in the kidney in this model. This corroborates previous studies showing that injecting PHZ to mice (0.2 mg/g body weight) results in renal tissue damage 48 h later, revealed by reduced ^{3}H-inulin clearance[30]. Our results are also coherent with previously published hemolytic models of stored erythrocyte transfusion in mice[16] and guinea pigs[17], which reported distorted renal tubule function as a primary cause for renal impairment during intravascular hemolysis[17].

Renal inflammation has rarely been studied in detail in hemolytic models. In PHZ-injected mice, we detected IL-6 as a marker of tissue inflammation, as previously reported in other hemolysis models[16]. IL-6 is produced by activated macrophages as well as kidney resident cells including podocytes, endothelial, mesangial, and tubular epithelial cells. IL-6 induces vasoconstriction and endothelial dysfunction, increases ROS production and tubular atrophy, and could thus contribute to renal injury during intravascular hemolysis. Heme and hemolysis (induced by PHZ at 0.1 mg/g, followed by a second injection 16 h later at 0.05 mg/g) triggered the processing and secretion of IL-1β by macrophages via NLRP3- and inflammasome-mediated signals[31].

Taken together, our results indicate that kidneys respond to hemolysis through increased inflammation. Furthermore, we found that hemolysis triggered pro-inflammatory changes in renal endothelial cells, with upregulated expression of P-selectin, E-selectin and ICAM-1. These adhesion molecules are thought to contribute to renal inflammation[30 32–34] and vascular injury[17 35]. Inflammation resolved rapidly and these markers returned to baseline between day 2 and 4 after PHZ administration.

Detailed examination of metabolic parameters and renal function in PHZ-treated mice revealed extensive anemia, but only minor alterations of uremia, and no difference in parameters related to glomerular filtration at 48 h. We detected increased proteinuria and severe, transient oliguria (at 24 h). These may be explained by reduced water uptake. We also

detected reduced food uptake, hence reduced excrements, with subsequently reduced body weight. Altogether, obtained results indicate that murine kidneys are relatively resistant to hemolysis-induced AKI. Local inflammation and tubular and vascular stress appeared on the first day after hemolysis but resolved rapidly. Therefore, extensive analyses of metabolic parameters must be performed in murine models of intravascular hemolysis, in order to determine to what extend renal injury is related to the alteration of kidney function, or merely to the altered fitness of the anemic animals.

The poor fitness of the PHZ- and heme-injected animals suggests that other organs may be more affected than kidneys. Indeed, we detected alteration of the gene expression markers also in the liver and spleen, as expected from alternative hemolysis models[16]. Moreover, analyses of urinary protein profiles revealed a surprising band of about 55 kDa in PHZ- and heme-injected mice, which was not present in PBS-injected controls. Mass spectrometry indicated that this band corresponds to pancreatic α–amylase, as confirmed by western blotting. The presence of pancreatic α-amylase in urine is not thought to be related to renal injury, but to acute pancreatitis[36]. Acute pancreatitis in our PHZ- and heme-injected mice was confirmed by increases in plasmatic amylase. Early oxidative protein modifications and free radicals, as found in intravascular hemolysis in humans, contribute to functional impairment of the pancreas[37]. Acute pancreatitis is a known complication of hemolytic diseases[38,39], and it was reported in a rat model of intravascular hemolysis involving a PHZ analogue[40–42]. In rats, pancreatic inflammation was made evident by increased TNF-α, IFN-γ, IL-18, ICAM-1 and MCP-1 levels subsequent to hemolysis[42]. Aggravated pancreatic injury and inflammation were detected when the main heme-degrading enzyme, HO-1, was inhibited. Detailed analyses, beyond the scope of this study, will reveal which other organs may be affected the most by intravascular hemolysis.

We sought to determine the contribution of hemolysis-derived products other than heme, to the adverse effects of intravascular hemolysis. Heme is a well-known TLR-4 ligand[11,43] that can activate cells [4,11,31,35,44,45]. Therefore it was tempting to speculate that the renal injury in PHZ-treated mice resulted from massive heme release. Contrary to this speculation, we found that injection of heme alone resulted only in a slight increase in tubular injury markers, such as NGAL gene expression and meprin-α in urine.

One may argue that the dose of heme injected was insufficient to trigger more significant kidney injury, or that part of it did not reach the circulation after intraperitoneal

injection. Indeed, crystalline heme is poorly soluble in aqueous solutions and prone to aggregation upon injection *in vivo* [5]. We could not measure free heme in plasma after heme administration, as it is technically very difficult to distinguish hemoglobin-bound, from protein-bound and protein-free heme. Indeed, the exact concentrations of free heme in hemolytic diseases remains debated. Heme tends to bind to a large number of circulating proteins [3] or remain associated to erythrocyte membrane microvesicles [44,46]. We tested the injection of purified Hx as a tool to investigate the contribution of free heme to renal inflammation. If heme drove stress gene overexpression, its scavenging by Hx would lead to decreased expression. The gene expression pattern we observed in PHZ-induced mice was not modified by Hx addition, despite the fact that Hx did reach the circulation (data not shown). These results agree with another model of hemolysis[16], where haptoglobin but not Hx decreased NGAL, Kim-1 and IL-6 genes expression, as well as Ki-67 staining in kidneys. These results suggested that heme alone does not trigger major change in renal phenotype, but induces only mild and transient changes in metabolic parameters. One potential explanation could be the rapid upregulation of HO-1. HO-1 is the principal physiological mechanism of heme degradation, and a defense mechanism against oxidative stress [27]. Heme and PHZ treatment induced a potent upregulation of HO-1 in mouse kidney tubules, in agreement with previous observations[10,47,48]. Therefore, the induction of HO-1 and its cytoprotective function may explain the rapid resolution of renal inflammation and the absence of severe AKI in our PHZ-injected mice [49–51]. Moreover, despite its side effects, heme arginate was approved as a drug for treatment of acute porphyria [52].

In multiple studies, heme injection was used for tissue preconditioning, as a mean to enhance HO-1 activity in order to protect organs from subsequent challenges. This was studied in animal models[53–55] and used in human clinical trials for renal transplantation[56]. On the other hand, heme promotes vaso-occlusions and contributes to the severity of crises in experimental models of sickle cell disease [11,12,57,58]. Hence, the potentially opposed effects of heme must be taken into account when choosing an experimental model in order to study intravascular hemolysis.

Conclusion

Our results, added to the literature, converge to suggest that the injection of heme remains a method of choice for targeted, mechanistic studies, or as a tool to provoke vaso-occlusions in murine models of SCD [11,12,57,58]. However PHZ-induced intravascular hemolysis bears several advantages over heme injection, as it emulates the pathological process of erythrocyte destruction, combined with hemoglobin and heme release. Also, it gives reproducible results and is easy to handle. PHZ administration, studied herein, may thus be preferred to study erythrocyte degradation in physiological processes and hemolytic diseases, to establish the benefits of HO-1-mediated therapy, or to investigate the effects of hemoglobin and heme scavengers.

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Authors' contributions

Study design: LTR, NM, JDD. Perform research: NM, AG, MLF, SC, RN, TRR, MR, SK, NB, TG, RD. Discussed the data: LTR, JDD, OBB, RD, NM, AG, SC, MLF, PH, MR, SM, OM, MF. LR, NM and JDD wrote the manuscript. All authors approved the submission.

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Table 1. Proteins, identified by mass spectrometry in each of the bands, excised from the gel of urinary electrophoresis. The number represent the UniProtKB ID of the protein. PHZ: phenylhydrazine. BSA: bovine serum albumin; MW: molecular weight

MW kDa	**Heme**	**PHZ**	**PBS**	**BSA**
Band 200	Meprin A subunit alpha **P28825**	Meprin A subunit alpha **P28825**	Uromodulin **Q91X17**	
Band 75		Serum albumin **P07724** Uromodelin **Q91X17**		
Band 70	Uromodulin **Q91X17**	Serotransferrin **Q0921I1** Serum albumin **P07724**	Serotransferrin **Q0921I1**	
Band 65		Serotransferrin **Q0921I1** Serum albumin **P07724**		
Band 60	Serum albumin **P07724**	Serum albumin **P07724**	Serum albumin **P07724**	Serum albumin **P02769**
Band 50 - 55	**Pancreatic alpha-amylase P00688**	**Serum albumin P07724** **Alpha-amylase 1 P00687**		
Band 40	Pancreatic alpha amylase **P00688**	Serum albumin **P07724**		

Band 38		Keratin, type II cytoskeletal 79 **Q8VED5** Alpha-1-antitrypsine 1-1 **P07758** Alpha-1-antitrypsine 1-2 **P22599** Alpha-1-antitrypsine 1-4 **Q00897**		
Band 35		Keratin, type I cytoskeletal 42 **Q6IFX2** Keratin, type I cytoskeletal 14 **Q61781**		
Band 25	Kallikrein **P15947**	Kallikrein **P15947** Protein AMBP **Q07456**	Kallikrein **P15947** Serum albumin **P07724**	

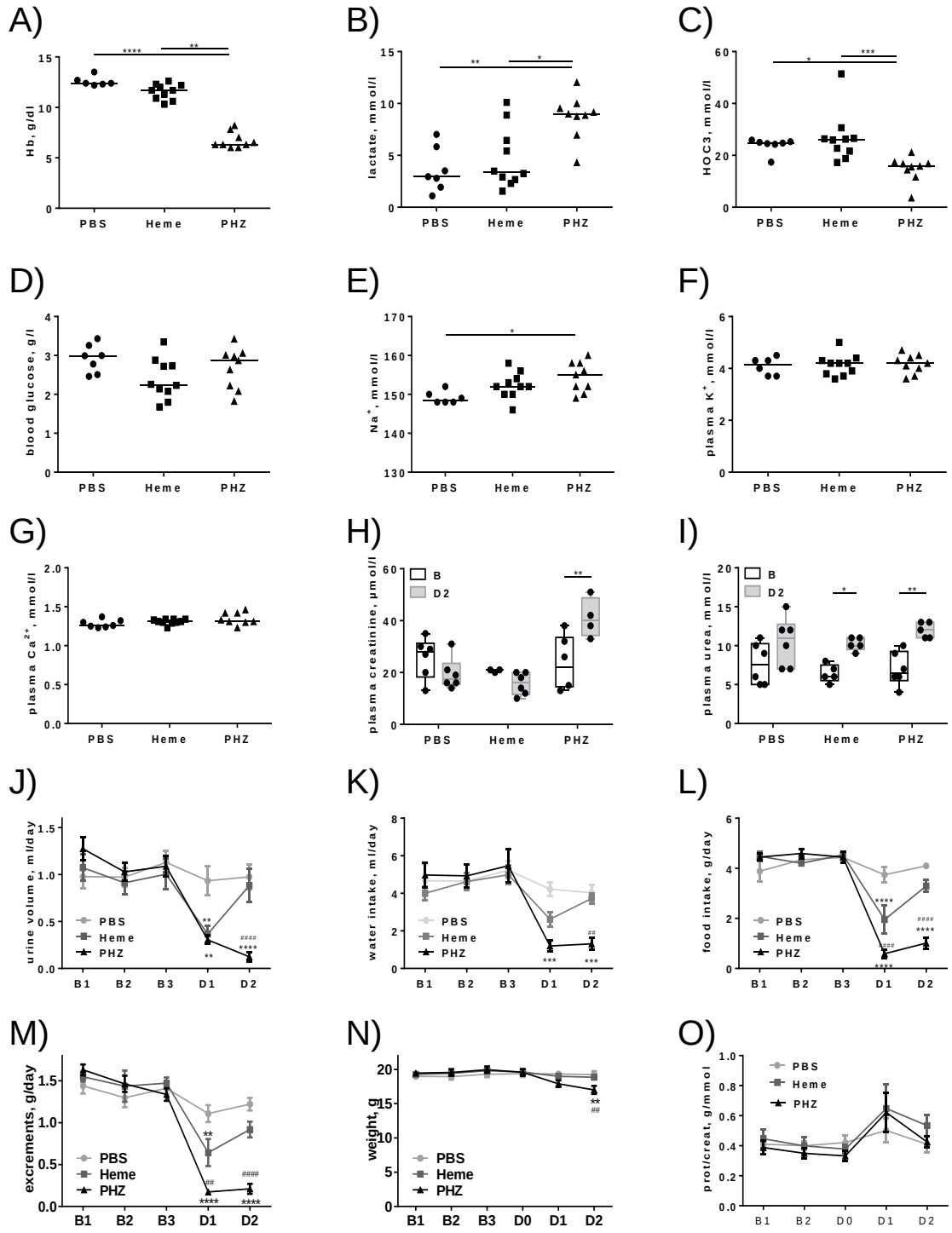

Figure 1: Evaluation of the renal function parameters in the PHZ and heme-injected mice in metabolic cages. A) Blood total hemoglobin, B) plasma lactate, C) plasma HCO_3^-, D) blood glucose, E) plasma sodium, F) plasma potassium, G) plasma calcium, H) plasma creatinine, I) plasma urea. * p<0.05, ** p<0.005, *** p<0.001, Kruskal-Wallis with Dunn's test for multiple pairwise comparisons (A-G) or performed by Two-Way ANOVA with Tukey's test for multiple pairwise comparisons (H, I). Kinetic evolutions of J) urine volume, K) water intake, L) food intake, M) excrements, N) weight, O) proteinuria/creatininuria ratio. B1, B2, B3 correspond to baseline measures 3 consecutive days before injection and D1, D2 correspond to mice follow-up 2 consecutive days after i.p. injection (for each measure, n≥6 per group). ** p<0.005, *** p<0.001, ****: p<0.0001 compared to PBS-treated mice, ##: p<0.005, ####: p<0.0001 compared to heme-treated mice, Two-Way ANOVA with Tukey's test for multiple pairwise comparisons.

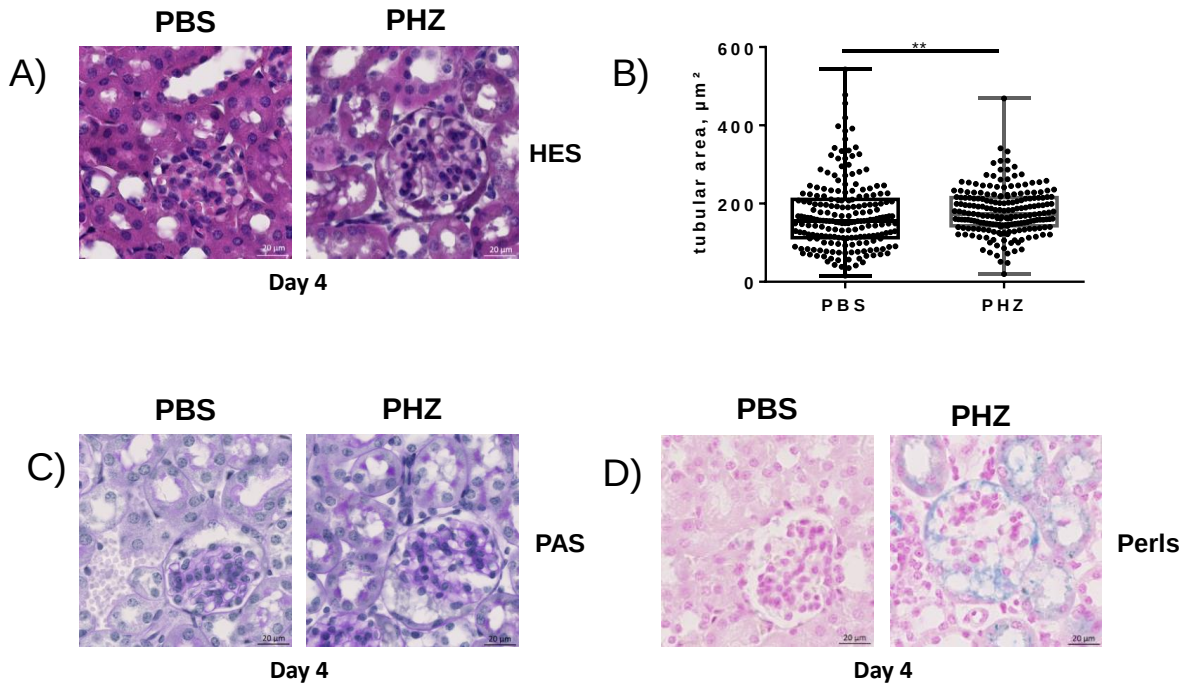

Figure 2: Renal histology of PHZ-treated mice. A) Fixed and paraffin-embedded kidneys from mouse sacrificed at day 4 were cut at 5µm and stained with hematoxylin-eosin (x15). B) Quantitative analysis of proximal tubular size, each point represents the size of one tubule (n=200). Mean +/- SD, **: p<0,005, Mann-Whitney test. C, D) Fixed and frozen kidneys (x20) from mice sacrificed at day 4 were cut at 5µm and stained with PAS (C), Perl's Prussian Blue (D).

Figure 3

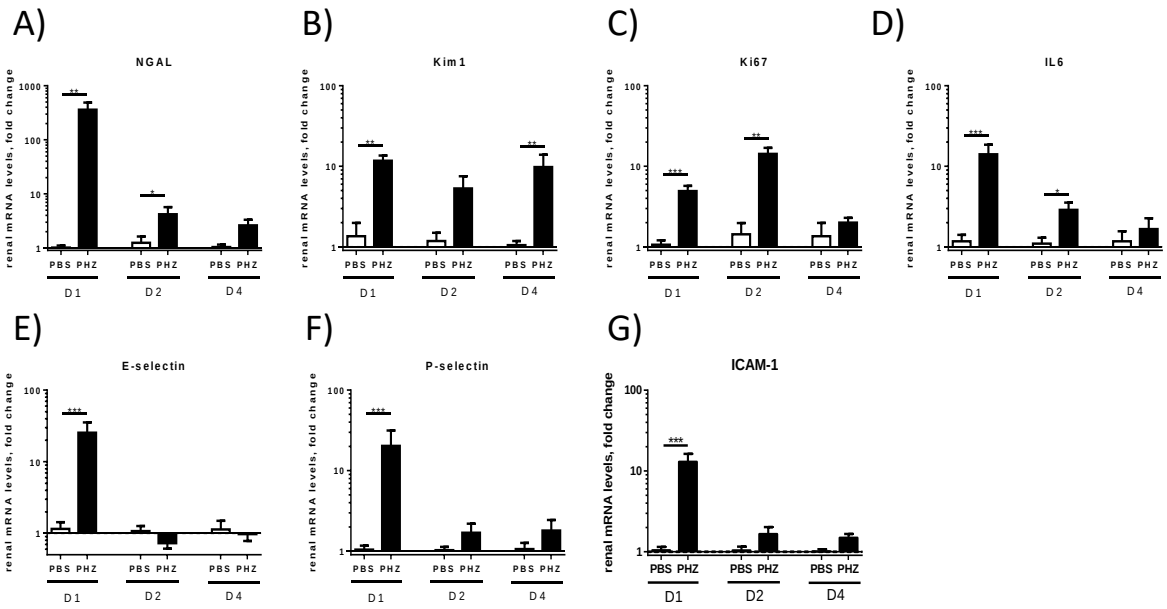

Figure 3: Hemolysis induces renal injury. Kinetics of gene expression in renal tissue of A) NGAL, B) Kim-1, C) Ki-67, D) IL-6 E), E-selectin F), P-selectin G), ICAM-1 at 1, 2 and 4 days after PBS or PHZ injection. Mean +/- SEM, *: p<0.05, **: p<0.005, ***: p<0.001, Mann-Whitney test.

Figure 4

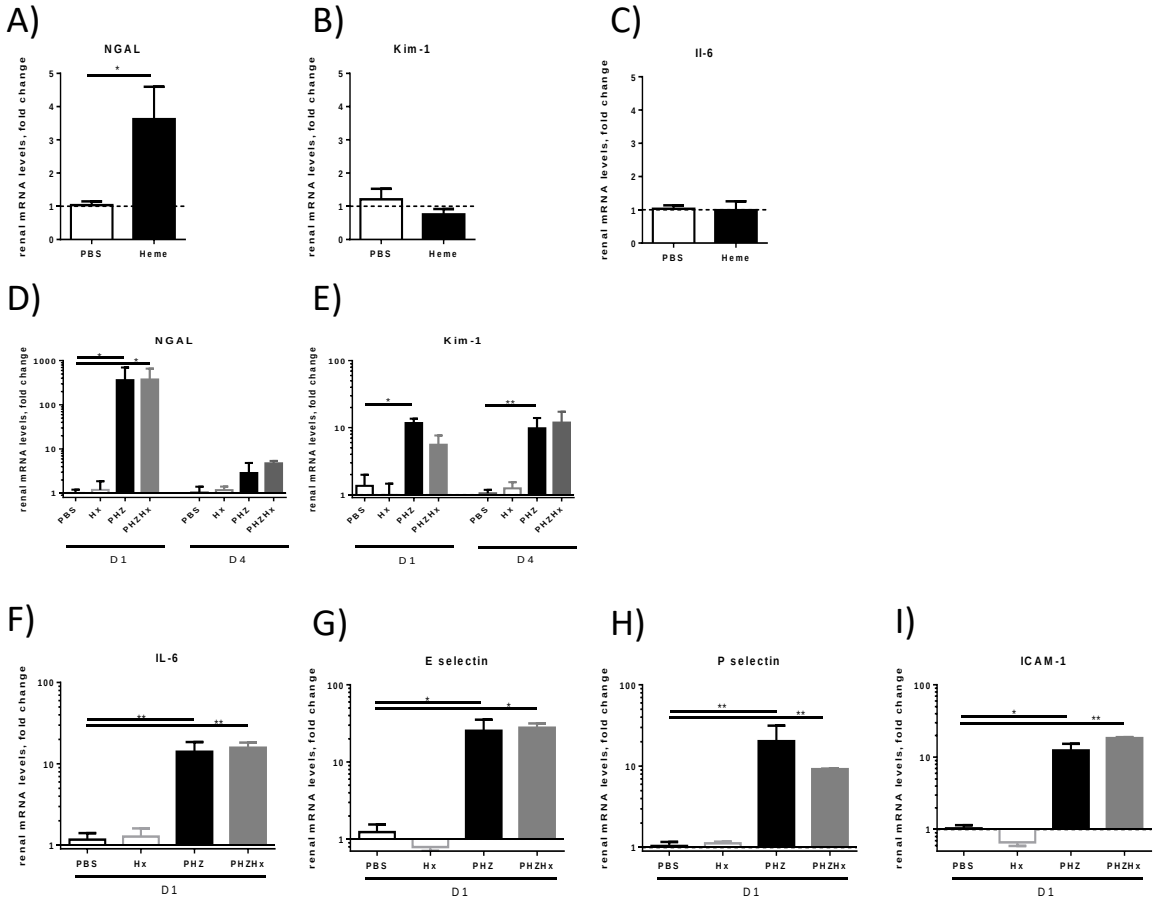

Figure 4: The renal injury is largely heme-independent. Gene expression in renal tissue after injection of heme: A) NGAL, B) Kim-1, C) Il-6. Influence of Hx on the levels of genes expression in the PHZ model, D) NGAL, E) Kim-1, F) IL-6, G) E-selectin, H) P-selectin and I) ICAM-1 *: $p<0.05$, **: $p<0.005$, Mann-Whitney test for A-C and Kruskal-Wallis with Dunn's test for multiple pairwise comparisons for D-I. Panels A-C compare genes expression after two injections of heme at day 0 and day 1. Panels D and E compare the genes expression at day 1 and day 4 (D1 and D4) after injection of PHZ+/-Hx. The remaining panels show results for D1 after injection of PHZ +/- Hx.

Figure 5

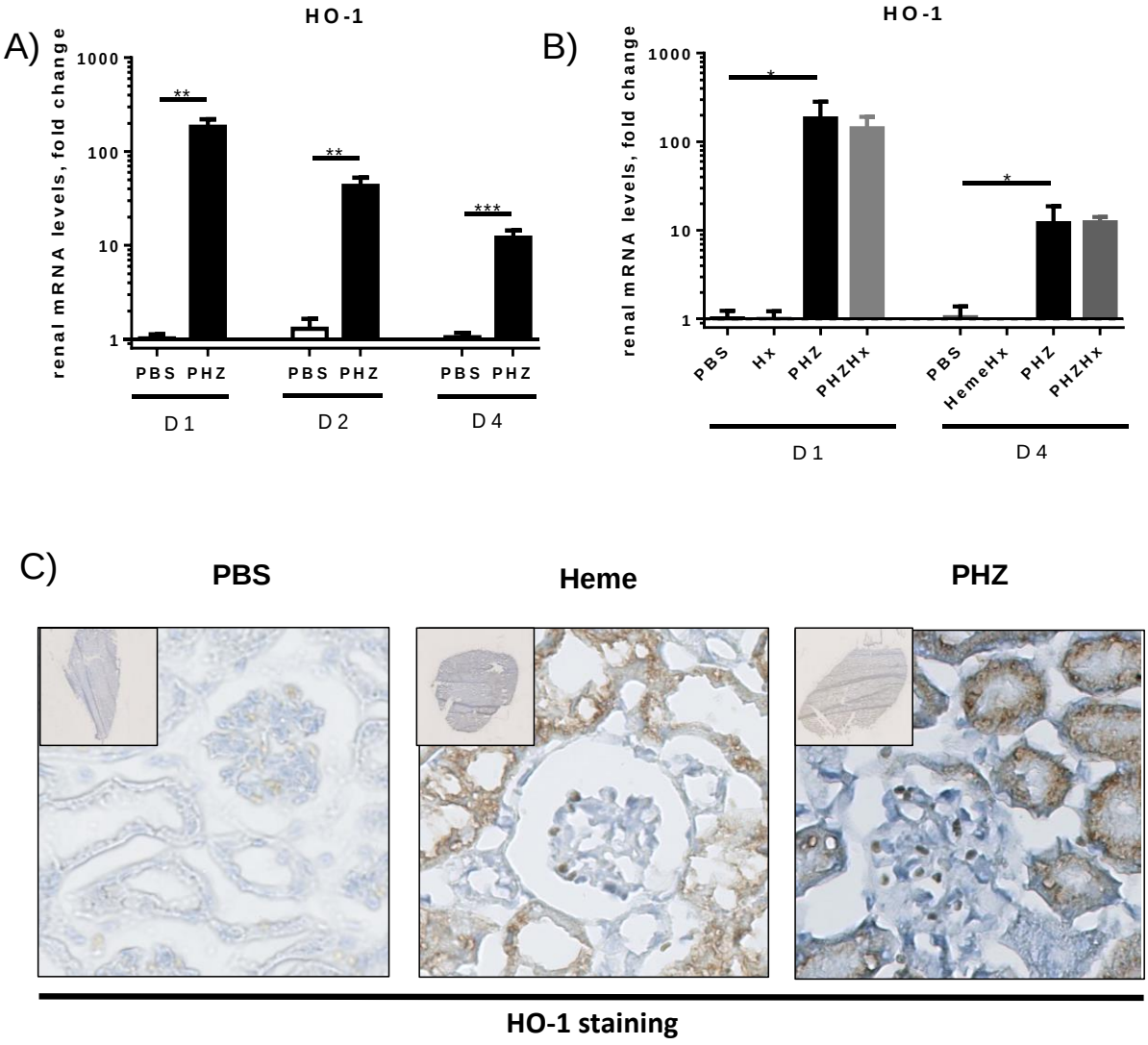

Figure 5: Heme oxygenase 1 expression is induced in tubular kidneys in response to hemolysis. A) Kinetics of gene expression in renal tissue of HO-1. **: p<0.005, ***: p<0.001, Mann-Whitney test. B) HO-1 expression at days 1 and 4 was not modulated by Hx; *: p<0.05, Kruskal-Wallis with Dunn's test for multiple pairwise comparisons C) Immunohistochemistry analysis of frozen kidney sections of mice (x20), injected with PHZ, heme or with the vehicle only. The staining for HO-1 appears in brown and nuclei in blue. Representative images from one out of three or five mice per group (staining performed in 3 independent experiments).

Figure 6

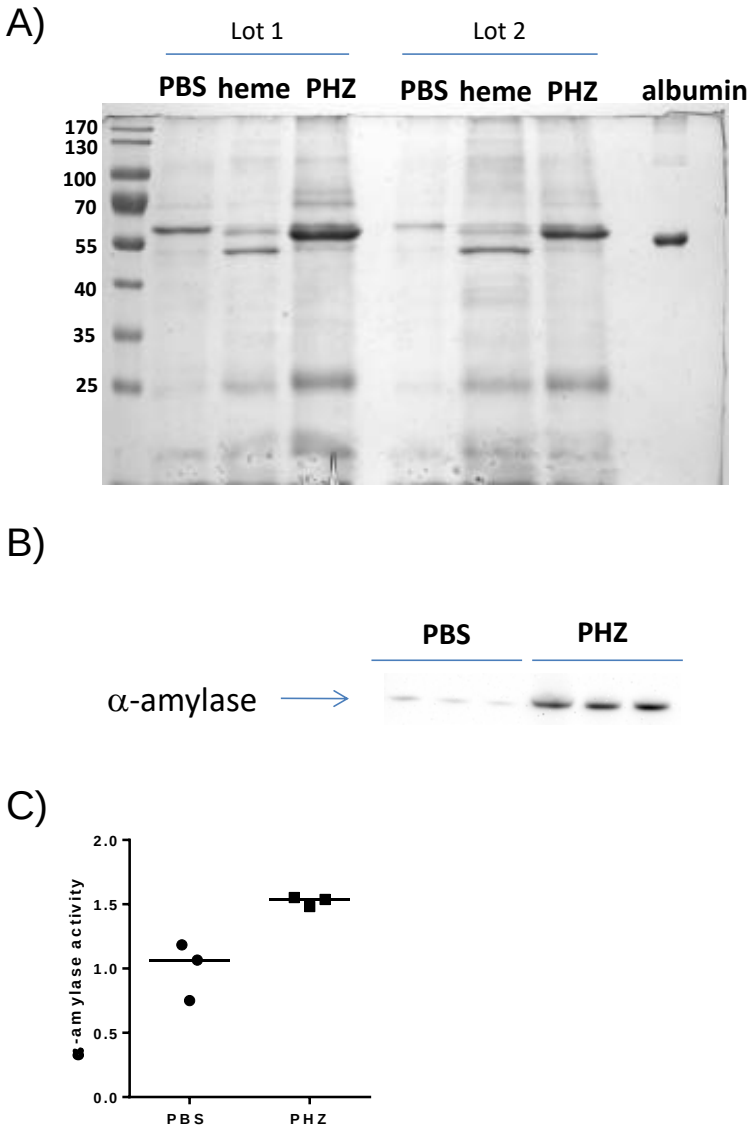

Figure 6: PHZ triggered pancreatic a-amylase activity. A) Proteinuria analysis by Coomassie blue staining of mice treated with PHZ, heme or vehicle only. B) Western Blot analysis of a-amylase excreted in urines of mice treated with PHZ, heme or vehicle only. C) Comparison of a-amylase activity in urines from mice treated with PHZ or vehicle only.

Manuscript 2: **Intravascular hemolysis induces complement system activation mediated by heme and heme-loaded erythrocyte microvesicles**

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Manuscript under revision at JCI Insight

Intravascular hemolysis induces complement system activation mediated by heme and heme-loaded erythrocyte microvesicles

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Conflict of interest

N.B., T.G. and S.M are employees of CSL Behring. LTR receives research funding from CSL Behring. The remaining authors declare no conflict of interest.

Abstract

In hemolytic diseases like sickle cell disease (SCD), intravascular hemolysis results in the release of hemoglobin, heme and heme-loaded membrane microvesicles in the bloodstream. Intravascular hemolysis is then associated with inflammation and organ injury. Our previous studies have demonstrated that the complement system can be activated by heme *in vitro*. However, the interrelation of intravascular hemolysis, complement activation and tissue damage remains unknown. Our objective was to investigate the mechanisms by which hemolysis and red blood cells degradation products trigger complement activation *in vivo*. We analyzed kidney biopsies of patients with SCD nephropathy and model mice with SCD. We detected significant tissue deposits of complement C3 and C5b-9. Moreover, drug-induced intravascular hemolysis or injection of heme in mice triggered C3 deposition, primarily in the kidneys. Red blood cells degradation products like heme-loaded microvesicles and free heme induced alternative and terminal complement pathway activation in normal serum and on EC surfaces, contrary to hemoglobin. Heme triggered rapid P-selectin, C3aR and C5aR expression and down-regulated CD46 expression on EC. Importantly, complement deposition was attenuated *in vivo* and *in vitro* by hemopexin, the potent heme scavenger. In conclusion, we describe for the first time that intravascular hemolysis triggers complement activation *in vivo*, and is associated with SCD nephropathy. Conversely, heme inhibition using hemopexin may provide a novel therapeutic opportunity to limit complement activation in hemolytic diseases.

Introduction

Intravascular hemolysis is a pathological event, leading to red blood cell (RBC) degradation and liberation of hemoglobin (Hb) in the bloodstream. Upon Hb oxidation, heme is released from the globin chains, but is rapidly scavenged by hemopexin (Hx) (1). Upon Hx saturation, labile cell-free heme mediates oxidative stress, vascular damage and inflammation (2).

Heme has been described as a key player in the pathogenesis of various diseases as malaria, sepsis, transfusion complications and sickle cell disease (SCD) (2). SCD is a severe genetic disease and a major public health concern in many countries, affecting around 6 million individuals in all continents (3). In SCD heme participates in vaso-occlusions (VOC), endothelial cell (EC) toxicity and reactive oxygen species (ROS) generation (4–6). SCD patients display high levels of heme-loaded RBC microvesicles (MVs), which distribute heme to tissues and contribute to EC damage and vaso-occlusions (5,7). Heme acts as a damage-associated molecular pattern and triggers activation of monocytes (8) and EC (4–6). *In vitro* studies suggested that heme activates the innate immune complement system in serum and on RBC surfaces (2,9–12). In addition, triggering complement by heme causes an inflammatory reaction, leading to the release of anaphylatoxins C3a and C5a, and the formation of the membrane attack complex C5b-9 on EC surfaces (12). Clinical observations showed a significant increase of pathological markers of complement activation in SCD serum, especially during painful VOCs (13–15), as well as increased levels of surface-bound C3 fragments on RBC (13,16).

Renal damage is a complication of intravascular hemolysis, including in SCD (3). Moreover, the kidney is particularly susceptible to complement attack (9). In SCD nephropathy, the exact mechanism and pathophysiological relevance of complement activation is not established, despite some early reports mentioning C3 fragment deposition in kidneys in man and in a mouse model of SCD (17–19). Further studies are needed to establish the exact link between complement activation, endothelial activation and renal injury in SCD.

Here we demonstrate that complement activation proceeds until the terminal pathway, in SCD nephropathy in man and in kidneys of SCD mouse model. Moreover, hemolysis-derived products, heme and SCD RBC-derived MV, activated complement in serum and on EC *in vitro*, and in a mouse model of intravascular hemolysis and induced complement-related stress markers on EC. *In vivo* and *in vitro,* C3 fragments were antagonized by Hx.

Results

Cohort of SCD nephropathy.

On light microscopy, predominant pattern of glomerular disease was specific SCD glomerulopathy (n=5), FSGS (n=3), MPGN (n=3) and TMA (n=2) (Table 1). Glomerular lesions were associated in 10/13 patients with chronic tubulointerstitial lesions with different degrees of interstitial fibrosis and tubular atrophy. Three patients had tubular casts and 6 patients showed inflammatory cell infiltrate (macrophages). Perls staining revealed hemosiderosis involving renal tubules in 7/8 tested patients. Chronic vascular lesions were observed in 10/13 patients resulting in hyaline arteriosclerosis (HA) in most of patients. One patient had TMA lesion involving extra glomerular small vessels. By immunofluorescence, C3 staining was positive in 9/11 in glomeruli (capillary walls and/or mesangium) and/or in vessels. Polyclonal immunoglobulin staining was positive in 5/11 patients in glomeruli.

Complement deposition is present in kidneys of patients and mice with SCD

C3 staining was positive in 9/11 (82%) of the SCD nephropathy patients' biopsies in glomeruli (capillary walls and/or mesangium) and/or in vessels (Figure 1A, Table 1, Supplemental results). C3 staining was negative in 10 kidney biopsies taken during the same time period for different reasons (excluding SCD), and evaluated by the pathologists as normal. Two of 3 tested SCD biopsies were positive for C9 staining (Figure 1B). Graft rejection biopsy is shown as a positive control for C3 and C9 staining. Polyclonal immunoglobulin staining was positive in 5/11 (45%) patients in glomeruli (Table 1).

A similar pattern of C3 and C5b-9 deposition was detected in transgenic SAD mouse kidneys. Staining for C3 fragments significantly increased in SAD versus WT littermate mice at 6 months of age (Figure 1C, D), while at 3 months staining was negative (data not shown). C3 deposition was mostly peritubular or intra-glomerular (Figure 1C). Moreover, complement activation partially co-localized with the EC marker CD31 in glomeruli (Figure 1E). Kidneys stained also positive for C5b-9 deposits (Figure 1F, G) and IgG (data not shown).

Characterization of a model of intravascular hemolysis

SCD nephropathy, where we detected C3 deposits (Figure 1A) is a chronic disease. In order to study whether the complement deposits observed in both sickle patients and in sickle mice were related to hemolysis, we set up a mouse model of drug-induced intravascular hemolysis to characterize the kidney injury and complement deposits.

Hemolysis induction by phenylhydrazine (PHZ) was evident from the darker color of the urine and organs (not shown), and by significantly decreased hematocrit in blood (Figure S1A). Plasma concentration of Hx in mice was about 0.15 mg/ml. Twenty-four hours after treatment, endogenous Hx was increased in the circulation (Figure S1B). Concentration of endogenous Hp significantly increased 6 hours after PHZ-treatment, and found in high concentration after 24 hours (Figure S1C). However, Hx-treatment did not induce an increased-concentration of Hx or of Hp (Figure S1B and S1C). Human Hx was found up to 0.4 mg/ml (more than two-fold over the basal level of endogenous Hx) 6 hours after Hx-injection and remained detectable after 24 hours (Figure S1D).

Immunohistochemistry performed on frozen kidney sections revealed no HO-1 expression in PBS and Hx-treated mice (Figure S2A). Perls Prussian blue staining for hemosiderin, a hallmark for hemolysis, was positive in the PHZ-injected mice (Figure S2B). The induction of hemolysis by PHZ injection increases HO-1 expression within 24 hours, which is not modified by Hx (Figure S2A). By light microscopy, analysis of kidney sections from PBS- and PHZ- treated WT mice revealed no modification of histology (Hematoxylin-Eosin staining) and glomeruli size (Figure S2C,D).

Intravascular hemolysis induced heme-dependent complement deposits in tissues, including kidneys and endothelium

After injection of PHZ, we detected significant acute effects subsequent to hemolysis by loss of weight and a peak of endogenous Hx and Hp at 24h (Supplementary Figure S1A-D). Little or no C3 fragment deposition was found by immunofluorescence (Figure 2A,B) and immunohistochemistry (Supplementary Figure S3A) in PBS-injected animals. Significant increase of C3 fragment deposition was observed in kidney sections from PHZ-treated mice, located in glomeruli or around blood vessels and tubules (Figures 2A,B), was measured by

immunofluorescence and confirmed by immunohistochemistry in an independent set of experiments. C3 deposits in glomeruli partially co-localized with EC marker vWF (Figure 2C).

To find out whether these changes are heme-dependent or occur due to upstream products (such as hemoglobin), a heme scavenger (human plasma-derived Hx), was injected i.p. (confirmed as reaching the circulation, Supplementary Figure S1E). Treating mice with Hx attenuated significantly and nearly completely the complement activation in kidneys (Figure 2A,B and S3A). Hx injection alone did not activate complement. Moreover, injection of free hemin reproduced the positive staining and the deposits were reduced by Hx (Figure 2D). C5b-9 staining was negative (Figures 2E).

The same pattern of complement activation was reproduced in the heart, although with a lower C3-staining intensity (Supplementary Figure S3B). Staining for C3b/iC3b on RBC from PBS, PHZ or hemin-injected mice was negative (data not shown).

Heme triggers rapid EC surface complement deposition in vitro, which is counteracted by Hx and to less extend by human serum albumin (HSA)

The protective effect of Hx against complement deposition on EC *in vivo* and *in vitro* could be related at least to two phenomena – acting against targets in serum and on EC. We compared the efficacy of HSA and Hx in in vitro experiments using HUVEC. Two settings were used: First heme was added with the serum-free culture medium and after 30 min NHS was added to the wells, while heme was still present (H+NHS condition). This condition evaluates the cumulative effect of the EC activation, which renders the cells susceptible to complement deposition together with the direct activation of complement in serum. In the second setting, EC were incubated with heme for 30 min and after that the heme-containing supernatant (SN) was removed before addition of NHS (SN removed condition). This condition evaluates the complement activation due to modification of the EC surface only.

Hemin, but not Hb, activated complement in fluid phase in serum and on the EC surface (Figure 3A,B). We observed that 5 µM of Hx inhibited the effects of up to 50 µM hemin on C3 and C5b-9 deposition, while HSA had no effect at this dose and was overall 8-10-fold less efficient compared to Hx (Figure 3C-F, Supplementary Figure S4A-E).

The inhibitory effect of different concentrations of Hx was compared to HSA, because of the high HSA concentrations in blood and its affinity, albeit low, for heme (Supplementary Figure S4A-C). Exposure of EC to heme with increased concentration of Hx prevented dose-dependently C3 fragments deposition on cell surface. Around 90% of inhibition of the deposition caused by 50 µM heme was obtained up to 10 µM of Hx in both conditions - when SN was removed and when NHS was directly added to medium in presence of heme (Figure S4A). However, higher amounts of HSA were needed to inhibit C3 fragments deposition, since 40% of C3 fragments deposition remained detectable with 80 µM of HSA (Figure S4A). Efficiency of HSA was similar when SN was removed or not as measured by HSA titration (Figure S4B,C). However, Hx was more efficient at low doses (≤5 µM) when SN was removed. In order to compare the potential inhibitory effect of 5 µM of Hx with HSA, HUVEC were treated with 5 µM of HSA with increased concentration of heme. SN was removed before addition of NHS. C3 fragment deposition and C5b-9 formation were not controlled by the addition of 5 µM of HSA in the medium (Figure S4D and S4E).

As previously observed by others (4,20), our experiments *in vitro* showed that we did not observe a one to one neutralizing effect between Hx and hemin. This can be explained by the poor solubility of hemin, which tends to aggregate in aqueous solutions, decreasing its active concentration (1,2).

Hx protects the EC from acquiring a complement activating phenotype.

So far we have seen that the complement deposition resulting from PHZ induced hemolysis was heme dependent and responded to Hx. We therefore aimed to study this phenomenon *in vitro* in a rapid timeframe (30 mins) and to compare the effect of hemin and hemoglobin as well as the scavenging by Hx and HSA. On the EC surface, hemin caused dose-dependent decrease of C3b regulator MCP expression (up to 50%), which was prevented by Hx (Figure 4A,B). In contrast, equimolar HSA had no effect (Supplementary Figure S4F). Moreover, hemin induced rapid, Hx-sensitive expression of P-selectin, a molecule known to recruit C3b on EC surfaces (Figure 4C).

We detected a 2 to 2,5 fold increase in cells with high expression of anaphylatoxin receptors C3aR and C5aR, within 30 mins, at 50 µM of hemin (Figure 4D,F). This effect was dose-dependent (Figure 4E,G). Hx at 5 µM inhibited this increase, while equimolar HSA had no

effect (Supplementary Figures S4G,H). The dotted line (F-H) represents the surface expression in absence of heme (0 heme in the titration curve).

RBC membranes induce complement activation in a heme-dependent manner

RBC MVs may represent a significant and pathological form of heme carrier in plasma, particularly during intravascular hemolysis, and most likely in SCD (5). We tested the potential capacity of RBC membrane fragments to activate complement, measured by Ba release, and the inhibitory effect of Hx. First, we compared the complement-activation capacity of RBC MVs generated *in vitro* using two different methods in order to trigger vesiculation: a Ca^{2+} ionophore or mechanical stimulation (shear stress). MVs prepared with the Ca^{2+} ionophore were selected for further studies due to the higher production yield (Supplementary Figure S5A).

Stimulating EC with these MVs induced a dose-dependent increase in both Ba and sC5b-9 release (Supplementary Figures S5B,C). SCD RBC MVs, (which carried 2 to 3 times more heme than control RBC MVs, as previously described (5)) significantly increased Ba and sC5b-9 release compared to HD levels (Figures 5A,B). There was a linear correlation of Ba with sC5b-9 release (Figure 5C). Hx significantly decreased complement activation for SCD and HD RBC MVs (Figures 5D,E and Supplementary Figure S5D). Of note, RBC MVs from SCD patients during vaso-occlusive crisis (VOC) or patients at steady state stimulated an equivalent release of Ba and sC5b-9 complexes (Supplementary Figure S5E,F).

We then tried to model RBC MVs with synthetic phospholipid multilayer vesicles (MLVs), composed of phosphatidylcholine and phosphatidylserine (PS), and artificially loaded with heme. Naked MLV activated complement, as illustrated by sC5b-9 release in NHS, in a Hx-insensitive fashion (Supplementary Figures S6A). However, heme-loaded MLV induced even stronger sC5b-9 release in fluid phase, which was blocked by Hx (Supplementary Figures S6B), similarly to the results with free heme (Supplementary Figures S6C).

We found significant increase of C3 deposition on endothelial surfaces in the presence of SCD MVs, compared to control MVs (Figure 5F,G). Hx prevented C3 deposition (Figure 5H). On endothelium, a concentration of 1000 SCD MV/µL had comparable effects to 6 µM

of free hemin. The concentration of heme previously reported in a similar preparation of RBC MV approximated 5 µM(5).

Hemolysis and heme activate complement by the alternative pathway (AP)

We confirmed here that hemin activates complement by the AP in NHS (11,12) (Supplementary figure S6C), since the sC5b-9 release in presence of Ca^{2+} chelator EGTA and Mg^{2+} (allowing activation of the AP only) was identical to that in the presence of Ca^{2+} and Mg^{2+} in the buffer (allowing activation of all pathways). Similar results were obtained for HD MVs and MLVs (Supplementary Figure S6A,B,D). Moreover, Ba, which is a specific marker of the AP, was released after addition of RBC MVs in serum, and is sensitive to Hx (Figure 5A,D).

To evaluate the contribution of the AP *in vivo*, we induced hemolysis in $C1q^{-/-}$ mice, which are unable to activate the classical pathway. C3 deposits, in kidneys were still present despite C1q deficiency (Supplementary Figure S6E).

Discussion

The results presented here help to decipher the mechanisms of complement activation during intravascular hemolysis. Complement activation in kidneys is a key factor for local inflammation, vascular and tissue damage in a large spectrum of renal diseases (21). The deposition of C3 fragments in tissues represents a durable signal of tissue inflammation and a sensitive and robust indicator of disease activity (22). We observed significant C3 deposits in human SCD nephropathy biopsies and SAD mouse kidneys, in agreement with previous observations (18,19).

Here, we demonstrate for the first time that intravascular hemolysis triggers complement activation *in vivo*, in a process dependent on free heme. The C3 fragment staining patterns in our drug-induced hemolysis and SAD mouse models were similar. For the first time, we also reveal glomerular C5b-9 deposits in human SCD and SAD mouse kidneys, demonstrating that complement activation progressed up to its terminal phase of C5b-9 complex assembly and

deposition in this disease. *In vitro* assays of complement activation, in serum or on EC surfaces, upon exposure to hemin or heme-loaded RBC MVs supported this conclusion. Furthermore, complement activation during hemolysis progressed through the AP, despite any suspected effects of heme on immunoglobulins and a potential activation of the classical pathway (2,20,23–25). This corroborates previous clinical reports of increased AP marker Bb and sC5b-9 complexes in SCD patients' serum (13–15).

To simulate the consequences of intravascular hemolysis, we studied complement activation in response to RBC degradation products like hemin, hemoglobin and RBC MVs, which represent a significant form of heme carriers in plasma during intravascular hemolysis and in SCD (5). RBC MVs levels increase about 3-fold during VOC in SCD (26,27). RBC MVs also contribute to inflammation in SCD and transfusion-related complications (25,28). RBC MVs can trigger vaso-occlusions in SAD mouse kidneys, in a heme-dependent and Hx-inhibited process (5,7). Here, RBC MVs from SCD patients activated the alternative and terminal complement pathways in a largely heme-dependent manner. This is confirmed by the similar complement activation produced by artificial heme-loaded MLVs.

In cultured EC, C3 and C5b-9 deposition after hemin exposure could be explained by a rapid cell surface expression of P-selectin, an inflammatory mediator and a C3b-ligand (29,30). These were completely inhibited by Hx. P-selectin is known to recruit C3b to the EC surface, and it represents an interesting therapeutic target, as a cell adhesion molecule that favors blood cell adhesion to vessel walls and vaso-occlusive crises in SCD (39,40). EC exposure to heme also resulted in a loss of the C3b-regulator, MCP and an increase in C3aR and C5aR expression, in line with the C3aR upregulation reported in transfusion-induced hemolysis (31). Therefore hemolysis and heme in particular, unlike Hb, may thus enhance the responsiveness of EC to complement-mediated inflammation. Released C3a and C5a can bind to C3aR and C5aR to mediate pro-inflammatory processes. We have previously shown that heme induces hydrolysis of C3 into C3(H2O) and provokes direct complement activation in the serum (12). C3b generated by the fluid phase C3 convertase will bind to the EC membrane and will form new AP C3 convertases that can locally auto-amplify and generate C3b and C5b-9 deposition. Taken together, our data establishes activation of the complement AP and EC sensitization to complement in response to intravascular hemolysis, *in vivo* and in SCD (Figure 7).

Our work points at new avenues in order to reduce inflammatory process during hemolysis, one of them being indirect complement inhibition by scavenging heme. The experimental injection of Hx just before PHZ administration in the model of intravascular hemolysis clearly attenuated complement deposits, demonstrating the causal role of free heme. *In vitro* experiments showed that Hx, and HSA at much higher doses, protected EC from heme-mediated stress and subsequent complement activation *in vitro*. HSA is present at higher levels in plasma compared to Hx and can contribute to heme scavenging and protection against complement activation. Nevertheless, Hx but not HSA showed efficacy in preventing tissue injury and inflammation in mouse models of hemolysis (38). Previous reports have also shown that hepatic overexpression of Hx inhibits inflammation and vascular stasis in murine SCD (32), while $Hx^{-/-}$ mice develop a severe renal damage after experimental hemolysis, pointing to Hx as a guardian of kidney function during intravascular hemolysis (33). Hx could be also useful in case of heart dysfunction subsequent to hemolysis. Indeed, in a model of repetitive injections of low doses of PHZ, cardiac oxidative stress was counteracted with preventive injections of Hx (34). The recent discovery of a specific antibody against heme, the 2H10, will provide further information about contribution of heme in pathogenesis of hemolytic diseases (35). Benefits of Hx are indirect evidences of the harmful effects of heme. Therapeutic Hx administration is under intense scrutiny to alleviate heme-induced inflammation, including in SCD (5,34,36–38).

However, Hx administration remained insufficient to prevent inflammation and renal injury in an old blood transfusion hemolysis model (38) as well as in our PHZ model (data not shown). In PHZ-induced hemolysis, a peak of Hx and haptoglobin levels was observed in plasma after 24 hours, as well as expression of the cytoprotective heme-degrading enzyme HO-1. However, despite this acute phase response, HO-1, and endogenous Hx and Hp failed to prevent complement deposition and exogenous Hx was needed. One explanation could be the acute and extensive nature of old blood transfusion and PHZ-induced hemolysis, as opposed to the chronic but lower grade hemolysis occurring in SCD patients. Moreover, acute hemolysis may generate other RBC-derived products (hemoglobin, MVs, etc) in quantities sufficient to sustain tissue injury independently from heme. Indeed, haptoglobin administration decreased significantly renal injury in the transfusion model (38). The acute and complex nature of the PHZ mouse model was useful for a mechanistic study, but it does not allow us to determine to what extend complement activation contribute to the pathological process of hemolytic diseases.

Evidence for the pathogenic role of complement comes from cases and models, where direct complement inhibition was tested in hemolytic diseases, including SCD. Targeting C5 with a blocking antibody was shown to inhibit vaso-occlusions in a mouse model of SCD (41), while inhibiting C3 with Compstatin CP40 is investigated *in vitro* in a model of malaria (42,43). However, CP40 remains still unavailable for clinical use. Complement inhibition downstream of C3 is now possible in the clinic, using the monoclonal, C5-blocking antibody Eculizumab, which abrogates terminal pathway activation. Cases of the efficacy of Eculizumab against delayed transfusion reaction and hemolysis in SCD are emerging (44), showing the importance of complement for the disease process.

In summary, our study demonstrates alternative and terminal complement pathways activation during intravascular hemolysis and in SCD. Further research for efficient complement inhibition is thus fully warranted. It also suggests that heme scavenging strategies like Hx supplementation could indirectly help target complement activation (Figure 7).

Methods

Reagents

The Fe^{3+} form of heme (hemin [ferriprotoporphyrin IX], designated as heme, (Frontier Scientific Inc. or Sigma-Aldrich) was dissolved to 20 mM in 50 mM NaOH and 145 mM NaCl, and further diluted in an appropriate vehicle just before use. Stock solution of phenylhydrazine (PHZ) of 25 mg/mL (Sigma-Aldrich) was prepared in PBS immediately before use. Plasma-purified Hx and human serum albumin (HSA) was provided by CSL Behring. Complement ELISA kits MicroView Ba and sC5b-9 were from Quidel. Microvesicles (MVs) were obtained from SCD patients or healthy donors (HD) RBC after vesiculation with calcium ionophore A23187 or mechanical vesiculation as previously described (7).

Patients

Patients and study approval

Kidney biopsies and corresponding clinical data from 13 SCD patients were retrieved from the archives of the Necker-Enfants Malades Hospital (n=10) and the Pathology Institute of the CHRU Lille (n=3) to constitute a cohort of SCD nephropathy (Table 1) A normal protocol kidney allograft biopsy, performed at 3 months, was used as a negative control. A biopsy of a patient with acute humoral rejection and another from a patient with lupus nephritis were used as positive controls for the staining. Patients provided their written informed consent for the biopsies, the use of clinical data and the secondary use of histological material for research. This study was performed in accordance with the Declaration of Helsinki.

Classification of SCD patients

Kidney biopsy of 13 SCD patients is detailed in Table 1. In 6 cases, kidney biopsies were performed because of chronic renal failure and in the remaining 7 cases, because of proteinuria. The grading scale used was: -for no lesion, + for rare or few, ++ for moderate, and +++ for severe lesions.

Staining for complement in patients' kidney biopsies

Routine staining protocol for C3 (polyclonal rabbit anti-C3c Human Complement/FITC (Dako, F0201)) was applied for the patients with missing archived images. For the 3 patients from CHRU Lille C9 staining was also available (rabbit anti-human C9 - Abcam, ab71330) used in Ventana XT autostainer (Ventana Medical systems, Benchmark XT, Strasbourg, France). Whole slides were scanned and analyzed by an Axio Scan (Zeiss) or Nanozoomer (Hamamatsu).

Animal experimentation

Study approval. All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with the approval APAFIS#3764-201601121739330v3 of the French Ministry of Agriculture.

Mouse treatment. C57Bl/6 mice were from Charles River Laboratories, (L'Arbresle, France) and $C1q^{-/-}$ were from an in house colony, which was a kind gift from Prof. Marina Botto (Imperial College, London). Eight week old female C57Bl/6 mice were injected i.p. with 200 µL of PBS (Gibco), human Hx alone (0.491 µmol/kg, corresponding to 28 µg/g body weight (36)), freshly prepared heme (40 µmol/kg, corresponding to 26 µg/g body weight (6)) repeated 24 hours later (day 0 and day 1). Alternatively, mice were injected only at day 0 and organs were recovered at day 1 or day 2. PHZ (900 µmol/kg, corresponding to 0.125 mg/g body weight) was injected i.p. at day 0 and C57Bl/6 mice were sacrificed at day 1, or $C1q^{-/-}$ mice were sacrificed at day 1. To test the efficacy of Hx, mice were pre-treated with i.p. injection of 40 µmol/kg of human Hx diluted in PBS one hour before i.p. injection of 0.491 µmol/kg of freshly prepared heme or 0.1 mg/g body weight of PHZ. Concentrations and route of administration were chosen as described (6,34,36). Blood was collected from the submandibular vein 3 days before the first injection (baseline) and before endpoint (day 1).

SAD mice. SAD mice carry a human Hb β-chain transgene with 3 mutations (b**S** b6Val, bS-**A**ntilles, b23Ile, and **D**-Punjab b121Glu) on a C57Bl/6J background and are a well-characterized model of SCD and SCD-associated renal injury (17). Frozen kidneys from 3 and 6 months-old SAD mice were used for this study.

Immunofluorescence on mouse tissue.

Five µm thick frozen sections of kidneys, hearts and spleens were cut with Cryostat Leica AS-LMD and fixed in acetone on ice for 10 min. Complement deposition was studied using rat anti-mouse C3 fragments (C3bi/C3b/C3c) IgG1 (Hycult Biotech, HM1065), visualized with goat anti-rat-AF488 (Invitrogen, A-11006) and rabbit anti-mouse C5b-9 (Abcam, ab55811), visualized with goat anti-rabbit IgG-AF488 (Invitrogen, A-11008). Double staining for C3 deposition and EC marker CD31 was performed using rabbit anti-mouse CD31 (Abcam ab28364), revealed by anti-rabbit AF555 (Invitrogen A-21429). Stained slides were scanned by Slide Scanner Axio Scan (Zeiss) or analyzed by confocal microscopy (Confocal LSM710, Zeiss). Quantification of the staining was performed using Visiopharm software (Visiopharm A/S, Hørsholm, Denmark) by measuring the fluorescence intensity of the staining within the cortex reported on the total cortical surface.

Immunohistochemistry on mouse tissues

Five µm thick frozen sections of kidneys and hearts were cut with Cryostat Leica AS-LMD and fixed in acetone on ice for 10 min. Complement deposition was studied using rat anti-mouse C3 fragments (C3bi/C3b/C3c) IgG1 (Hycult Biotech, HM1065), followed by a goat anti-rat polyclonal IgG-HRP (R&D system). HO-1 expression was studied using rabbit anti-mouse HO-1 (Abcam, Ab13243) followed by a polymer anti-rabbit (DAKO, K4003). Both staining were revealed with DAB solution. Slides were scanned by Nanozoomer (Hamamatsu). Hematoxylin-Eosin and Perls coloration were performed by routine procedures using sections of paraffin-embedded kidneys. Coloration of slides was scanned by Slide Scanner Axio Scan (Zeiss).

Flow cytometry assessment of C3b/iC3b on mouse RBC

Blood from PBS, PHZ or heme-injected mice were recovered 24 hours after injection and RBC were purified. These cells were stained for C3 fragments deposition using rat anti-mouse C3bi/C3b/C3c IgG1 (Hycult Biotech, HM1065), visualized with goat anti-rat-AF488 (Invitrogen, A-11006). Alternatively, RBC from naïve mice were purified and incubated at $1x10^6$ cells/tube for 30 min with 50 µM heme, followed by addition of normal mouse serum for 30 min. Cells were washed and probed for C3 fragments deposits by flow cytometry (BD LSR II), and further analyzed by FlowJo X.

Haptoglobin and Hemopexin quantification

Ten µL of plasma sample was placed into a clean Eppendorf tube followed by the addition of 75 µL MeOH to precipitate the protein. The methanol was removed after centrifugation and the pellet was air-dried and afterwards resuspended in 50 mM NH4HCO3/0.16% ProteaseMAX containing heavy-isoptope labeled peptides, which are specific for human hemopexin or endogenous haptoglobin/hemopexin and used as internal standards. Samples were incubated at 56°C/550 rpm for 45 min, samples were reduced by adding 0.5 M DTT (56°C/550 rpm for 20 min) and afterwards alkylated by adding 0.5 M IAA and incubated for 20 min at RT protected from light. Tryptic digestion was carried out at 37°C/550 rpm for 3 h and the samples were separated immediately on a C18 column (AdvanceBio Peptide Mapping, 2.1 x 250 mm). The measurements were conducted using Agilent 6550 iFunnel QTOF mass spectrometer connected to an Agilent 1290 Infinity II HPLC instrument.

Data was analyzed by calculating the peak area of the analyte and the internal standard using Agilent MassHunter Quant software. A standard curve was created by Agilent MassHunter Quant; where the average ratio of the analyte response to the internal standard response against concentration was plotted.

Complement activation and complement receptors on EC

Flow cytometry. Primary human umbilical vein endothelial cells (HUVEC) were cultured in 24-wells plates as previously described (12,45), exposed to heme or RBC MVs at the indicated doses for 30 mins at 37°C in serum-free M199 cell culture medium (Gibco) in presence or absence of Hx or HSA. For evaluation of complement activation, HUVEC either washed and further exposed to normal human serum (NHS) diluted to 33% in M199 medium or serum was added to final dilution of 33% to the wells, already containing heme+/-hemopexin, for 30 mins at 37°C. HUVEC were used for experiments until passage 4. Cells were washed, detached, labeled and fixed in 0.5% formaldehyde. Cells were analyzed by flow cytometry (BD LSR II), and further analyzed by FlowJo X. The following antibodies were used for staining: anti-human MCP-RPE (BIORAD, MCA2113PE), anti-human C3aR-PE (BioLegend, 345804), anti-human C5aR-APC (BioLegend, 344310), in addition to the corresponding isotype controls. After incubation with serum, staining was performed with anti-C3c mouse monoclonal IgG1 antibody (Quidel, A205), or with anti-C5b-9 mouse

monoclonal IgG1 (kindly provided by Prof. Paul Morgan, Cardiff University) or an isotype control and revealed by anti-mouse-IgG1-PE secondary antibody (Beckman Coulter, IM0551).

Immunofluoresence. HUVEC cells were cultured on slides in 24-well plates. Cells were incubated for 5 mins in presence of 50 µM of heme, with or without 5 µM of Hx. Cells were fixed in paraformaldehyde 4% after staining with anti-human P-selectin primary antibody (BIORAD, MCA796) or an isotype control and revealed with AF-488 secondary antibody (Invitrogen, A21204), and DAPI (to stain nuclei). Staining was analyzed by Axiovert 200M microscope (Zeiss).

Complement activation in serum.

MVs, generated from healthy donors (HD) or SCD patients' RBCs, as well as artificial multilayer vesicles (MLV) loaded or not with heme were incubated with buffer, Hx or HSA for 10 mins, followed by 33% NHS during 30 mins at 37°C. MVs were diluted at a final concentration of 1000 MV/µL, 2500 MV/µl or 5000 MV/µl in TBS with 4 mM $CaCl_2$ and 4mM $MgCl_2$, allowing activation of the three complement pathways. Or MV's were diluted in TBS with 10mM EGTA and 4mM $MgCl_2$, thus allowing activation of the alternative pathway (AP) only. The contribution of the AP was determined by the difference in the signal between the two conditions. Ba and sC5b-9 levels in the serum were evaluated according to the ELISA kit instructions (Quidel). EDTA was used as a negative control to block complement activation.

Statistics

Results were analyzed using a statistical software package (GraphPad Prism 5) as indicated in the figures legends.

Authors' contributions

Study design: LTR and NM. Perform research: NM, AG, HR, VG, MLF, SK, SB, DC, RN, TRR, TG, MR, SC. Care for the patients and provided patients material: MF, SLJ, MR. Provided purified hemopexin and instructions for its use: NB, SM. Discussed the data: LTR, OBB, JDD, VFB, NM, AG, VG, MF, SLJ, PH, SC, MR. All authors wrote of the manuscript and approved the submission.

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Table 1. Description of the renal biopsies of patients with SCD nephropathy

Patient n°	1	2	3	4	5	6	7	8	9	10	11	12	13
Light microscopy													
Nb of glomeruli	17	15	15	25	na	18	7	22	22	9	12	10	36
Nb of sclerotic glomeruli (%)	0 (0)	5 (33)	10 (66)	8 (32)	na	4 (22)	3 (43)	5 (23)	6 (27)	2 (22)	3 (25)	1 (10)	6 (17)
Glomerular lesions													
Glomerular hypertrophy	++	++	+	++	+++	+	+	-	na	++	++	++	+
Mesangial hypertrophy	+	++	+	++	-	++	+++	-	++	++	++	+	+
Mesangial proliferation		-	++	+	-	+	-	-	-	-	-	-	-
Duplication of GBM	-	++	-	++	+	+++	+	++	-	+	+	-	-
FSGS	-	-	++	-	+	+	+	+	++	-	+	+	++
Capillaries distention	+	na	-	-	+	+	-	-	-	+	++	++	na
Intra capillary Thrombus	-	-	-	-	-	+	-	-	-	+	-	+	+
Tubulo interstitial lesions													
Interstitial fibrosis	-	+++	+++	+++	-	+++	-	+++	++	+	+++	++	+++
Tubular atrophy	-	+++	++	+++	-	+++	-	+++	++	+	+++	++	+++
Cellular infiltration	-	-	-	-	-	-	-	+	+	+	+	+	+++
Casts	-	+	+	-	-	-	-	-	-	-	+	-	na
Vascular lesions	HA	HA	HA	HA	NS	NS, HA	HA	NS, HA	HA	TMA	-	-	NS
Perls staining	na	+	na	na	+	na	-	na	+	+	+	+	+
Immunofluorescence													
C3	na	CW,Mes	Mes	Vessel	-	na	Mes	vessel	Mes	CW, vessel	Mes, CW	-	Mes
fibrin	na	-	-	CW	-	na	-	-	-	-	-	-	-
Ig	na	CW	Mes	CW	-	na	CW	-	Mes	-	-	-	-
Predominant histological pattern	SCDG	MPGN	FSGS	SCDG	SCDG	MPGN	MPGN	TMA	FSGS	TMA	SCDG	SCDG	FSGS

Abbreviations: CW, capillary wall; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; HA, hyaline arteriosclerosis; Ig, immunoglobulin; MPGN, membranoproliferative glomerulonephritis, Mes, mesangium ; na, not available; NS, nephrosclerosis; SCDG, sickle cell disease glomerulopathy; TMA, thrombotic microangiopathy lesions

Figure 1

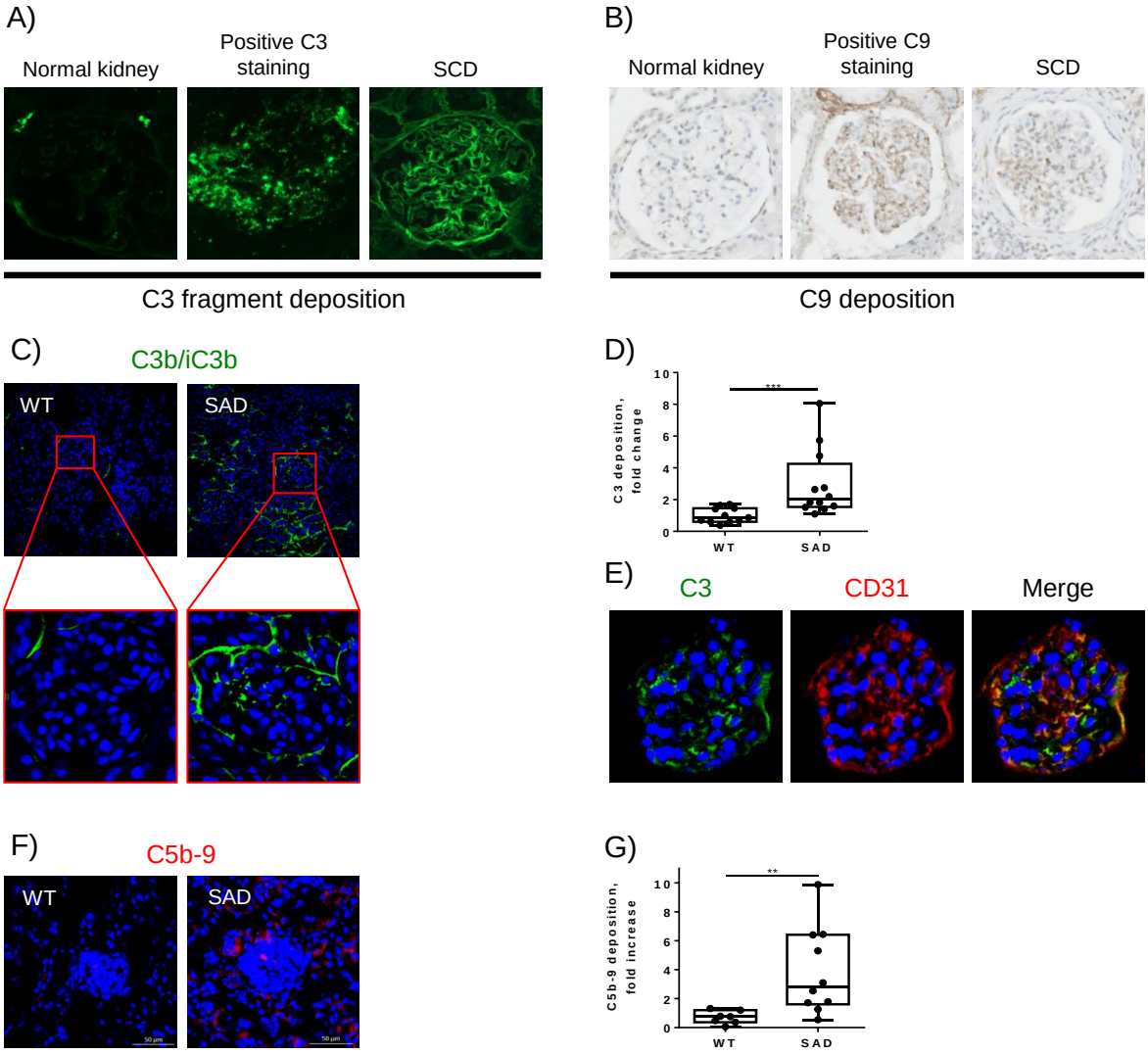


Figure 1: Patients suffering from SCD nephropathy and SCD mice have complement deposition within kidneys. A) C3 fragment staining (false color green) and B) C9 staining (brown) of kidney biopsies of 1/3 patients with SCD nephropathy, performed by immunofluorescence on frozen tissue and by immunohistochemistry on paraffin-embedded tissues respectively. A normal protocol kidney allograft biopsy performed at 3 months was used as a negative control and a biopsy of a patient with acute humoral rejection as a positive control for the staining. C) C3b/iC3b staining (false color green) of kidney sections of SAD mice (n=12) and WT littermates (n=11) at 6 months of age. The bottom panel represents a zoom on one representative glomerulus. DAPI staining of nuclei is blue. D) Quantification of the staining in SAD mouse kidneys and WT littermates. E) Double staining of SAD mouse kidney section for C3b/iC3b (false color green) and endothelial marker CD31 (false color red). The merge indicating co-localization is in orange. Focus on one glomerulus. F) C5b-9 staining (false color red) in a representative glomerulus of WT and SAD mice. G) Quantification of the staining in SAD mouse (n=10) kidneys and WT littermates (n=7). **: p<0,005; ***: p<0,001, Mann-Whitney test.

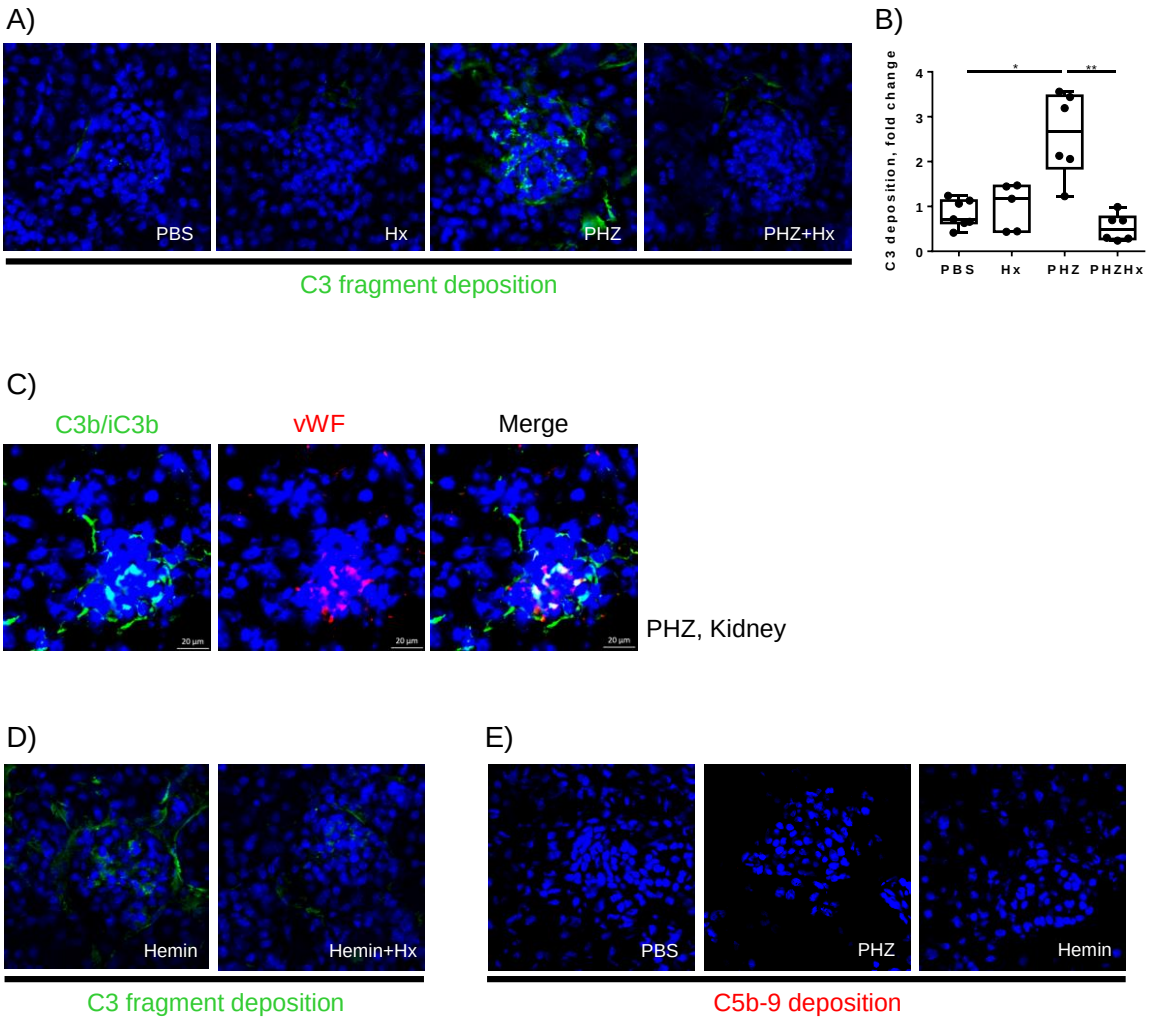


Figure 2: Hemolysis induced heme-dependent complement deposition in kidney *in vivo*. A) Confocal analysis of C3b/iC3b staining of frozen kidney (x40) sections of mice, injected with PBS, or PHZ, with or without Hx (n=6, N=3). B) Quantification of the staining was performed using Visiopharm software. C) Double staining of PHZ-injected mouse kidney section for C3b/iC3b (false color green) and endothelial marker vWF (false color red). The merge indicating co-localization is in orange. D) C3b/iC3b staining (false color green) of kidney sections of mice, injected with hemin, with or without Hx. E) Absence of C5b-9 staining (false color red) in a representative glomerulus of PBS, PHZ or hemin-injected mice. * $p<0.05$, ** $p<0.005$, Kruskal-Wallis with Dunn's test for multiple pairwise comparisons.

Figure 3

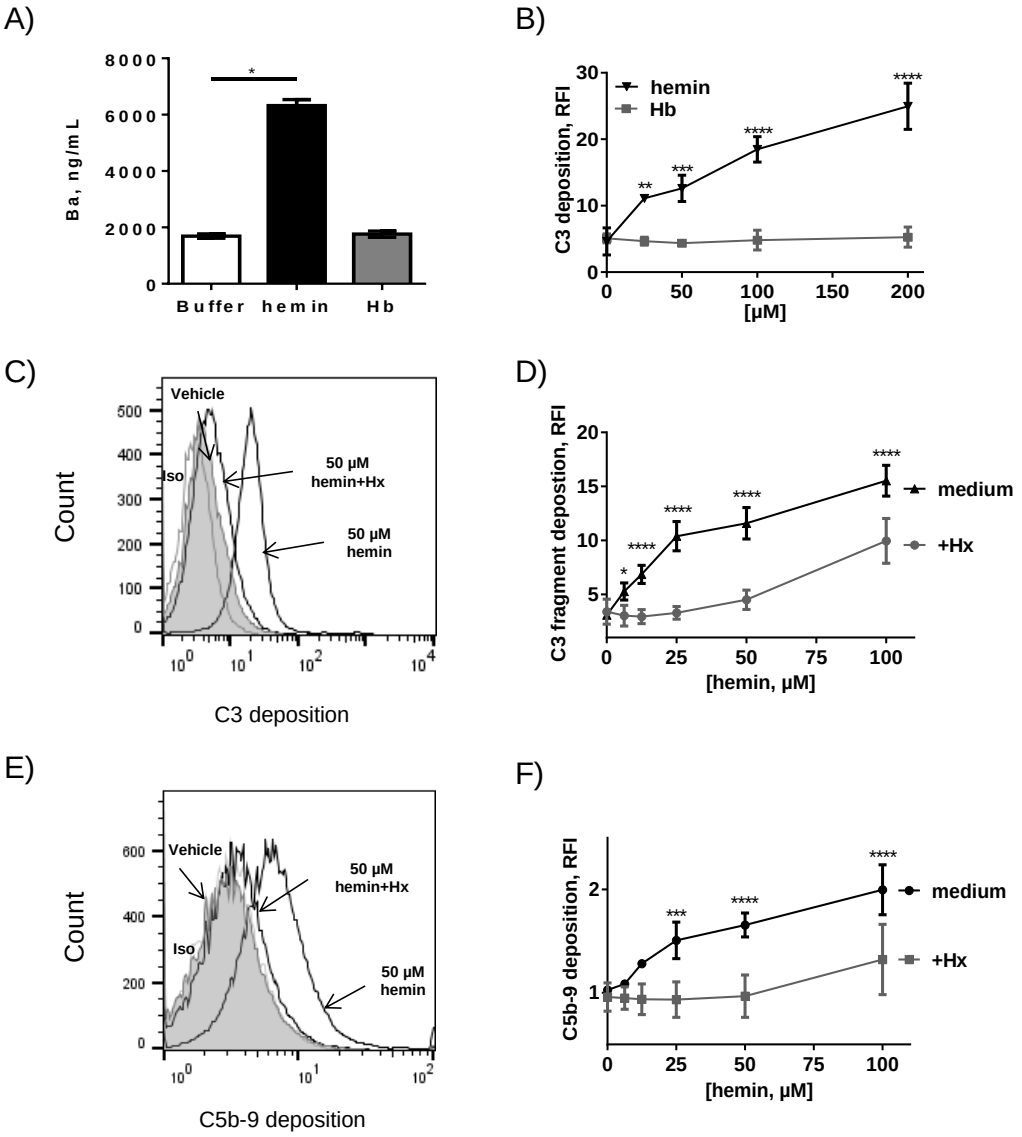


Figure 3: **Heme and not Hb induced complement activation in serum and on EC surface, which was controlled by Hx.** A) NHS was incubated with 50µM of hemin, 12,5µM of Hb (one molecule of Hb contains 4 heme molecules) diluted in TBS. After a 30 min incubation time at 37°C, the level of released Ba was measured by ELISA. *: p<0.005, Kruskal-Wallis with Dunn's test for multiple pairwise comparisons. B) HUVEC were treated with increasing concentrations of hemin or diluted in FCS-free culture medium (M199) for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in the same medium) for 30 mins at 37°C. Cells were detached and stained for C3 deposition by flow cytometry. **: p<0.005, Two-Way ANOVA with Sidak's test for multiple comparisons. C-F) HUVEC were treated with increased concentration of hemin with or without 5µM of Hx for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free) for 30 mins at 37°C. Cells were detached and stained for C3 deposition (D,E), or C5b-9 formation (F,G) by flow cytometry. D) and F) show representative flow cytometry histograms and E) and G) – quantitative analyses. *: p<0.05, **: p<0.005, ***: p<0.001, ****: p<0.0001, Two-Way ANOVA with Sidak's test for multiple comparisons.

Figure 4

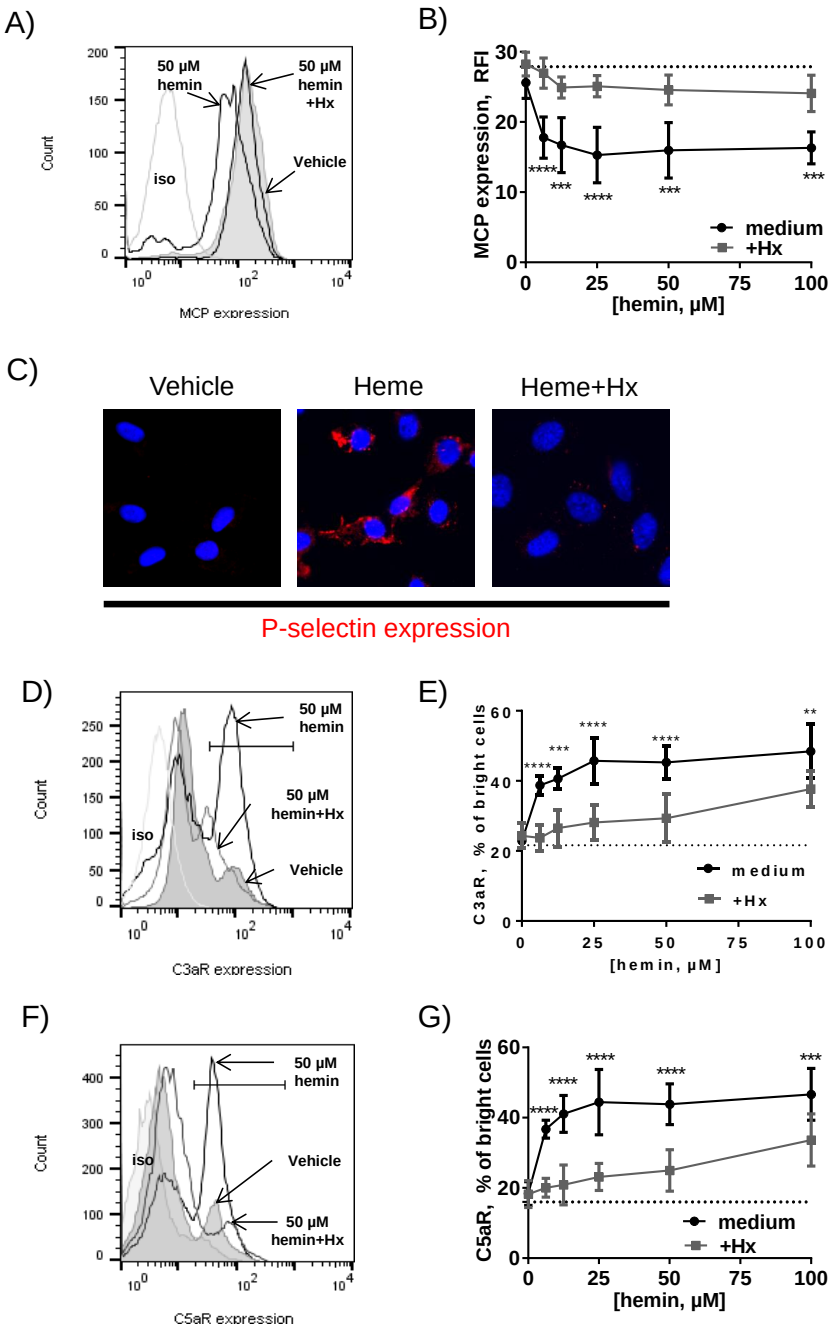


Figure 4: Heme induced complement-related stress markers on the endothelial cell surface, which can be controlled by Hx. HUVEC were treated with increasing concentration of hemin with or without 5µM of Hx for 30 mins at 37°C. Cells were detached and stained for MCP (A,B), C3aR (D,E) or C5aR (F,G) expression by flow cytometry. *: p<0.05, **: p<0.005, ***: p<0.001, Two-Way ANOVA with Sidak's test for multiple comparisons. Left panels (A,D,F) represent histograms from flow cytometry and right panels (B,E,G) – quantitative analyses. C) HUVEC were treated with 50µM of heme with or without 5µM of Hx. Expression of P-selectin (false red color) was stained and studied by immunofluorescence. Nuclei are stained with DAPI (blue).

Figure 5

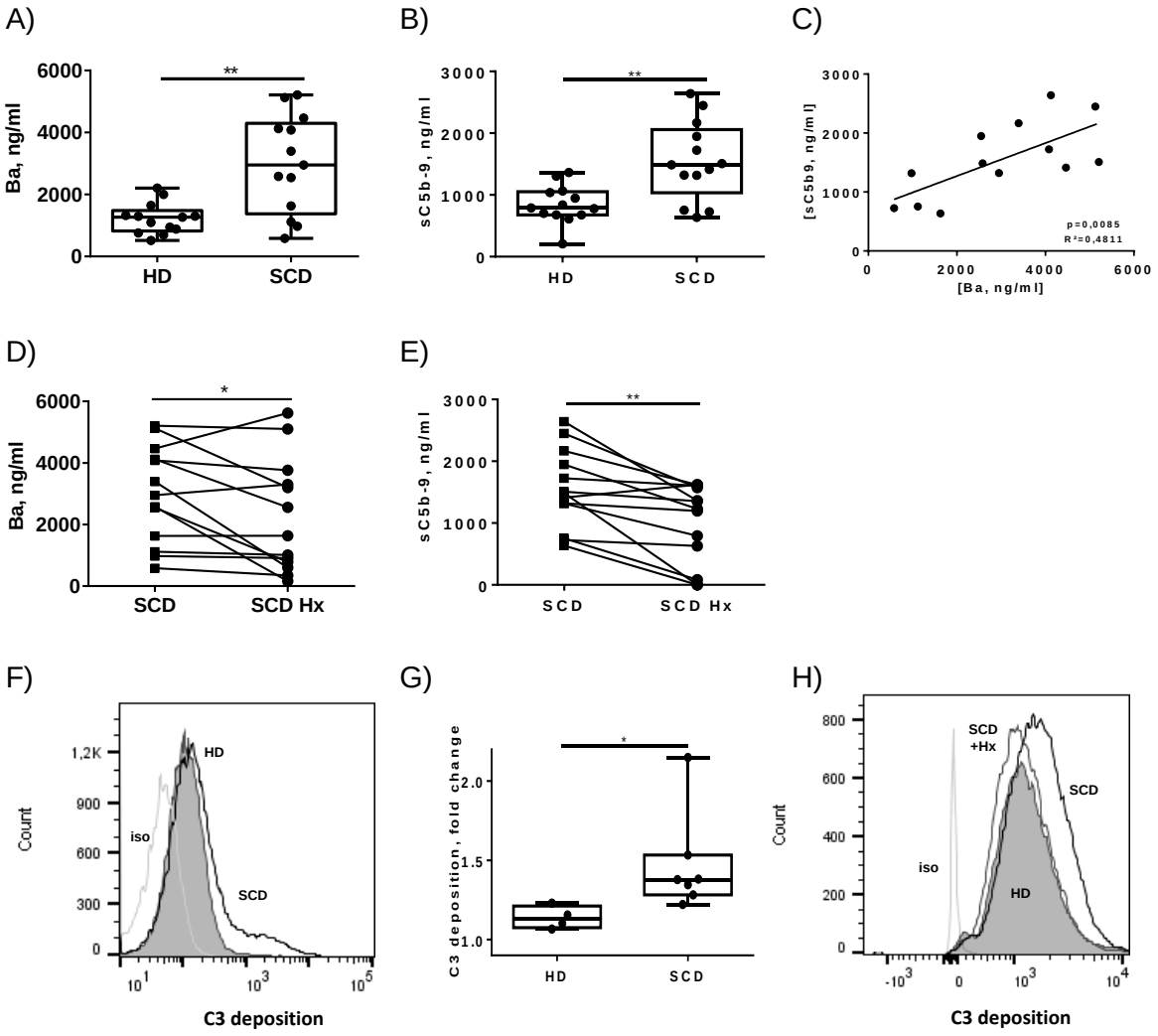


Figure 5: Erythrocytes MVs from SCD patients activated complement in a heme-dependent, Hx sensitive manner. A, B) NHS was incubated with 1000MP/µL of RBC MVs from HD (n=13) or SCD patients (n=13) diluted in TBS. After 30 mins at 37°C, the level of released Ba (A) and sC5b-9 (B) were measured by ELISA. *: p<0.05, **: p<0.005, Mann-Whitney test. C) Linear correlation between measured Ba and sC5b-9 levels with RBC MVs from SCD (n=13) in tested samples. R^2=0.4811; p=0.0085. D,E) NHS was incubated with 1000MP/µL of MVs from SCD patients (n=13) diluted in TBS with or without 25µM Hx. After 30 mins at 37°C, the level of released Ba (D) and sC5b-9 (E) were measured by ELISA. *: p<0.05, **: p<0.005, Wilcoxon test. (F, G) HUVEC were treated with 1000MPs/µL of RBC MVs from HD (n=4) or SCD patient (n=7) diluted in M199 medium for 30 mins at 37°C. Without removing the supernatant, HUVEC were exposed to NHS (final concentration at 33% diluted in M199 FCS-free) for 30 mins at 37°C. Cells were detached and stained for C3 deposition by flow cytometry. F) Flow cytometry histograms, G) Quantitative analyses. *: p<0.05, Mann-Whitney test. H) HUVEC were treated with 1000MPs/µL of RBC MVs from one SCD patient with or without 5µM Hx. As above, NHS was added and cells were stained for C3 deposition by flow cytometry.

Figure 6

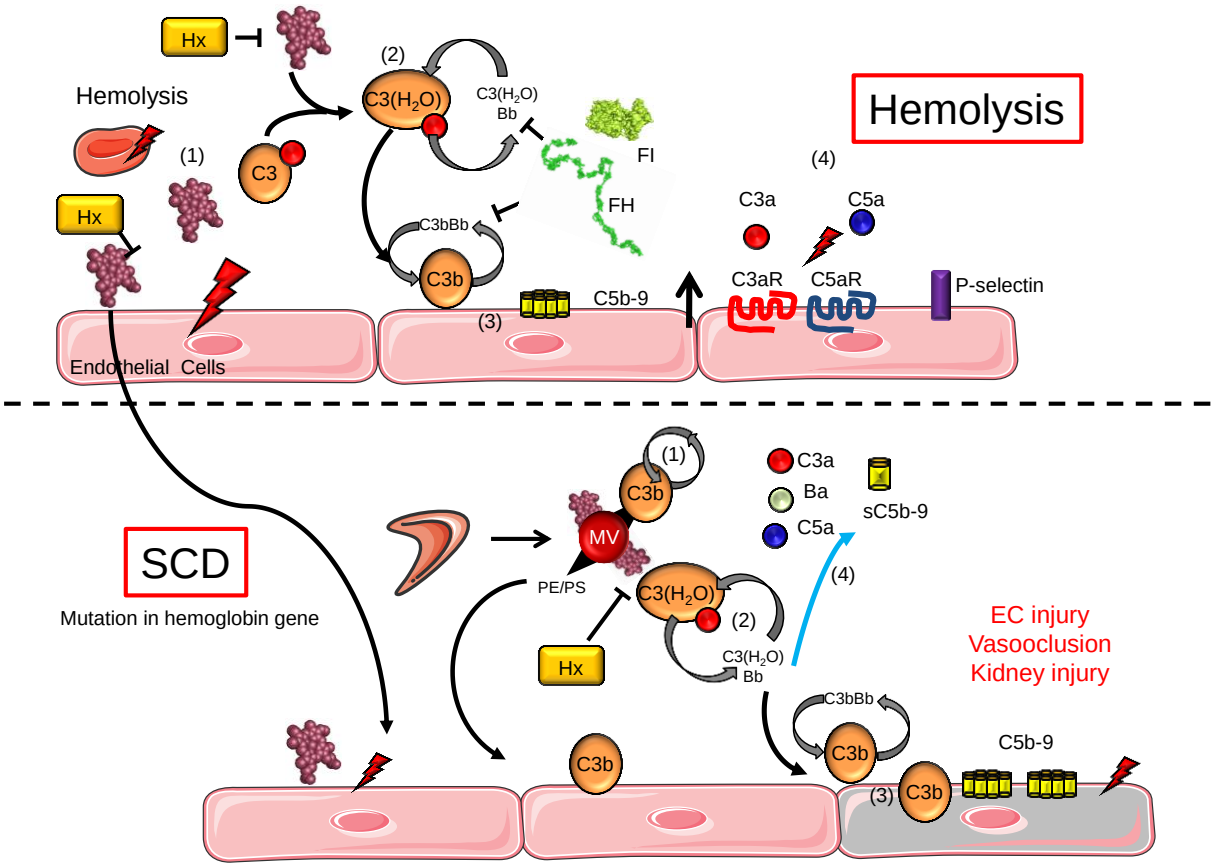
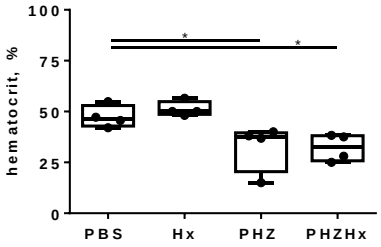


Figure 6: Schematic view of different non-exclusive mechanisms leading to complement activation during hemolysis. Extracellular heme can serve as an enhancer of the pathological process in sickle cell disease (SCD) and other hemolytic diseases by: (1) Heme activates endothelial cells (EC) by its pro-inflammatory and pro-oxidant effects. (2) Heme induces hydrolysis of C3 into C3(H_2O) and provokes direct complement activation in the serum. (3) C3b generated by the fluid phase C3 convertase will bind to the EC membrane and will form new AP C3 convertases that can locally auto-amplify and generate C3b and C5b-9 deposition. (4) Released C3a and C5a can bind to C3aR and C5aR to mediate pro-inflammatory processes. In the context of SCD, sickle RBC will generate microvesicles (MVs) expressing phosphatidylserin (PS) and phosphatidylethanolamin (PE) that carry heme. They carry heme, which will activate complement in serum and also on the EC surface. We hypothesize that this complement activation will contribute to the vascular injury and tissue damage in SCD. Hemopexin (Hx) was able to block partially or completely, the hemolysis-induced complement activation *in vitro* and *in vivo*.

A)



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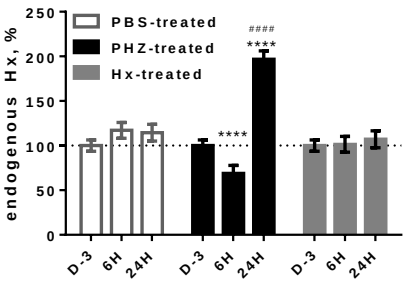
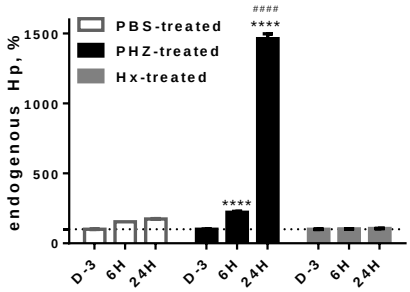
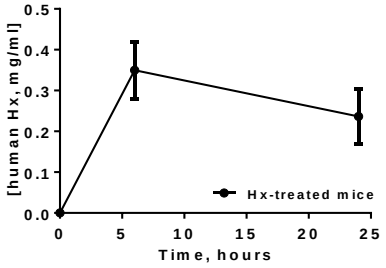


C)

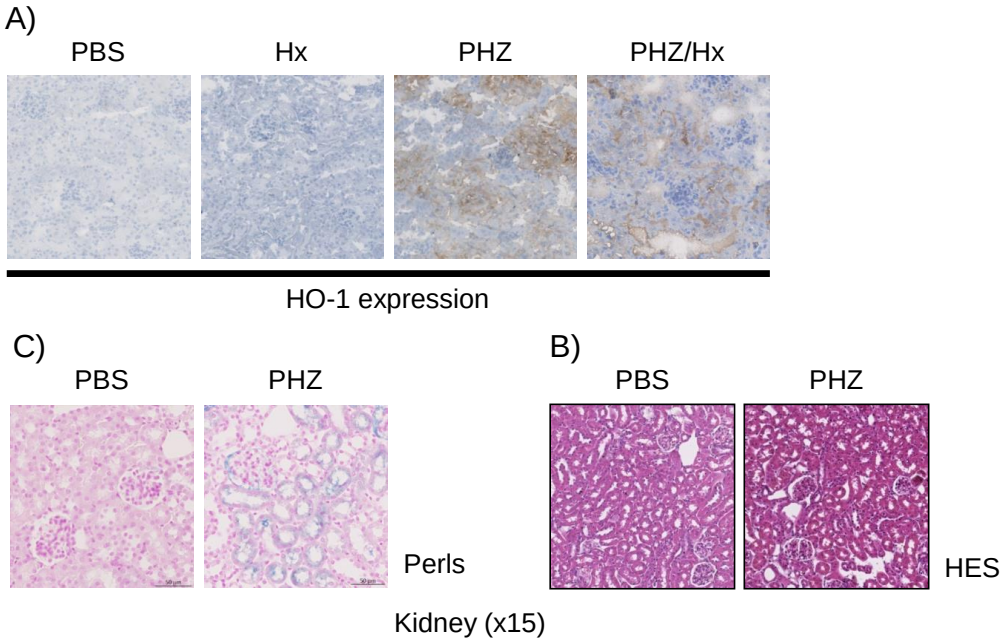


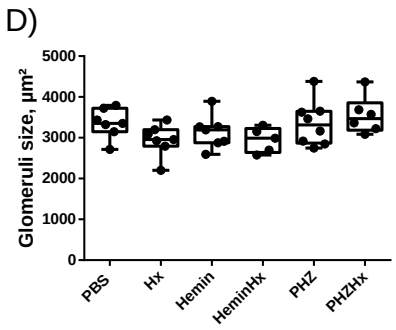
D)



Supplementary figure 1: PHZ injection triggered hemolysis and modulated endogenous Hx and Hp expression. A) Hematocrit percentage of PBS, Hx, PHZ and PHZHx-treated mice (n=4). *: p<0,05 performed by Kruskal-Wallis with Dunn's test for multiple comparisons. B) Quantification of endogenous Hx from plasma of PBS, PHZ and Hx-treated mice (n=4). C) Quantification of endogenous β-chain Hp from plasma of PBS, PHZ and Hx-treated mice (n=4). ****: p<0,0001 compared to D-3; ####: p<0,0001 compared to 6H; performed by Two-Way ANOVA with Tukey's test for multiple comparisons. D) Quantification of human Hx in plasma of Hx-treated mouse 6 hours and 24 hours after injection (n=4).

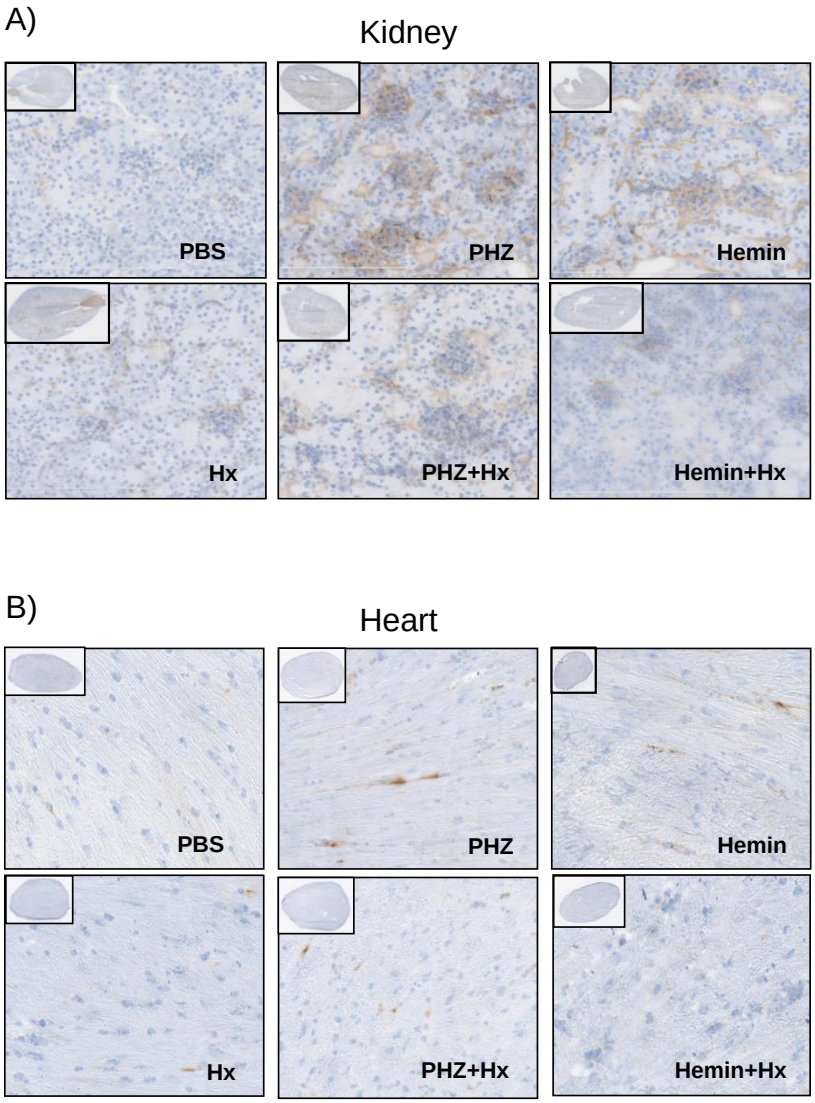
Figure S2





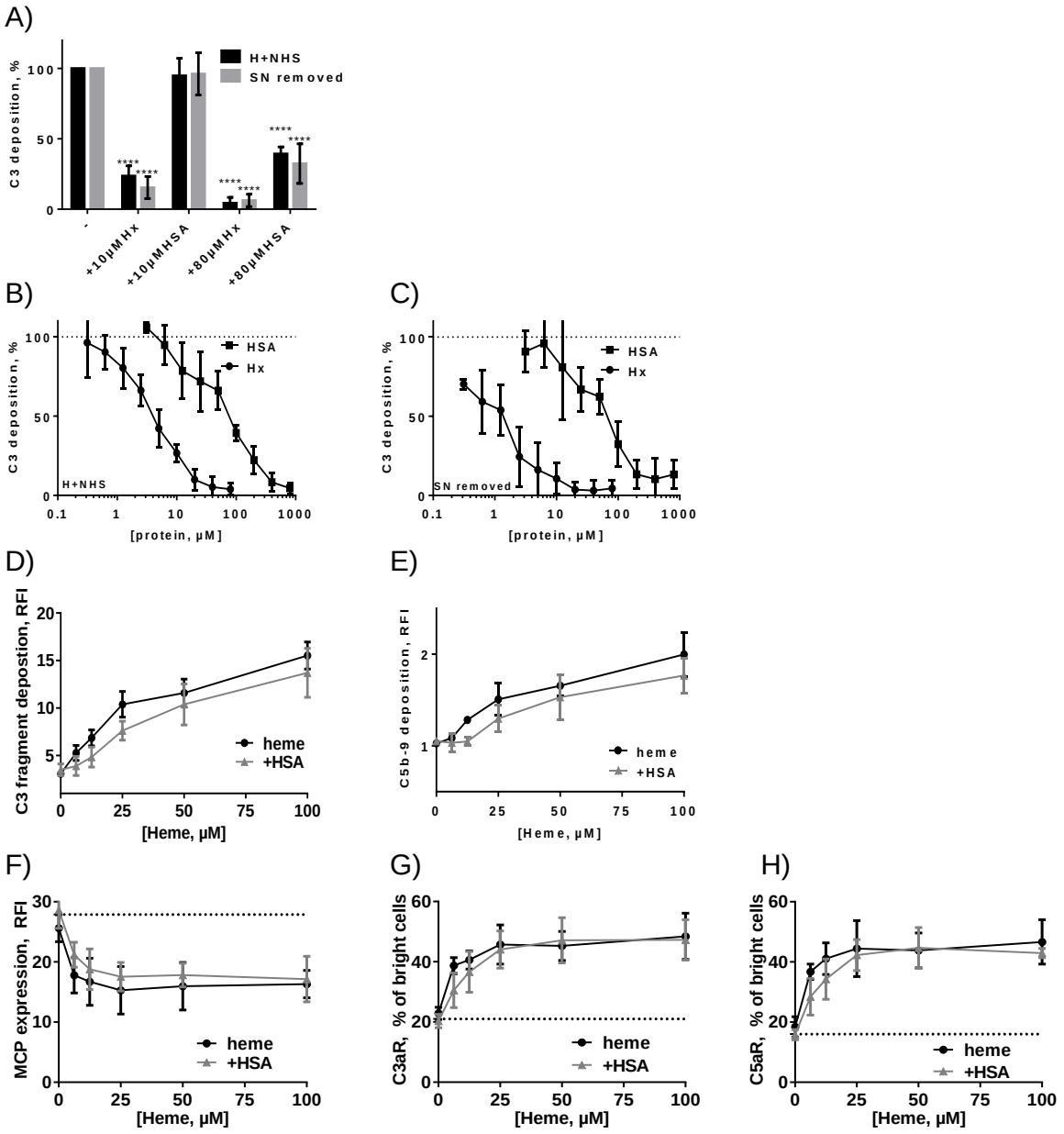
Supplementary figure 2: Renal histology of PHZ-injected mice. A) Immunohistochemistry analysis of frozen kidney sections of mice (x20), injected with PHZ or with the vehicle only, with or without Hx. The staining for HO-1 appears in brown and nuclei in blue. Representative images from one out of three or five mice per group (n=5, N=3). B,C) Fixed and paraffin-embedded kidneys were cut at 5µm and stained with Perls Prussian Blue (B) and hematoxylin-eosin (C). D) Quantitative analysis of glomerular size, each point represents the average size of glomeruli in one mouse (n=7).

Figure S3



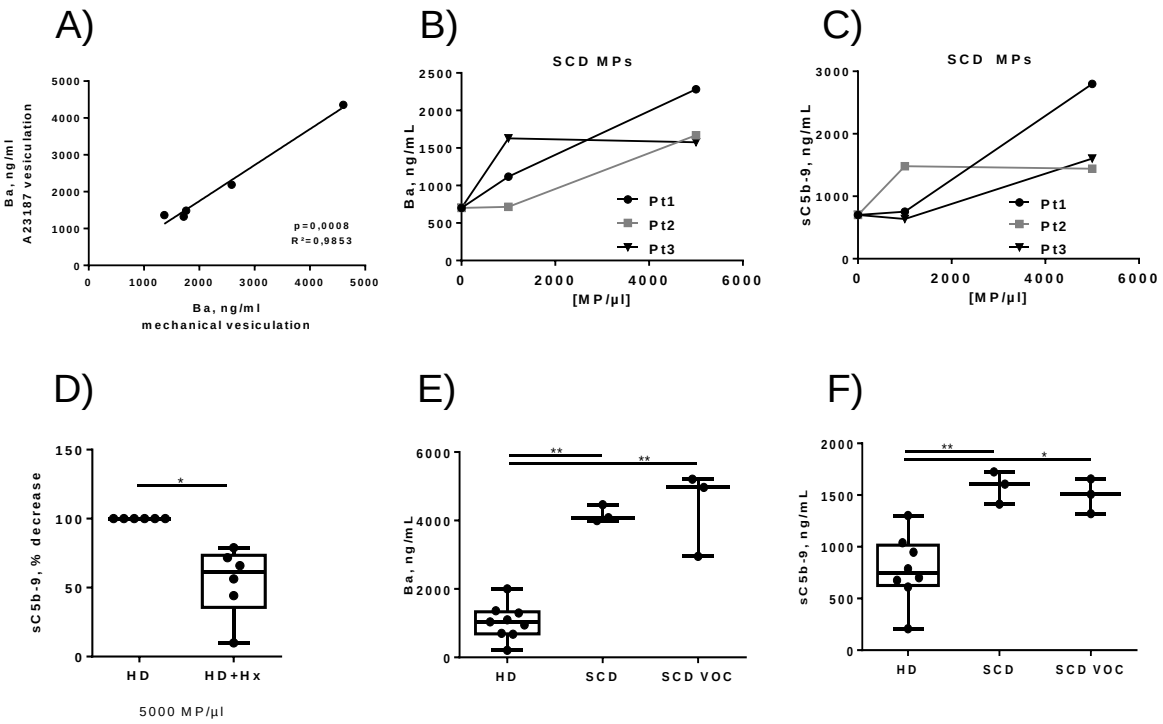
Supplementary figure 3: Complement activation is induced *in vivo* under hemolytic conditions and differentially affects kidneys and heart. Immunohistochemistry analysis of (A) kidney (x20) and (B) heart (x10) sections of mice, injected with PHZ, heme, or with the vehicle only, with or without Hx. The staining for C3 activation fragments deposition appears in brown and nuclei in blue. Representative images from one out of 5 mice per group repeated in 3 independent experiments (n=5, N=3).

Figure S4



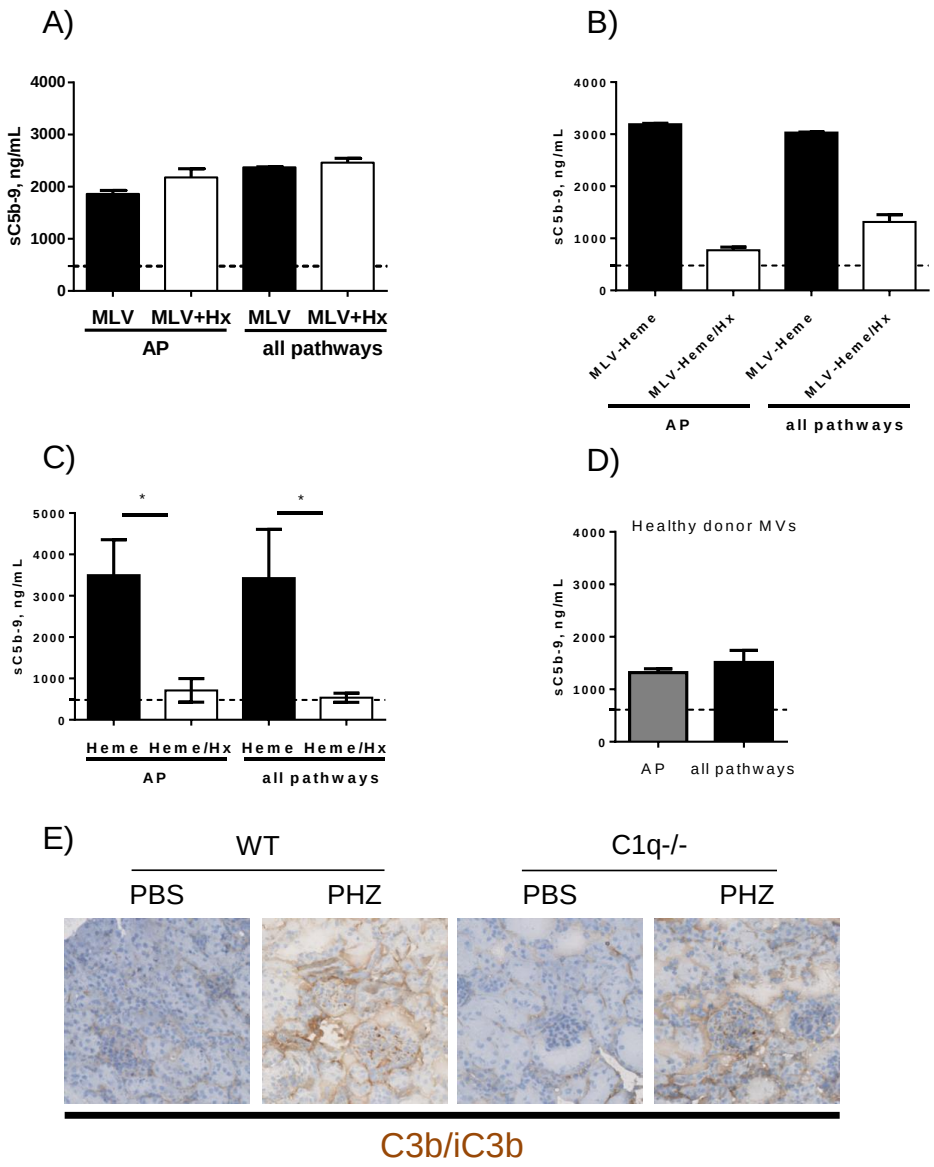
Supplementary figure 4: Albumin was less efficient than Hx to prevent heme effects. A, B, C) HUVEC were treated with increased concentration of albumin or Hx and 50 µM of heme, diluted in M199 FCS-free medium for 30 minutes at 37°C. HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free medium) after removing the supernatant (SN removed) (B), or not (H+NHS) (C), for 30 minutes at 37°C. Cells were detached and stained for C3 deposition by flow cytometry (n=4). D, E) HUVEC were treated with increased concentration of heme with or without 5 µM of albumin following the same protocol. Cells were detached and stained for (D) C3 deposition or (E) C5b-9 formation by flow cytometry. F) HUVEC were treated with increased concentration of heme with equimolar concentration of albumin, diluted in FCS-free medium for 30 minutes at 37°C. HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free medium) after removing the supernatant. Cells were detached and stained for C3 deposition by flow cytometry (n=4) G, H, I) HUVEC were treated with increased concentration of heme with or without 5µM of Hx for 30 minutes at 37°C. Cells were detached and stained for (G) MCP, (H) C3aR or (I) C5aR expression. Detection was done by flow cytometry (for each measure, n≥3). *: p<0,05, ***: p<0,001, ****: p<0.0001, performed by Two-Way ANOVA with Sidak's test for multiple comparisons.

Figure S5



Supplementary figure 5: RBC macrovesicles (MVs) from stable SCD patients or in vaso-occlusive crisis (VOC) induce equivalent complement activation. NHS was incubated with increased concentration of RBC MPs from healthy donors (HD) or SCD patients, diluted in TBS. After 30 minutes incubation time at 37°C, the level of released Ba and sC5b-9 was measured by ELISA. A) Comparison of Ba release induced by RBC MVs from two different preparations, generated by A23187 ionophore and by mechanical vesiculation. NHS was incubated with 0, 1000 or 5000 MVs/µL of RBC MVs from SCD patients (Pt), diluted in TBS, and (B) Ba and (C) sC5b-9 were measured. D) NHS was incubated with 5000 MVs/µL from HD (n=6) with or without 25µM of Hx. Release of sC5b-9 was measured. *: p<0,05, performed by Mann-Whitney test. E, F) NHS was incubated with RBC MVs from HD, SCD patients or SCD patients in VOC and release of (E) Ba and (F) sC5b-9 were measured. *: p<0,05, **: p<0,005, performed by Kruskal-Wallis with Dunn's test for multiple comparisons.

Figure S6



Supplementary figure 6: Microvesicles (MVs) induce alternative pathway activation dependently and independently on heme. sC5b-9 release was analyzed by ELISA kit. (A) Artificial multilayer vesicles (MLV) or (B) heme-loaded MLV (MLV-heme) were incubated during 30 minutes at 37°C with 33% NHS, diluted at a final concentration of 5000MV/µL with or without 25µM of Hx. Two dilution buffers were used: complement alternative pathway (AP), TBS with 10mM EGTA and 4mM $MgCl_2$ or permissive for activation of all complement pathways (all pathways), TBS with 5 mM $CaCl_2$ and $MgCl_2$. Illustrative results of two independent repetitions. (C) Heme was incubated with 33% NHS for 30 minutes at 37°C with or without 25µM of Hx, diluted in AP buffer or a buffer allowing activation of all pathways. (D) sC5b-9 release, induced by MVs from healthy donors, incubated at 5000 MV/µL in 33% NHS in the two different buffers (n=4). (A-D). The dotted line represent the level of sC5b-9 in absence of MVs in serum (vehicle only). E) Immunohistochemistry analysis of frozen kidney sections of WT and $C1q^{-/-}$ mice, injected with PHZ or with the vehicle only. The staining for C3 activation fragments deposition appears in brown and nuclei in blue. Representative images from one out of five mice per group. *: p<0,05, performed by Kruskal-Wallis with Dunn's test for multiple comparisons.

Manuscript 3: **Intravascular hemolysis induces complement attack on endothelium in a TLR4-dependent manner**

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Manuscript in preparation

Intravascular hemolysis induces complement attack on endothelium in a TLR4-dependent manner

Nicolas S. Merle[1,2,3], Romain Paule[1,3,4], Olivia May[1,2,3,5], Rémi Noé[1], Tania Robe-Rybkine[1,2,3], Amélie DiGiovanni[1,3], Jordan Dimitrov[1,2,3], Véronique Frémeaux-Bacchi[1,2,3,6], Lubka T. Roumenina[1,2,3]

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Abstract

Complement system is a complex and tightly regulated protein cascade. Our recent studies have demonstrated that hemolysis derived product heme is able to activate complement in serum, on endothelial cells (EC) and within tissues, providing a rational for scrutinizing the impact of complement system in hemolytic diseases. However, the mechanism of acquisition of a complement activing phenotype by the host cells remains unclear. The objective of this study was to understand the underlying mechanism that promotes complement activation on EC. We used *in vitro* EC model, applied a mouse model of drug-induced hemolysis on WT and $TLR4^{-/-}$ mice and analyzed complement deposition on blood vessels. Using a specific inhibitor of TLR4 transduction, TAK-242, we demonstrate that about 50% of complement deposition, that is induced by heme, is dependent on TLR4 signaling on EC *in vitro*. Moreover, quantification of C3 deposition on blood vessels of hemolytic and heme-injected WT and $TLR4^{-/-}$ mice demonstrated the importance of TLR4 signaling for complement activation on endothelium. Our results of protein interactions in real time and the use of blocking antibodies incriminate P-selectin, whose expression is subsequent to TLR4 activation by heme, as a major anchoring protein for C3(H_2O) and C3b on cell membrane. These results contribute to understand the mechanism leading to the acquisition of a complement sensitive surface by EC during hemolysis. Moreover, targeting TLR4 or P-selectin will provide a beneficial effect by preventing complement attack on EC, relevant for hemolytic diseases.

Introduction

Intravascular hemolysis is a pathological event in numerous diseases, including sickle-cell disease (SCD), thalassemias, microangiopathic hemolytic anemias, paroxysmal nocturnal hemoglobinuria, ABO mismatch transfusion reaction, autoimmune hemolytic anemia, malaria, etc[1]. Intravascular hemolysis leads to red blood cells (RBC) degradation, releasing hemoglobin (Hb) in the bloodstream. The accumulation of cell-free Hb and its degradation products overwhelms the capacity of scavenger systems to counteract hemolysis, resulting in a presence of a large amount of free heme in the circulation[2,3]. Increasing evidences demonstrate that heme is a Danger-Associated Molecular Pattern (DAMP) that mediates multiple pro-inflammatory and pro-thrombotic activities[4]. Indeed, heme modulates the activity of multiple plasma systems[4], such as the complement[5–9] and coagulation cascades[10–13], as well as the antibody reactivity[14–17]. Moreover, heme promotes recruitment and activation of neutrophils until NET formation[18–20], stimulation of macrophages, phenotype switch[21] and synthesis of pro-inflammatory mediators as tumor necrosis factor α (TNFα)[22], as well as activation of endothelial cells (EC) and rapid mobilization of EC-specific granules, Weibel-Palade Bodies (WPB), resulting in von Willebrand Factor and P-selectin externalization[6,23]. The exact mechanisms of cell activation by heme are not fully understood but it is well documented that it induces Toll-like Receptor 4 (TLR4) signaling transduction[3,23]. By contrast to lipopolysaccharide (LPS), the main ligand of TLR4[24], the TLR4 activation by heme was demonstrated to be CD14-dependent but MD-2-independent[22], suggesting different signaling and effector functions.

Under hemolytic conditions there is a concomitant activation of the plasma systems and the exposed cells, but the cross-talk between these processes is poorly studied[4,25]. We have previously demonstrated complement deposits on heme-activated endothelial cells[6]. Complement system is a powerful first line of defense against pathogens, but can induce severe cell damage when inappropriately activated in host tissues[26,27]. Complement activation stimulates pro-inflammatory and pro-thrombotic phenotype of EC by inducing expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin[28] and P-selectin[25,29,30]. Therefore, hemolysis and complement would synergize to induce EC activation and damage[4]. Here we sought to investigate the mechanisms by which heme renders the endothelial cells susceptible to complement activation.

In this study, we show that complement deposition on EC *in vitro* and *in vivo* in mice is dependent on TLR4 activation by heme, providing a direct link between TLR4 and complement system activation on EC surface. Furthermore, the TLR4-dependent surface expression of P-selectin is crucial for anchoring C3 activation fragments and subsequent MAC formation on EC membranes. These results are explaining at least in part, the mechanism of complement deposits on EC *in vitro* and *in vivo*.

Methods

Reagents

The Fe^{3+} form of heme (hemin [ferriprotoporphyrin IX], designated as heme, (Sigma-Aldrich)) was dissolved to 20 mM in 50 mM NaOH and 145 mM NaCl, and further diluted in an appropriate vehicle just before use. Stock solution of phenylhydrazine (PHZ) of 25 mg/mL (Sigma-Aldrich) was prepared in PBS immediately before use. Inhibition of TLR4 was performed using TAK-242 molecule diluted in PBS to a final concentration of 400nM (Calbiochem, CAS 243984-11-4).

Animal experimentation

Mouse treatment. All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with the approval APAFIS#7135-2016100520465430v5 of the French Ministry of Agriculture. C57Bl/6 mice were from Charles River Laboratories, (L'Arbresle, France) and $TLR4^{-/-}$ were from an in house colony, which was a kind gift from Dr Benedicte Manoury (Cochin Hospital, Paris). Eight week old C57Bl/6 and $TLR4^{-/-}$ mice were injected i.p. with 200 µL of PBS (Gibco) or with freshly prepared heme (40 µmol/kg, corresponding to 28 µg/g body weight[23]) repeated 24 hours later (day 0 and day 1). Alternatively, mice were only injected i.p. at day 1 with PHZ (900 µmol/kg, corresponding to 0.125 mg/g body weight) and organs were recovered at day 2. Concentrations and route of administration were chosen as described[31]. Blood was collected from the submandibular vein 3 days before the first injection (baseline) and before endpoints (day 2). At the endpoint, organs were recovered and frozen in nitric liquid and stock to -80°C.

We used male and female C57Bl6/J and TLR4$^{-/-}$ mice, group sexe were matched for each experiment.

Immunofluorescence on mouse tissue.

Five µm thick frozen sections of kidneys, hearts and spleens were cut with Cryostat Leica AS-LMD and fixed in acetone on ice for 10 min. Complement deposition was studied using rat anti-mouse C3 fragments (C3bi/C3b/C3c) (Hycult, HM1078), visualized with goat anti-rat-AF555 (Thermoscientific, A-21434). Endothelial cells were visualized with EC marker vWF using polyclonal sheep anti-rabbit vWF (Abcam, ab11713) revealed by donkey anti-sheep (Thermoscientific, A-21434). Stained slides were scanned by Slide Scanner Axio Scan (Zeiss). Quantification of the staining was performed using Visiopharm software (Visiopharm A/S, Hørsholm, Denmark). The colocalization of vWF and the C3 activation fragments was used to quantify the complement deposits particularly on the large vessels in the liver. This organ was selected because of the presence of easily identifiable high amount of macro-vessels to allow robust analyses.

Complement activation and complement receptors on EC

Flow cytometry. Primary human umbilical vein endothelial cells (HUVEC) were cultured in 24-wells plates as previously described[6,32], exposed to heme or RBC MVs at the indicated doses for 30 mins at 37°C in serum-free M199 cell culture medium (Gibco) in presence or absence of Hx or HSA. For evaluation of complement activation, HUVEC either washed and further exposed to normal human serum (NHS) diluted to 33% in M199 medium or serum was added to final dilution of 25% to the wells, already containing heme+/-TAK-242, for 30 mins at 37°C. HUVEC were used for experiments until passage 4. Cells were washed, detached, labeled and fixed in 0.5% formaldehyde. Cells were analyzed by flow cytometry (BD LSR II), and further analyzed by FlowJo X. The following antibodies were used for staining: anti-human MCP-RPE (BIORAD, MCA2113PE), anti-human C3aR-PE (BioLegend, 345804), anti-human C5aR-APC (BioLegend, 344310), in addition to the corresponding isotype controls. After incubation with serum, staining was performed with anti-properdin mouse monoclonal IgG1 (Quidel, A233), anti-FH mouse monoclonal IgG1 (Quidel, A254), anti-C3c mouse monoclonal IgG1 antibody (Quidel, A205), or with anti-C5b-9 mouse monoclonal IgG1 (kindly provided by Prof. Paul Morgan, Cardiff University) or an

isotype control and revealed by anti-mouse-IgG1-PE secondary antibody (Beckman Coulter, IM0551). Blocking antibody against P-selectin (R&D systems, AF137) was diluted in M199 FCS-free medium supplemented with 10mM EGTA and 2 mM $MgCl_2$ to a final concentration of 25 µg/ml or with a polyclonal sheep antibody as an irrelevant antibody (R&D systems, 5-001). HUVEC were stained for C3 deposition and C5b-9 formation as described below.

Immunofluoresence. HUVEC were cultured on slides in 24-well plates. Cells were fixed in paraformaldehyde 4% after staining of P-selectin, C3 activation fragments, CD31, vWF, p38, p65. Respectively, staining was performed with anti-human P-selectin primary antibody (BIORAD, MCA796), rabbit anti-human C3 FITC (DAKO, F020102-2), rabbit anti-human vWF (DAKO, A0082), mouse anti-human CD31 (Ancell, 180-020), phospho-p38 (Thr180/Tyr182) (#9211, Cell Signaling), phospho-p65 (Ser536) (#3033, Cell Signaling) or matched isotype controls. Markers were revealed with rabbit anti-mouse AF-488 secondary antibody (ThermoFisher, A21204) or goat anti-rabbit AF555 (ThermoFisher, A21429) and DAPI (to stain nuclei). Staining was analyzed by Axiovert 200M microscope (Zeiss).

Cellular ELISA. HUVEC cells were cultured on slides in 24-well plates. HUVEC were exposed to 200 ng/mL LPS for 1 hour. Cells were washed and stained for vWF HRP (DAKO, P022602) or P-selectin primary antibody (BIORAD, MCA796) and revealed with secondary anti-mouse HRP antibody (Santa Cruz, sc-2005).

Western Blot. Cells were treated with 25 µM of heme from 30 mins to overnight, 100 ng/mL of LPS as positive controls. Cells were washed, detached and incubated 30 mins at 4°C in RIPA lysis buffer. Phosphorylation of p65 was confirmed by WB using Phospho-65 (Ser536) (#3033, Cell Signaling), controlled with total NF-κB p65 (D14E12) (#8242, Cell Signaling) using the SNAP technology (Merk Millipore), followed by Anti-rabbit IgG, HRP-linked Antibody (#7074, Cell Signaling). The signal was revealed by chemiluminescence.

Surface Plasmon Resonnance. Binding studies were performed using the ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories), based on SPR technology. Recombinant human P-selectin (R&D Systems) was covalently coupled via amine coupling in 50 mM sodium acetate at pH 4,8 to a sensor chip. The immobilization of concentration of P-selectin resulted in 1,600 resonance units, corresponding to a surface density of 1,3 ng/mm^2. The binding assays were performed in TrisHCl 10mM, pH 7.4 with 150mM NaCl, 1,2 mM $CaCl_2$ and 1,2 mM $MgCl_2$ at 25 C with a flow rate of 30 µl/min. The binding of the C3b to the

P-selectin–coated channel was corrected for non- specific binding to the control channel (no protein; between 5 and 10%). P-Selectin binding at equilibrium was determined at increased concentrations of C3b (A112, CompTech), and freeze/thaw C3 (A113, CompTech) (0.0625, 0.125, 0.25, 0.5 and 1 µM). The set of sensorgrams were best fit to the 1:1 Langmuir binding model with drifting baseline using the BIAevaluation software (version 3.0) provided by the manufacturer.

Statistics

Results were analyzed using a statistical software package (GraphPad Prism 5) as indicated in the figures legends.

Results

Heme triggers TLR4 activation on endothelial cells

In 2013, our team reported complement activation on endothelial cell in presence of heme[6]. We therefore aimed to study whether this phenomenon is dependent on TLR4. Investigation of TLR4 expression on EC by flow cytometry showed a positive staining on resting cells (Figure 1A). After 30 min incubation time with heme, TLR4 expression remains stable compare to basal level (Figure 1B). Pre-treatment of the cells with TAK-242 did not alter the expression of TLR4. In comparison, LPS did not alter expression of TLR4 expression within 30 min on EC surface (Figure S1A,B). Study of CD14 by flow cytometry revealed a double pick of expression on EC on resting state, distributed at 50% of both dim cells ($CD14^{low}$) and bright cells ($CD14^{high}$) (Figure 1C). Heme-treatment induces an increase of bright cell (80%) at the expense of dim cells (20%). Inhibition of TLR4 by TAK-242 only partially prevented increased expression of CD14 (Figure 1D). TLR4 activation was studied by phosphorylation of intermediates of signaling pathway by Western Blot and immunofluorescence. Phosphorylation of p-38 increased in total cell lysate within 6 hours in presence of 25 µM of heme in a time-dependent manner western blot (Figure 1E). This was confirmed by immunofluorescence (Figure 1F). Moreover, translocation of phosphorylated-p65 in the nucleus was found increased up to 6 hours (Figure 1G) and was confirmed by IF (Figure 1H).

LPS treatment was used as a positive control and also induced an increase of p38 and p65 phosphorylation.

Inhibition of TLR4 with TAK-242 partially prevents heme-induced complement deposits on EC.

Exposure of EC to heme and serum resulted in an increase of C3 fragments and C5b-9 deposition in presence of heme within 30 min (Figures 2A,C). Inhibition of TLR4 by the use of TAK-242 prevented about 50% of both C3 fragments and C5b-9 deposition on cell surface (Figure 2B,D). However, LPS treatment, with or without TAK-242, did not induce complement activation on EC surface (Figure S2).

Inhibition of TLR4 did not protect from complement activating phenotype.

The partial protective effect of TAK-242 against complement deposition on EC could be related to prevention of modification of expression of complement-related protein. A defect of complement regulation could be related to the TLR4-dependent C3b deposition. Thus, we studied the impact of heme-treatment on FH binding on EC and MCP expression, the two major complement regulators, as well as the expression of C3a and C5a receptors (C3aR and C5aR, respectively) within 30 min.

Binding of factor H (FH) increased on EC surface in presence of free heme, which was not prevented by TAK-242 (Figures 3A,B). Heme caused a dose-dependent decrease of MCP (up to 50%)[6]. Here, we confirmed a decrease of MCP expression, which was not prevented by TAK-242 (Figures 3C,D). Treatment with LPS did not modulate FH binding and MCP expression (Figures S3A-D).

Expression of both C3aR and C5aR revealed a double peak expression on resting state, distributing 50% of both dim cells and bright cells (Figures 3E,G). Heme-treatment rapidly increased both C3aR and C5aR expressions, enhancing bright cell population (Figures 3F,H). However, neither C3aR- nor C5aR-increased expression were prevented by inhibition of TLR4 signaling by TAK-242 (Figures 3H,J). In comparison, LPS-treatment did not produce any effect on C3aR and C5aR expression (Figures S3E-H).

Rapid increase of P-selectin expression is the causal link between TLR4 and complement activation in presence of heme.

P-selectin expression induced by heme could participate to complement deposition on EC, since P-selectin has been shown to anchor C3b on cell membrane[33]. First, we observed that both LPS and heme induce WPB mobilization within 30 min, translated by the release of vWF and the rapid expression of P-selectin (Figure 4A, S4A-D). Moreover, expression of P-selectin colocalized with C3 deposition (Figure 4B). We did not detect properdin binding on EC (Figure S4 E,F).

Staining of C3b and P-selectin by immunofluorescence on HUVEC revealed a colocalization of these two proteins in presence of heme (Figure 4B). Investigation of the interaction between C3b and P-selectin by surface plasmon resonance revealed a Kd about 100 nM (Figure 4C). Moreover, C3(H_2O) was also able to bind recombinant P-selectin (Figure 4D). C3(H_2O) was generated by freezing/thawing native C3. For this reason, the exact quantity of C3(H_2O) could not be determined, thus preventing calculation of binding affinity. Presence of heme did not affect the binding of both C3b and C3(H_2O) on P-selectin (Figures S5A,B).

In order to know whether these interactions have functional consequences on EC, we tested the capacity of a blocking antibody against P-selectin to prevent complement deposition. Blocking of P-selectin prevented 50% of C3 activation fragments deposition, compared to cells treated with an irrelevant antibody. This inhibition was equivalent to the blocking of TLR4 signaling by TAK-242 (Figure 4E). Moreover, no additive effects of incubation with TAK-242 with antibody against P-selectin were observed (Figure 4F).

Hemolysis-induced complement deposition on macrovascular EC is dependent on TLR4.

To further investigate the implication of TLR4 activation by heme on complement deposition, we studied complement activation on macrovessels in response to drug-induced hemolysis or heme injection in WT and $TLR4^{-/-}$ mice. EC and C3 fragment deposition were stained in parallel in order to study complement deposition on endothelium under hemolysis (Figure 5).

In liver, treatment with PHZ or heme significantly increased co-localization between C3 fragments and vWF compared to treatment with PBS in WT mice (Figure 5A,B). However in TLR4$^{-/-}$ mice, co-localization of C3 and vWF appears significantly reduced compare to WT mice despite treatments (Figure 5A,B).

As in liver, co-localization of C3 and vWF within kidneys of WT mice was significantly increased in both PHZ- and heme-treated mice compare to PBS controls, which was significantly attenuated in TLR4$^{-/-}$ mice as shown in the quantification (Figure S5A,B).

Discussion

In the current study, we provide a mechanistic insight into the acquisition of a complement activating phenotype by EC during hemolysis *in vitro* and *in vivo*. Obtained results demonstrate a direct link between heme-triggered TLR4 signaling and complement system activation on EC, which is mediated by P-selectin expression and recruitment of C3b and C3(H2O) on the cell surface.

Multiple lines of evidences show that hemolysis triggers heme-dependent complement activation *in vitro*[5,6,9] and *in vivo* (Merle et al, under revision), including on endothelial surface, but the mechanism remained poorly understood. Our results demonstrated that this process is dependent on TLR4 signaling, triggered by heme. This is evidenced by the use of TLR4 inhibitor TAK-242 *in vitro*, which attenuated both C3 fragments and C5b-9 deposits on heme-exposed EC. Moreover, we have discovered C3 activation fragments deposits on vascular endothelium *in vivo*, in a mouse model of intravascular hemolysis. These deposits were much weaker on the endothelium on TLR4$^{-/-}$ mice compare to WT. Moreover, the same effect was reproduced after injection of heme. Therefore, these results demonstrate that hemolysis-induced complement activation on endothelium is a heme-dependent, TLR4-mediated process.

Heme triggered EC activation and WPB mobilization[6]. The role of TLR4 in this process was elegantly demonstrated by Belcher et al. *in vitro* and *in vivo*[23]. We confirmed the phosphorylation and translocation of NFkB to the nucleus and found p38 and Erk1/2 phosphorylation by heme, consistent with TLR4 signaling. We also confirmed that both free heme and LPS lead to TLR4-mediated WPB mobilization, translated by exocytosis of vWF

and rapid expression of P-selectin on EC surfaces[23]. Interestingly, we detected a rapid increase in the expression of CD14, while the expression of TLR4 remained stable after 30 minutes of exposure to heme or LPS, suggesting a MyD88-signaling pathway activation, using TIRAP as an adaptor. Indeed, in case of MyD88-independent signaling, upregulation of CD14 would lead to TLR4 internalization and recruitment of the adaptor TRAM, as happens in macrophages[34]. EC are known to lack TRAM[35], which can explain the inability to internalize TLR4. The rapid increase of CD14 expression, partially dependent on TLR4 activation, and our immunofluorescence analyses (not shown) indicate the existence of CD14-containing granules in EC. Such storage and TLR-4 mediated upregulation of CD14 is described in azurophilic granules of neutrophils[36,37]. Despite the large body of data in the literature, no link between TLR4 and complement system have been established under hemolytic conditions, so far.

Interestingly, both P-selectin[30,33] and VWF[38–40] are discussed as factors, modulating complement activation. P-selectin has been suggested to promote local complement system activation by anchoring C3b to EC membrane[30,33]. Study of the interaction between P-selectin and C3b revealed a high binding affinity, around 100nM, echoing previous publications[30,33]. We have reported previously an unexpected binding of C3(H_2O) – the fluid phase activation product of C3, critical for the so called "tick over" of the alternative pathway[41] - to EC surface upon treatment with heme[6]. The mechanism behind this phenomenon remained unclear. Here we demonstrate that C3(H_2O) can interact with P-selectin[42], explaining why this typically plasma protein, which is not able to covalently bind to cell surface, contrary to C3b, is found on EC membrane. Moreover, neither C3b nor C3(H_2O) binding to P-selectin were disturbed by the presence of heme, suggesting that they are robust and operational in hemolytic conditions. Therefore, our data demonstrated the relevance of P-selectin as a platform for the C3 activation fragments recruitment on EC. Another platform molecule for recruitment of C3 fragments is properdin[43,44]. It serves as a pattern recognition molecule, binding to damaged cells and activating complement. Here we did not detect any properdin binding on heme-exposed EC, excluding its contribution to complement activation. Moreover, the inhibition of the C3b binding with a blocking antibody against P-selectin underlined the relevance of the C3b/P-selectin interaction on EC surface. The decrease in the C3 fragments deposition on the cells was similar to the one obtained with the TLR4 inhibitor, pointing towards the conclusion that the contribution of TLR4 is entirely mediated by P-selectin. P-selectin plays a critical role in vaso-occlusion in patients with sickle cell disease[45–48]. P-selectin inhibitor crizanlizumab

was associated with a lower frequency of sickle cell–related pain crises in patients with sickle cell disease[49]. Complement activation is also observed in sickle cell disease[50–53] and its blockade prevents stasis in a mouse model[54]. We hypothesize that one additional mechanism of action of P-selectin blockade will be the reduced complement activation in patients with sickle cell disease.

Yet, it is surprising to note that treatment with LPS did not induce complement activation on EC *in vitro*, contrary to what was observed with heme, especially when our results clearly show involvement of TLR4 and possibility to express P-selectin. The incapacity of LPS alone to induce complement activation on EC has already been described on marrow microvascular EC[55] as well as *in vivo* in mice[30]. LPS alone is known to activate complement, when immobilized on plastic or added in serum, but large doses are needed compared to the trace amounts sufficient for cytokine production[56,57]. Heme activate complement in serum and generates $C3(H_2O)$[6]. The concomitant expression of P-selectin (serving as a platform) and the fluid phase complement activation by heme (providing the ligand to be recruited on the platform) may explain its capacity to activate complement on endothelium contrary to LPS.

Our in vitro results indicated that TLR4 stimulation was not the only mechanism, contributing to the complement-activating phenotype of heme-exposed EC. TLR4 inhibition did not prevent the loss of the membrane regulator MCP and DAF, which we reported previously[6], as well as LPS was unable to decrease their expression. These data suggested a TLR4-independent mechanism, which could pass through lowering the regulatory capacity of the cell. Moreover, increased FH binding to the heme-exposed cell surface remained unchanged in presence of TLR4 inhibitor, suggesting a direct effect of the oxidative stress. Indeed, FH protects cells, suffering from oxidative stress by binds to oxidized lipids[58,59].

Another event, which was TLR4 inhibition independent and not induced by LPS, was the rapid up-regulation of the membrane C3aR and C5aR. Immunofluorescence staining revealed that EC contained an important intracellular pool of both C3aR and C5aR in still unidentified granules, suggesting exocytosis[60]. C3aR and C5aR are not contained in WPB, since inhibiting TLR4 signaling did not prevent their up-regulation by heme. Both C3aR and C5aR have been found in lysosomes of T cells, sustaining T cell homeostasis[61]. We have previously reported that heme generates C3a and C5a in serum[6]. This, together with the local generation of these anaphylatoxins on the EC surface will induce C3aR and C5aR signaling. C3aR and C5aR engagement promotes complement deposition on EC[30,60], including dependent on P-selectin[30].

Therefore, the complement activation, initiated by heme in serum and on EC, will be perpetuated by a vicious circle. In it C3a and C5a will activate the EC, will trigger more P-selectin and hence – more complement activation. Such an amplification loop of complement-mediated inflammation will be exacerbated in infections hemolytic diseases, such as Shiga toxin-mediated hemolytic uremic syndrome (HUS)[30,62], possibly in malaria[5] as well as in genetically driven HUS, associated with complement overactivation[6,63,64].

Interestingly, concomitant activation of both TLR4 and complement system have already been observed in response to bacterial infections[65] and in sepsis[66]. The adverse effects of inflammation in these models were attenuated by concomitant complement and TLR4 inhibition using C3 inhibitor compstatin and anti-CD14 blocker, respectively. However, activation of TLR4 and complement system in these cases were independent events, since terminal complement complex is still formed when only TLR4 signaling is blocked[65]. In hemolytic conditions, interrelation between TLR4 and complement activations highlighted the potential benefit of TLR4 blockade to prevent harmful processes mediated by both TLR4 and by complement system

In conclusion, our study demonstrates a new mechanism of acquisition of a complement-sensitive phenotype by endothelium. Moreover, it highlights a potential benefit of a therapeutic use of inhibitors of the axis TLR4/P-selectin on the complement-mediated inflammation under hemolytic conditions.

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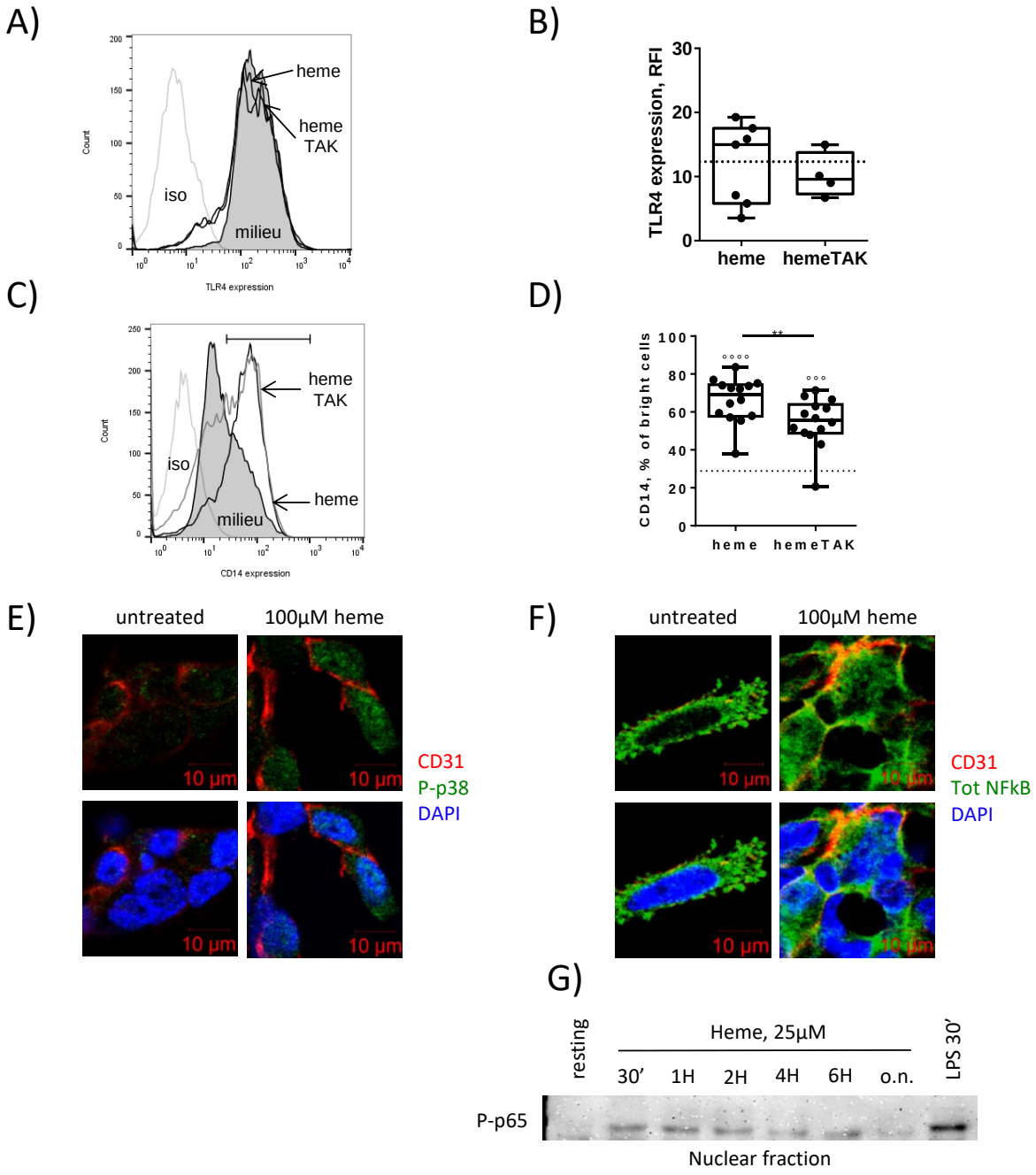


Figure 1: Heme induced TLR4 signaling activation. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were detached and stained for TLR4 (n=4) (A,B) and CD14 (n=14) (C,D) by flow cytometry. *: p<0.05, **: p<0.005, ***: p<0.001, One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality. E,F) Cells were incubated for 30 mins in presence of 100 µM of heme. Phosphorylation of p38 (E) and NFkB (F) was stained and studied by immunofluorescence (green). Nuclei were stained with DAPI (blue). HUVEC were treated with 25µM of heme are recovered after 30 mins, 1h, 2h, 4h and 6h. After centrifugation, phosphorylation p65 (G) was studied by WB in total cell lysate and nuclear fraction, respectively. HUVEC were treated with 100µM of heme for 30 mins at 37°C.

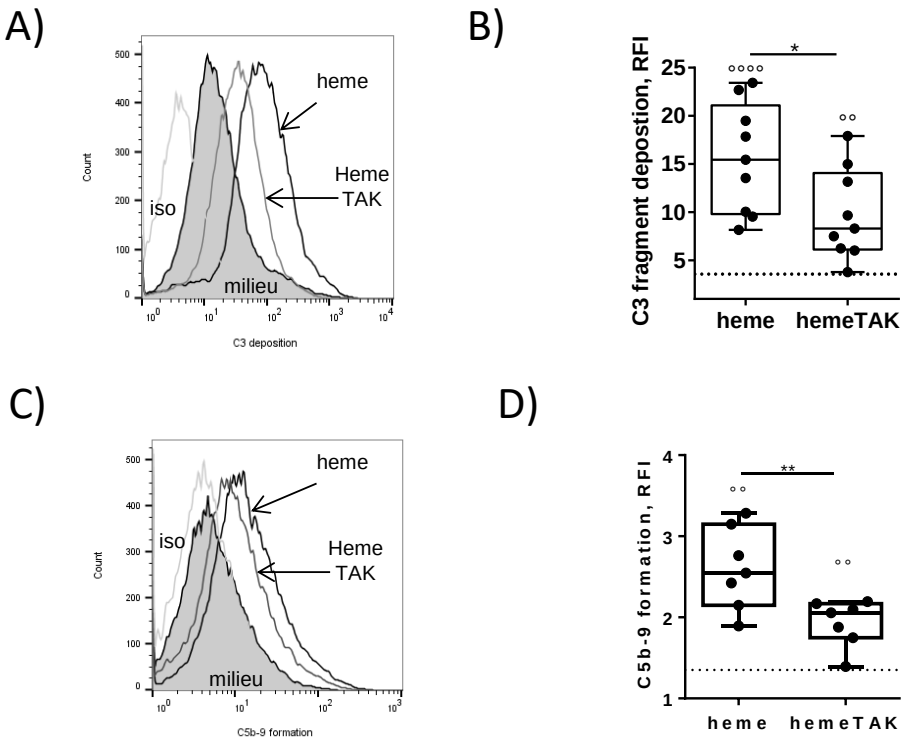


Figure 2: Heme-mediated complement deposition on EC is partially dependent on TLR4 activation. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free) for 30 mins at 37°C. Cells were detached and stained for C3 deposition (A,B), or C5b-9 formation (C,D) by flow cytometry. ***: p<0.001, One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality.

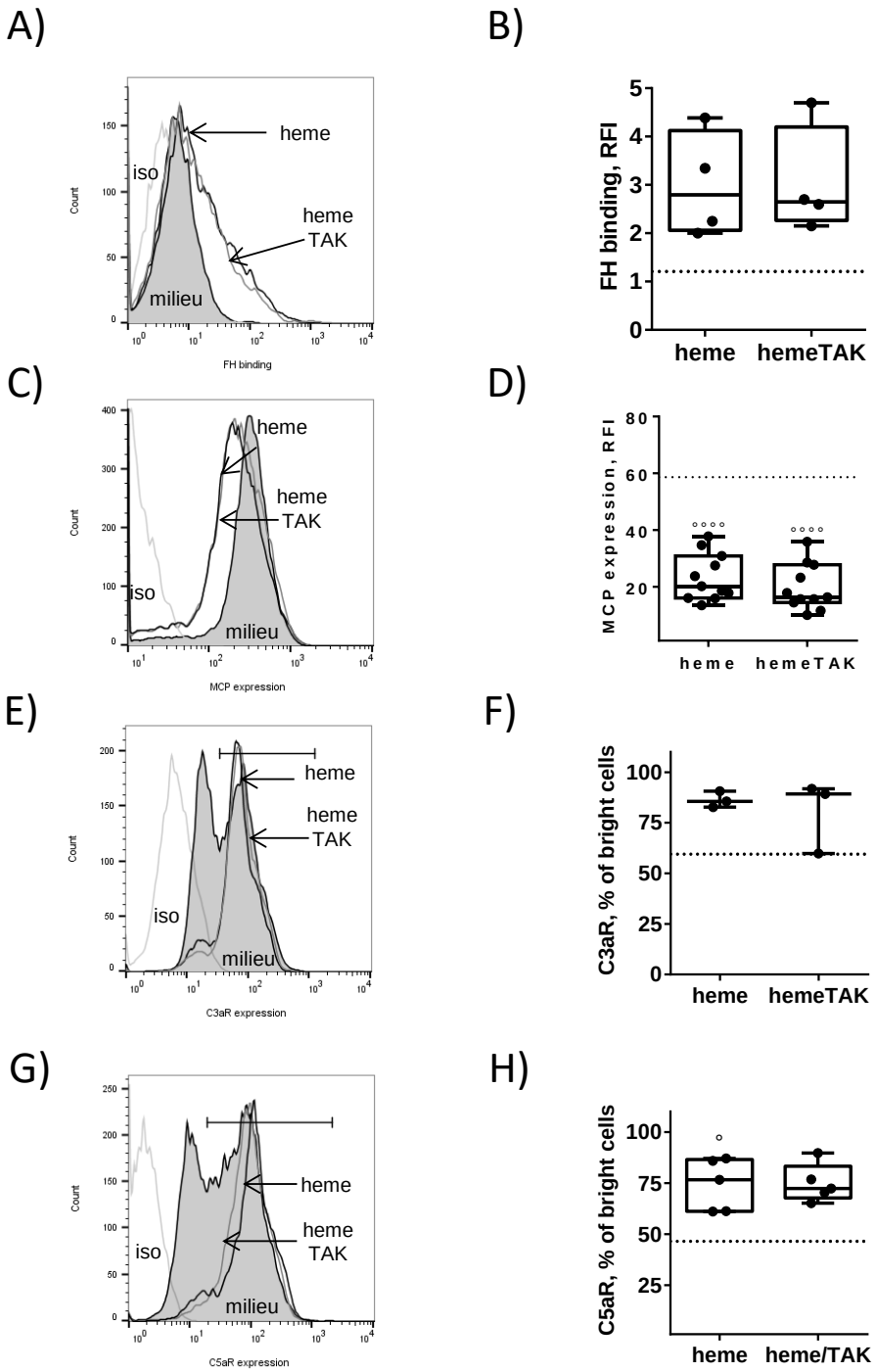


Figure 3: Heme altered expression of complement-related proteins on EC surface in a TLR4-independent manner. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free) for 30 mins at 37°C. Cells were detached and stained for FH (n=5) (A,B). HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were detached and stained for MCP (n=13) (C,D), C3aR (n=3) (E,F) of C5aR (n=5) (G,H) by flow cytometry. **: $p<0.005$, ***: $p<0.001$, One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality.

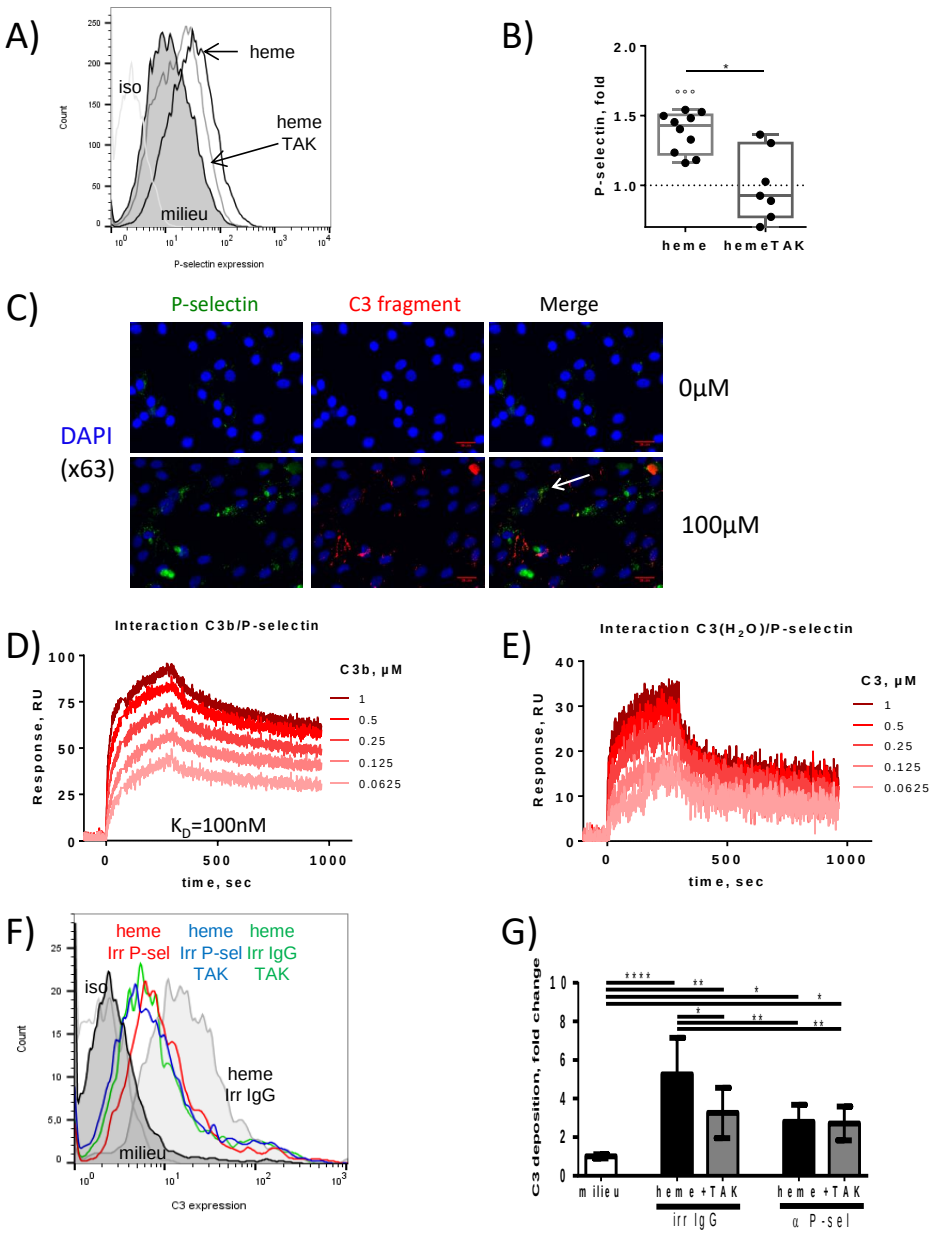


Figure 4: P-selectin is secondary induced to heme-mediated TLR4 activation and serves as an anchoring factor for C3 activation fragment deposition. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were detached and stained for P-selectin (n=9) (A,B) by flow cytometry. C) HUVEC were treated for 5 mins with 100µM of heme. Double staining of P-selectin (false green color) and C3 fragment deposition (false color red) were studied by immunofluorescence. Nuclei were stained with DAPI (blue). Interaction between C3b/P-selectin and C3(H_2O)/P-selectin were studied by surface plasmon resonance by injecting increased concentration of C3b (E) or freeze/thaw native C3 (F) on P-selectin-coated chip. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free supplemented with 10mM EGTA and 2mM $MgCl_2$) for 30 mins at 37°C. Cells were detached and stained for C3 fragment deposition (n=3) (F,G) by flow cytometry. **: p<0.005, ***: p<0.001, One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality.

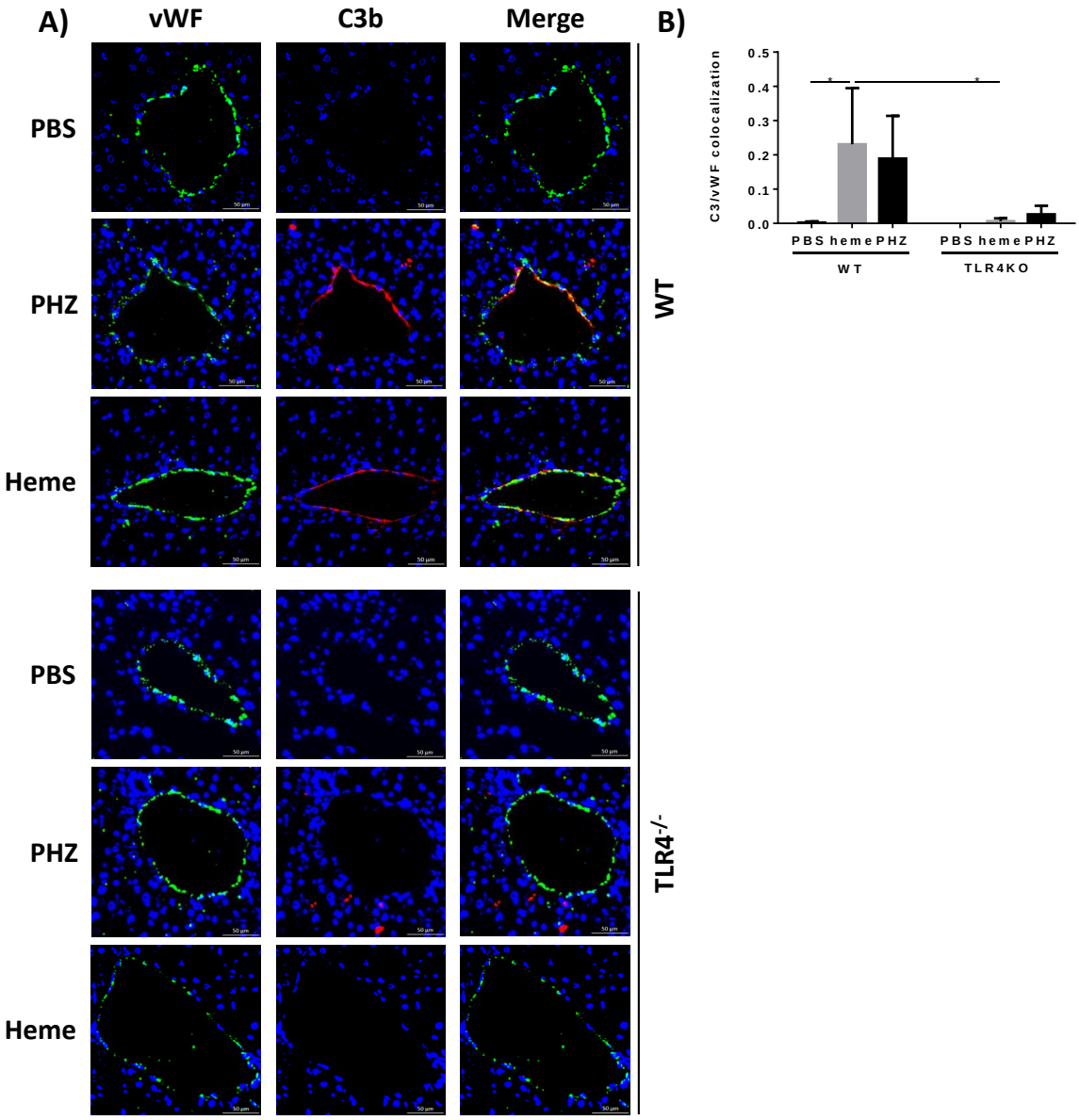


Figure 5: TLR4 activation by hemolysis induces complement deposition on liver endothelium. A) Double staining of WT and TLR4$^{-/-}$ mice liver frozen sections for vWF (false color green) and C3b/iC3b staining (false color red). The merge indicating co-localization is in orange. Focus on macrovessels. B) Staining quantification of WT (PBS=2; heme=4; PHZ=5) and TLR4$^{-/-}$ (PBS=1; heme=5: PHZ=6) mice. *: $p<0.05$; **: $p<0.005$, ***: $p<0.001$, Two way ANOVA with Tukey's test for multiple comparisons.

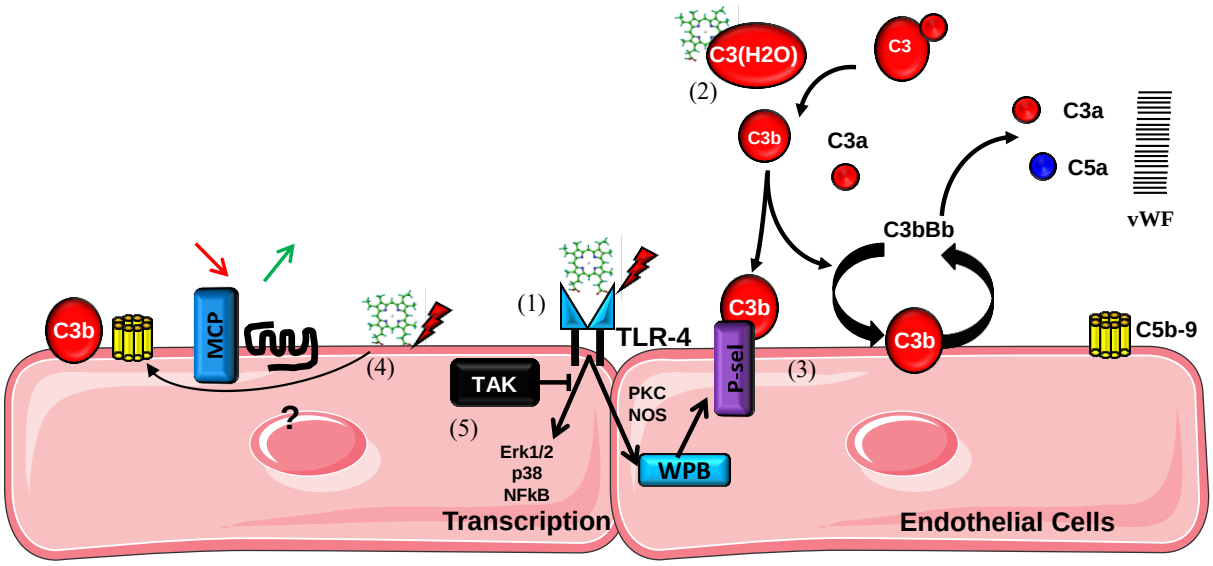
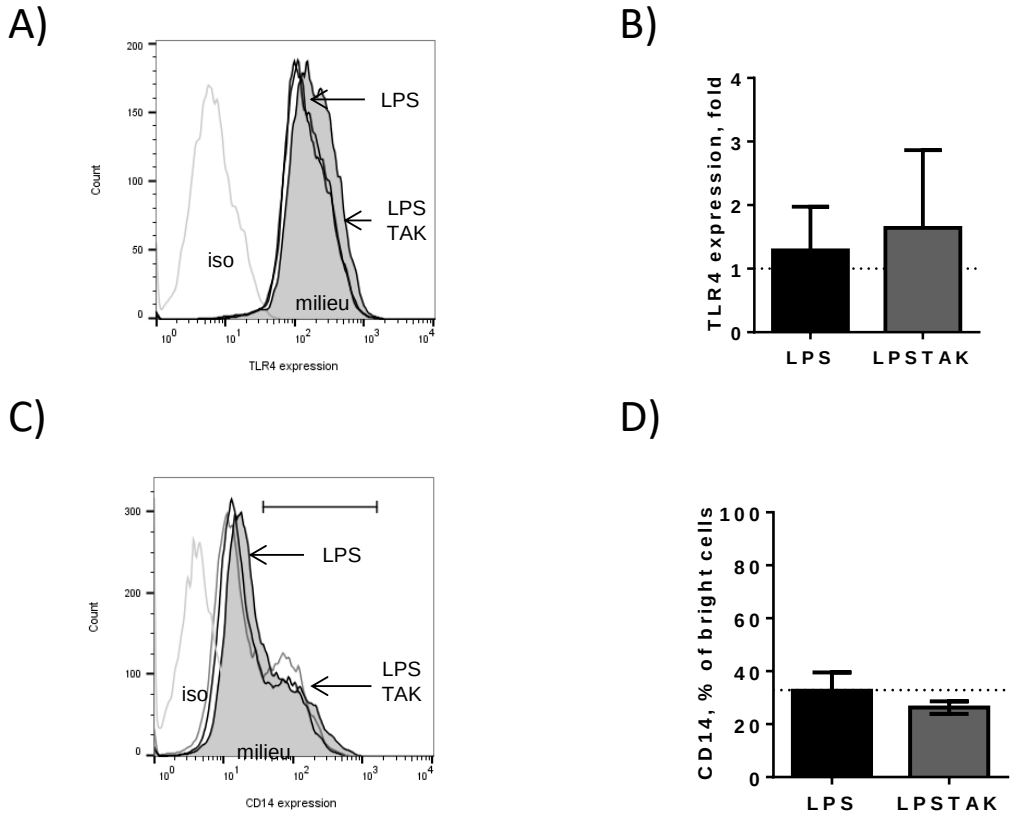


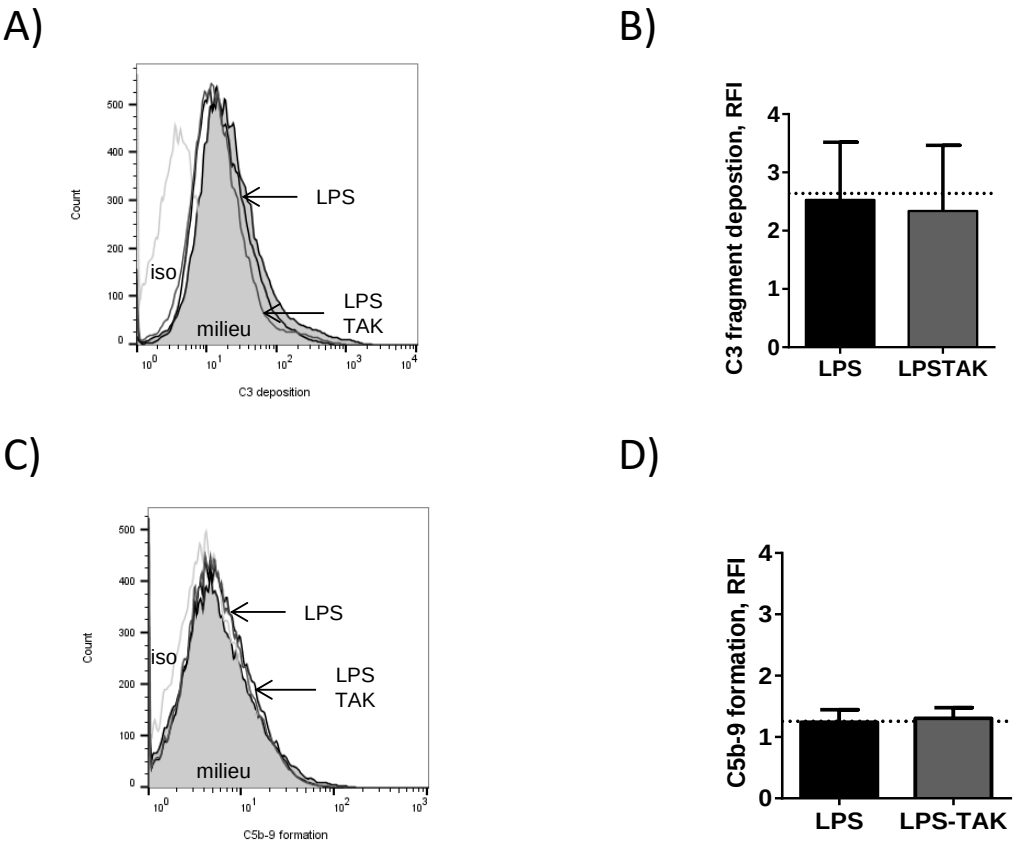
Figure 6: Schematic view of complement activation mechanism on EC during hemolysis. (1) Heme released from hemolysis binds to TLR4, expressed on EC, which induces to Erk1/2, p38 and NFκB pathways transduction as well as rapid exocytosis of Weibel Palade bodies (WPB), leading to von Willebrand factor (vWF) release and P-selectin (P-sel) expression. (2) In parallel, heme promotes formation of alternative C3 convertases in fluid phase, which cleave native C3 from serum. (3) C3(H_2O) and generated C3b are anchored on cell membrane, allowing local formation of C3 convertase, release of anaphylatoxins C3a and C5a, till C5b-9 formation. (4) However, an undetermined mechanism, probably linked to the variation of MCP, C3aR and C5aR expressions still promote complement deposits. (5) Treatment with TAK-242 inhibits the heme-mediating TLR4 activation, preventing about 50% of complement deposits.

Figure S1



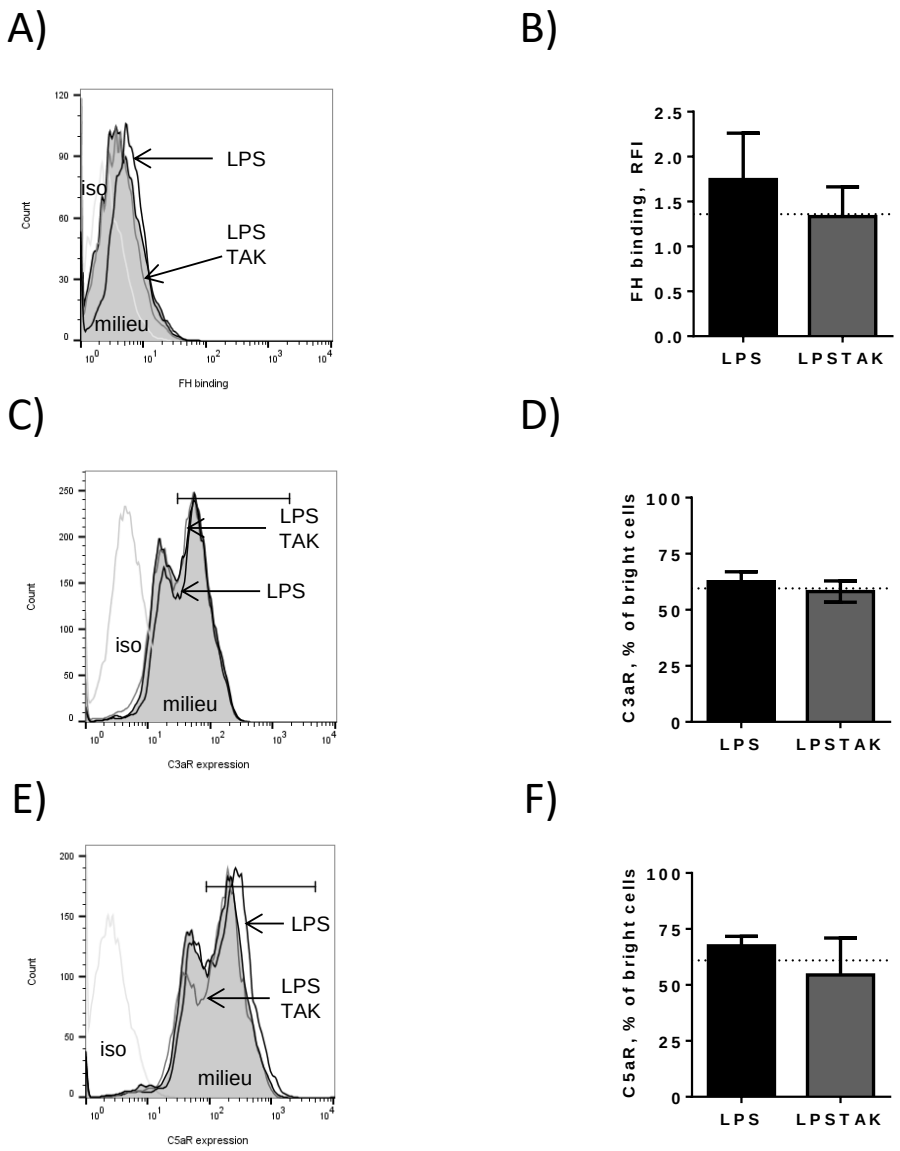
Supplementary figure 1: LPS does not alter expression of TLR4 and CD14. HUVEC were treated with 200ng/ml of LPS with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were detached and stained for TLR4 (n=2) (A,B) and CD14 (n=4) (C,D) by flow cytometry.

Figure S2



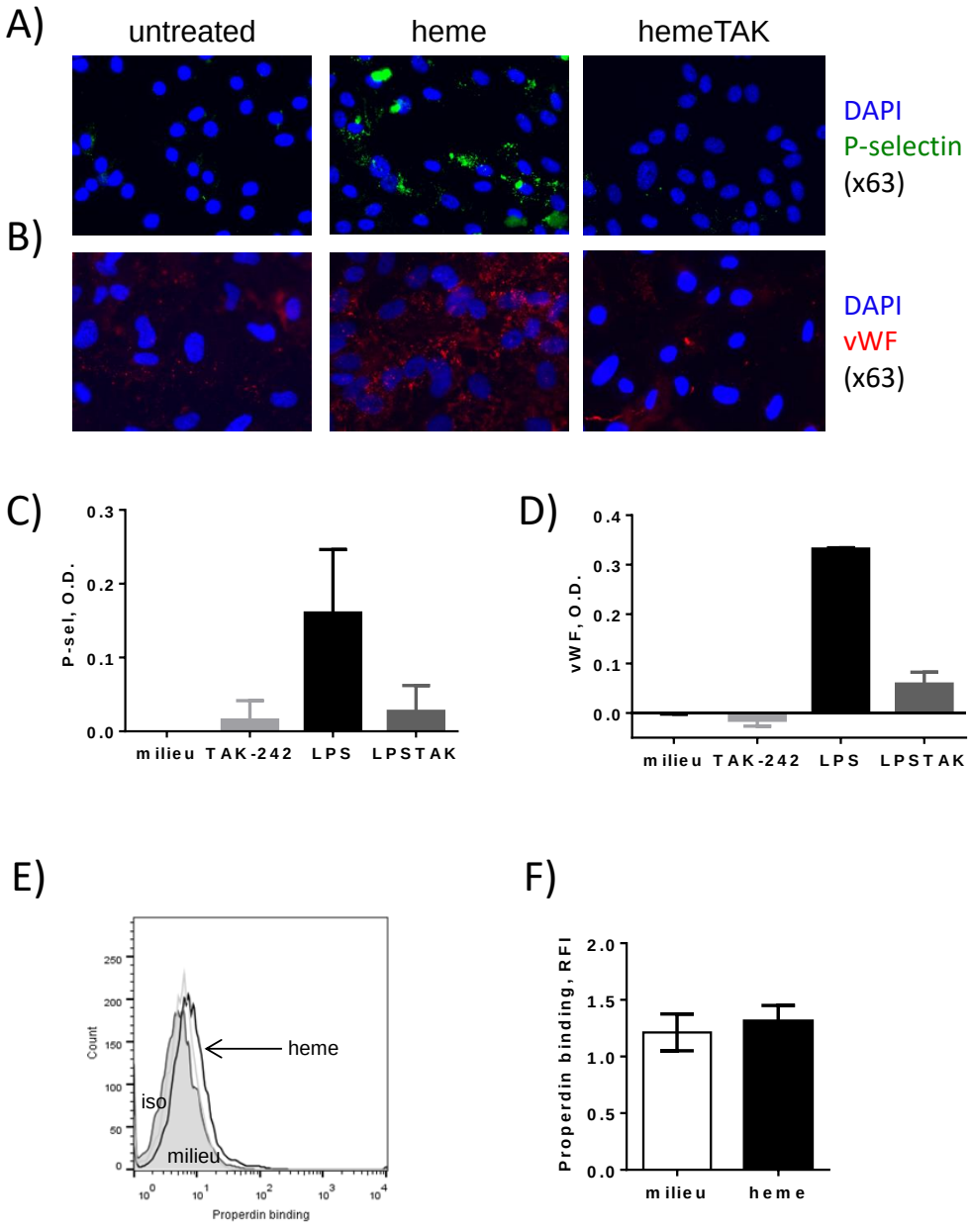
Supplementary figure 2: LPS is not capable of inducing complement deposition on EC. HUVEC were treated with 200ng/ml of LPS with or without 400nM of TAK-242 for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free) for 30 mins at 37°C. Cells were detached and stained for C3 deposition (A,B), or C5b-9 formation (C,D) by flow cytometry. One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality.

Figure S3



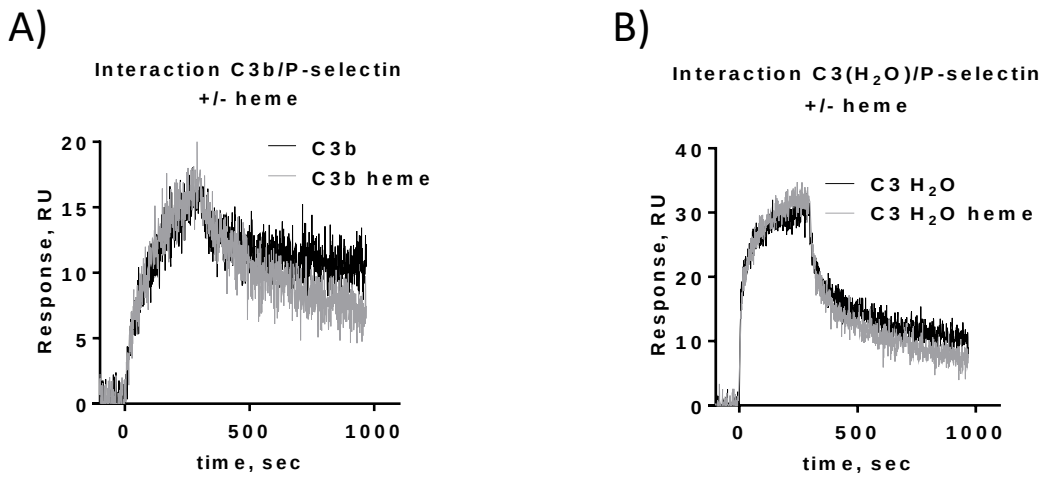
Supplementary figure 3: LPS does not alter expression of complement related proteins on EC surfaces. HUVEC were treated with 200ng/ml of LPS with or without 400nM of TAK-242 for 30 mins at 37°C. After removing the supernatant, HUVEC were exposed to NHS (33% final concentration diluted in M199 FCS-free) for 30 mins at 37°C. Cells were detached and stained for FH (n=3) (A,B). HUVEC were treated with 200ng/ml of LPS with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were detached and stained for C3aR (n=3) (E,F) of C5aR (n=3) (G,H) by flow cytometry. **: $p<0.005$, ***: $p<0.001$, One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality.

Figure S4



Supplementary figure 4: Both heme and LPS induce expression of P-selectin and release of vWF. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were stained for P-selectin (false color green) (A) or vWF (false color red) (B) by colorimetric. Nuclei were stained with DAPI (blue). HUVEC were treated with 200ng/ml of LPS for 1 hour at 37°C. Exocytosis of P-selectin (n=3) (C) and vWF (n=3) (D) were measured by cellular ELISA.

Figure S5



Supplementary figure 5: Presence of heme did not modify binding of C3b and C3(H2O) on P-selectin. Interaction between C3b/P-selectin and C3(H_2O)/P-selectin in presence of 100µM of heme were studied by SPR by injecting increased concentration of C3b/heme (A) or freeze/thaw native C3/heme (B) on P-selectin-coated chip.

ANNEXES

Annex 1: Study of complement deposition in organs

Background:

We previously observed an exclusive heme-dependent complement deposition within kidney, raising the question of such heme-dependent mechanism in other organs.

Goal:

The aim of this study was to investigate how hemolysis promotes complement deposits in hearts, liver and spleen.

Methods:

Mouse treatment. C57Bl/6 mice were from Charles River Laboratories, (L'Arbresle, France). Eight week old female C57Bl/6 mice were injected i.p. with 200 µL of PBS (Gibco), human Hx alone (0.491 µmol/kg, corresponding to 28µ g/g body weight), freshly prepared heme (40µmol/kg, corresponding to 26µg/g body weight) repeated 24 hours later (day 0 and day 1). Alternatively, mice were injected only at day 0 and organs were recovered at day 1 or day 2. PHZ (900µmol/kg, corresponding to 0,125 mg/g body weight) was injected i.p. at day 0 and C57Bl/6 mice were sacrificed at day 1. To test the efficacy of Hx, mice were pre-treated with i.p. injection of 40 µmol/kg of human Hx diluted in PBS one hour before i.p. injection of 0.491 µmol/kg of freshly prepared heme or 0.1 mg/g body weight of PHZ. Concentrations and route of administration were chosen as described.

Immunohistochemistry. Five µm thick frozen sections of kidneys, hearts, livers and spleens cut with Cryostat Leica AS-LMD and fixed in acetone on ice for 10 min. Complement deposition was studied using rat anti-mouse C3 fragments (C3bi/C3b/C3c), followed by a goat anti-rat polyclonal IgG-HRP (R&D system). Staining was revealed with DAB solution. Slides were scanned by Nanozoomer (Hamamatsu). Slides were scanned by Slide Scanner Axio Scan (Zeiss).

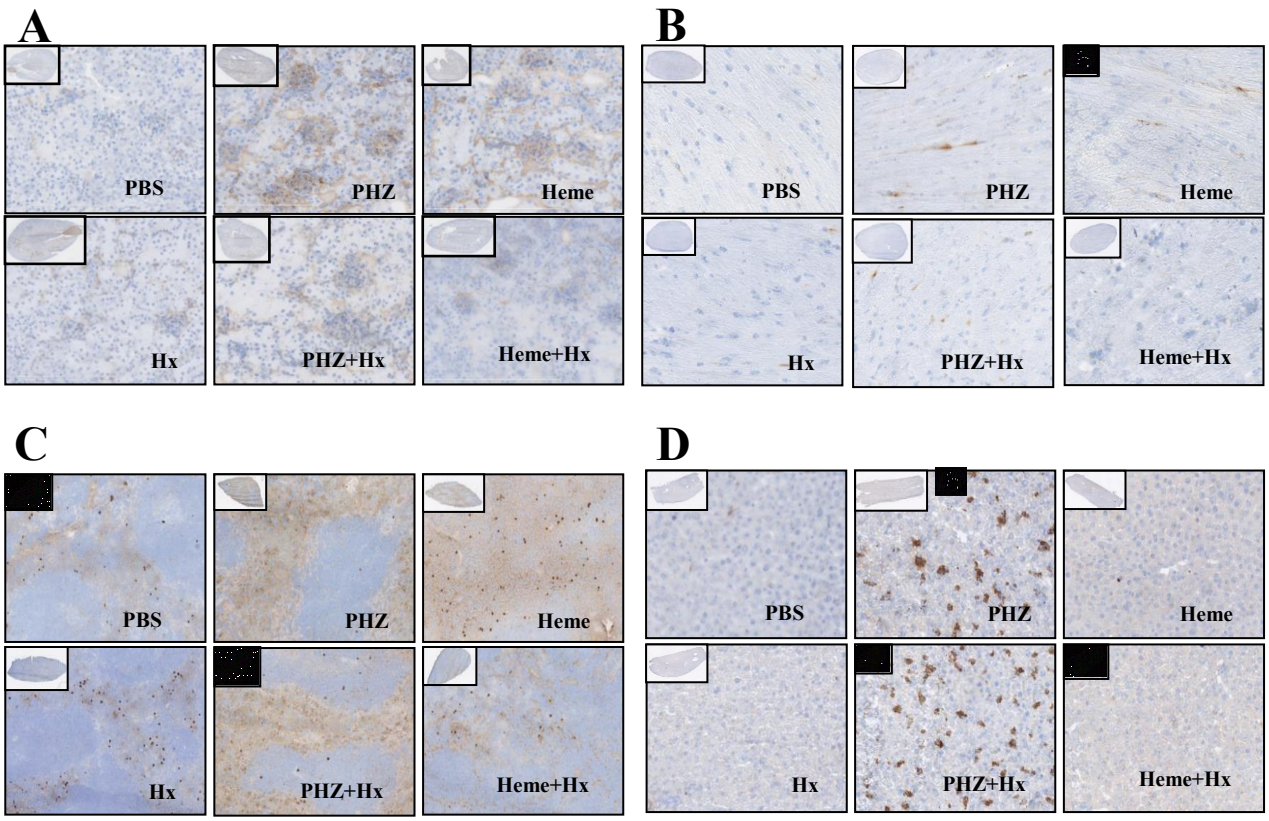


Figure 20: Study of complement deposits in kidneys, heart, liver and spleen. Immunohistochemistry analyses of C3 fragments deposition of frozen kidney (x20) (A), heart (x30) (B), liver (x20) (C) and spleen (x20) (D) sections from mice injected with PHZ, heme or vehicle only, with or without Hx. The staining of C3 fragments deposition appears in brown and nuclei in blue.

Results:

Staining on frozen kidney sections demonstrated no or few C3 fragments deposition deposition in PBS and Hx basal conditions. Increase of C3 fragment deposition specifically localized on inside glomeruli, on Bowman capsules, blood vessels and in the tubular basement membrane was observed in PHZ- and heme-injected mice. Treatment of the mice with Hx prevented this complement activation in both conditions (Figure 20A).

Study of heart sections showed no C3 staining on PBS and Hx treated mice. A weak increase of C3 -fragment deposition was observed on PHZ and heme injected mice. As observed in the kidneys, pre-treatment of the mice with Hx prevent complement activation on the organ, suggesting that C3 deposition is heme-dependent in this organ (Figure 20B).

Study of the spleen revealed an exclusive C3 staining in the red pulp. PBS and Hx injected mice present a punctate staining defined as normal for this organ. Heme treated mice showed an increased C3 staining in the red pulp, which was completely inhibited by injection of Hx, reaching the C3 level of control mice. PHZ-induced hemolysis increased C3 staining in the spleen, which was not normalized after injection of Hx (Figure 20C).

No C3 staining was detected on liver frozen section in mice control groups. PHZ but not heme injection increased C3 staining in the liver. Hx injection in this group of mice did not have inhibitory effects (Figure 20D). Of note, we detected complement deposition in both PHZ and heme-treated mice on blood vessels by immunofluoresence (Manuscript 3).

Conclusion:

These results highlight an organ-dependent mechanism during hemolysis. Kidneys and hearts can be classified in a heme-dependent complement activation group, while the liver clearly shows hemolysis-dependent, heme-independent complement activation. Complement deposition in the spleen responds to both hemolysis- and heme-dependent mechanism. These result must be considered and compare to mRNA expression, as we described in the third article. The heme-independent mechanism observed in liver and spleen can be explained by their capacity to clear dying erythrocytes, which promotes complement deposition to favor phagocytosis (Schroit et al., 1985).

Further investigations must be performed to decrypt the exact mechanism leading to complement activation in these organs in vivo. Thus, it will allow understanding the different behavior of kidney, heart, liver and spleen in response to hemolysis.

Annex 2: Study of the heme binding sites on TLR4

Background:

Binding of heme on TLR4 has been described in several studies (Belcher et al., 2014; Fernandez et al., 2010; Figueiredo et al., 2007). During this work, we provided evidence that TLR4 activation by heme promotes P-selectin activation and then lead to complement activation on EC surface. However, the unexpected activation of TLR4 by heme in MD2-independent manner remains misunderstood.

Goal:

The aim of this study was to investigate how heme can trigger TLR4 by investigating the potential binding sites *in silico* and by SPR. This work has been done in collaboration with Céline M. Labbé, and Maria A. Miteva, MTi, Inserm U973 – Univ. Paris Diderot

Methods:

Molecular docking. Docking experiments of the heme molecule into the TLR4-MD-2-LPS complex structure were performed using Autodock v4.2.6 (Trott and Olson, 2009). The X-ray structure (PDB ID 3FXI (Park et al., 2009)) was used for all docking experiments. First blind docking was performed on the entire surface of the TLR4 monomer and TLR4 dimer complex containing MD2 and LPS (table 1). The preferred zone in term of heme binding energies to TLR4 were then additionally explored by docking. In addition, we performed docking into the interface zone between the two chains of TLR4 of the dimer complex. For the last case, we replaced the side chain positions of His 458 of the two monomer chains of Asp 433 of the two monomer chains of TLR4 using the implemented rotamer libraries of PyMOL (The PyMOL Molecular Graphics System, Version 1.5 Schrödinger, LLC.) in order to create a cavity allowing to the heme to enter between the two chains of TLR4.

The docking was performed using the Lamarckian genetic algorithm (GA) with 2500000 evaluations generate orientations/conformations of the ligand. Fifty docking runs were performed with an initial population of 150 random individuals. The used grid parameters for all docking runs are shown in Table 1. All torsions of the heme were allowed to rotate while

the protein was retained rigid during docking. The heme was protonated and charged using the script prepare_ligand4.py available in ADT tools v1.5.6 (Sanner, 1999). TLR4, MD-2 and LPS were protonated and charged using the script prepare_receptor4.py available in ADT tools v1.5.6 (Sanner, 1999). We added the two Mg^{2+} atoms into the dimer complex.

Table 1.

Docking run	**Receptor**	**Number of grid points**	**spacing**	**center coordinate (x, y, z)**
Blind docking	TLR4 monomer	126, 126, 126	0.792	6.583, 13.333, 46.250
Blind docking	TLR4 dimer + MD-2 + LPS	126, 126, 126	0.742	11.059, -8.142, -7.528
Zoom in zone 1	TLR4 monomer	126, 126, 126	0.375	4.0, 6.0, 22.0
Zoom in zone 2	TLR4 dimer + MD-2+LPS	82, 68, 60	0.375	11.0, -26.0, 1.0
Zoom in zone 3	TLR4 dimer + MD-2+LPS	126, 104, 116	0.375	8.0, -3.0, 14.0
Zoom in the interface zone.	TLR4 dimer + MD-2+LPS	72, 72, 72	0.375	10.0, 2.0 -7.0

Surface plasmon resonance. TLR4 and TLR4-MD2 were immobilized on the GLC sensor chips of ProteOn XPR36 equipment according to the manufacturer (BioRad). The immobilization of concentration of TLR4 and TLR-MD2 resulted in 4000 resonance units, corresponding to a surface density of 3,33 ng/mm^2. Six different concentrations of heme (starting with 1mM) were injected as analytes. The flow rate was 30 mL/min with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) running buffer (10 mM HEPES, 150 mM NaCl, Tween 0.005%; pH 7.3). Each interaction was tested on at least two different chips at least twice per chip. Data were double referenced and analyzed by using ProteOn manager software.

Results:

We performed docking experiments of the heme molecule into the TLR4-MD-2-LPS complex structure. First, we performed blind docking on the entire surface of the TLR4 monomer and on the entire surface of the dimer complex containing TLR4 monomer, MD-2 and LPS. The three preferred zones in terms of predicted binding energies of the heme obtained after the blind docking were then additionally explored by docking. In addition, we performed docking into the interface zone (zone 4) between the two chains of TLR4 of the dimer complex close to His 456 and His 458 as they have been previously suggested to be required for the activation of TLR4 by Ni^{2+} (Schmidt et al., 2010).

In order to find possible binding sites of the heme into the TLR4-MD-2-LPS complex, we first carried out blind docking into the TRL4 as a monomer and then into the TLR4-MD-2-LPS dimer complex. The blind docking results into the TLR4 monomer suggested two prefered binding zones, zones 1 and 3 (see figure 1). The blind docking results into the TLR4-MD-2-LPS dimer complex confirmed the zone 3 and suggested a new zone 2.

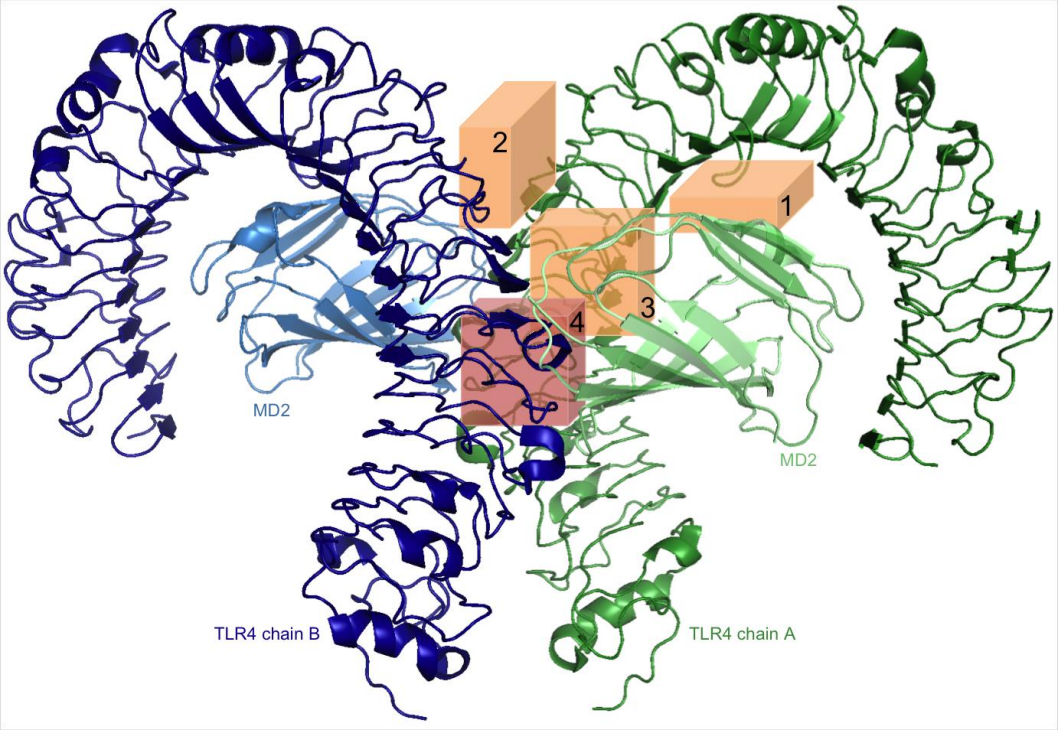


Figure 21: Three possible binding zones identified by blind docking. The chains A and B of TLR4 are shown in green and blue, respectively, by cartoon representation. The two chains of MD-2 are shown in green and blue, respectively, by transparent cartoon representation. The three proposed binding zones are shown in orange. The interface zone is also shown as zone 4 in red.

Then we performed docking experiments in these four specific zones.

Zone 1

For zone 1, the best docking pose into the TLR4 monomer alone showed binding energy of -8.50 kcal/mol and the heme was placed of the binding site where MD2:LPS complex is known to bind TLR4 (Figure 22). The two heme carboxylic groups may be involved in salt bridges with the side chains of Lys230, Arg257, Arg289 of TLR4.

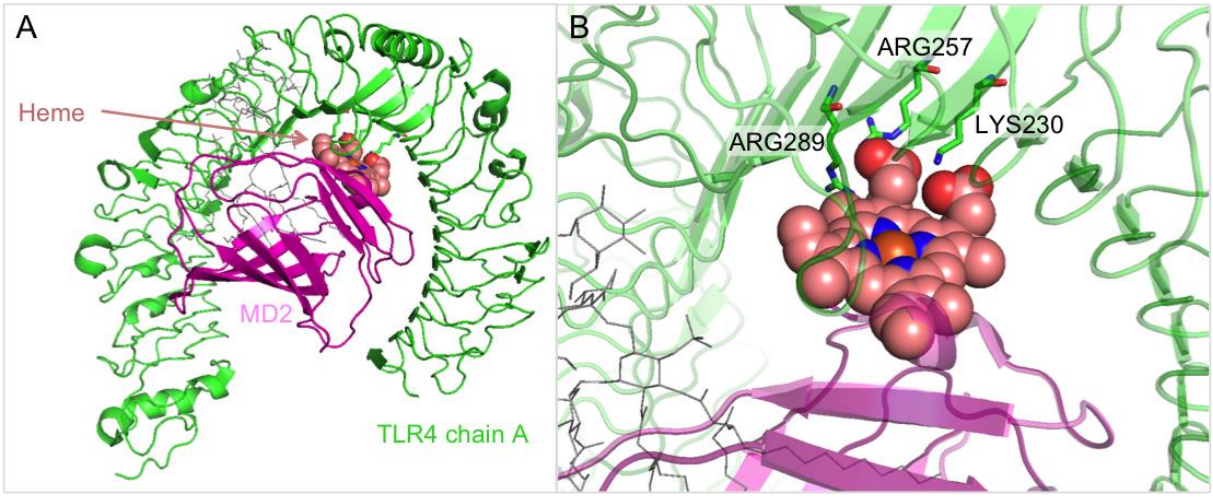


Figure 22: The monomer TLR4 and MD-2 are shown in green and pink cartoon, respectively. The heme is shown as spheres. **A**. Global view of the heme bound to the TLR4 monomer. MD-2 is shown only for illustration of its place without being part of this docking. **B**. The residues of the TLR4 monomer interacting with the heme are shown as stick colored by atom type. The heme is partially on the MD-2 binding site suggesting a possible competition with it.

Zone 2

The best energy pose obtained in the zone 2 after docking into the dimer complex TLR4-MD-2-LPS was with binding energy of -8.14 kcal/mol. After interactive analysis we selected another pose with binding energy of -7.78 kcal/mol (Figure 23). In fact, in this selected position, the two heme carboxylic groups can be involved in salt bridges with the side chains of the two Lys324 of both TLR4 chains. The Fe atom is in contact with the two side chain oxygen atoms of Gln344 of the chains of TLR4 (Figure 23).

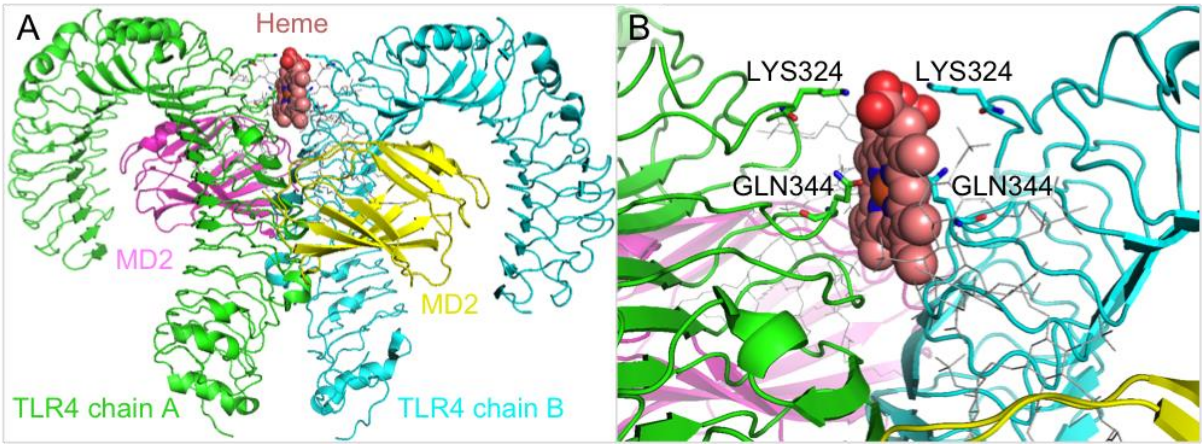


Figure 23: The two chains of TLR4 are shown in cartoon and colored in green and cyan, respectively. The two MD-2 chains are shown as pink and yellow cartoon, respectively. LPS are shown as grey lines. The heme is shown as spheres colored by atom type. The residues of the two chains of TLR4 interacting with the heme are shown as stick colored by atom type. **A**. General view of the heme bound to the dimer complex TLR4-MD-2-LPS. **B**. Specific interactions between the heme and LYS 324 and GLN 344 of the two chains of TLR4.

Zone 3

The best energy pose obtained from either docking the heme into the dimer complex TLR4-MD-2-LPS, either into the dimer of the TLR4 only was with binding energy of -9.99 kcal/mol (Figure 24). In such position, the two heme carboxylic groups are involved in salt bridges with Arg382 (chain A) and Lys 435 (chain B). The three histidines 431, 456, 458 are closely placed to one of the heme carboxylic groups. The Fe atom of heme is in contact with the carbonyl oxygen atom of Arg 382 (chain A of TLR4) (Figure 24).

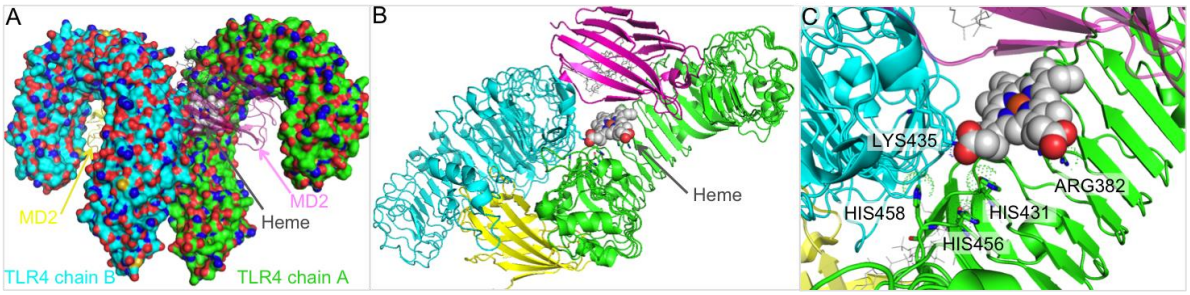


Figure 24: **A**. Global view of the heme binding into the zone 3 of the TLR4 dimer. The two chains of TLR4 are shown in surface and colored in green and cyan by atom type, respectively. **B**. The two chains of TLR4 are shown in cartoon and colored in green and cyan, respectively. The two MD-2 chains are shown as pink and yellow cartoon, respectively. LPS are shown as grey lines. The heme is shown as spheres colored by atom type. **C**. The residues of both chains of TLR4 interacting with the heme are shown as sticks and dots colored by atom type.

Zone 4

The best energy pose obtained when performing docking into the interface zone between the two chains of TLR4 of the dimer complex was with predicted binding energy of -9.39 kcal/mol (Figure 25). Moreover, His 456 and His 458 previously suggested to be required for the activation of TLR4 by Ni^{2+} (Schmidt et al., 2010) are in contact with the heme.

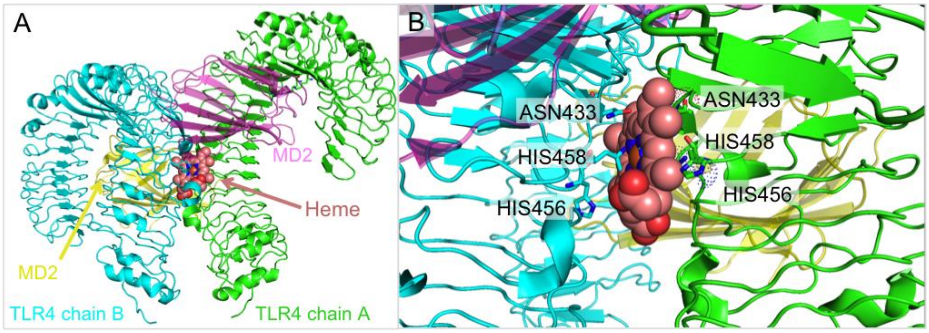


Figure 25: The two chains of TLR4 are shown in cartoon and colored in green and cyan, respectively. The two MD-2 chains are shown as pink and yellow cartoon, respectively. The heme is shown as spheres colored by atom type **A**. Global view of the heme binding into the TLR4 dimer. **B**. Zoom of the heme bound between the two chains of TLR4. The iron atom of the heme is in contact with each His 458 of both chain of TLR4. The His 456 and 458 and the Asp 433 are shown as stick colored by atom type for the chain B of TLR4 and stick and dots colored by atom type for the chain A of TLR4.

Taken together, *in silico* analyses allow speculating a possible competition between heme and MD2 on TLR4. To go further, we tested the capacity of heme to bind TLR4 with or without the presence of MD2. We detected a specific interaction of heme on TLR4 monomer, up to 80 RU. We also detected a specific interaction of heme on TLR4-MD2 complex. However, this interaction was twice time weaker (up to 40 RU), compare to the binding of heme to TLR4 monomer.

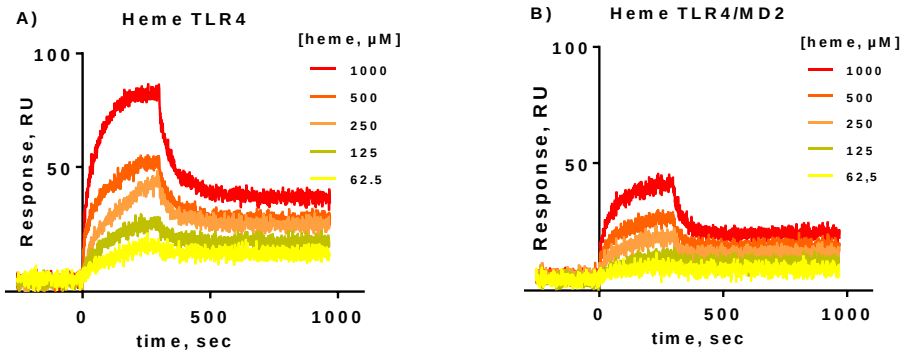


Figure 26: **Interaction of heme to TLR4 decreased in presence of MD2.** SPR analysis of the binding of different concentrations of heme to (A) monomer of TLR4 or (B) heterodimer TLR4-MD2 fragments immobilized on the chip.

Conclusion:

To sum up, we detected four binding areas. The first one (zone 1) is located at site of MD2-LPS interaction with a monomer of TLR4, suggesting a potential competition with the complex. Zone 2, by interacting with two Lys 234, could stabilize the TLR4 homodimer complex. Zones 3 and 4 obtained were the two best binding engergies < -9 kcal/mol and were located in the same area, excluding co-existing binding sites on TLR4. However, due to their localization close to His 456 and His 458, previously described as being essential for TLR4 stabilization by a Ni^{2+} atom, suggests a strong stabilization role of the TLR4 complex. Moreover, our SPR experiment tends to confirm a competition between MD2 and heme, and confirm several binding area of heme on TLR4.

To further evaluate the relevance of this study, based on our *in silico* analysis, we could measure the potential of activation of heme on TLR4 expressing single mutation *in vitro*.

Annex 3: Study of GPCR implication in the heme-induced complement activation on EC surface

Backgound:

We demonstrated that complement activation on EC is partially dependent on TLR4 activation by heme. However, the residual complement deposition highlights the contribution of another mechanism(s) in this process. Heme has been shown to promote activation and recruitment of neutrophils through activation of GPCR receptor (Graça-Souza et al., 2002; Porto et al., 2007). Furthermore, these receptors, which are ubiquitously expressed, have been associated to activation of pro-inflammatory pathways and suggested to closely interact with TLR4 in several models (Abdulkhalek et al., 2012; Liu et al., 2013; Loniewski et al., 2008).

Goal:

The aim of this study was to investigate whether at least one GPCR is involved in the heme-mediated complement activation process on EC.

Methods:

Cell culture. Primary human umbilical vein endothelial cells (HUVEC) were cultured in 24-wells plates as previously described (Frimat et al., 2013), exposed to heme at the indicated doses for 30 mins at 37°C in serum-free M199 cell culture medium (Gibco) in presence or absence of 1 ng/mL of PT. For evaluation of complement activation, HUVEC either washed and further exposed to normal human serum (NHS) diluted to 33% in M199 medium or serum was added to final dilution of 33% to the wells, already containing heme+/-PT, for 30 mins at 37°C. HUVEC were used for experiments until passage 4.

Proteomic analysis. Cells were treated with 25 µM of heme from 30 mins to 6 hours, 100 ng/mL of LPS, or TNFα at 0,001 UI as positive controles. Cells were washed, detached and incubated 30 mins at 4°C in RIPA lysis buffer. **Proteome Profiler.** Cytoplasmic fractions of cell lysis were put on Proteome Profiler™ Antibody Arrays (ARY003B, R&D Systems) overnight. **Western Blot.** Membrane was revealed following the protocol provided by the company. Phosphorylation of Akt was confirmed by WB using Phospho-Akt (Thr308) (D25E6) XP® Rabbit mAb (#13038, Cell Signaling) controlled with total Akt A (#9272, Cell

Signaling) using the SNAP technology (Merk Millipore), followed be Anti-rabbit IgG, HRP-linked Ab (#7074, Cell Signaling). The signal was revealed by chemiluminescence.

Flow cytometry. Cells were washed, detached, labeled and fixed in 0.5% formaldehyde. Cells were analyzed by flow cytometry (BD LSR II), and further analyzed by FlowJo X. The following antibodies were used for staining: anti-human MCP-RPE (BIORAD, MCA2113PE), anti-human C3aR-PE (BioLegend, 345804), anti-human C5aR-APC (BioLegend, 344310), in addition to the corresponding isotype controls. After incubation with serum, staining was performed with anti-C3c mouse monoclonal IgG1 Ab (Quidel, A205), or with anti-C5b-9 mouse monoclonal IgG1 (kindly provided by Prof. Paul Morgan, Cardiff University) or an isotype control and revealed by anti-mouse-IgG1-PE secondary Ab (Beckman Coulter, IM0551).

Results:

Proteome profiler analysis revealed an increased phosphorylation of several pathway intermediates related to TLR4 (in green) and GPCR (in red) activation (Figure 1A). We further confirmed by western blot experiment an increase of Akt phosphorylation, which is the first downstream of GPCR activation (Figure 1B). Phosphorylation of Akt incrased from 30 mins and remained highly activated up to 6 hours.

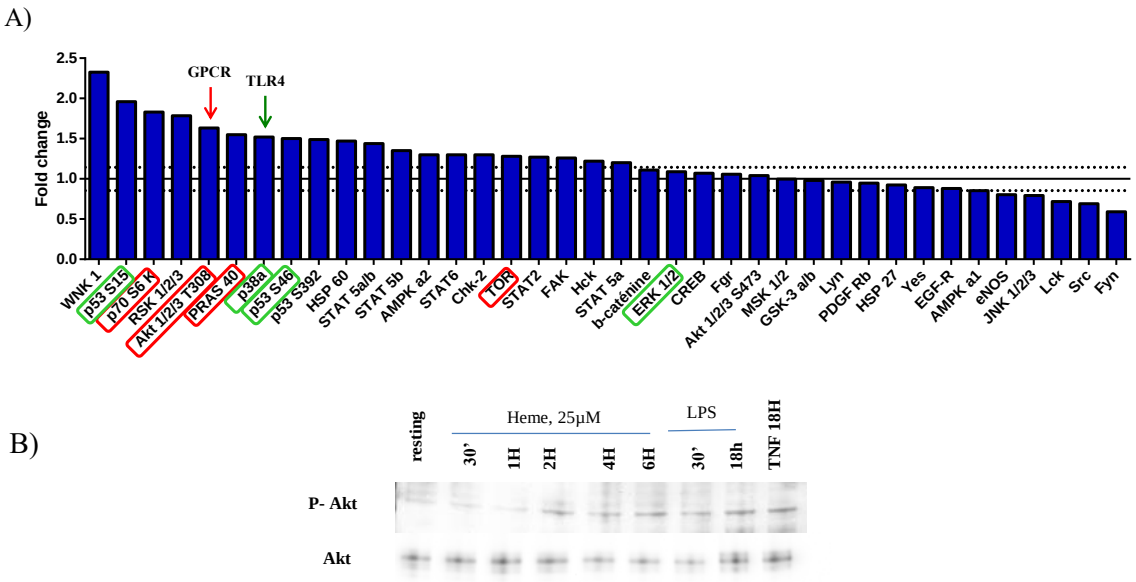


Figure 27: Heme induces Akt phosphorylation. (A) Cells were treated with 25 µM of free heme for 30 mins at 37°C. Phosphorylation of intermediates of signaling pathways analyzed by Human Phospho-Kinase Antibody Array kit (R&D System). Dotted lines represent the SD obtained for the control (FC=1), calculated according to the method provided by the compagny. (B) Time-response of Akt phosphorylation in HUVEC in presence of heme revealed by Western blot.

Study of complement activation on HUVEC surface was achieved on heme-treated cells followed by addition of NHS for 30 min. Evaluation of complement activation by flow cytometry revealed an increase of both C3 fragments deposition and C5b-9 formation on cell surface in presence of heme, which was prevented about 80% by pre-treatment with PT. (Figure 28 A,B,C).

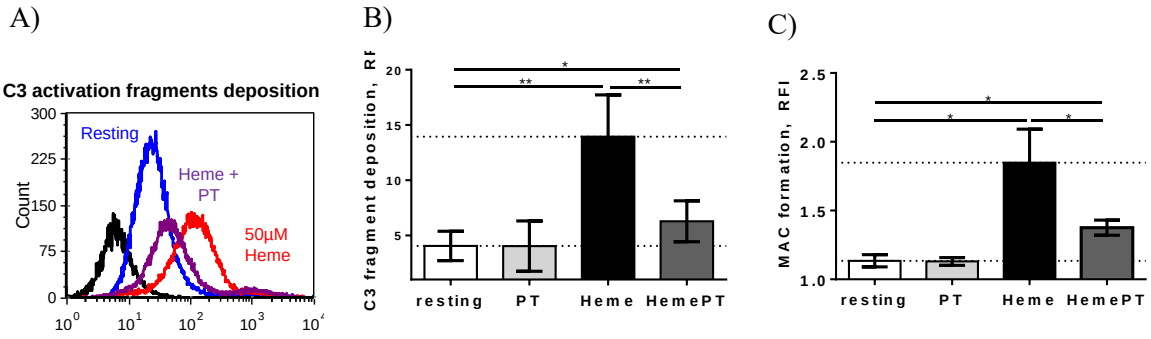


Figure 28: Heme induces complement activation on EC in a GPCR-dependent manner. HUVEC were exposed to PT overnight followed by incubation of free heme for 30 mins at 37°C. After removing the supernatant, HUVEC were incubated with 33% NHS for 30 mins at 37°C. Cells were detached and stained with Ab described in methods. (A) Representative histogram of flow cytometry. C3 deposits (B) and C5b-9 formation (C) in presence of 50 µM of heme and/or 1 ng/mL of PT. *: $p<0.05$; **: $p<0.005$, Mann-Whitney test.

We previously described that expression of complement related marker on EC are modified upon treatment with free heme (Frimat et al., 2013). However, expression variation of these markers remains unclear. Expression of MCP rapidly decreased about 40% upon treatment with heme, which was not prevented by pre-treatment with PT (Figure 29A). Both C3aR and C5aR expression rapidly increase on EC surface upon treatment with heme. Pre-treatment with PT partially prevented elevation of C3aR and C5aR expressions (Figure 29B,C).

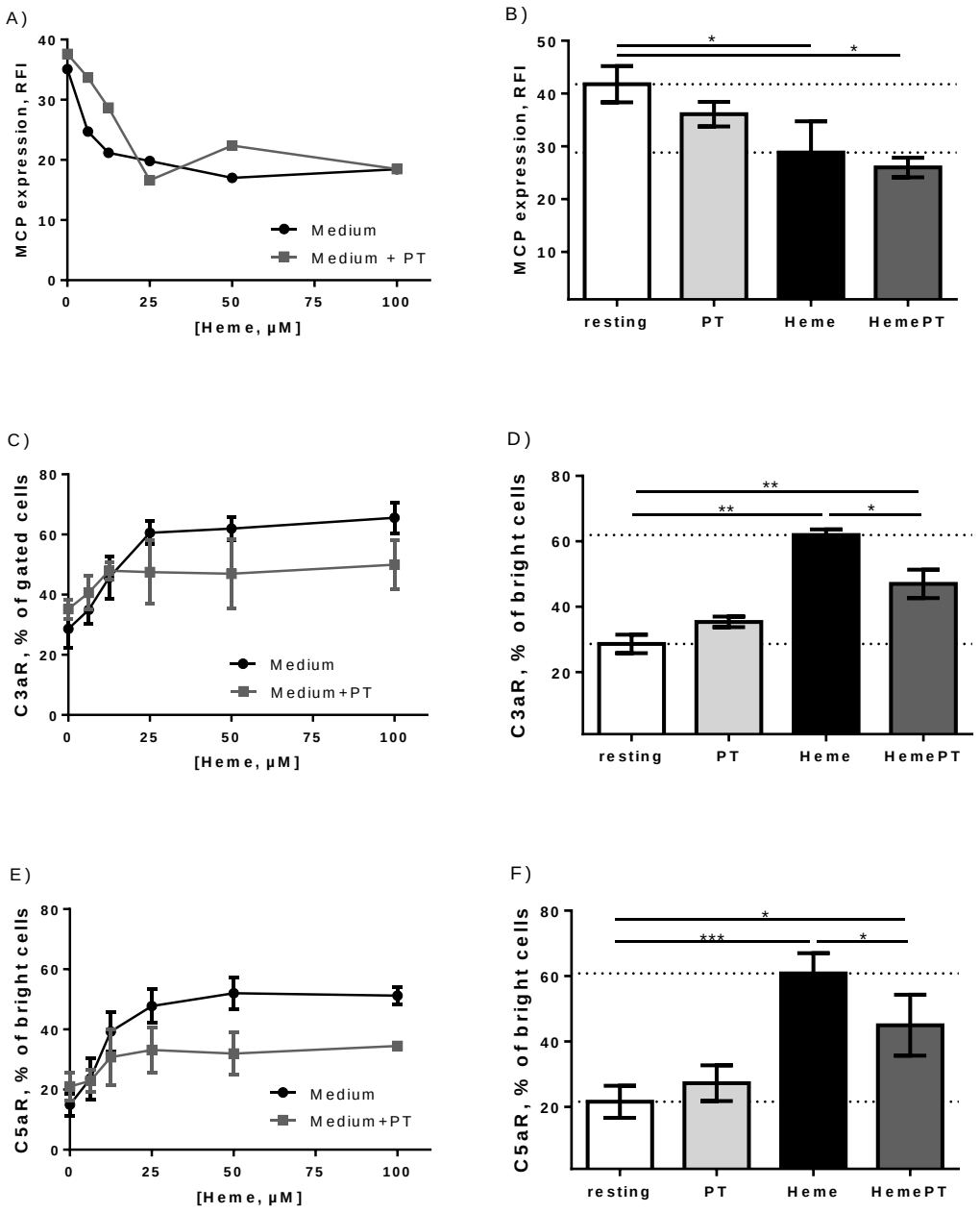


Figure 29: C3aR and C5aR expression are partially controlled by GPCR activation induced by heme. HUVEC were exposed to PT overnight followed by incubation of free heme for 30 mins at 37°C. Cells were detached and stained with Ab described in methods. Histogram of dose-response of heme of MCP (A), C3aR (C) and C5aR (E). Graphs representation of MCP (B), C3aR (D) and C5aR (F) in presence of 50 µM of heme and/or 1 ng/mL of PT. *: $p<0.05$; **: $p<0.005$; ***: $p<0.001$, Mann-Whitney test.

Conclusion:

Heme-induced complement deposits on EC are, at least in part, controlled by GPCR activation. In addition to the previous results obtained with TAK-242, these results ask the question about a potential cooperation between TLR4 and GPCR activation by heme. Moreover, the capacity of PT to inhibit 80% of complement deposits underlines a potential role of GPCR to act as an upstream of TLR4 activation by heme. Cooperation of TLR4 and GPCR has been suggested at the level of intracellular signaling pathway as well as a direct linkage of these receptors on the cell membrane. To assess this cooperation at the intracellular level, we could first perform an experiment by incubating both TAK-242 and PT. No additive effect of these two inhibitors would provide evidence of the dependence of TLR4 to GPCR activation. At the cell membrane, heterodimer formation between TLR4 and GPCR could be assessed by performing co-immunoprecipitation of TLR4. Thenafter, mass spectrometry experiment could allow discovering what is the GPCR implicated in this process.

Interestingly, if decrease of MCP expression was not controlled by GPCR blocking, increase of both C3aR and C5aR expression were partially prevented by PT. Anaphylatoxins receptors belong to the GPCR family, and their activation can be prevented by the use of PT. In parallel of increased expression of C3aR and C5aR, we demonstrated that heme-induced complement activation led to release of C3a and C5a in serum. Together, these results suggest that activation of C3aR and C5aR by their own ligand induce a positive feedback that maintains a high expression on EC surface.

Annex 4: Study of the heme-induced complement activation in aHUS *in vitro* model

Background:

During this work, we confirmed the capacity of heme to activate complement system on EC (Frimat et al., 2013). However, our *in vivo* results tend to demonstrate that complement activation is not associated to endothelium injury, since the natural heme scavenger prevents complement deposition without preventing acute mRNA stress makers. Furthermore, this correlated with undetected C5b-9 on endothelium, indicating no complement-mediated injury. Thus, we hypothesized that heme alone is not enough to promote sufficient complement activation that could overpass the regulation on the cell membrane.

Goal:

In this study, we aimed to evaluate the capacity of heme to drive complement deposits in a complement deregulated model. Moreover, we aimed to evaluate the capacity of Hx to prevent complement activation.

Methods:

Cell culture. HUVEC were cultured in 24-wells plates as previously described (Frimat et al., 2013), exposed to 5 µM of HSA or Hx then incubated with increased concentration of heme during 30 min at 37°C. Supernatant was removed and cells were incubated in 25% NHS, or 25% ΔFI human serum, or 500 µg/mL of blocking Ab against FH (ox24) in 25% NHS, diluted in EGTA/Mg^{2+} (M199, 10 mM EGTA, 4 mM Mg^{2+}) medium for 30 min at 37°C. HUVEC were used for experiments until passage 4.

Flow cytometry. Cells were washed, detached and stained for C3 deposition with anti-C3c monoclonal Ab (Quidel, A205) and revealed by anti-mouse IgG1 PE secondary Ab (Beckman Coulter, IM0551) was analyzed by flow cytometry.

Results:

To test whether Hx can control heme-induced complement activation in a model of complement-related disease, two aHUS *in vitro* models were developed. Human serum depleted in FI mimics mutations occurring in aHUS patients, whereas blocking Ab against FH (ox-24) mimics a subtype of auto-immune HUS where patients develop anti-FH antibodies (Blanc et al., 2015). Our study was performed in EGTA/Mg^{2+} medium in order to block classical (CP) and lectin pathways, and avoid potential CP activation through C1q in the presence of Ox-24. Heme-induced complement activation was similar in presence or absence of EGTA/Mg^{2+} (not shown). C3 deposition increased 5 fold in presence of 50 µM heme, compared to resting cells (Figure 30A). Adding HSA did not influence complement activation, whereas Hx completely prevented heme-induced complement fragment deposition (Figure 30A).

Depleted FI serum increased C3 deposition on resting cells compared to NHS-treated cells (2.5 fold), and up to the maximal levels obtained at 50 µM heme. However, the curves showed that a plateau was reached at 25 µM of heme with FI-depleted serum (at 4-fold lower heme concentration compared to NHS). HSA failed to decrease C3 deposition (data not shown), but 5 µM Hx completely inhibited heme-dependent C3 deposition (up to 50 µM heme; Figure 30B).

Ox-24 increased C3 deposition on resting cells compared to NHS-treated cells (RFI~6 versus ~10) (Figure 30C). Heme induced a dose-dependent increase of C3 deposition on HUVEC, and reached a plateau at 50 µM. In this experimental model, Hx prevented up to 25 µM heme, and only partially inhibited heme-induced complement deposition at 50 µM heme (Figure 30C). HSA did not significantly prevent the effects of heme on C3 deposition (data not shown). Comparative analyses at 50 µM of heme are shown at Figure 30D.

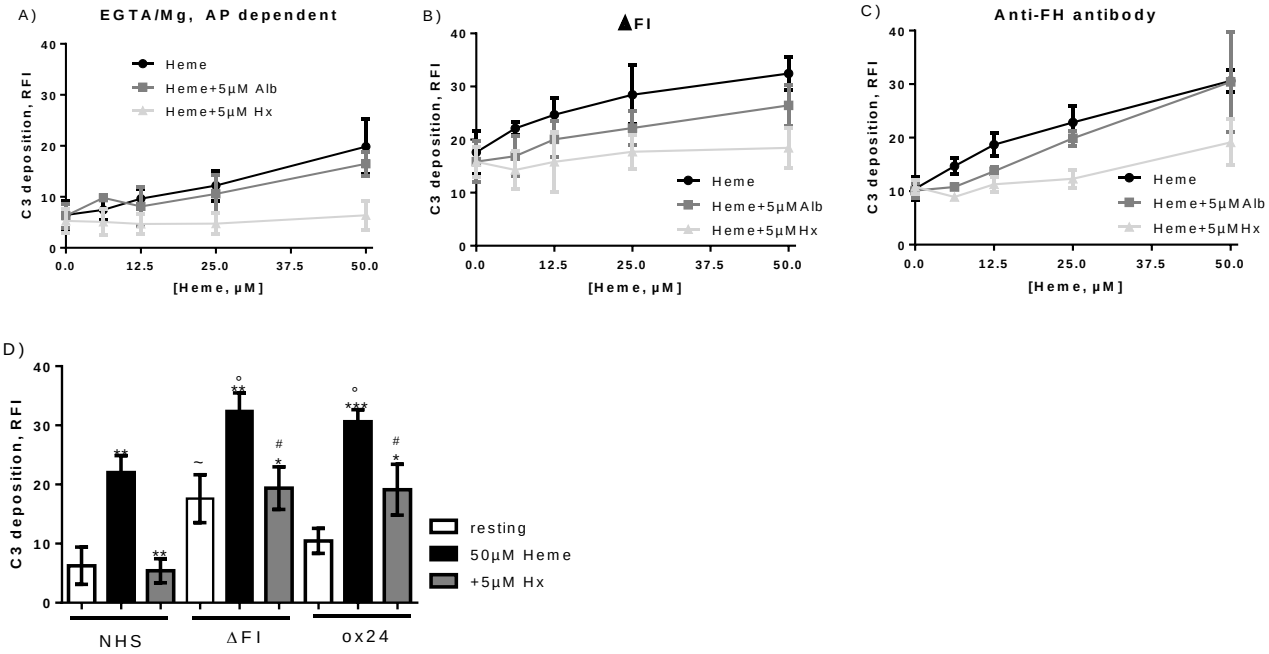


Figure 30: Hx inhibits heme induced complement deposition on HUVEC in aHUS *in vitro* model. At panels (A), (B) and (C), the heme-Hx curves were all significantly different compared to heme ($p<0.0001$, two way Anova), while HSA-heme curves reached statistical significance only at panels (B) and (C) ($p=0.0005$ and $p=0.045$ respectively, two way anova test). (D) Graph representation of statistical differences between resting cells, 50 µM heme treatment with or without 5 µM of Hx in NHS, ΔFI or ox24 serum. *represents statistical difference with previous column in the same serum subgroup. ~ represents statistical difference between resting cells, ° represents statistical difference between heme-treated cells, # represents statistical difference between heme+Hx treated cells crossed between serum subgroups. Statistical significance * $p<0.05$, ** $p<0.005$, *** $p<0.001$ (paired t-test); ~, °, # $p<0.05$, (Mann-Whitney).

Conclusion

Heme-induced complement activation is exacerbated in human serum lacking regulation of complement system. Natural heme scavenger Hx still provides inhibitory effect regarding C3 deposition. To go further, C5b-9 formation should be performed to evaluate the relevant application of Hx in this model. Then after, renal function should be assessed in PHZ-induced hemolysis in an aHUS mouse model, as well as the capacity of Hx to prevent complement deposition.

DISCUSSION

Emerging studies demonstrating the contribution of complement to the pathogenesis of a large spectrum of diseases have highlighted the crucial importance of this previously neglected ancestral branch of the immune system. The deleterious impact of complement on the RBC destruction in PNH (Logue et al., 1973) and the remarkable efficacy of the C5 blocking Ab, eculizumab, demonstrated a strong and direct link between complement and hemolytic diseases (Brodsky, 2017; Hillmen et al., 2006, 2013). The rationale of the present work was based on another, much less studied phenomenon – the capacity of the hemolysis-derived product, heme, to activate complement in serum (Frimat et al., 2013; Lindorfer et al., 2016; Pawluczkowycz et al., 2007) as well as to render EC surface prone to complement attack (Frimat et al., 2013).

Indeed, in 2013 our team provided evidence that heme works as an activator of the AP *in vitro* (Frimat et al., 2013) and thus encouraged our investigations about the relationship between hemolysis and complement activation *in vivo,* in mouse models and in patients with SCD. During the three years of this thesis, I developed a mouse model of PHZ-induced intravascular hemolysis and characterized the organ injury, especially injury in the kidney, and used it to demonstrate that hemolysis induces complement activation *in vivo,* especially in the kidney, in a heme-dependent manner (Manuscript 1). *In vivo,* cell free heme associates with proteins and particles including RBC microvesicles. I found that RBC microvesicles indeed activated complement in serum partially in a heme-dependent manner (Manuscript 2). These results are particularly relevant for SCD because the SCD erythrocyte microvesicles carry 3x more heme and more strongly activated complement compared to normal ones. Complement in hemolytic conditions was activated on EC *in vitro* and *in vivo*. I found that the molecular and cellular mechanisms by which heme causes complement deposition on endothelium is related to direct binding to and stimulation of TLR4 and a still unidentified GPCR (Manuscript 3 + additional data). I detected a signaling pathway (differing from the pathway in which LPS is engaged) that results in p38 phosphorylation and NFκB translocation to the nucleus, leading to gene expression from one side, and rapid mobilization of EC granules containing P-selectin and vWF on the otherside. I found that P-selectin binds C3b and C3(H_2O) and thus initiates complement activation on EC surface. These processes are demonstrated *in vitro* and the importance of TLR4 for vascular C3 deposits was shown *in vivo,* using $TLR4^{-/-}$ mice. Finally, I found that the plasma heme scavenger Hx, which is under

development as a potential therapeutic agent for SCD, prevents completely heme-mediated complement activation (Manuscript 2).

Despite the capacity of heme to trigger the AP in serum and on EC *in vitro*, (Frimat et al., 2013; Pawluczkowycz et al., 2007), hemolysis-associated complement depositions within organs have been under-estimated and poorly studied. Yet, work in the past decades have attributed to complement multiple harmful effects including EC injury (Tedesco et al., 1997) and renal disorders like aHUS and C3G (Zuber et al., 2012). In SCD, hemolysis has been associated to painful crisis, vaso-occlusion and organ injuries, including in kidneys. However, studies about complement deposition and renal injury in SCD are lacking and C3 staining is reported in only a few papers from the 70s. In this study, kidney biopsies of patients suffering from SCD nephropathy stained positive for both C3 and C9 deposits in the majority of the tested cases. In addition, I confirmed this observation in a mouse model of SCD, which is associated to nephropathy (De Paepe and Trudel, 1994). These results demonstrate a presence of complement deposits in a hemolytic disease, in which there are no complement abnormalities (as is the case of aHUS). What remains to be studied is whether these deposits are pathogenic or just a bystander, non-specific effect, unrelated to the pathology. Having in mind the strong pro-inflammatory role of complement anaphylatoxins and deposited C5b-9, I suggest a contribution of complement system in nephropathogenesis in SCD. To investigate this, we should explore the effect of complement blockade using a C3 inhibitor or BB5.1 (murine analog of eculizumab) in SAD mouse model on proteinuria. The difficulties in the breeding of the SAD mice and the difficulties in obtaining BB5.1 precluded such experiments in this thesis. Nevertheless, a recent communication has demonstrated a complete disappearance of stasis in heme-treated SAD mice treated with BB5.1, suggesting a crucial role of complement in triggering vaso-occlusion in SCD (Schaid et al., 2016). Based on the fact that VOC occurs upon binding of sickle RBC on stressed endothelium, I hypothesized that the involvement of complement system was related at least in part to the induction of endothelium stress.

During these three years I aimed to find out whether hemolysis triggers complement activation *in vivo*, what the molecular and cellular mechanisms of this process are and to what extent it is associated with organ injury. To study how hemolysis drives complement activation, I set up a mouse model of drug-induced hemolysis. These results clearly demonstrate that hemolysis is able to promote complement activation within the four tested organs: kidneys, heart, liver and spleen. Surprisingly, the mechanism behind the observed C3

activation fragments deposits was organ-dependent. In kidneys and heart it was heme-mediated (reproduced by free heme injection and prevented by Hx in the PHZ model). In the spleen both heme-dependent and independent pathways operate, since injection of heme caused C3 deposits, but Hx was inefficient to abrogate them. In contrast, in the liver the mechanism was completely heme-independent (not reproduced by heme and not prevented by Hx). The suppression of the heme-dependent complement activation in these organs can be explained by the strong upregulation of the heme-degrading enzyme HO-1, which we detected at gene expression level (and by immunohistochemistry, data of O. May). In contrast, HO-1 was not expressed in the glomeruli – the primary site of heme-dependent complement deposits in the kidney.

Having shown the heme-dependent complement activation during hemolysis in mouse kidney and heart, I sought to investigate the existence of heme-independent mechanisms of complement activation by RBC membranes *in vitro* and *in vivo*. Physiologically, both spleen in first line, and liver in second line, are known to work as clearance organs for senescent and damaged RBC, or lysed RBC membranes (Schroit et al., 1985). To this regard, generation of RBC MVs in PHZ-treated mice can explain the heme-independent mechanism detected in both spleen and liver. Indeed, since I demonstrated that neither OxyHb nor MetHb have the capacity to induce complement activation *in vitro*, it is plausible that RBC MVs mediate complement activation in heme-independent mechanism in those organs. In the past decade, more and more data show the implication of RBC fragments and MVs from RBC in pro-inflammatory processes, especially in the context of SCD and of transfusion-related complications (Alaarg et al., 2013). RBC ectosomes were shown to bind C1q, leading to C3 fragment deposition (Sadallah et al., 2008). Moreover, C1q is known to bind PS (Païdassi et al., 2008, 2011). Therefore, I hypothesized that pieces of RBC membranes generated by PHZ could activate the classical pathway in a heme-independent manner. However, experiments managed in $C1q^{-/-}$ mice did not reveal any differences regarding complement activation in PHZ-treated mice. Moreover, the complement activation by RBC MVs was entirely AP-mediated. These data suggested that another mechanism can induce complement activation in the red pulp of the spleen. In another study, RBC MV did bind iC3b but not C1q (Zecher et al., 2014). In this case the complement deposition was suggested to be mediated by thrombin and could not occur in complement AP buffer, containing EGTA-Mg. In our hands, RBC MV generated from normal and SCD RBC activated complement by the AP *in vitro*, using EGTA-Mg to a level, identical with the one in Ca^{2+} and Mg^{2+} containing buffer, allowing activation

of all complement pathways. This data is in agreement with yet another study which described RBC MVs that expose PS and phosphatidylethanolamine (PE) on their membrane and can activate the AP (Wang et al., 1993). All of these studies show that RBC-derived MV, independent of the mode of generation, activate complement albeit the differences in the proposed mechanism. A clear role of the surface-exposed PS was demonstrated in this process. PS-mediated complement activation by the AP can explain our observations for C1q-independent and heme-independent deposits in the liver and spleen of the PHZ-treated mice.

Our *in vivo* and *in vitro* data pointed towards a major contribution of RBC MVs for the complement activation. The pool of labile free heme is always associated to plasma proteins, lipoproteins (LDL and HDL), or RBC MVs to mediate pro-inflammatory processes (Alaarg et al., 2013; Camejo et al., 1998). Interestingly, RBC MVs from SCD patients carry three times more heme on their surface compared to healthy donor MVs, and induce VOC in SAD mouse kidneys in a heme-dependent manner (Camus et al., 2015). Here, I discovered that RBC MVs from SCD patients do exert a trigger capacity on complement system in serum in both heme-dependent and heme-independent manner. These results corroborate and provide an explanation of the complement activation observed in SCD patients sera (Chapin et al., 2016; Chudwin et al., 1985; Gavriilaki et al., 2017; Mold et al., 1995). Furthermore, I show that these RBC MVs promotes complement activation on EC in a heme-dependent mechanism, thus supporting a rationale for studying complement activation within organs. Intriguingly, I observed only a partial inhibition of complement activation in serum when Hx was added to RBC MVs from SCD patients. This was explained by PS/PE expression on erythrocyte microvesicles (Chudwin et al., 1994; Tomasko and Chudwin, 1988; Wang et al., 1993). We confirmed this phenomenon by modeling RBC MVs with MLVs expressing PS/PE. The ensemble of these results allowed us to conclude that hemolysis, in part via generation of RBC MVs, can trigger complement activation in serum and in tissues by two mechanisms – heme-dependent and heme-independent (most likely PS-dependent). We collaborated with Dr. Olivier Blanc-Brude (Paris, INSERM UMRS970) to study the contribution of PS from the MV to complement activation. Taken together, these phenomena can explain the presence of C3 deposits that I detected in the kidneys of SCD in mice and men as well as the presence of complement-activation fragments, such as Bb and sC5b-9 in SCD patients' plasma.

Having demonstrated the *in vivo* relevance on the heme-mediated complement activation, I sought to decipher the mechanisms by which heme renders the EC complement-activator surface at a cellular level. Evidence from the literature suggesting that heme is a

potent activator of membrane receptors led us to investigate the possibility of the implication of TLR4 (Belcher et al., 2014; Figueiredo et al., 2007) and GPCRs (Graça-Souza et al., 2002; Porto et al., 2007). My results demonstrated a key contribution of TLR4. Indeed, inhibition of TLR4 prevented about 50% of complement deposits on EC surfaces. This result provides evidence for a direct relationship between complement and TLR4. Indeed, many studies have documented the cross-talk between these two pathways (Gustavsen et al., 2016; Huber-Lang et al., 2014; Skjeflo et al., 2014), but this study demonstrated for the first time a direct implication of TLR4 activation on complement deposition on host cell surfaces. However, the exact signaling mechanism via TLR and the binding site(s) of heme on this receptor remain undetermined. Intriguingly in 2007, Figueiredo et al highlighted a capacity of heme to mediate TLR4 signaling on macrophages through a CD14-dependent but MD2-independent mechanism (Figueiredo et al., 2007; Piazza et al., 2011). This was a surprising result, since MD2 is absolutely required for forming a quaternary complex of two TLR4:MD2 heterodimers, necessary to promote TLR4 signaling in response to the classical TLR4 ligand LPS. Thus, I hypothesized the existence of a potential heme-binding site, localized in the binding area of MD2 on TLR4. Indeed, our SPR results clearly show a decreased capacity of free heme to bind TLR4:MD2 complex compared to TLR4 alone, suggesting a competition. However, the residual heme binding activity on TLR4:MD2 complexes led me to suspect existence of at least one other binding site.

Further, *in silico* molecular modelling of heme binding on TLR4, in collaboration with Dr. Maria Miteva (INSERM UMRS973), revealed four potential binding areas, three engaging TLR4 monomers and one interface zone, making a link between two monomers of TLR4. The third and fourth areas have the highest energy and are therefore the most probable. Free heme showed an affinity for Lys 435 (Zone 3) or His 458 (zone 4), which are at the interface between two monomers of TLR4 and could then stabilize TLR4 dimers. These two areas are overlapping and hence cannot co-exist. Based on the binding energies, which are very similar, it is impossible to determine which one is the most predictable interaction. Molecular dynamic analyses are needed to reveal with higher accuracy the potential binding site of heme in this part of TLR4. The fourth zone, in which heme is binding to His 458 and His 456 of the two TLR4 chains, involving the Fe^{2+} ion of heme, would be a good candidate since it has been shown that protoporphyrin IX lacking Fe^{2+} is not capable of inducing TLR4 activation (Figueiredo et al., 2007). To investigate this possibility, testing the interaction between protoporphyrin IX and monomer of TLR4 could be relevant. The first binding site

(zone 1) on the TLR4 monomer predicted a binding close to the Arg 264 of TLR4, supporting our hypothesis of a competition between binding sites of MD2 and heme, observed by SPR. Indeed, Arg 264 is an amino acid contained in the B patch of TLR4 responsible for the primary interaction between TLR4 and MD2 (Park et al., 2009). This interaction of free heme on monomer of TR4 alone could mask Arg 264 and then competes with MD2. Interestingly, Mg^{2+} was captured from crystallization medium near this LPS binding site, suggesting a potential role of divalent ion to stabilize the interaction. Thus, heme iron would not prevent, but could even drive a synergistic effect with LPS, as it has already been suggested to do on macrophages (Fernandez et al., 2010; Lin et al., 2010; Piazza et al., 2011). The last binding site (zone 2), predicts a salt bridge and involves the Fe^{2+} ion that can also stabilize the TLR4 homodimer formation. All together, these results strongly suggest that multiple heme molecules can act in concert and by-pass the TLR4:MD2:LPS complex to form and stabilize a homodimer of TLR4 without LPS. These results of no overlap between LPS and the predicted heme binding sites are in agreement with the different activation profile driven by these two TLR4 activators. Indeed, different cytokine production was observed in heme-treated macrophages compared to LPS treatment (Figueiredo et al., 2007). Nonetheless, additional experiments must be performed to fill in our understanding of this interaction. To do this, further experiments should be performed by expressing specific mutants of TLR4, selected based on our *in silico* approach, and to measure the activation of cells. Alternatively, soluble mutants of TLR4 have to be expressed and purified and their affinity for heme measured by SPR and/or by spectroscopy. Finally, a co-crystal of heme with TLR4 can provide details of the binding on the atomic level. This work is ongoing in the laboratory of Prof. Marcelo Bozza (Rio de Janeiro, Brazil) and his collaborators. We will be involved in the testing of the heme binding to the mutants of TLR4 by SPR.

The 50% remaining C3 deposition, which was not prevented by the treatment with TAK-242, raises the question about the implication of another receptor and another pathway in this process. Using a proteomic assay, we detected increased phosphorylation of intermediates of signaling pathways related to TLR4, as expected, but also related to GPCR activation. This corroborated previous studies, highlighting the potential role of heme as a ligand for a still unidentified GPCR (Graça-Souza et al., 2002; Porto et al., 2007). PT showed an inhibitory effect on complement deposition, supporting the contribution of at least one GPCR. However, the decrease of about 80% of C3 deposition on EC surface suggested that GPCR activation may function as an upstream event of TLR4. Based on this observation we

could formulate two hypotheses; 1) GPCR and TLR4 collaborate through intracellular signaling intermediates, where TLR4 would be only an enhancer and 2) GPCR and TLR4 can directly interact at the cell surface. In fact, both interactions between GPCR and TLR4 activation (through signaling pathways and physically) have been reported (Abdulkhalek et al., 2012; Cattaneo et al., 2014; Huber-Lang et al., 2014; Liu et al., 2013; Serezani et al., 2011; Wang et al., 2014). For instance, activation of TLR4 has been shown to be essential for maintaining CCR2 expression on monocytes – a GPCR receptor for the chemokine MCP-1 – thus playing a key role in the recruitment of monocytes (Liu et al., 2013). On the other hand, direct interactions between NMBR (neuromedin B receptor), a GPCR, with TLR4, TLR7 and TLR9 have been reported (Abdulkhalek and Szewczuk, 2013; Abdulkhalek et al., 2012). In this study, authors revealed in RAW-blue macrophages a new mechanism in which NMBR potentiates MMP9 to induce Neu1 activation. Activated Neu1 removes sialic acid from TLR4 and then promotes TLR dimerization to facilitate MyD88 recruitment and promote NFκB signaling pathway.

Finally, in our model, activation of complement results in a release of anaphylatoxins C3a and C5a (Frimat et al., 2013), which can in turn activate their own receptors on EC (Monsinjon et al., 2003; Morigi et al., 2011), which are GPCRs. Thereby, we can speculate that hemolysis and heme, in part via TLR4 stimulation, can induce complement activation, which, in turn, could provide C3a and C5a to trigger C3aR and/or C5aR signaling. Moreover, this is compatible with the increased expression of C3aR and C5aR observed on EC in our studies, which was prevented by PT. Interestingly, interaction between C5a/C5aR axis and TLR4/CD14 has been reported in a sepsis model (Huber-Lang et al., 2014). However, further studies must be performed in order to determine which GPCR(s) are involved and to understand how it (they) interact with TLR4. To assess this question, an experiment with double treatment with both TAK-242 and PT will be done first. Results with no additional effect would confirm the relation between TLR4 and GPCR. Physical interaction could be studied by performing co-immunoprecipitation of TLR4 and detect by mass spectrometry the associated protein. Moreover, mass spectrometry would allow determining what is (are) the GPCR bound to TLR4. Multiple attempts to do this were unsuccessful since, in all protein extraction conditions tested, I failed to detect co-precipitation of CD14 with TLR4, which served as positive control for a well-known interaction partner. Resorting to the DiscoverX system could provide a first evidence of the activation of GPCR by heme. Indeed, this technique allows screening a large number of GPCR activated by a ligand on HEK cells. For

instance, a recent study discovered the new receptors for C4a, PAR1 and PAR4, two GPCR expressed on EC (Ricklin et al., 2017).

To go further, I explored the relevance of the heme sensing by TLR4 for the complement activation *in vivo*. Using the same model of drug-induced hemolysis with PHZ, we detected decreased C3 fragment deposition in $TLR4^{-/-}$ compared to WT mice on endothelium of the liver vessels. I selected the liver as a test organ, due to the presence of many, easily distinguishable, large blood vessels. Of note, I observed the same effect in heme-treated mice. Firstly, these results demonstrate an implication of TLR4 and heme in the C3 fragments deposition on blood vessels. Secondly, I confirm that TLR4 is sensing heme *in vivo*, in agreement with the heme-injection model established in SAD mouse (Belcher et al., 2014; Vinchi et al., 2015). Furthermore, the direct effect of heme tends to indicate that neither OxyHb nor MetHb are involved in TLR4 activation, in agreement with *in vitro* datas. This, though, is in contradiction with a single study, showing that 7 mg/mL of MetHb is able to trigger TLR4 in microglia cell lines in a model of subarachnoid hemorrhage (Kwon et al., 2015). In our model, we excluded a potential role of MetHb as a ligand for TLR4, since MetHb was not able to promote complement deposition on EC up to 800 µM (data not shown), corresponding to 13 mg/mL. One explanation of the observed difference is the particular microenvironment in the subarachnoid hemorrhage model and the nature of the studied cells. In numerous other studies hemoglobin was shown to be unable to activate TLR4.

To find out what makes the EC so particular to attract complement deposits after exposure to hemolysis and heme *in vivo* or to heme *in vitro*, I studied the stress response of HUVEC to a rapid (30min-1h) exposure to heme. Apart from the triggered signaling pathways, I detected a rapid mobilization of a unique type of EC intracellular granules, the WPB. This resulted in surface expression of P-selectin and release of vWF, in agreement with previous studies in the lab (Frimat et al., 2013) and later confirmed by others (Belcher et al., 2014). Based on these results and on previous studies (del Conde et al., 2005; Morigi et al., 2011), we expected that C3 fragment deposition and P-selectin will interact with each other on the endothelium in a heme- and TLR4- dependent manner. Indeed, I detected the direct interaction by SPR and demonstrated this importance on EC surface using a blocking Ab. These results demonstrate that P-selectin (but not properdin, data not shown) serves as a platform for recruitment of C3b and $C3(H_2O)$, enhancing thus the complement activation. To further characterize this interaction and its relevance, we should perform co-localization

between C3 fragments and P-selectin in mouse blood vessels and confirm my preliminary data for co-localization on HUVEC *in vitro*. Moreover, the use of a blocking Ab against P-selectin should inhibit complement deposition without inhibiting P-selectin expression in organs.

The results presented until now have allowed us to shed light on whether and how hemolysis induces complement activation *in vitro* and *in vivo*. I also detected associations of hemolysis and complement deposits in SCD patients' kidney biopsies. So the next step was to figure out to what extend hemolysis and complement deposits are pathogenic in our models. To address this question, I first focused our attention on the kidney, since it was the organ where I found the strongest heme-dependent C3 deposits. In collaboration with Prof. Pascal Houillier and Dr. Marie-Lucile Figueres from our research center (Paris, INSERM UMRS1138) a nephrologist-master student in the lab, Anne Grunenwald, evaluated the alterations of the renal function in PHZ and heme-injected mice, compared to PBS-treated controls, using metabolic cages. Despite the reduced fitness of the animals due to the anemia and reduced food and water intake, the renal function remained normal. These results showed that mouse kidneys are resistant to major functional alterations under hemolytic conditions and that more sensitive markers have to be tested in order to evaluate kidney injury. Therefore, I set up a low density array assay for relatively high throughout assessment of gene expression in mouse tissues. It revealed an increase of mRNA related to acute stressed response, tubular injury, and vascular and tissue inflammation during hemolysis. Surprisingly, this renal inflammation was not reproduced by heme and counteracted by Hx, while this natural heme scavenger inhibited complement deposits. This observation tends to demonstrate that renal inflammation is not dependent on heme and complement. Similar analyses are ongoing for the $TLR4^{-/-}$ mice. If indeed renal injury and inflammation triggered by hemolysis are heme-independent, the levels of genes expression should be increased in the $TLR4^{-/-}$ mice injected with PHZ to the same levels as in the WT. These observations correlate with a recent study showing, in a model of prolonged storage RBC administration in mice, that only Hp but not Hx, prevented increase of mRNA stressed markers (Graw et al., 2016). However, this is in contradiction with an earlier study, showing that $Hx^{-/-}$ mice develop severe renal damage after PHZ-mediated acute hemolysis, highlighting the role of Hx as a guardian of kidney function against the products of intravascular hemolysis (Tolosano et al., 1999). A definitive answer about the role of complement in the induction of kidney damage will be an experiment where

the gene expression will be measured in $C3^{-/-}$ mice which were injected with heme or PHZ. These experiments are in progress.

Complement deposits in kidneys are considered a pathogenic factor, so it was surprising to find no consequence. One explanation could be related to the fact that in our model, we did not detect any C5b-9 deposition within kidneys of PHZ-treated mice. To explain this, I suppose that hemolysis is not powerful enough to promote a complete activation of the complement system. C3 deposits and C3a, in such an acute model, may not be powerful enough to induce disease manifestations. Moreover, evaluation of endogenous Hx in mice clearly shows a residual presence of Hx 6 hours post hemolysis. This would argue that heme effects are still controlled, limiting its pro-inflammatory activity, and thus limiting C3 deposition on cell membranes. Cumulating with the recent discoveries about the need to have a high density of C3 deposition on surface to switch from the C3 to the C5 convertase (Berends et al., 2015; Jore et al., 2016), this could explain why we did not detect C5b-9 deposition. Furthermore, this would also explain why I did not detect severe renal damages, since C5b-9 deposition has been shown to promote EC injury and further kidney injury such as in aHUS (Tedesco et al., 1997; Zuber et al., 2012). Therefore, acute hemolysis should be combined with a second defect, (mutations in Hb gene as in SCD, or even complement mutations as in aHUS) the combination of which would be deleterious and would probably induce breakdown of kidney function. To assess this question, we should first evaluate complement deposition, in particular C5b-9, within kidneys of $Hx^{-/-}$ compared to WT under PHZ treatment. Secondly, if C5b-9 deposition is observed, evaluation of renal damages of $Hx^{-/-}$ mice treated with PHZ, with or without administration of complement inhibitor would determine the role of complement system in the alteration of the kidney function.

During the characterization of the PHZ- and heme-injected mouse models, I did detect signs of acute pancreatitis, namely presence of pancreatic α-amylase as revealed by mass spectrometry of urine performed by Prof. Regis Daniel (University of Evry val d'Essonne), our western blot and plasmatic α-amylase measurement. This result corroborates previous studies, showing that acute hemolysis can drive a transient acute pancreatitis (Druml et al., 1991; Saruç et al., 2003, 2007). Moreover, in 2007, among RBC degradation products, authors demonstrated the role of free heme in this process (Saruç et al., 2007). Despite these interesting results, the difficulty to work with pancreatic tissue in mice precluded further studies of this phenomenon.

The last organ that I analyzed was the heart. Our results revealed heme-dependent C3 deposition also in hearts. I have not evaluated the genes expression in this organ yet in any of our models. Nevertheless, it will be interesting to complete the study based on the flowing arguments from the literature: In SCD, cardiac complications represent a major concern because of its high association to morbidity and mortality (Gladwin and Sachdev, 2012). Although described in rare cases, concomitant iron deposition to cardiac dysfunction has been documented (Darbari et al., 2006). In line with this study, both heme and PHZ treatment in $Hx^{-/-}$ mice were shown to affect cardiac function, which was prevented by Hx (Ingoglia et al., 2017). Actually, systolic dysfunction was attributed to heme-driven ROS in cardiomyocytes, impairing Ca^{2+} homeostasis. Cumulated to my result, this heme-derived effect allows to hypothesize that complement could play a role in this model. Moreover, recent studies pointed towards a critical impact of complement activation on cardiac dysfunction during sepsis (Fattahi et al., 2017; Kalbitz et al., 2016), thus supporting the idea that complement activation could participate to cardiac dysfunction. Actually, in aHUS, extra-renal manifestations (neurological and cardiac) occurs in about 20% of cases, and they also depend on C5b-9 formation (Hofer et al., 2014). Indeed, as depicted for kidney injury, long-term treatment with eculizumab has been shown to improve cardiopathy and neurological complications observed in patients suffering from aHUS (Emirova et al., 2016; Loirat et al., 2016; Pape et al., 2015; Vilalta et al., 2012). This evidence highlights the need to investigate to what extend complement deposits found within the heart under hemolytic conditions, are pathologically relevant and if relevant, potentially try to explore anti-complement therapeutic strategy. Furthermore, we should measure mRNA expression levels of stress markers in order to determine whether heart injury is associated with complement activation.

Taken together, these analyses of the organ injury in the PHZ model revealed multivisceral damage, but free heme release was only clearly related to this in the spleen. During intravascular hemolysis, and in our PHZ model, RBC rupture results in release of MVs and cell-free Hb. These might be the key drivers of organ injury. I am testing the contribution of Hb using a direct Hb injection as well as administration of Hb scavenger Hp before the PHZ treatment. These experiments are in progress. The role of microvesicles can be tested by injection of *in vitro* generated RBC MVs into the mice. Further studies are needed to determine whether and to what extend complement activation induced by hemolysis contributes to the tissue injury.

Because renal damage (as well as injury to other organs) are multifactorial, hemolysis often acts as a co-factor and aggravates damage induced by a primary hit/pathology. This additional 'hit' could be an underlining genetic abnormality, such as Hb mutation in SCD or a mutation in a complement gene as in aHUS. This primary factor can contribute to the initial complement activation – i.e. the presence of MVs in SCD or the overactive AP C3 convertase and deficient regulation in aHUS. Hemolysis and heme will thus serve as amplifiers of the complement activation, engaging a vicious circle of complement overactivation and EC and other tissue damage, overwhelming the resistance mechanisms and tolerable cell stress. This hypothesis is in agreement with our observation that despite the very strong hemolysis in the PHZ model, we did not detect C5b-9 deposits in the kidney. In contrast, in both human and mice with SCD, C5b-9 was deposited in the kidney. I suggest that in acute settings complement needs to proceed until the terminal pathway in order to contribute to tissue injury. *In vivo*, even under hemolysis, the regulatory mechanisms, at least in mice, are strong enough and are able to prevent it. In order to induce a disease phenotype, the C3 deposits have to be chronic, such as in the case of $FH^{-/-}$ mice, where young mice are disease-free and C3G-like pathology appear over time (Pickering et al., 2002).

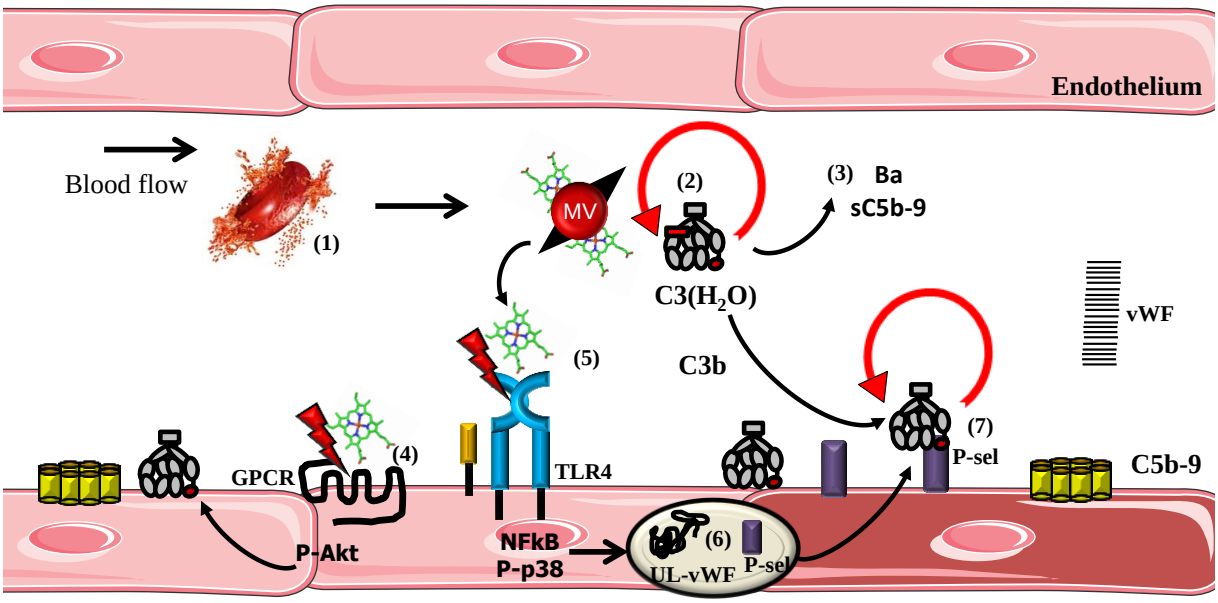


Figure 30: Hemolysis drives complement activation in bloodstream. Upon RBC lysis (1), MVs expressing PS and carrying heme promote complement activation in serum (2) that leads to the release of Ba and sC5b-9 activation fragments (3). Heme induces both G Protein-Coupled Receptor (GPCR) (4) and Toll-like Receptor 4 (TLR4) (5) activation on EC membrane. TLR4 activation promotes mobilization of Weibel Palade bodies (WPB) (6), which rapidly releases von Willebrand Factor (vWF) and provokes P-selectin (P-sel) expression. Thereafter, C3b binds to P-sel and promotes local complement activation until formation of C5b-9 on cell surface (7).

During these three years, I aimed to investigate the relationship between hemolysis and complement activation. I first demonstrated that hemolysis drives complement activation in a heme-dependent manner. I described that erythrocytes MV from SCD patients carrying heme play a key role in complement activation in serum and on EC surface, thus linking hemolysis and complement system in SCD. On the cell membrane, I revealed that TLR4 activation by heme is a crucial event for sensitizing the endothelium to complement attack. Indeed, I brought evidence that TLR4 activation by heme induces p38 and NFkB signaling pathways and leads to WPB mobilization. Further, degranulation of these EC-specific granules promotes release of vWF and rapid expression of P-selectin on EC membrane, which serves as an anchoring platform for complement deposition. Interestingly, I depicted an organ-specific profile of complement activation under hemolytic conditions, as well as a particular mRNA signature. Hence, I found a particular tropism for kidneys of heme-mediated complement activation. According to all this work, I highlighted the potential benefit of Hx and/or TAK-242 administration for controlling complement deposition in hemolytic diseases related to kidney disorders.

PERSPECTIVES

The results obtained during these three years brought some answers, but also raised novel questions. Since my results indicated that hemolysis is not powerful enough by itself to induce terminal complement pathway activation within the kidney, I suggested that an additional genetic abnormality would be necessary to proceed to C5b-9 formation, which will contribute to organ disorder. To test this hypothesis, I will further investigate the implication of hemolysis in a mouse model, prone to TMA lesions, recapitulating the signs of human aHUS. To this end, I will use mice lacking expression of FH in liver (Vernon et al., 2015). Indeed, those mice present a decreased plasma FH concentration with about 80%, compared to controls. They are healthy at resting conditions, but bypass complement regulation upon triggering renal injury with nephrotoxic serum, leading to a complement-mediated TMA phenotype. Thus, these mice represent a suitable model for studying the impact of hemolysis on the background of an imbalanced complement system. The Cfh(loxP/loxP) mice were kindly provided by Prof. Matthew Pickering (Imperial College London). I have already crossed them with Alb-Cre mice to generate different study groups: $Cre^{+/+}Lox^{-/-}$ (susceptible to TMA) and $Cre^{+/+}Lox^{+/+}$ as well as $Cre^{-/-}Lox^{+/+}$ (controls). Two experimental settings will be tested. In the first, heme will be injected or hemolysis will be triggered by PHZ (Figure 31). In the second, nephrotoxic nephritis will be triggered as described by Vernon et al (Vernon et al., 2015). Renal injury will be followed by hematuria, serum urea, hematocrit and glomerular thrombosis. Moreover evaluation of the presence of proteins, including NGAL, in mouse urine will be tested by WB, and mRNA expression of the previously selected markers will be measured within kidneys. These will include NGAL and KIM-1 for tubular injury; also P-selectin, ICAM, VCAM, E-selectin as EC stressed markers. These markers will be correlated with complement deposits (C3 and C5b-9) within kidneys. If I succeed to provoke severe renal injury, this result will provide a rationale for considering hemolysis as a second trigger of the disease, as our team hypothesized previously (Frimat et al., 2013; Roumenina et al., 2016a, 2016b). To go further, I will test the efficacy of both Hx and TAK-242 as therapeutic strategies, with respect to the renal function. This will allow to establish whether preventing free heme-mediating complement and TLR4 activation is a relevant alternative strategy to control organ injury. Finally, it would bring a critical rationale for assessment of complement-mediated organ injury in diseases associated with hemolytic events, such as SCD, malaria, rhabdomyolysis, etc.

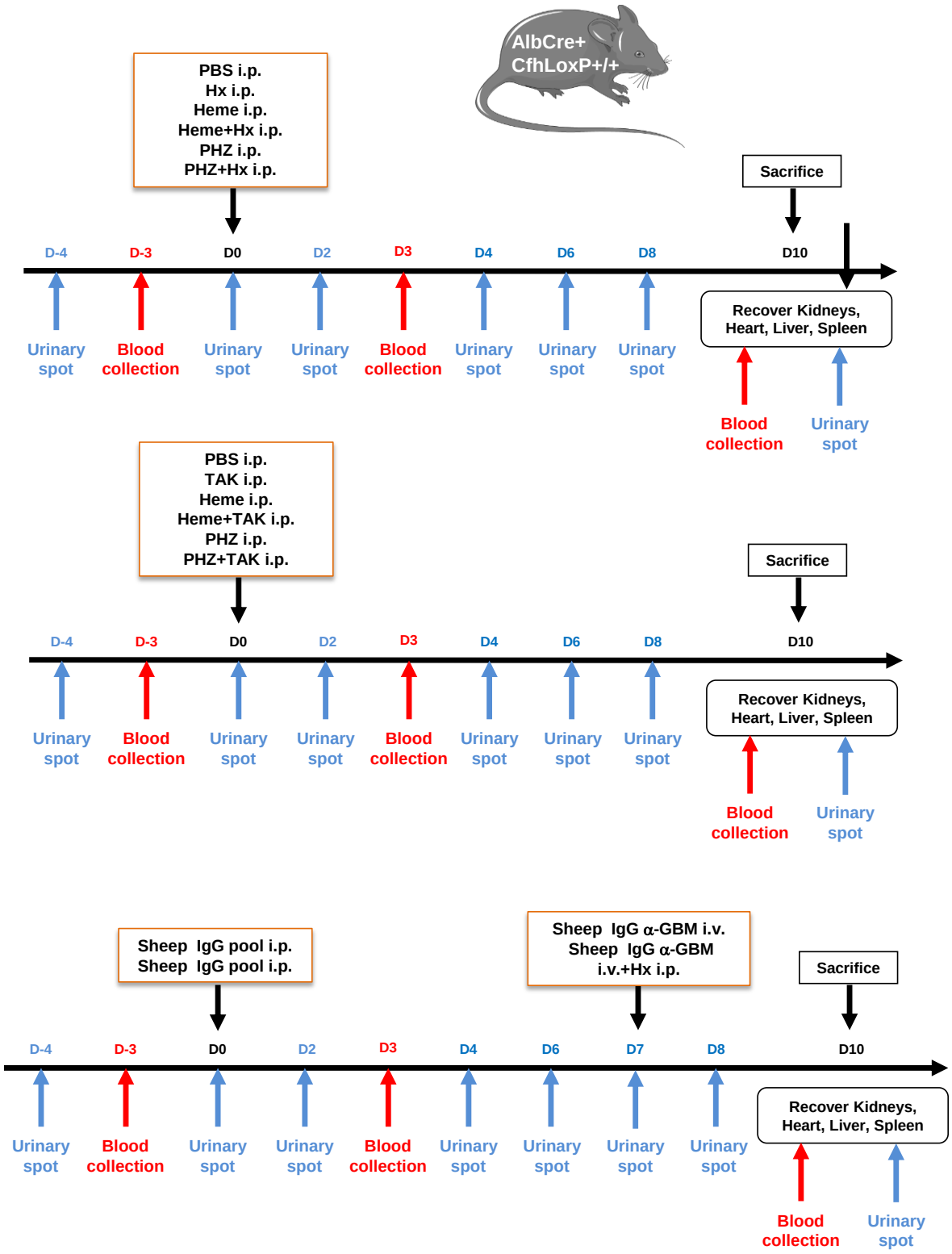


Figure 31: Experiment setting to be performed on Cfh(loxP/loxP) mice

In terms of therapeutic strategy to prevent organ injury, the Hb scavenger, Hp, seems to be a more efficient therapeutic solution, as suggested by a recent study explaining a MetHb-dependent stress gene expression (Graw et al., 2016). Indeed, by acting at the OxyHb/MetHb level, administration of Hp is expected to prevent both acute stress protein expression as well as heme-dependent effects, such as P-selectin expression and complement deposition. Therefore, Hp will be tested in all experimental settings and its effect will be compared to the one that we have described for Hx. CSL Behring (Bern, Switzerland) has provided us with plasma-derived Hx and Hp, as well as research funding to carry out these experiments.

I can only speculate about the potential use of Hx, Hp or TAK-242 as therapeutic options for hemolytic diseases, where complement is involved. The use of TAK-242 has already proven its efficacy by counteracting stasis, occurring in SCD mice treated with heme, thus revealing the crucial impact of TLR4 in VOC process (Belcher et al., 2014). More recently, using the mouse model, authors showed the same capacity of anti-complement treatment to improve stasis (Schaid et al., 2016). Here, these datas showed the implication of TLR4 on complement activation occurring on EC in response to hemolysis. Altogether, these results tend to demonstrate a close link between TLR4 and complement activation under hemolytic conditions. Thus, efficacy of TAK-242 would be mediated by the decrease of P-selectin-mediated leucocyte recruitment as well as a decrease of complement activation on EC, reducing endothelium injury. To go further, efficacy of TAK-242 should be scrutinized in a mouse model of aHUS regarding effects on kidney damage, where complement deposits on EC drive renal injury. However, such a strategy would only block complement deposits from occurring on EC, whereas local hemolysis could still maintain complement activation in serum and mediate pro-inflammatory processes on EC via C3a/C3aR axis (Morigi et al., 2011) (Figure 32).

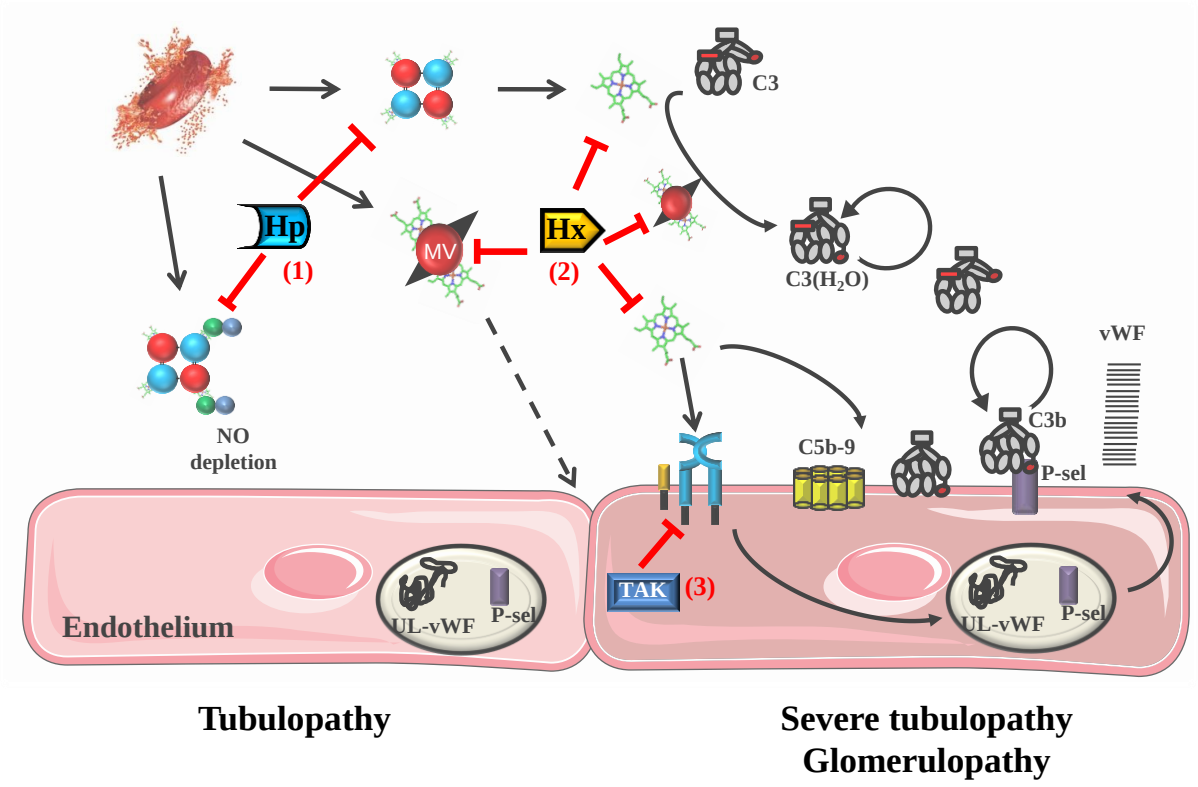


Figure 32: Schematic representation of the beneficial effect provide by Hp, Hx and TAK-242. (1) Hp can prevent the first step of hemolysis by scavenging Hb. (2) Hx prevents at a second step, by scavenging free heme and MVs carrying heme, thus preventing complement activation in fluid phase and on EC. (3) TAK-242 inhibits TLR4/P-selectin axis activation by heme, thus preventing complement deposition dependent on P-selectin.

Nowadays, if hydroxyurea treatment limits painful crises during VOC (Alvarez et al., 2012), the mechanism leading to AKI remains unclear. However, multiple studies agree on correlation between acute drop in hemoglobin and kidney injury in SCD (Baddam et al., 2017; Nath et al., 2000b, 2013). Basically, I determined that complement activation occurs on endothelium during hemolysis and I found also a potential association between C5b-9 and nephropathy in SCD. Based on previous observations on aHUS patients describing that C5b-9 is responsible for renal disorders (Zuber et al., 2012), we can anticipate a contribution of complement system in the nephropathogenesis process in SCD. Thus, resorting to new therapeutic strategies against complement system would provide a beneficial effect to those patients. For instance, anti-C5 strategy and TT30 would be of interest, since studies provide evidence about their beneficial effect on complement activation, organ injury and complement-mediated hemolysis in aHUS, PNH and SAD mouse models (Cofiell et al., 2015; Emirova et al., 2016; Gavriilaki et al., 2017; Hillmen et al., 2013; Pape et al., 2015; Risitano

et al., 2012; Schaid et al., 2016; Smith-Jackson et al., 2017; Vilalta et al., 2012). However, such a strategy would potentially lead to higher sensitivity to infections (McNamara et al., 2017). Since I succeeded in preventing complement activation using Hx in our drug-induced hemolysis model, it might be of interest to investigate the potential of Hx, combined or not combined with an anti-complement strategy, in a mouse model of a complement-related disease such as aHUS. Results would bring evidences of the crucial impact of hemolysis on this disease and would claim the use of Hx as a blocker of complement activation in hemolytic disease. Further, regarding our results and recent study asserting the beneficial impact of anti-C5 strategy in SAD mouse model on stasis (Schaid et al., 2016), testing Hx and/or anti-C5 treatments in SAD mouse model would highlight the involvement of complement activation on nephropathogenesis in SCD.

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REVIEW
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Complement system part I – molecular mechanisms of activation and regulation

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Complement is a complex innate immune surveillance system, playing a key role in defense against pathogens and in host homeostasis. The complement system is initiated by conformational changes in recognition molecular complexes upon sensing danger signals. The subsequent cascade of enzymatic reactions is tightly regulated to assure that complement is activated only at specific locations requiring defense against pathogens, thus avoiding host tissue damage. Here, we discuss the recent advances describing the molecular and structural basis of activation and regulation of the complement pathways and their implication on physiology and pathology. This article will review the mechanisms of activation of alternative, classical, and lectin pathways, the formation of C3 and C5 convertases, the action of anaphylatoxins, and the membrane-attack-complex. We will also discuss the importance of structure–function relationships using the example of atypical hemolytic uremic syndrome. Lastly, we will discuss the development and benefits of therapies using complement inhibitors.

Keywords: complement system proteins, complement regulatory proteins, structure–function relationships, anaphylatoxins, membrane-attack-complex, classical complement pathway, alternative complement pathway, endothelial cells

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Introduction

Complement is a central part of the innate immunity that serves as a first line of defense against foreign and altered host cells (1). The complement system is composed of plasma proteins produced mainly by the liver or membrane proteins expressed on cell surface. Complement operates in plasma, in tissues, or within cells (2). Complement proteins collaborate as a cascade to opsonize pathogens and induce a series of inflammatory responses helping immune cells to fight infection and maintain homeostasis. The complement system can be initiated depending on the context by three distinct pathways – classical (CP), lectin (LP), and alternative (AP), each leading to a common terminal pathway. In a healthy individual, the AP is permanently active at low levels to survey for presence of pathogens (**Figure 1A**). Healthy host cells are protected against complement attack and are resistant to persistent low-grade activation. The three pathways are activated on the surface of apoptotic cells, which are constantly generated within the body during normal cellular homeostasis

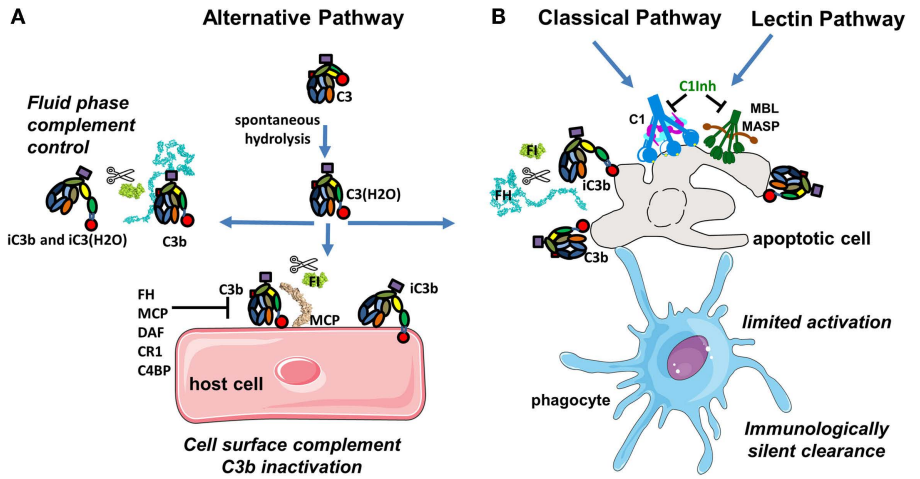

FIGURE 1 | Complement activation in physiological conditions. (A) The alternative pathway is permanently active due to spontaneous transformation of bio-inactive molecule C3 to bioactive C3(H_2O). This allows generation of C3b, which is rapidly inactivated by FH and FI in fluid phase or is covalently bound to the surface and then inactivated on host cells. **(B)** Classical and lectin pathway recognition molecules bind to apoptotic cells and together with C3b from the alternative pathway induce a low level of complement activation. Apoptotic cells are not lysed, but rapidly cleared by phagocytes in an immunologically silent manner. Host cells and plasma contain multiple regulatory proteins to assure that complement activation will be limited in physiological conditions.

(**Figure 1B**). This complement activation is tightly regulated to eliminate dying cells without further activation of other innate or adaptive immune components. Complement is only fully activated in cases of pathogen infection. During an infection, complement leads to inflammation, opsonization, phagocytosis, and destruction of the pathogen and ultimately results in activation of the adaptive immune response (**Figure 2**). Both inefficient and over stimulation of complement can be detrimental for the host and are associated with increased susceptibility to infections or non-infectious diseases, including autoimmunity, chronic inflammation, thrombotic microangiopathy, graft rejection, and cancer.

In this review, we discuss recent advances in the molecular and structural basis of activation and regulation of the complement pathways followed by the discussion of one complement-mediated disease – atypical hemolytic uremic syndrome (aHUS) to illustrate how the knowledge of the structure–function relationships between complement proteins helps to understand aHUS physiopathology and aid in the development of targeted therapy. In the second part of this review, published in the same issue of Frontiers in Immunology, we provide a detailed review of the literature related to the role of the complement system in immunity (3).

Complement Activation During Normal Homeostasis and Pathogen Infection

The central component of the complement system is C3. The activation of each of the three pathways (CP, LP, and AP) results in cleavage of inactive C3 protein into the functional fragments C3a and C3b. C3a is an inflammation mediator and C3b is an opsonin, which can bind covalently and tag any surface in the immediate proximity to the site of its generation.

Complement Tick-Over in the Alternative Pathway

In the plasma, during normal physiological conditions, the dominant active complement pathway is the AP (**Figure 1A**). The AP monitors for pathogen invasion by maintaining a low level of constitutive activation by a process known as tick-over (4). Tick-over is the spontaneous hydrolysis of a labile thioester bond, which converts C3 to a bioactive form C3(H_2O) in the fluid phase (5). The rate of hydrolysis of C3 to C3(H_2O) can be accelerated by interactions between C3 and a number of biological and artificial interfaces, including gas bubbles, biomaterial surfaces, and lipid surfaces and complexes (6). Upon hydrolysis, the thioester domain (TED) of C3 undergoes a dramatic structural change that exposes a binding site for another member of the AP called Factor B (FB). The C3(H_2O)-bound FB is then cleaved by a serine protease (SP) Factor D (FD) allowing formation of a fluid phase C3 convertase complex C3(H_2O)Bb. C3(H_2O)Bb is able to interact and cleave native C3 molecules to C3a and C3b (5, 7–11). During normal physiological conditions, this C3 convertase constantly generates small amounts of C3b, which is able to bind covalently via its TED domain to any adjacent surface containing hydroxyl groups. Nevertheless, not all hydroxyl groups attract equally C3b (12). The -OH in the 6th position appears to be more reactive than the average -OH group in sugars. Therefore, the particular sugars composition of the pathogen surface will determine the efficacy of complement activation. C3b will bind covalently to a surface that is located within about 60 nm from the convertase, due to the fact that the half-life of the thioester in C3b is ≈60 μs with a poor attachment efficiency of 10% (13). On host cells, bound C3b molecules are rapidly inactivated by an army of membrane-expressed or fluid phase-recruited complement regulators (described in detail below). A tick-over

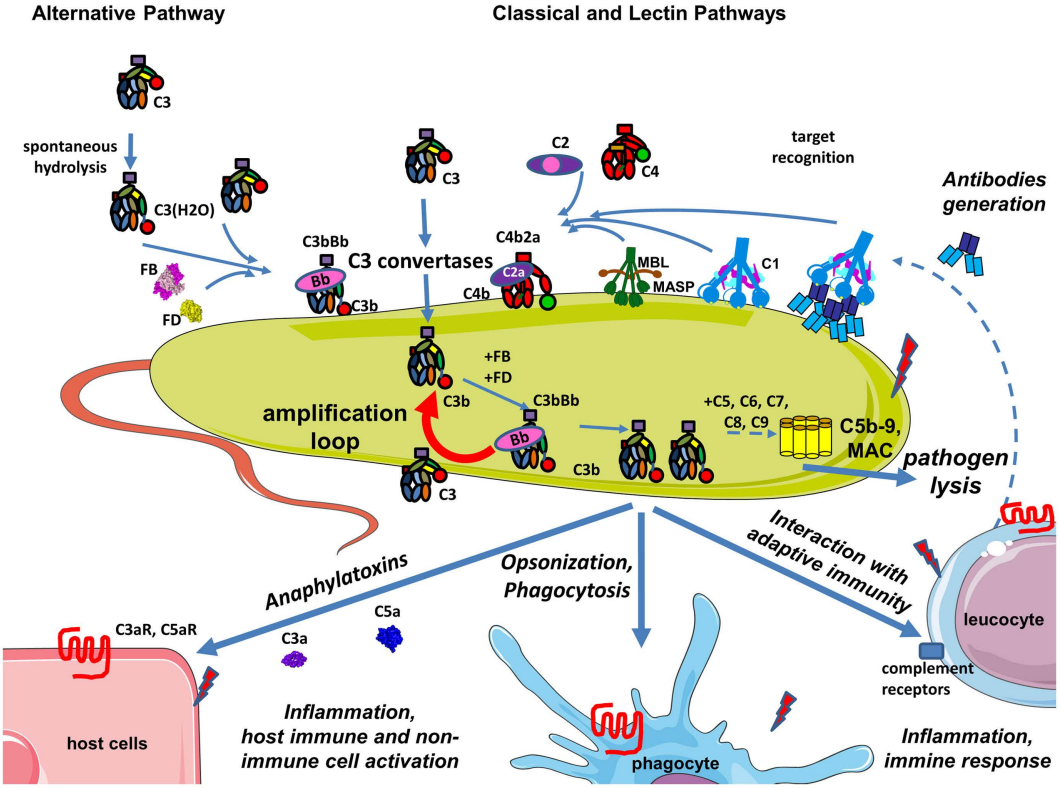

FIGURE 2 | **Complement during infection with a pathogen**. The permanent activity of the alternative pathway allows it to immediately identify pathogens that are not specifically protected against complement. Danger-associated molecular patterns on its surface of pathogens are recognized by C1q, MBL, and ficolins allowing classical and lectin pathway activation, C3 convertase, C4b2a generation, and C3 cleavage. Opsonization due to covalent binding of C3b to the target is accelerated by the amplification loop of the complement pathways. The effector functions resulting from this complement activation are: pathogen lysis by C5b-9 membrane attack complex, opsonization and phagocytosis of the pathogen, activation of host immune and non-immune cells by complement anaphylatoxins, inflammation, stimulation of an adaptive immune response, and antibody generation. Secreted antibodies will bind to the pathogen and create immune complexes that will be recognized by C1q and will activate the classical pathway. Altogether these mechanisms contribute to pathogen elimination.

mechanism for spontaneous activation of the CP has also been suggested, but the molecular interactions of this process are not well understood (14).

Clearance of Apoptotic Cells

Apoptosis, programed cellular death, is a process of normal cellular homeostasis and in healthy individuals everyday billions of cells die by this mechanism. Complement activation occurs on apoptotic cells with low levels of C3b deposition to facilitate their elimination without releasing danger signals, which could lead to further immune responses (15, 16) (**Figure 1B**). This complement activation occurs by membrane alterations and by decreased expression of complement regulators on the membrane of apoptotic compared to resting cells. The silent clearance of the apoptotic cells is assured by the binding of the initiators of the CP (C1q) and LP [Mannose-Binding Lectin (MBL) and ficolins]. These initiator proteins interact with receptors on phagocytic cells (immature dendritic cells or macrophages), which elicit immune tolerance and prevent immune responses toward self-antigens (17–20).

Pathogen Elimination

On pathogens that lack specific regulators of complement, C3b interacts with FB and FD to form a surface-bound C3 convertase as part of the AP, which cleaves C3 into C3a and C3b. Maximum complement activation is achieved during pathogen recognition leading to a pro-inflammatory milieu, contributing to generation of an adaptive immune response and rapid elimination of the pathogen (**Figure 2**). Complement-derived anaphylatoxins have potent inflammatory mechanisms including recruitment of phagocytes to the site of infection and activation of leukocytes, endothelial cells, or platelets. Upon activation, terminal complement components also have direct lytic capacity to kill pathogens.

The CP and LP have a critical role in pathogen recognition and initiation of the complement cascade. However, the AP assures more than 80% of the terminal complement activity during pathogen recognition (21). Additional AP C3 convertases are formed on the C3b molecules generated either by CP activation or the AP C3 convertases. This chain reaction amplifies opsonization of the target and increases generation of anaphylatoxins. This

amplification loop augments the effect of all pathways and is the heart of the complement cascade (22).

Structural Basis of Complement Activation and Regulation

Target Recognition and Initiation of Complement Pathways

The CP and LP have clearly identified recognition molecules, C1q, MBL, and ficolins, which trigger each pathway only when and where it is necessary. The recognition event induces a structural change in the recognition molecule, which in turn induces the activation of enzymes able to cleave the subsequent molecules in the cascade and generate the central enzymatic complexes of complement, CP and AP C3 convertases. The AP lacks a traditional target recognition molecule as an initiator. However, several molecules, such as properdin and P-selectin, can recruit C3(H_2O) and C3b to the cell surface and serve as local initiators of the AP.

Recognition Molecules of the Classical and Lectin Complement Pathways

Complement pathway and LP are triggered after interaction of a pattern-recognition molecule with the target structure. The recognition molecule of the CP, C1q, has an extra-hepatic origin and is produced mainly by immature dendritic cells, monocytes, and macrophages (23). It has a complex, described as a "bouquet of flowers" topology (**Figure 3A**), composed of 18 chains of three types (A, B, and C), forming six globular target recognition domains (gC1q) attached to a collagen-like region (CLR). Each gC1q domain carries a Ca^{2+} ion, which maintains domain stability and regulates the electrostatic field (24). C1q recognizes mostly charged patterns and can bind to more than 100 different target molecules, including IgG and IgM containing immune complexes and surface-bound pentraxins [C-reactive protein (CRP), pentraxin 3 (PTX-3)] (25). Therefore, CP can be activated in either an immune complex-dependent and -independent manner. Mapping of the target molecule-binding sites on gC1q by a site-directed mutagenesis, revealed that charged residues on the apex of the gC1q heterotrimer (with participation of all three chains), as well as, the side of the B-chain are crucial for binding to IgG, IgM, CRP, and PTX-3. These binding sites are not identical, but partially overlapping (24, 26–29). C1q recognizes pathogen-associated molecular patterns including lipopolysaccharide (LPS) (30) and bacterial porins (31). The gC1q domain also recognizes molecules, exposed on the surface of dying cells (32, 33), including phosphatidylserine (34, 35), double stranded DNA (36, 37), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (38), annexins A2 and A5 (39), and calreticulin (35, 40–42). The most well-characterized target recognition molecule of the LP is the MBL, which recognizes carbohydrates (43). MBL has a similar overall structure to C1q, but exists in multiple oligomeric forms (trimers, tetramers, and higher ordered oligomers) (**Figure 3B**). C1q and MBL associate in a Ca-dependent manner with SP complexes, consisting of C1r and C1s for the CP (44, 45) and MBL-associated serine proteases (MASP) for the LP (46, 47). In absence of Ca^{2+} ions (such as in plasma samples collected in EDTA), C1q and MBL cannot interact with $C1r_2C1s_2$ and MASPs, respectively and CP and LP activation is prevented. In the presence of Ca^{2+} ions, after activation, SPs cleave subsequent complement components C4 and C2. The resulting complex C4b2a is the C3 convertase for CP and LP. This C3 convertase has enzymatic activity and is able to cleave the central complement component C3 to bioactive fragments C3a and C3b.

Mechanism of Activation of the Classical Pathway

Recent studies have shed light on the molecular mechanisms of activation of CP and LP. It has long been established that C1q requires one surface-bound IgM or several IgG molecules in close proximity in order to interact with several of its globular domains and to activate complement. However, the molecular mechanisms and the C1-antibody stoichiometry required for optimal activation remain poorly understood (48). IgM is a planar polymeric molecule (pentamer or hexamer), in which C1q-binding sites are hidden. A conformational change occurs upon binding to an antigen (staple conformation), leading to exposure of C1q-binding sites. Contrary to IgM, IgG is a monomer and despite the presence of the C1q-binding sites, only very low affinity binding can be achieved. The epitope distribution of the antigen and the density of the IgG binding determine the level of complement activation, however the molecular mechanisms were unknown until recently. Diebolder and colleagues demonstrated that specific non-covalent interactions between Fc fragments of IgG and formation of ordered antibody hexamers on the antigen surface are needed for efficient C1q-binding (**Figure 3A**) (49). Their proposed model could explain the strong antigen and epitope dependency of complement activation. Efficient C1q-binding could only occur upon formation of a platform of IgG Fc fragments with a steric compatibility for gC1q domains. Clustering of IgG molecules on the antigen surface could be affected by antigen size, density, and fluidity (50, 51) such that smaller antigen-antibody complexes will allow only moderate complement activation. In addition, binding stoichiometry is further complicated by antibodies fluidity on surfaces of regularly spaced epitopes. It has been demonstrated that IgG exhibit "bipedal" stochastic walking forming transient clusters that might serve as docking sites for the C1q-binding and complement activation (52).

Once C1q binds to its target surface, a conformational change is required to transmit the signal from the gC1q domain via the CLR to induce auto-activation of C1r (**Figure 4A**) (53). Molecular modeling, mutagenesis, and disease-associated mutation analysis revealed the structure of the $C1r_2C1s_2$ binding site in the cone of collagenous arms of C1q (44, 54, 55). The $C1r_2C1s_2$ proenzyme tetramer within the C1 complex in a resting state adopts an eight-shaped form (**Figure 4B**). Upon activation, conformational changes in the tetramer allow transition to an S-shaped active form, passing through a transition state. This conformational change allows auto-activation of the C1r SP domain. Subsequently, activated C1r will cleave and activate C1s. The driving force for this auto-activation of C1r is an increase of the angle between the collagen stalks of C1q (48). However, the mechanism for this structural change is poorly understood. Mutagenesis experiments have revealed that residues on the apex and the lateral surface of the B-chain of gC1q are important for IgG, IgM, CRP, or PTX-3

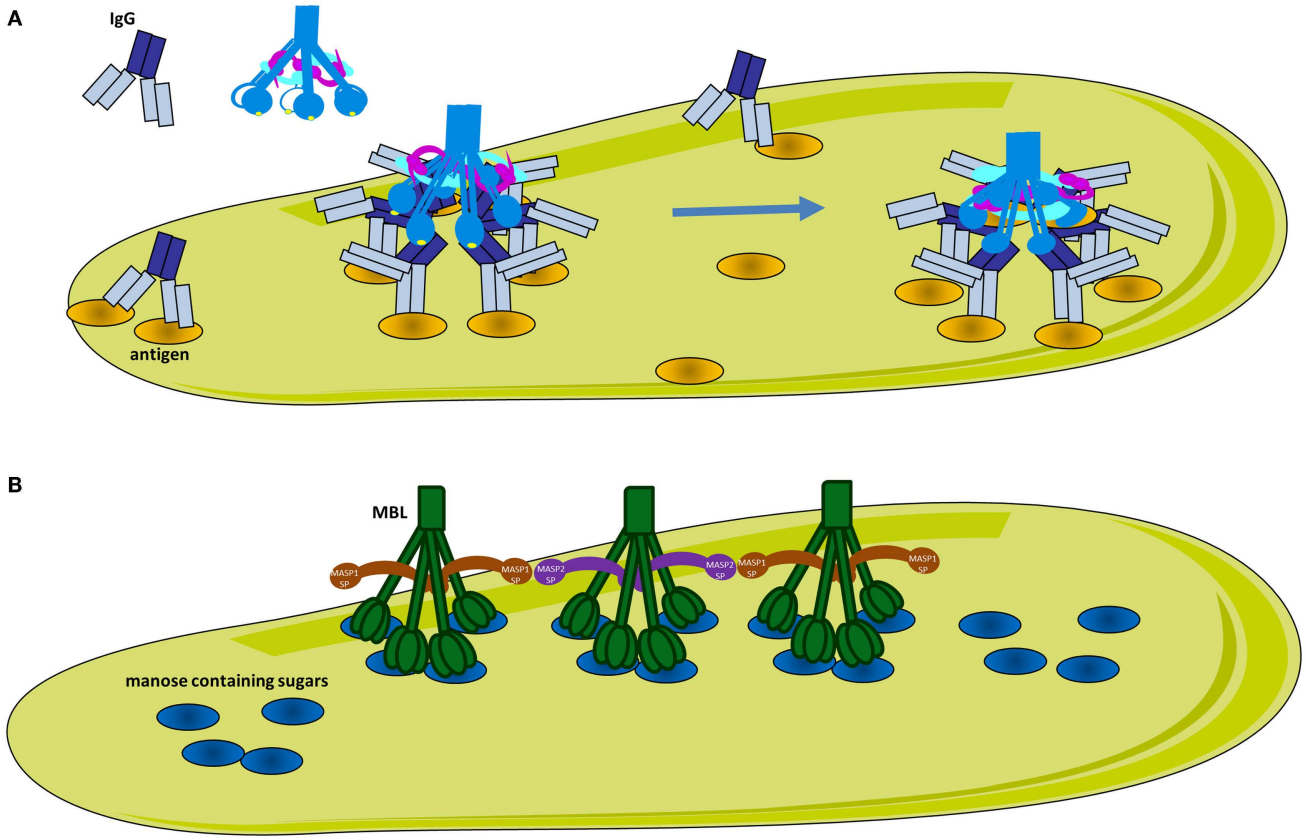

FIGURE 3 | Classical and lectin pathway activation. **(A)** Activation of the classical pathway. Multiple adjacent IgG molecules are needed to bind C1q. IgG interacts with its target antigen forming specific circular structures. A single FAB binds to the antigen, while the other does not. The movement of the Fc domain exposes the C1q-binding sites allowing complementarity with the six globular domains of C1q (gC1q). The number of engaged IgG molecules will determine the compatibility of the immune complex with C1q and hence the strength of classical pathway activation. C1q circulates in plasma-associated with the serine proteases C1r and C1s, forming inactive C1 complex. After binding, the target C1q undergoes a conformational change to increase the angle between its collagenous stalks (CLR). This conformational change activates C1r, which in turn activates C1s. **(B)** Activation of the lectin pathway. MBL recognizes mannose containing sugars on pathogens. MBL circulates associated with serine proteases MASP-1 or MASP-2. Upon target binding, juxtaposition of MASP-2 and MASP-1 containing MBL complexes is required for MASP-1 to activate MASP-2.

interactions (27–29). Taking into account the surface morphology of gC1q and its targets, it is difficult to contemplate how these residues can engage simultaneously in binding. One possibility is that these residues form binding sites, which make contact with the target subsequently and not simultaneously (**Figure 4A**). These data, combined with the importance of the Ca^{2+} ions for the electrostatic field of C1q (24), and the induced conformational change leading to an increased angle between the collagenous stalks by gC1q and target interaction (48, 56–60) has led to the proposal of an electrostatic model for the activation of the C1 complex (**Figure 4A**). This model suggests that the increase in the angle between the collagen stalks occurs because of a rotation of the gC1q domains, driven by electrostatic interactions between gC1q and the target molecule (IgG, IgM, or CRP) (24). Interactions between the negatively charged binding sites on the target may cause a removal of the Ca^{2+} ion from gC1q. Loss of Ca^{2+} changes dramatically the size and orientation of the electric moment vector of gC1q. This electrostatic change can induce the rotation of gC1q allowing it to engage the lateral surface of the B-chain. This rotation may provide the mechanical stress necessary for the transition of the $C1r_2C1s_2$ complex from closed, inactive eight-shaped conformation to an active, S-shaped conformation, allowing C1r auto-stimulation and further C1s activation by C1r. In this active form allows the tetramer to unfold and to extend its C1s ends outside the C1q cone for interaction with C4 and C2. Cleave of C4 and C2 by C1s allows formation of the CP C3 convertase in the immediate proximity to the C1 complex-binding site (44, 45, 53, 61). It is a matter of debate whether the C1s catalytic domain faces the exterior or the interior of the C1q cone. If the recognition and cleavage of C4 occurs inside the cage-like structure of the cone of C1 (62), this may increase the efficacy of the covalent binding of the bioactive cleavage product C4b to the surface. However, it is still unclear what would be the driving force to assure the entrance of both C4 and C2, as well as the substrate molecules of C3, in this confined space. These data imply that one C1 complex will have limited efficacy, generating only one or two C3 convertases as a result of steric hindrance. It is too few compared to the experimental evidence that one activated C1 complex generates about 35 C4 molecules during its lifespan (63).

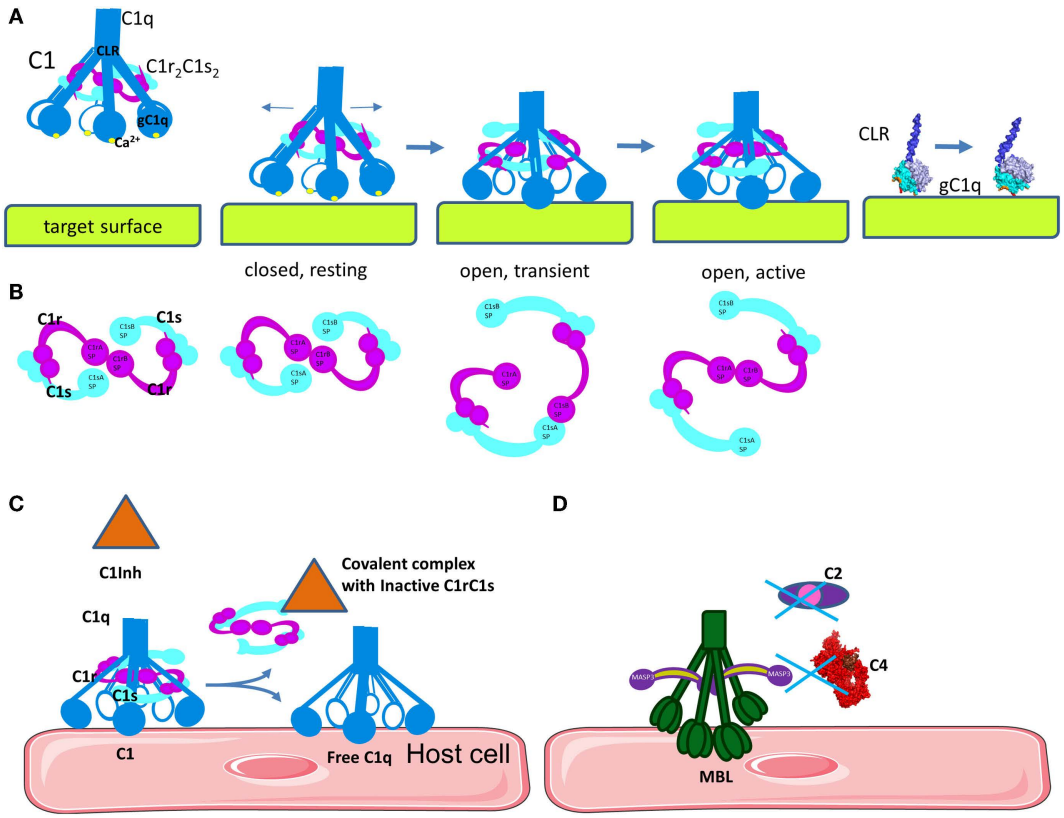

FIGURE 4 | Mechanism of C1 classical pathway activation and regulation. **(A)** Structural changes in C1q are necessary to induce auto-activation of C1r and activation of C1s. Upon binding of the inactive, closed C1 complex, electrostatic interactions with a target surface may alter the electrostatic field of the domain. This will induce a rotation of gC1q, leading to opening of the angle between the CLRs. A part of the binding site on gC1q apex will be lost, but new links will be formed with the side surface of the B-chain. **(B)** Concomitant with the structural changes in C1q, $C1r_2C1s_2$ complex will pass from closed, inactive eight-shaped conformation to an active, S-shaped conformation, allowing C1r auto-activation and further C1s activation by C1r. **(C)** The C1 inhibitor is a serpin that binds covalently to the active site of C1r and C1s, blocking their function. It also dissociates $C1r_2C1s_2$ from C1, releasing free C1q. C1 inhibitor also inhibits the lectin pathway by binding to MASP-1 and MASP-2. **(D)** MBL can bind to MASP-3, MAp44, or MAp19, which cannot cleave C4 and C2.

In order to understand the exact architecture of the C1 complex, further investigation will be required.

Mechanism of Activation of the Lectin Pathway

The pattern-recognition molecules of the LP are MBL, collectins, as well as ficolins H, L, and M (64, 65). The LP pattern-recognition molecules have an N-terminal collagenous region similar to C1q, however their C-terminal domains differ from gC1q. Collectins contain carbohydrate recognition domains, which recognize sugar patterns. MBL, which belongs to the collectin family, recognizes terminal monosaccharide exposing horizontal 3′- and 4′-OH groups (glucose, mannose, and *N*-acetyl-glucosamine) in a Ca-dependent manner. These sugars are rarely present on host proteins and cell surfaces, but frequently expressed on bacteria, viruses, and dying cells. Ficolins are associated with MASPs protein in the circulation and have C-terminal recognition fibrinogen-like domains, which are able to bind acetyl groups, such as *N*-acetyl-glucosamine, on the surface of bacteria. Following binding, MASPs associated with MBL or ficolins are activated and result in the cleavage of C4 and C2 (66, 67). Similar to C1q, stable binding can be achieved only when the ligands are clustered on the surface forming a specific pattern. This complex can simultaneously engage several carbohydrate recognition domains or fibrinogen-like domains for collectins and ficolins respectively.

Despite the similarity between the architecture of the C1 and MBL/MASP complexes, the mechanism of activation of the LP is different than the classical one (64). While in the CP each C1 complex carries both C1r and C1s, the majority (~70%) of the MBL molecules present in plasma are associated with only one homodimer of either MASP-1 or MASP-2 to assure their separation (**Figure 3B**) (68). In physiological conditions, MASP-1 is required for the activation of MASP-2 and both activated proteases can cleave C2 while MASP-2 can also cleave C4. Auto-activation properties confer to MASP-1 a fluctuating state between inactive and active-like conformations, giving it a key role in LP activation (69–74). Auto-activation of MASP-2 provides a residual capacity (~10%) to cleave its natural substrate C4 in zymogen form (75). Since MASP-1 and 2 are associated with different MBL or ficolin molecules, they are required to juxtapose their recognition molecules on ligand surfaces to facilitate activation of different

MASPs (76). Therefore, MASP-1 from one complex will activate MASP-2 from the adjacent complex, allowing C4 cleavage (77). MASP-3 also influences LP activation.

The described mechanisms of activation of the CP and LP illustrate two of the key characteristics of the complement cascade. Complement activation relies on the versatility of the target patterns recognition molecules (C1q, MBL, ficolins) that can discriminate between self and non-self and bind to pathogen- or danger-associated molecules. These molecular patterns are often generated after a specific conformational changes, such as with IgM or particular clustering, such as with IgG, CRP, or pathogen-associated carbohydrates. Complement is driven by these conformational changes that transmit a signal as a result of the recognition event to the subsequent complement components, activating them or modulating their function.

Regulation of the Classical and Lectin Pathways Initiation

Activation of the CP is controlled by a serpin molecule, C1 Inhibitor (C1Inh). C1Inh binds and inactivates C1r and C1s, leading to dissociation of the C1 complex and liberation of free C1q leaving an inactive covalent complexes between C1Inh and C1r or C1s (**Figure 4C**) (78, 79). C1Inh is thought to bind to and stabilize unactivated C1r and C1s in the C1 complex thus retarding their spontaneous activation (80), but this function is poorly studied. C1Inh has additional functions outside complement inactivation, related to kinin pathway (a plasma system related to inflammation, vasodilatation, and pain). Angioedema is disease caused by hereditary or acquired C1Inh deficiency. The edemas are triggered by increased permeability of the blood vessels in response to elevated levels of bradykinin as a result of the C1Inh deficiency. Recombinant and plasma-derived C1Inh are approved therapeutic agents for hereditary angioedema (81).

C1q inhibitors released under physiological or pathological conditions such as chondroitin-4 sulfate proteoglycan and the hemolysis derivative heme can bind to C1q and inhibit the CP (55, 82). The mechanism of action of heme, as well as a number of synthetic C1q inhibitors, rely on binding to gC1q and alteration of its electrostatic properties (55, 83). Calreticulin, released during cell death or from parasites can also act as an inhibitor of C1q to prevent CP activation (84, 85).

Inhibition of the LP is influenced by MASP-3, MAp44, and MAp19 proteins, which share high sequence homology with MASP-1 and -2 and have similar binding affinity to MBL and ficolins (70, 86). These proteins may compete with MASP-1 and -2, but are unable to cleave MASP, C2, and C4 preventing further activation of the LP cascade (**Figure 4D**). In addition, C1Inh is able to control LP activation by inhibiting MASP-1 and MASP-2 but not MASP-3 activity (87, 88).

Platforms for Surface Assembly of the Alternative Pathway C3 Convertase

C3b can bind to the cell surface not only via its own thioester bond, but also by interacting with surface molecules that serve as platforms for C3b recruitment (**Figure 5**). Recently, it has been observed that C3b and C3(H_2O) can also bind to the cell surface by these platforms resulting in local activation of the AP. Although activated platelets are the predominant cell type that binds C3(H_2O) (89, 90), the exact molecules it binds are not yet well defined. Nevertheless, several molecules are likely candidates for activated C3 recruitment on different cell types.

Properdin, which stabilizes the alternative C3 convertase (91), is able to bind pathogens, activated or damaged host cells to induce stimulation of the AP (**Figure 5A**) (92, 93). Properdin recognizes heparin and heparan sulfate on tubular cells leading to complement activation (94). Properdin contributes to the AP activation on human neutrophils and has been observed in neutrophil-mediated diseases (95). Degranulation stimuli on neutrophils induce low properdin release and deposition, triggering AP by recruiting C3b and promoting C3 convertase formation. Plasma properdin is also required for an efficient C3b feedback loop and amplified AP activation (95). Generation of C5a also further stimulates properdin release and amplifies complement activation and neutrophil stimulation. Moreover, it has been demonstrated that myeloperoxidase secreted by neutrophils during degranulation binds to properdin and leads to the AP activation (96). Properdin released by activated neutrophils can also bind to activated platelets. In the absence of C3, properdin can also bind directly to platelets after interaction with a strong agonist and serves as a platform for recruitment of C3b or C3(H_2O) and C3 convertase formation (**Figure 5B**) (90). This complement activation may contribute to localization of inflammation at sites of vascular injury and thrombosis.

Another protein, which can recruit C3b to a cell surface is complement Factor H (FH)-related protein 4A (CFHR4A). This protein shares sequence homology with FH and is able to bind C3b via its C-terminal domain. CFHR4A lacks regulatory domains and cannot inactivate C3b. Even more, it has been suggested that CFHR1A can serve as a platform for the alternative C3 convertase formation. Formed convertase on CFHR4A had a higher resistance to FH-mediated decay.

P-selectin (CD62P) recruits leukocytes via binding to P-selectin glycoprotein ligand 1 (PSGL-1) (97) and has been described to bind C3b on the cell surface leading to the activation of AP (**Figure 5C**) (98, 99). Morigi et al. showed the effect of P-selectin as a platform for C3 convertase formation *in vitro* and in a murine model of Shiga toxin (Stx2)/LPS-induced hemolytic uremic syndrome (HUS) (99). P-selectin expression was partially triggered by the anaphylatoxin C3a contributing to a vicious circle of complement activation aggravating microvascular thrombosis HUS pathology (99).

Another activator of C3 convertase, heme, is released from hemoglobin during hemolysis, where it stimulates the AP. Heme induces deposition of C3 activation product in erythrocytes and has been shown to play a role in malaria pathogenesis (100, 101). Heme binds C3 (not C3b), likely near to the TED domain, leading to the generation of C3(H_2O) and homophilic C3 complexes associated with overactive C3/C5 convertases (102). Furthermore, *in vitro* experiments on human EC have shown that heme-induced mobilization of specific EC granules that store von Willebrand Factor and P-selectin called Weibel Palade bodies, is at least in part induced by TLR4 (102, 103). This TLR4 stimulation lead to degranulation of P-selectin accompanied by C3b and C3(H_2O) binding to the cell surface of EC. Heme is a hydrophobic molecule that binds to lipid bilayers and it is hypothesized that cell-bound heme may serve as a platform to recruit C3(H_2O) (102).

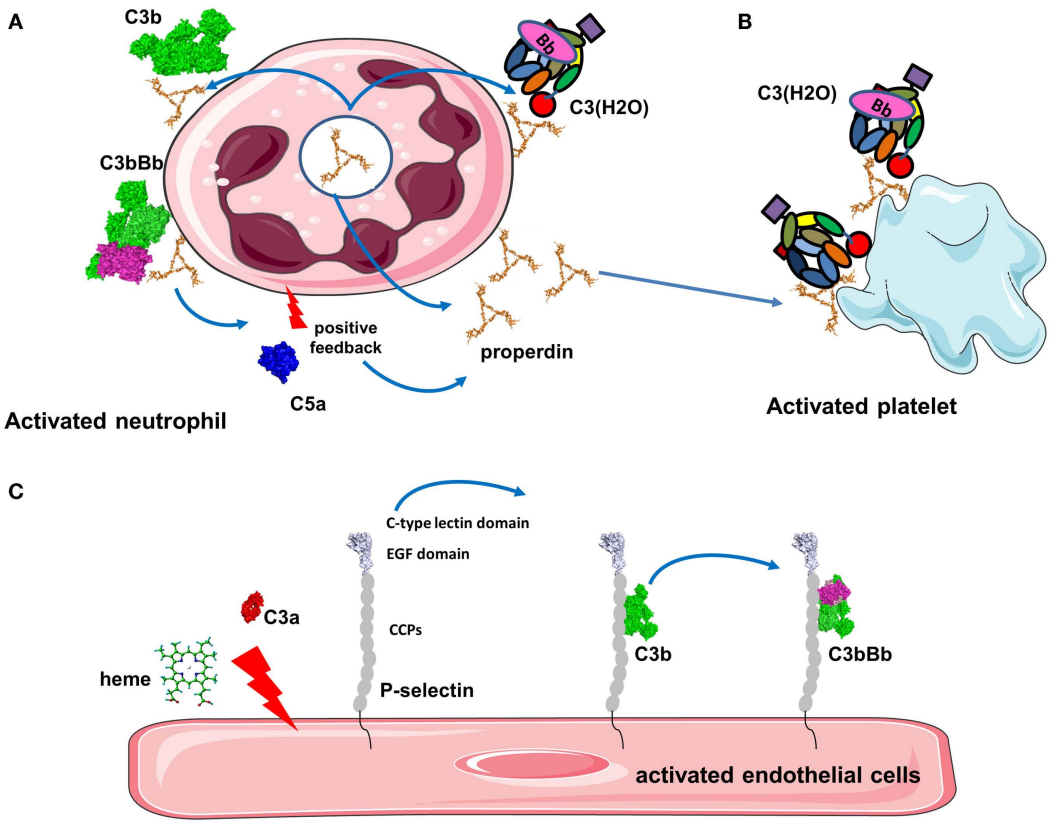

FIGURE 5 | Platforms for complement activation. **(A)** Properdin is released from activated neutrophils and is bound to the cell membrane where it recruits C3b to form the alternative pathway C3 convertases. C5a then activates additionally neutrophils and they secrete more properdin. This installs a vicious cycle of neutrophil and complement activation. **(B)** Properdin released from neutrophils or in the plasma binds to activated platelets promoting C3(H_2O) recruitment and complement activation. **(C)** Stimulation of endothelial cells with C3a, heme, or other agonists induces expression of P-selectin. P-selectin contains CCP domains and binds C3b, promoting formation of C3 convertases that generate more C3a to stimulate cells.

Collectively, these examples lead us to propose a general mechanism for a positive feedback loop implicating protein platforms in tissue damage. An initial trigger will stimulate the cell to either express a platform protein (properdin for neutrophils or P-selectin for EC and platelets) or to bind molecules from the fluid phase (properdin, CFHR4A, or heme in case of hemolysis). The type of the platform will likely depend on the cell type, location of activation, and other yet undiscovered factors. C3(H_2O) will bind to these platforms and will initiate local complement activation and C3b deposition. The amplification loop will generate C3a and C5a, which upon binding to their receptors (described below) will augment cell activation and increase expression of platform proteins stored in intracellular granules or recruited from the plasma. These events will form an intensified circle resulting in local inflammation, thrombosis, and tissue damage.

Structure and Function of the C3 Convertases

Alternative Pathway C3 Convertase

The structure and function of the AP C3 convertase has been dissected during the few last years. Upon cleavage and removal of C3a, C3b undergoes a dramatic structural change (**Figure 6A**) leading to exposure of novel binding sites. This allows recruitment of FB which binds in a Mg^{2+}-dependent manner and yields the pro-convertase C3bB (**Figure 6B**) (104). This interaction occurs via the Von Willebrand Factor A-type (VWF-A) domain and three complement control protein (CCP1-3) domains of FB (104, 105). The catalytic SP domain of FB undergoes large conformational changes oscillating between a closed (loading) and an open (activation) forms (**Figure 6B**) (104–106). In the open (activation) conformation, the scissile bond is exposed and the FD binding site is formed correctly.

Factor D is synthesized in an inactive pro-FD enzyme lacking proteolytic activity (107). It was suggested that this zymogen form can be cleaved by MASP-1/3 into a form with limited activation to support the basal levels found in the AP (108, 109) and becomes fully activated only upon binding to C3bB open complex. The physiological relevance of MASPs-mediated cleavage of pro-FD is still being debated. MASPs cleavage is not the only mechanism for FD activation, since mice deficient in MASP-1/3 have reduced but detectable AP activity (110) and the only patient found to be deficient in MASP-1 and -3 was reported to have a normal AP activity (111). Further studies are needed to elucidate the

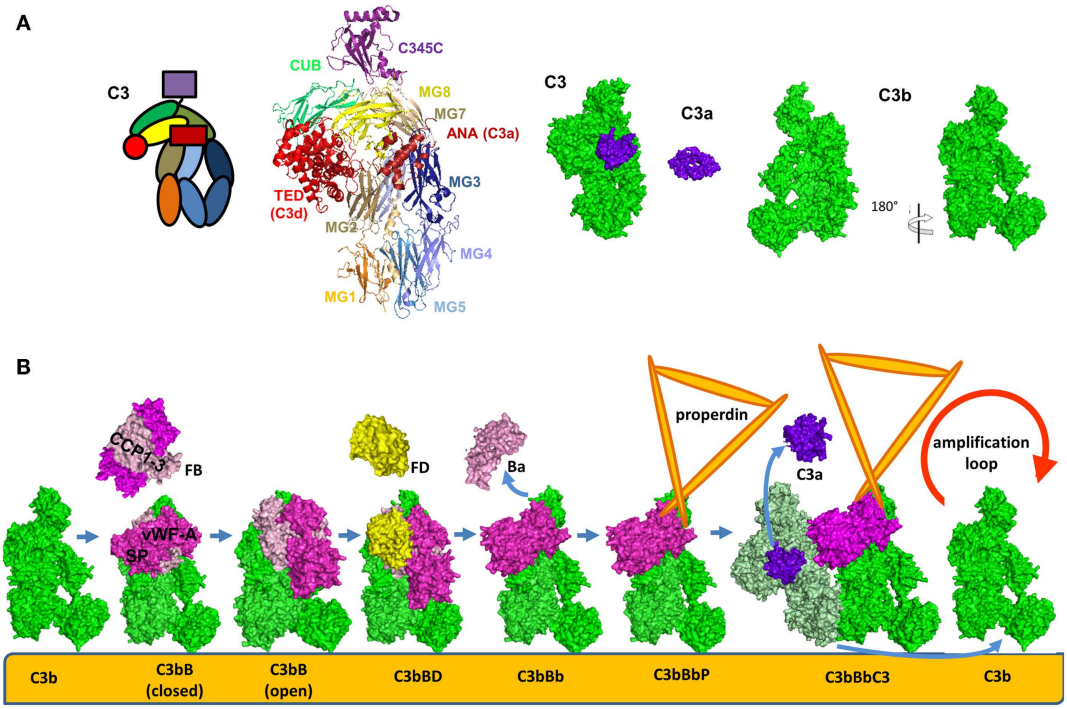

FIGURE 6 | Alternative pathway C3 convertase. **(A)** Structure and domain organization of the central complement component C3 and its cleavage fragments C3b and C3a. C3b is shown in two orientations to illustrate the surfaces containing the ANA domain and the opposite surface, carrying FB and FH binding sites. **(B)** Steps of formation of the alternative pathway C3 convertase. C3b is shown in green, FB in magenta, FD in yellow, C3a is in violet, and the substrate molecule C3 – in light green. For these molecules, the available crystal structures were used for the visualization. The C3bBbC3 complex is visualized based on molecular modeling. Properdin, for which a crystal structure is not available, is depicted in orange.

mechanism of FD activation in mice and men. Insights into this pathway could help lead to the development of MASP-1 inhibitors as a strategy to treat renal diseases associated with uncontrolled AP activation.

Upon activation, FD binds to and cleaves C3b-bound FB releasing the N-terminal fragment Ba (comprising the CCP1-3 and the αL helix). This results in the formation of the C3 convertase of the AP C3bBb (**Figure 6B**) (112). The Bb fragment consists of a VWF-A and a SP domain. The SP domain undergoes a new structural change and is positioned in a conformation, similar to the closed form of the pro-convertase (**Figure 6B**) (113). A substrate molecule of C3 binds then to the alternative C3 convertase and is cleaved generating new C3b and C3a molecules (**Figure 6B**) (113).

Factor B binds in a similar manner to C3(H_2O) to form the alternative C3 convertase [C3(H_2O)Bb] in the fluid phase (114). Even if FB has a higher affinity to C3(H_2O) than to C3b, the convertase activity remains lower than the C3bBb complex, as measured by C3a released. The fluid phase convertase C3(H_2O)Bb also has higher resistance to decay by complement inhibitors (114).

Classical Pathway C3 Convertase

The structure and mode of action of the CP C3 convertase is not well understood. C4 and C2 share high degree of sequence and structure homology to C3 and FB respectively, thus the mechanism of formation of the classical C3 convertase may be similar to the well-studied AP C3 convertase, described above (**Figure 6**). C4 is cleaved by activated C1s or MASP-2 to bioactive fragment C4b and a small fragment C4a (**Figures 7A,B**). C4b contains an internal thioester bond, similar to that in C3b, and forms covalent amide or ester linkages with the antigen–antibody complex or the adjacent surface of a cell. C4b binds C2 in Mg^{2+}-dependent manner. C2 is then cleaved by C1s or MASPs. Since the concentration of C2 is about 20–30-fold lower compared to C4, one active C1 complex can cleave about 35 C4 molecules, while only 4 C2 will be cleaved for the same time (63). The larger fragment C2a remains bound to C4b and forms the CP C3 convertase C4b2a (**Figure 7C**) (115) and the smaller fragment C2b is released in the circulation. Historically, C2b was considered to have kinin-like properties, but recent reports failed to confirm this function (116, 117).

The C3 convertases are an excellent example of a general mechanism that governs different steps of the complement cascade. Each subsequent step can only occur after a conformational change, triggered by the preceding step, thus assuring the temporal and specific control of this powerful destruction cascade (118).

Stabilization of the Alternative Pathway C3 Convertase

The C3bBb is a short-lived complex with a half-life of about 90 s (119) and, therefore a stabilization of this complex is required to assure

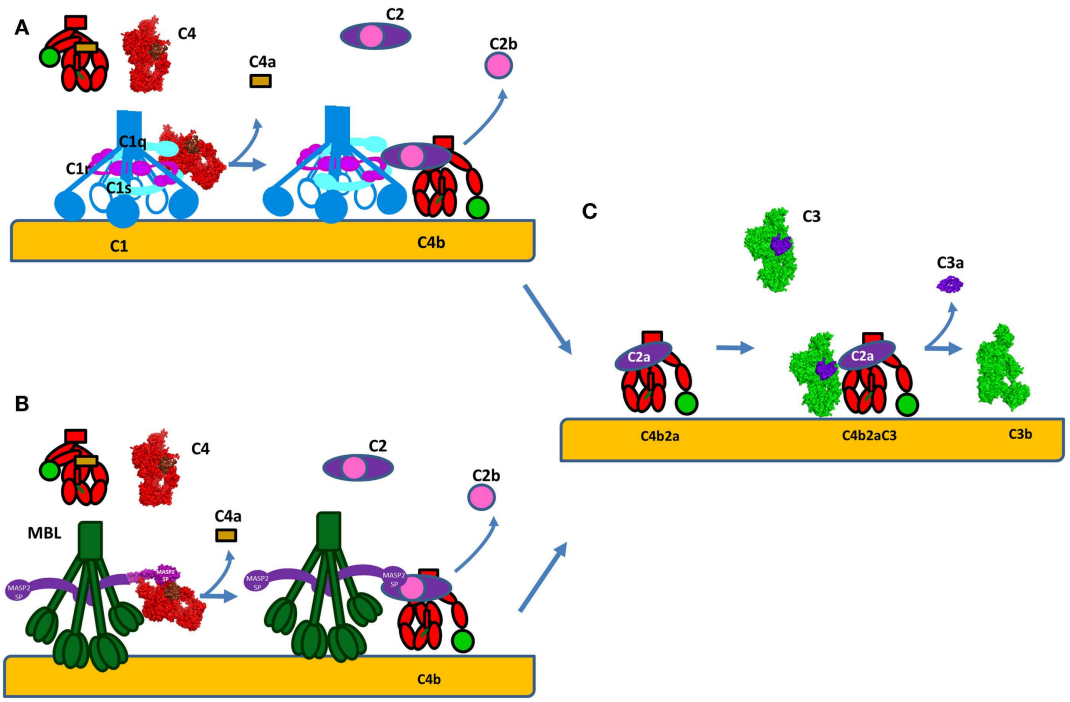

FIGURE 7 | Formation of the C3 convertase by the classical and lectin pathways. **(A)** C1s or **(B)** MASP-2 will cleave C4 into bioactive fragment C4b that bind covalently to the surface of cells and interacts with C2. The small fragment C4a is released. Following, the same enzyme will cleave C2 to generate the classical pathway C3 convertase C4b2a. **(C)** C4b2a will interact with C3 cleaving it and releasing the bioactive fragments C3a and C3b. C3b will bind covalently to the surface and allow formation of alternative pathway C3 convertases C3bBb via the amplification loop. The C3a generated is a pro-inflammatory anaphylatoxin. The C4 molecule is presented in red, with brow colored the ANA domain, which will become C4a after cleavage and in green – the TED domain, which will become C4d after cleavage. The crystal strictures of C4, C4b, and C2a were used for the representation. The CP C3 convertase C4b2a is modeled based on the structure of the AP convertase C3bBb, with which it shares high homology.

efficient host defense (**Figure 6B**) (93). The AP C3 convertase is stabilized 5- to 10-fold by association with properdin (91). Properdin (120) is secreted by monocytes/macrophages and T lymphocytes (121, 122) and is stored in secondary granules of neutrophils (95, 123) and mast cells (124). Properdin is composed of identical rod-like protein subunits (125) that are associated head-to-tail to form cyclic dimers, trimers, and tetramers that resemble rods, triangles, and squares, respectively. The function of properdin is dependent on its level of polymerization, with the tetramer being approximately 10-fold more active than the dimer (126). Purified properdin results in aggregates and has artificially high binding activity (127, 128). Properdin binds C3bBb, as well as, the pro-convertase C3bB and C3b (92). Properdin interacts both with the C345C domain of C3b and the VWF-A domain of Bb, in order to stabilize the convertase (129). Interestingly, electron microscopy studies visualized that properdin binding induces a large displacement of the TED and CUB domain of C3b (129). These structural changes distort the FH binding site (130–132), which may explain the relative resistance of the stabilized C3 convertase to decay by FH.

Recent studies of Hourcade and colleagues demonstrated that properdin is not merely a stabilizer of the C3 convertase, but also a pattern-recognition molecule that binds to microbial surfaces including glycosaminoglycans (GAGs), apoptotic, and necrotic cells providing a platform for C3 convertases assembly (93).

Negative Regulation of the C3 Convertases

The amplification loop is the balance between two competing cycles, acting on the C3b–C3 convertase formation, which enhances both amplification and downregulation via the C3 breakdown cycle. Complement amplification depends on the balance between binding rates of each reaction (22). To regulate activation, several inhibitors of complement pathways, primarily against AP, exist in the fluid phase and on host cells. Complement inhibitors have overlapping functionality. FH works as a soluble inhibitor of the alternative C3 convertase, while membrane cofactor protein (MCP), decay acceleration factor (DAF), and complement receptor 1 (CR1) work as membrane inhibitors. FH is a specific cofactor for C3b and C4 binding protein (C4BP) primarily is a cofactor for C4b, while MCP and CR1 act as cofactors for the inactivation of both C3b and C4b via Factor I (FI).

Inactivation of C3b and C4b by Factor I

Factor I is a SP found in the plasma that cleaves C3b in presence of different cofactor molecules, such as FH, MCP, CR1, or C4BP (**Figure 9A**). The protease activity of FI leads to the generation of degradation product of C3b, iC3b, which is unable to bind FB (133). A zymogen form of FI has not been detected in the

circulation. In fact, FI circulates in a proteolytic form but in inhibited conformation. The activity of the light chain of FI is allosterically inhibited in the circulation by a non-catalytic heavy-chain (134). In presence of its cofactors, the non-catalytic heavy-chain of FI is released and it is able to cleave C3b between Arg1281 and Ser1282 to give the iC3b fragment (134, 135). Molecular modeling suggested that FI binds FH1–4 and C3b simultaneously in a groove between two proteins, which is in agreement with the hypothesis that FI binds CCP1–3 of FH and C345C of C3b (132).

Factor I Cofactors

Membrane cofactor protein, DAF, and CR1 (CD35) serve as cofactors for FI-mediated proteolysis of C4b and C3b. MCP is composed of 4 extracellular CCP domains expressed on all nucleated cells (136) and CR1 contains 30 extracellular CCP domains and is expressed on leukocytes, erythrocytes, and glomerular podocytes. MCP N-glycosylation on CCP2 and CCP4 is essential for MCP inhibitory activity (137). CCP1–4 of MCP binds to C3b and C4b and is structurally similar to the four N-terminal domains of FH (described below), both proteins serve as cofactors for FI for C3b inactivation (**Figure 9A**) (132, 138–140). FH only induces C3b-degradation and is inefficient for C4b. CCP-3 and -4 of MCP are responsible for binding to C3b and C4b, while CCP1–2 only interacts with C4b (**Figure 8A**) (141). The binding site for MCP on C3b is partially overlapping with the site for CCP-3–4 on FH (142).

The first 28 CCP of CR1 are 4 long homologous repeats (LHR) for 7 CCP domains containing the binding sites for C3b and C4b (143, 144). C3b and C4b-binding sites located in CCP8–10 (LHR2) and CCP15–17 (LHR3), respectively, are responsible for FI cofactor activity (143, 145). CCP15–17 has a major role in C3b/C4b inactivation (146). CCP15 carries a positively charged region essential for the C4b-binding and a basic region in the CCP16 that is necessary for C3b-binding. Although the architecture of the CR1/C3b and CR1/C4b is not well defined, it is known that CR1 interacts with the α′NT region of C3b (residues 727–767), which overlaps with the FH CCP1-binding site, but not MCP-binding site (132, 133, 142, 147).

C4b can be inactivated by the action of C4BP, a plasmatic cofactor for FI (**Figure 8B**) (148–151). C4BP has a complex, octopus-like structure comprised of CCP domains containing α- and β-chains (152, 153). The first three CCP domains of each α-chain are involved in cofactor and convertase dissociation functions. A

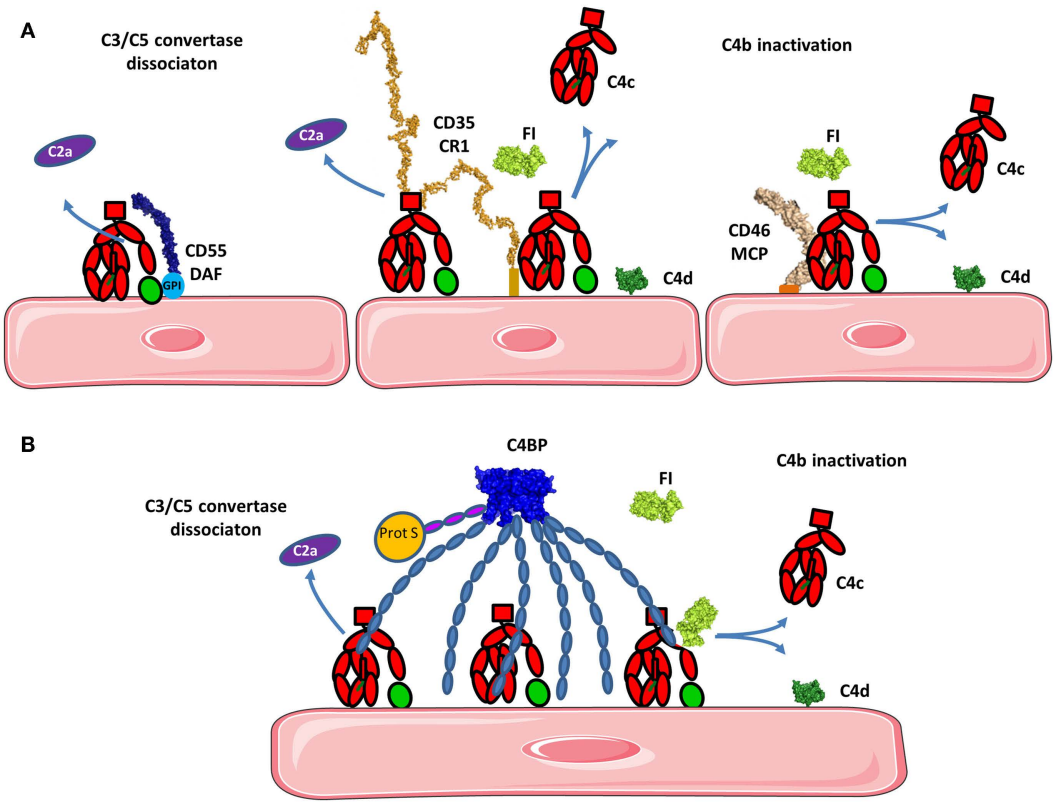

FIGURE 8 | Regulation of the classical and lectin pathways C3 convertase. To avoid overactivation, the CP and LP tightly regulate signaling. **(A)** If a C4b2a C3 convertase is formed, it will be rapidly dissociated by DAF and/or CR1 depending on the cell type. Bound C4b will be inactivated by FI in presence of cofactors such as CR1 and/or MCP. C4d will remain bound to the surface and C4c will be released. **(B)** C4BP can act in fluid phase as well as on the cell surface. C4BP has an octopus structure and interacts with several C4b molecules. It dissociates the C3 convertase and serves as a cofactor for FI in the cleavage of C4b to inactive fragments C4c and C4d. The structures of the complexes of C4b with the regulatory proteins have not been resolved yet, therefore the proteins are depicted in proximity one to another, represented by their known structures, but no complex could be reliably modeled.

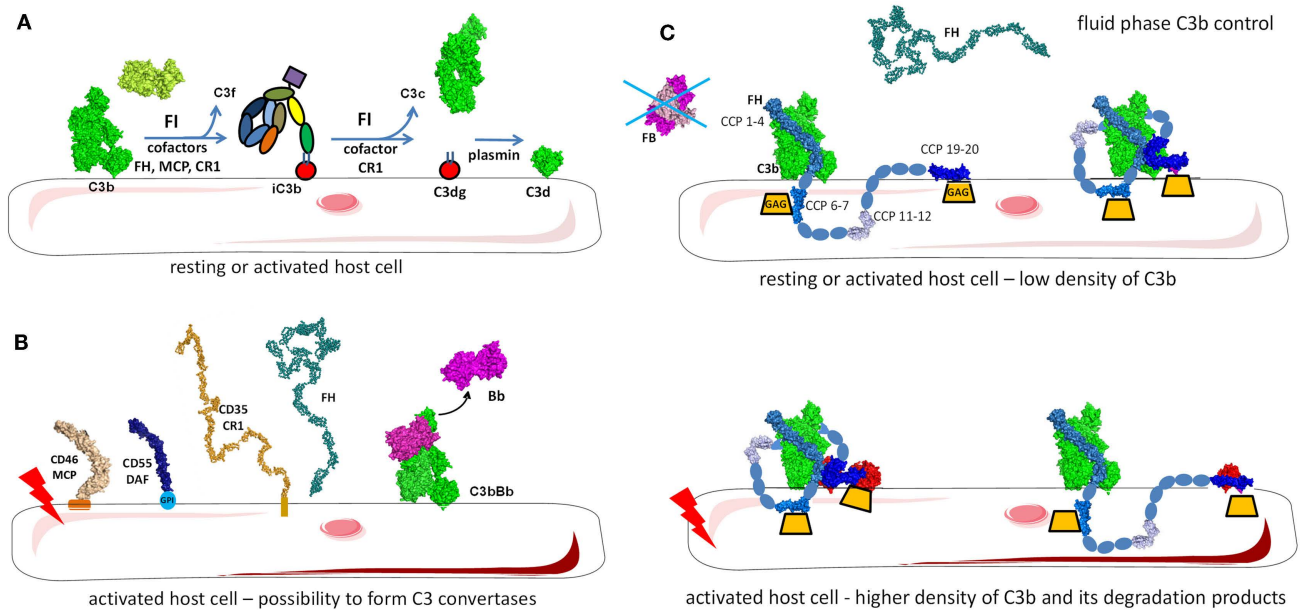

FIGURE 9 | Regulation of the alternative pathway. **(A)** FH as a master regulator of C3b in the fluid phase and on the cell surface. FH binds to C3b in fluid phase preventing novel convertase formation. FH may bind to C3b and GAGs on the cell surface and the architecture of the complex depends on the level of activation of the cell and the density of deposited C3 fragments. Resting cells have only a few C3b molecules that are deposited and FH binds to them with the regulatory domains CCP1-4. CCP7 and CCP20 interact with GAG on the membrane. Alternately, CCP19 may bind to the TED domain of C3b allowing CCP20 to interact with GAGs. If the cell is activated and C3b and C3d (or two C3b molecules) are deposited in close proximity, FH may bind to two of these molecules, allowing GAG binding by CCP20. **(B)** On resting cells, C3b will immediately be inactivated to iC3b by the action of FI and the assistance of cofactors (FH, MCP, CR1). iC3b cannot bind FB and forms C3 convertases. Only the cofactor CR1 allows FI to execute a second cleavage generating C3c (released in the fluid phase) and C3dg, which remains bound to the cell. C3dg is rapidly transformed to C3d by tissue proteases. **(C)** If the host cell is activated, the complement control will not be sufficient to prevent any complement deposition and C3 convertases could be formed. To avoid cell damage, these convertases need to be dissociated. Multiple complement regulators such as DAF, CR1, and FH decay the C3bBb complex formed on host cells. Remaining C3b will be inactivated by FI, using FH, MCP, or CR1.

maximum of four C4b molecules can simultaneously interact with the α-chains of one C4BP molecule (154). The β-chain binds coagulation protein S and contributes to regulation of the coagulation cascade (155).

Complement receptor 1 is a unique cofactor of FI due to its ability to induce further cleavage of iC3b, generating C3c and C3dg degradation fragments (**Figure 9A**) (133), where FH, MCP, and C4BP only induce the cleavage of C3b to iC3b.

Several proteins have been shown to enhance FI-mediated cleavage in the presence of cofactors. Thrombomodulin binds to C3b and FH and negatively regulates complement by accelerating FI-mediated inactivation of C3b in the presence of FH and C4BP (156). Von Willebrand factor also enhances the efficacy of FH as a cofactor for FI (157). In specific *in vitro* buffer conditions, Von Willebrand factor has been suggested to have direct cofactor activity, but the physiological function of this interaction requires further validation (158).

C3 Convertase Dissociation

The AP C3 convertase is dissociated and Bb is released primarily by the action of CCP1–4 in FH (described below). CR1 dissociates the C3 and C5 convertases. CCP1–3 of CR1 carries a binding site for C4b allowing it to induce accelerated decay of the C3 convertase in the CP and AP (**Figures 8A** and **9B**) (143, 159). C4BP prevents the formation of the classical C3 and C5 convertases (148–151).

Decay acceleration factor accelerates decay of the C3 and C5 convertases in the CP and AP (**Figures 8A** and **9B**). DAF has four extracellular CCP domains, a highly glycosylated domain and a GPI anchor (160). DAF inhibits the AP C3 convertase by binding to Bb on the vWA domain with its CCP2 domain (161). CCP2 of DAF has a higher affinity for Bb compared to the intact FB. As a consequence, the active convertase is more sensitive to rapid decay, compared to C3bB (162, 163). DAF also binds to the C3 convertase on C3b via its CCP4 domain (163) and CCP-3 contributes to the accelerated decay function (164).

Factor H – The Master Regulator of the Alternative Pathway

Factor H regulates the AP and the amplification loop of the complement pathways (**Figure 9**). It is a soluble inhibitor of the C3 convertase competing with FB for binding to C3b (**Figure 1A**) (165). It also serves as a cofactor for C3b inactivation by FI (**Figure 9A**) and induces C3bBb complex dissociation (**Figure 9B**) (166, 167). FH is composed of 20 CCP domains arranged as beads on a string (168) (**Figure 9C**). CFHL1 is a shorter protein, containing the seven

N-terminal domains of FH, generated via alternative splicing from the same gene (169) and functions as a fluid phase complement regulator. The fluid phase convertase C3(H_2O)Bb is more resistant than the alternative cell-bound convertase and is less susceptible to regulation by FH (114). FH has two main ligands, C3b and the GAG, found on host cells surface. Recent crystallographic and mutation analysis resulted in precise mapping of the C3b and GAG-binding sites in FH.

Factor H binds to C3b and C3(H_2O), but not to uncleaved C3. The conformational changes in the TED and CUB domains accompanying the transition from C3 to C3b expose the FH-binding sites. FH binds to C3b in at least two regions, at the N-terminus and the C-terminus of the protein. The N-terminal four CCPs contain the complement regulatory activity (132) and the CCP1–4 interacts with the MG ring of C3b. CCP-3 interacts with the CUB domain and CCP4 interacts with the TED domains (132, 142). CCP1–2 competes with FB causing its dissociation from C3b (**Figure 9C**). Both Bb and CCP1–2 are negatively charged leading to electrostatic repulsion. CCP19–20 carry a second binding site for C3b and can also interact with C3d (130, 131, 170, 171).

It has been difficult to unravel questions regarding the stoichiometry and architecture of the C3b–FH complex in the physiological fluid phase and on the surface of cell (**Figure 9C**). Does FH interact in the same way with C3b and C3(H_2O) or with C3b in fluid phase and on cell surface? Could FH bind with its N- or C-terminal domains on the same C3b molecule or can it interact with two adjacent C3b or C3b and C3d molecules or with one C3b molecule via CCP1–4 at the C-terminus and engage in cell membrane binding? If it is assumed that FH CCP1–4 and CCP19–20 bind to the same C3b molecule and if there is a liner arrangement of the C-terminal CCPs, could there be a steric clash between CCP18 and CCP4? The crystal structure of CCP18–20 indicates a bend-back conformation of CCP18, allowing binding on the TED domain of both CCPs 1–4 and CCPs 19–20 (172). Studies of the C3b/C3d binding site on CCP19–20 showed that it may be overlapping, but it is not identical with the GAG-binding site (173–177). The C3b/C3d-binding site is extended toward the CCP19 and the GAG binding is extended toward the apex of the CCP20. It is possible that FH19–20 domains may bind both C3d and GAG at the same time (131, 172, 174, 177). Site-directed mutagenesis of the FH19–20 domains indicates that a ternary complex between C3d/FH19–20/heparin can be formed and is essential for the functional activity of FH (177). The formation of a ternary complex was confirmed by the crystal structure of FH19–20 with C3d and a model sialylated trisaccharide, where a surface area extending from SCR19 to the beginning of CCP20 binds C3d and CCP20 carries a is highly conserved binding site, which may accommodate GAGs and sialic acid containing glycans (174). Structural analysis of the complex of FH19–20 with C3d showed that CCP20 also may interact with C3d suggesting potential competition between C3d and GAG at this site of FH (130). Analysis of published PDB files indicates that this CCP20–C3d interaction is present in the other FH CCP19–20 crystals, but was considered a crystallization artifact. Nevertheless, mutations in CCP20 appeared to affect the interaction with C3b and C3d, suggesting that a C3d–CCP20 interaction is possible. Based on the accumulating structural and functional data, it can be hypothesized that the architecture of the C3b–FH complex is governed by the target surface and the density of the C3b and C3d molecules (**Figure 9C**). On host cells, one isolated C3b molecule will bind CCP1–4, while SCR7 and the C-terminus will interact with the GAG of the cell membrane. Since FH may circulate in plasma in a folded back hairpin conformation (178–181), simultaneous interactions with the N- and C-termini to the same C3b molecule could be possible, while CCP7 and 20 bind to the GAGs of the membrane. Indeed, CCPs 10–13 are involved in the inclination of FH, allowing both CCP1–4 and CCP19–20 binding to C3b (182). Using crystal structures of CCP10–11, CCP11–12, and CCP12–13, the authors also demonstrated that a tilt of 80–100° occurs allowing a hairpin structure formation. The compact architecture of the C3b–FH complex is supported by the existence of a third binding interface involving CCP6–10 in FH and the C3c portion of C3b (171, 183, 184).

Glycosaminoglycans are an important constituent of the cell membrane and play a critical role in complement regulation. In addition to the GAG-binding site in CCP20, FH carries another GAG-binding site located in CCP7 (183, 185–189). These GAG-binding sites in FH allow it to recognize negatively charged heparan sulfate moieties on the membrane and may explain the differences in the affinity of FH binding and a dependence on the expression of GAG and sialic acid on the cell surface (190). The difference in the susceptibility of sheep and rabbit erythrocytes to lysis by human complement, which are at the basis of the classical hemolytic tests for complement activation, is expression level of sialic acid on the surface of these cells. This allows them to bind (sheep) or not to bind (rabbit) to FH (165, 191). Together with the low affinity of properdin for heparin and heparan sulfate (94), GAG expression is a powerful regulator of the complement homeostasis between negative regulation and stimulation of the complement pathways. The two regions anchoring FH to the cell membrane, CCP7 and CCP20, are specialized in binding to unique GAG, expressed in different types of cells, however both are necessary to assure functional activity of FH on the cell surface (189). CCP7 containing construct CCP6–8 binds stronger to heparin than CCP20 containing construct CCP19–20 (192), while CCP6–8 and CCP19–20 do not recognize the same sulfate GAG. GAG and sialic acid are expressed in multiple human organs with different subpopulations and distinct structures that may provide a variation of the binding affinity of complement regulators (193). These differences can potentially explain why polymorphisms or mutations in these regions are associated with complement-mediated diseases.

Mutations or polymorphisms in the GAG-binding sites of FH may create an imbalance in the homeostasis of complement regulation and could explain its association with different diseases. For example, a polymorphism in CCP7 leading to a modification from a tyrosine to histidine at amino acid 402 (Y402H) is the strongest genetic susceptibility factor for age-related macular degeneration (AMD) (194–197). Detailed analysis of this mechanism revealed that CCP7 in FH binds not only to GAG, but also to oxidized lipids, including malondialdehyde (MDA) (198–200). Y402H binds more weakly to MDA and oxidized phospholipids expressed on retinal pigment epithelium compared to the non-mutated protein (198, 200).

Another ligand of CCP7 and CCP19–20 in FH is the CRP, which is secreted by the liver during inflammation acute phase. Binding of FH to CRP can enhance complement inhibition, particularly on apoptotic or damaged cells during inflammatory conditions (201).

The interaction of FH with different cell surfaces is controlled by CFHR proteins. CFHRs belong to the FH family and comprise five different members (CFHR1–5). CFHRs are composed of five to nine CCP domains, present a high sequence homology with FH, and are recently described in detail (202). CFHR1, CFHR2, and CFHR5 share high homology in their two N-terminal domains, allowing them to form homo- and heterodimers (**Figure 10**) (203, 204). CFHR3 and CFHR4 do not form dimers, the C-termini of each share a high sequence homology with FH leading to competition between CFHRs and FH for binding to C3b, C3d, and GAG on the cell surface (203, 205, 206). Therefore, the CFHRs will enhance complement activation, preventing the action of FH.

C5 Convertases and the Terminal Complement Pathway

C3b binds to the C3 convertase to form a new enzymatic complex – C5 convertase which cleaves C5 to bioactive fragments C5a and C5b (**Figure 11**). C5b recruits complement components C6, C7, C8, and C9 which polymerize to form the membrane-attack-complex (MAC) ring (**Figure 12**) (207). The structures of individual components and overall architecture of the C5b-9 complex are starting to be elucidated, while, the structure and the mechanism of action of the CP and AP C5 convertases is not fully understood and require further investigation.

C5 Convertases

The C3 convertases C4b2a and C3bBb are the precursors of the C5 convertases. The C3 convertases are able to bind C5, but with a very low affinity and cleavage rate. These C3 convertases switch their specificity and start to bind and cleave C5 only after binding of an additional C3b molecule in the immediate proximity or most likely over the C3 convertase itself (**Figure 11**) (208). The structure of this trimolecular complex is unknown, but has been suggested that the covalent C3b-binding site on C4b is the residue Ser1217 (p.Ser1236) within the TED domain (209). This residue is not conserved in C3b and the binding site seems to be located outside of the TED domain, in the α43 fragment (residues 1268–1641) (210), suggesting different topology of the two trimolecular complexes. Whether the newly bound C3b molecule interacts with C5 or affects the conformation of the C3 convertase subunits is currently unknown. Laursen and colleagues propose a model by which the MG1, MG4, MG5, and TED domains of C3b will be able to contact the CUB and TED domains of C4b. The CUB, MG2, MG6, and MG3 domains appear to be capable of reaching mainly the rest of C4b, while the MG7, MG8, and C345C domains potentially could be in direct contact with C5 (**Figure 11**) (211).

The dramatic conformational change of C4 upon release of C4a is very similar to C3 (212). TED domain, MG8, and CUB are exposed after C4 cleavage, allowing covalent bond with Gln1013. The crystal structure of C4b confirms the implication of the flexibles residues 1231–1255 in the interaction with the TED domain of C3b (209, 212). This model supports the idea that C3b has no direct interaction with C5 in the classical (and most likely in the alternative) C5 convertase (**Figure 11**). C4b and CVF-C5 structures suggest that C4b-binding area for C5 is within the domains MG4, MG5, and MG7, supporting Laursen hypothesis.

The loss of affinity to C3 and the acquisition of affinity to C5 results in cessation of C3b opsonization and initiation of MAC-induced membrane damage. Upon covalent binding of a C3b molecule to a CP convertase C4b2a, there is a formation of a trimolecular complex with about 1000-fold increased enzymatic activity toward C5 cleavage (213) compared to the bimolecular complex. The CP C3 convertase cleaves on average 1.5 C5 molecules per minute. The gained activity of the trimolecular complex

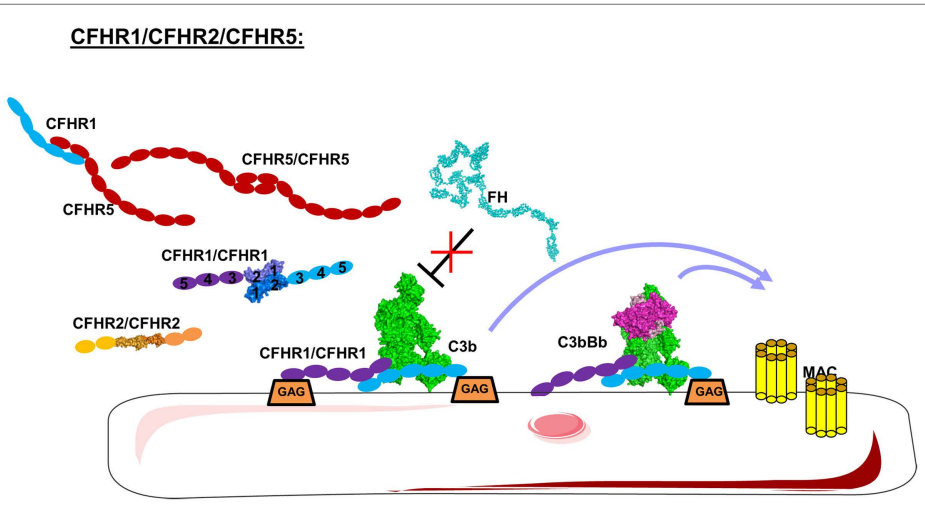

FIGURE 10 | Domain organization and mechanism of CFHRs. CFHR1, 2, and 5 carry dimerization N-terminal domains allowing them to form homo and heterodimers. These CFHRs, particularly CFHR1, are downregulators of FH, competing with FH for C3b and binding on the cell surface. This allow C3 convertases and MAC formation.

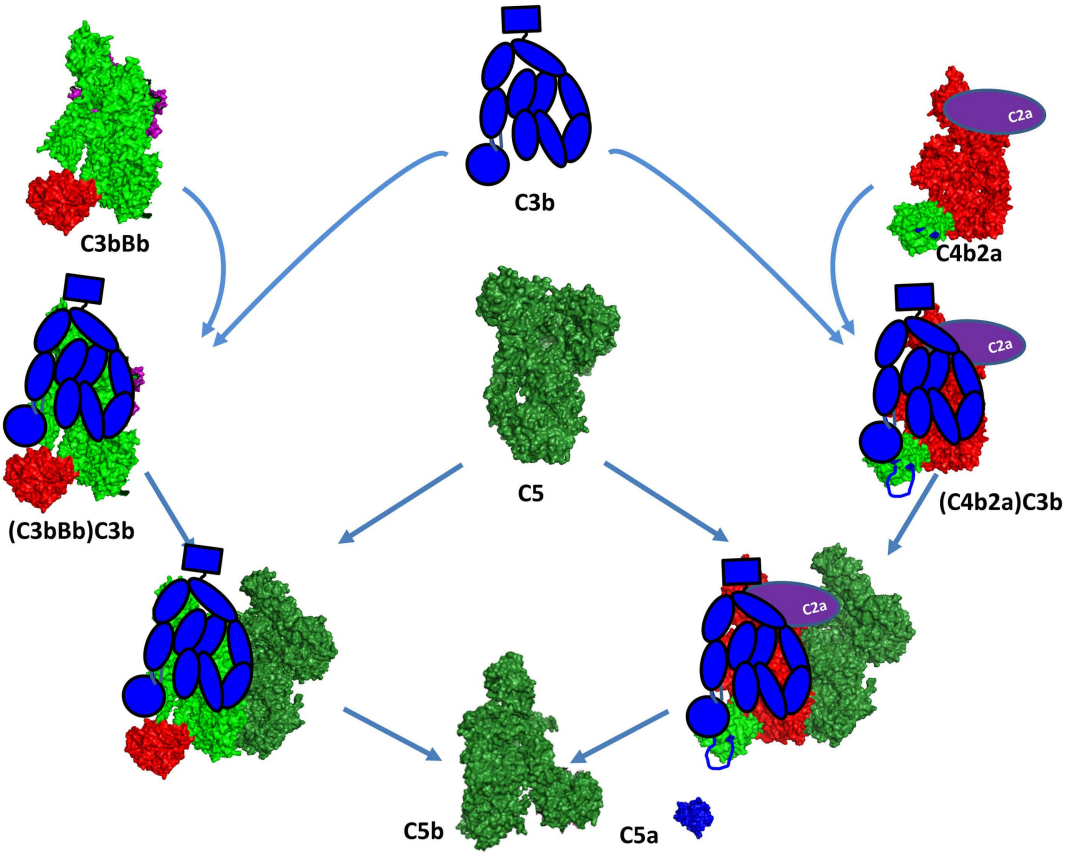

FIGURE 11 | C5 convertase formation. A C5 convertase is generated when a C3b molecule binds covalently in the vicinity or directly to a C4b or C3b, already engaged in a C3 convertase. This new enzyme loses its capacity to cleave C3 and starts to cleave C5. The binding site of the second C3 molecule is unclear, but it has been suggested to bind to the TED domain of C4d and to the CUB domain of C3b. Since the atomic coordinates of the two C5 convertases have not been published yet, this figure represents the current model for their organization. The CP C3 convertase is modeled here on the basis of the structures of C4b, C2a, and the AP C3 convertase C3bBb. The second C3b molecule is depicted in a schematic representation in blue to be distinguished from the C3b molecule interacting with FB to form the C3bBb complex. The Bb and C2a fragments are depicted in magenta and violet, respectively. They are partially visible behind the C3b and C4b molecules.

of the AP C3bBbC3b is six to ninefold weaker compared to the classical, cleaving less than 0.3 C5 molecules per minute (214). To compensate for the weaker efficacy, the AP C5 convertase can be stabilized by properdin (215, 216), which increases its half-life from approximately 3–30 min. This is different than the classical C5 convertase, for which a natural stabilizer has not been described. Together with the fact that the AP amplification loop generates a large number of C3b molecules makes the AP C5 convertase the main source of the terminal pathway complex C5b-9. In 2002, Pangburn and Rawal proposed a ring model for the amplification of the complement activation on the cell surface (213). In this model, any deposited C3b molecule coming from the classical or AP initiation can form an AP C3 convertase that will cleave one or several molecules of C3 (depending on the stabilization by properdin). A ring of new C3 convertases is formed or C3b binds on top of the C3 convertase itself to form a C5 convertase. As the activation spreads, the older enzymes will be inactivated by FH and FI leaving an expanding inner core of inactivated C3b surrounded by an active band of C5 convertases. The outermost band of newly formed C3 convertases is responsible for the growth of the site as it expands across the surface of the target.

Complement-Independent Cleavage of C5 and C3

Accumulating evidence suggests the existence of additional complement activation pathways in the plasma (217) aside from the three established pathways. Thrombin, human coagulation factors IXa, XIa, Xa, and plasmin were all found to effectively cleave C3 and C5 (218). C5a and C5b can be generated from C5 via thrombin, independently of the plasma complement system (217, 219). Thrombin cleaves C5 poorly at R751, which is the site of action of the C5 convertase (220). However, it does cleave C5 at an alternate site, R947, generating intermediates C5T and C5bT. These activation fragments will be generated at sites of activation of the coagulation cascade. Interestingly, C5bT formed a C5bT-9 MAC with significantly more lytic activity than with C5b-9.

Complement-independent cleavage of C3 by plasmin has also been suggested in the literature (221–223). The fragments generated by plasmin-mediated cleavage (C3a-like, C3b-like, iC3b-like,

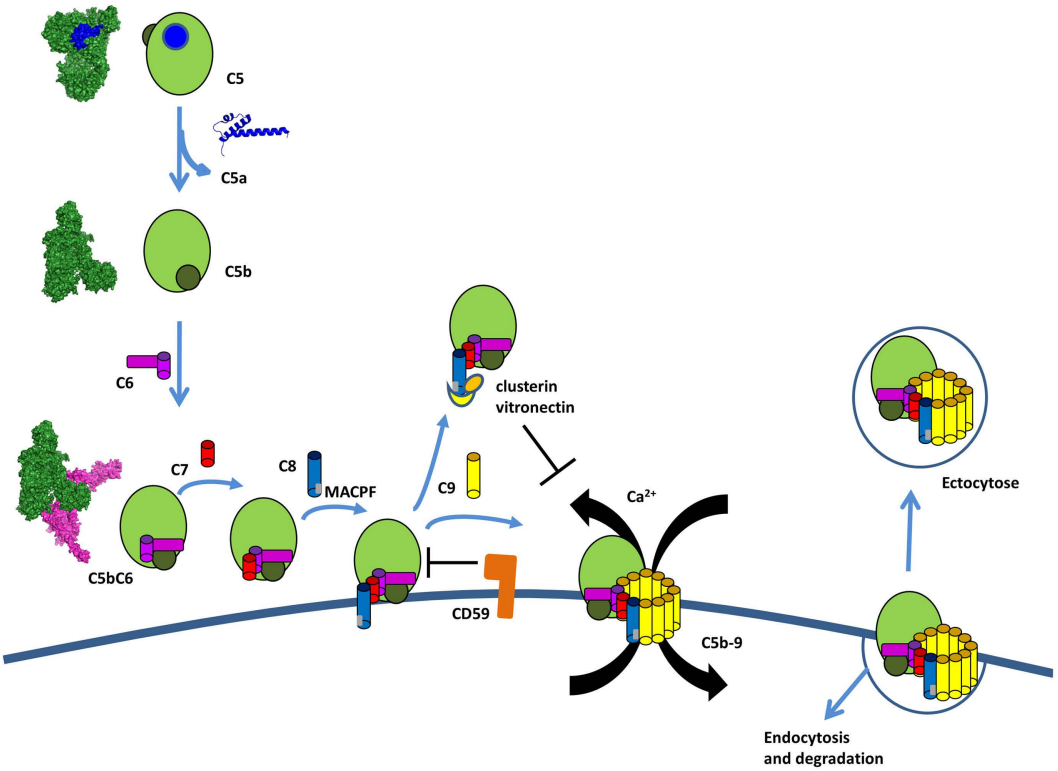

FIGURE 12 | The terminal complement pathway. The C5 convertase cleaves an inert molecule of C5 into a potent anaphylatoxin, C5a, and a bioactive fragment C5b. C5b interacts with C6, C7, C8, and multiple copies of C9 to form the membrane attack complex C5b-9 (MAC). C5b-8 inserts into the membrane and C9 polymerize to form a pore inducing Ca flux and pathogen lysis. Host cells are protected from lysis by expression of CD59, which prevents the insertion and by clusterin and vitronectin, which bind to C8 and prevents insertion in the membrane. If MAC is bound to the membrane, host cells can internalize it or remove it by ectocytose.

C3c-like, and C3dg-like) are similar, but not identical to fragments generated by the complement cascade and are biologically active.

Membrane-Attack-Complex C5b-9 Formation

Activation of the terminal complement pathway results in formation of MAC that form large, 10 nm wide, pores in the target membrane (207). These complexes are formed when C5 is cleaved into C5b by the C5 convertase. Upon cleavage, C5b undergoes a dramatic conformational change, similar to C3b, but with a TED domain ending up only halfway the distance to the MG ring (**Figure 12**) (224). This conformation of C5b interacts with C6, which wraps around the TED domain of C5b. C6 to C9 are homologous proteins that share similar central membrane-insertion domains called MACPF. Binding of C7, C8, and multiple copies of C9 results in MAC formation with C5b–C6–C7–C8β–C8αγ–C9 making an arc with a protrusion at the beginning by C5b. C5b-7 is lipophilic and binds to the cell membrane (225). C8 is the first component to penetrate the lipid bilayer upon interaction with the forming MAC. The structure of the MACPF domain of C8α (226) shows homology to perforin, a pore-forming protein released by cytotoxic T and NK cells, as well as, to bacterial cholesterol-dependent cytolysins. This resemblance suggests a common membrane perforation mechanism for MAC, perforin in the mammalian immune system, and bacterial pore-forming proteins (226). A single MAC contains up to 18 C9 molecules forming a tubular channel. However, only one or two C9 molecules are sufficient to form functional pores (227, 228).

Each functional MAC is sufficient to lyse by colloid osmosis in the membrane of metabolically inert cells, like erythrocytes or liposomes (229). Gram-negative bacteria are also susceptible to complement killing, in particular the meningitis causing *Neisseria* species (230, 231). Individuals deficient in terminal complement components are at increased risk for recurrent meningitis. Gram-positive bacteria have an extremely thick cell wall that MAC cannot penetrate leaving them resistant to complement elimination. Metabolically active nucleated cells are also resistant to lysis by complement (228, 232). In order to induce killing in these cells, multiple MACs must be inserted in the cell membrane together with coordination of calcium flux and not well-understood signal transduction (233). Once MACs have inserted in these cells, calcium flux is induced in the pore from the extracellular space or is released from intracellular stores (234). Subsequently, multiple still not well-known signaling pathways are activated leading to cell proliferation or apoptosis, which is dependent on the targeted cell type and experimental conditions.

Membrane-Attack-Complex Regulation

Membrane-attack-complex formation is tightly regulated to avoid accidental host cell damage and activation (**Figure 12**). C8 was suggested to play a dual role in MAC formation and regulation. In the absence of a cell membrane, the binding of C8 to C5b-7 induces conformation changes that result in a loss of ability to form pores, causing it to act as a MAC inhibitor (235). If soluble dead-end products sC5b-7, sC5b-8, and sC5b-9 are generated in fluid phase and do not bind to a membrane, they are scavenged by clusterin and vitronectin. These two regulators bind below the C5b-9 arc rendering it water soluble and preventing membrane binding (224, 236–238).

The GPI anchored protein, CD59 is expressed on most cell types (239, 240) and blocks membrane perforation by C5b-8 and C5b-9 (241, 242). CD59 does not bind free C8 and C9, but does interact with the MACPF domain of each protein upon conformational changes associated with C5b-9 complex formation (243–245). Furthermore, the lytic terminal complex of complement C5b-9 can be removed within minutes of its deposition on the membrane of target cells either by shedding via membrane vesicles (exocytosis) or by internalization and degradation (246–249).

Complement Anaphylatoxins

The anaphylatoxins, C3a and C5a, are constantly released during complement activation. These small (10–14 kDa) peptides play a critical role in supporting inflammation and activation of cells that express anaphylatoxin receptors (250). To enhance inflammation, anaphylatoxins recruit immune cells to the site of complement activation and induce oxidative bursts on macrophages (251, 252), eosinophils (253), and neutrophils (254, 255). However, some studies challenged the concept for the pro-inflammatory role of C3a. C3a has a more complex function, depending on the context, with a balance between pro- and anti-inflammatory roles. The highlight anti-inflammatory properties of C3a re-evaluate its physiological role during inflammation (256). Furthermore, C3a and C5a induce histamine production by basophils (257, 258) and mast cells (259) to provoke vasodilatation. C5a also recruits T-cells (260) and myeloid-derived suppressor cells (261) that constitutively express C5aR. Although the functional activity of C4a is debated, it has been reported to activate macrophage and monocytes (262, 263). However, a lack of cognate C4a receptor identification and unreproducible data (264) warrant further studies to determine the physiological role of C4a.

Structural data show that both human C3a and C5a adopt an alpha-helical conformation with four- and three-helical bundles, respectively (**Figure 13**). The C5a crystal structure has a core domain constituted as an antiparallel alpha-helical bundle and the C-terminal domain links the core domain by a short loop containing two adjacent arginines in position 62 and 74 (265) that both interact at the same binding site on the receptor. In human plasma, these two fragments are rapidly converted by carboxypeptidase N to C3a desArg and C5a desArg by cleavage at the C-terminal arginine (266, 267). C3a desArg has a very similar structure to C3a (268), but is incapable of binding to C3aR. The alpha1-helix of C5a desArg is detached at the three others alpha-helices (269). Due to this conformational change, human C5a desArg has 90% weaker pro-inflammatory activity compared to C5a (270). In contrast, murine C5a desArg is as potent as the murine C5a upon binding to C5aR on murine cells (271), which could be explained by the lack of major structural changes in the murine C5a desArg, compared to C5a. Both murine proteins form a four-helix bundle, contrary to the human C5a desArg, which adopts a three-helix bundle conformation upon cleavage of the terminal Arg residue. These inter-species differences need to be taken into account during analysis of *in vivo* experiments.

C3aR, C5aR, and C5aL2 belong to the G protein-coupled receptor (GPCR) family that contain seven transmembrane domains that are able to interact with C3a and C5a (**Figure 13**). The C3a-binding site of C3aR is located in the large second extracellular loop that contains a sulfotyrosine 174, which is critical for C3a docking (272). This interaction induces phosphorylation of intracellular pathways including PI3K, Akt, and MAPK leading to chemokine synthesis (273).

There are two sites in C5aR that are essential for C5a binding. The first sight consists of basic residues from human C5a that interact with sequences rich in aspartic residues located on N-terminal extracellular domain of C5aR (274). The second site is in a binding pocket located near the fifth transmembrane domain (275) and interacts with the C-terminal region of human C5a (276). Then, two distinct clusters of hydrophobic residues allow a molecular switch in C5aR leading to G protein activation (277). This mechanism exposes preserved residues clustered in two intracellular and two transmembrane domains that participate to the initial interaction with G proteins (278). The second extracellular loop plays a role of a negative regulator of C5aR activation and may stabilize the inactivated form of the receptor (279). By binding human C5a, C5aR induces downstream effects including activation of PI3K-γ (280, 281), phospholipase Cβ2 (282), phospholipase D (283), and Raf-1/B-Raf-mediated activation of MEK-1 (284).

Human C5a and C5a desArg are also able to bind C5L2 (285). C5L2 is again composed of seven transmembrane domains, however it is not coupled with G protein due to an amino acid alteration at the end of the third transmembrane in the DRY sequence (286). C5a has lower affinity to C5L2 compared to C5a desArg (287) and recently it has been demonstrated that C5L2 is a negative regulator of anaphylatoxin activity (288). It has also been reported that C5L2 and C5aR form a heterodimer (289) and this complex induces internalization of C5aR upon C5a binding (290). This internalization is essential for the induction of the late stage of ERK signaling (291, 292).

Complement Receptors for C3 Activation Fragments

Complement participates actively in the opsonization of pathogens and dying host cells, in addition to the clearance of immune complexes. Recognition molecules in the CP and LP, as well as cleavage fragments of C3, opsonize the target structure and serve as bridging molecules with receptors on the surface of the phagocytes. Depending on the type of the opsonin present (C3b, iC3b, or C3d), the phagocyte will generate a pro-inflammatory response or tolerogenic suppression.

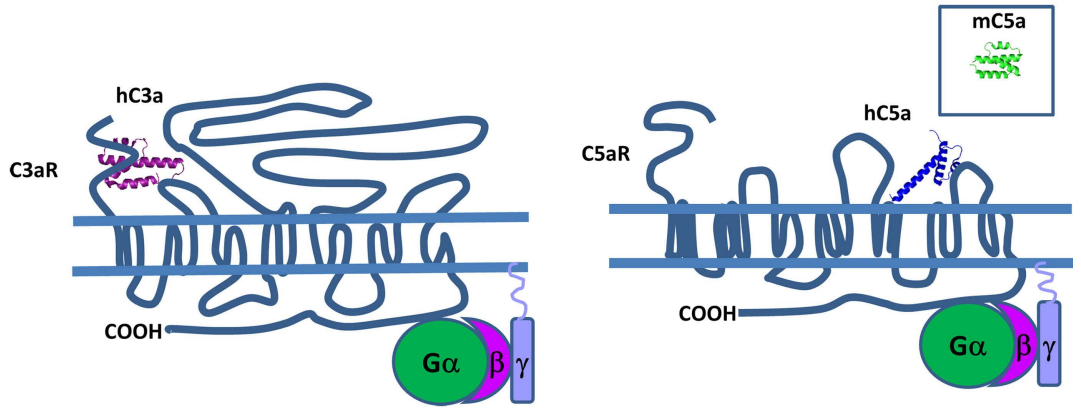

FIGURE 13 | Complement anaphylatoxins. C3a and C5a have a four and three helical bundle topology. Mouse C5a (in the square) is different from its human counterpart, because it has four helical bundle structure. These anaphylatoxins bind to G protein-coupled receptors C3aR and C5aR and stimulate pro-inflammatory signaling pathways.

Pathogens, immune complexes, and cell debris opsonized by C3 cleavage fragments can be recognized by CR (**Figure 14**) with three different structural organizations: containing CCP modules (CR1 and CR2), integrin family members (CR3 and CR4), and the immunoglobulin superfamily member (CRIg) (293). CR1 is expressed on monocytes, macrophages, neutrophils, erythrocytes, and renal podocytes, CR2 is found on B-cells, CR3 and CR4 are expressed by macrophages, monocytes, dendritic cells, neutrophils, and NK cells and CRIg has restricted expression and is found mainly on Kupffer cells in the liver and resident tissue macrophages (294). Interestingly, the expression of CRIg on macrophages in inflamed tissue is lower compared to macrophages outside of an inflammatory area (295).

Complement Receptor 2

Complement receptor2 (CD21) is expressed on B-cells interacting with C3d and iC3b on the surface antigens (**Figure 14**) forming a co-receptor complex with CD19 and CD81 (296). C3d serves as a molecular adjuvant by lowering the threshold for B-cell activation by 1000–10,000-fold (297). The TED domain of C3 has a completely different conformational environment in the native protein as compared to its degradation products C3b, iC3b, C3dg, and C3d. Recent studies showed that the binding site for CR2 in iC3b and C3d lies within the common TED domain (298). The C3d-binding site is located in the two N-terminal CCP domains of CR2 (299). Two different crystal structures had been proposed for the complex CR2:C3d; the first one, described in 2001 by Szakonyi et al. (300), showed that only the CCP2 domain of CR2 interacts with C3d. Contrary to this result, biochemical studies showed that mutations on several basic residues on CCP1 domain affected C3d binding to CR2 (301). In 2010, a second structure was proposed (302) in agreement with the mutagenesis data (301) where both CCP1 and CCP2 are involved in the interaction (**Figure 14**). One possible explanation for the discrepancy between structures could be due to the high concentration of zinc in the crystallization buffer from 2001 leading to a non-physiological complex.

Integrin Family Complement Receptors CR3 and CR4

Integrin family CR3 (also known as CD11b/CD18, $\alpha_M\beta_2$ or Mac-1) and CR4 (also known as CD11c/CD18 or $\alpha_X\beta_2$) are heterodimeric transmembrane complexes, composed of a unique α-chain and a common β-chain. They bind multiple ligands participating in phagocytosis, cell adhesion to the extracellular matrix, leukocyte trafficking, synapse formation, and co-stimulation. Ligand binding and signaling through integrin receptors is governed by a complex cascade of conformational changes, known as inside-out signaling (303). A receptor in its inactive, bend-closed conformation can respond to a cytoplasmic signal, transmitted inside-out through the β-chain, passing to a low affinity binding extended-closed and high affinity binding extended-open conformation. Upon ligand binding, another signal is transmitted outside-in, leading to raid cellular response, including actin remodeling, phagocytosis, degranulation, or slow responses involving protein neosynthesis. CR3 and to lesser extent CR4 are essential for phagocytosis of C3 fragments, opsonized immune complexes, and pathogens (304). CR3 and CR4 differ in their profile of recognized C3 fragments because both receptors bind to iC3b, but CR3 recognizes C3d, while CR4 binds to C3c, suggesting that the two receptors have distinct binding sites on the iC3b molecule (**Figure 14**) (305, 306). The iC3b and C3d binding site of CR3 is located in the VWF-A domain of the α-chain, also called αI domain, and binds in a divalent metal ion-dependent manner (307). This type of domain is present also in FB, which requires divalent ions (Mg^{2+}) in order to interact with C3b. However, the two VWF-A containing molecules do not bind to the same fragments of C3b (113, 305). FB interacts only with C3b, while CR3 binds to iC3b and C3d and the binding sites for these VWF-A domains are distinct (**Figure 14**). VWF-A domain of CR3 binds to the TED domain (C3d part) of iC3b and the CUB and C345C domains of iC3b may also contribute to the interaction with the β-propeller and βI domains of the α-chain of CR3 (305).

In contrast to CR3, CR4 binds to the C3c portion of iC3b (**Figure 14**). The architecture of the complex has only been observed at low resolution, by electron microscopy and displays

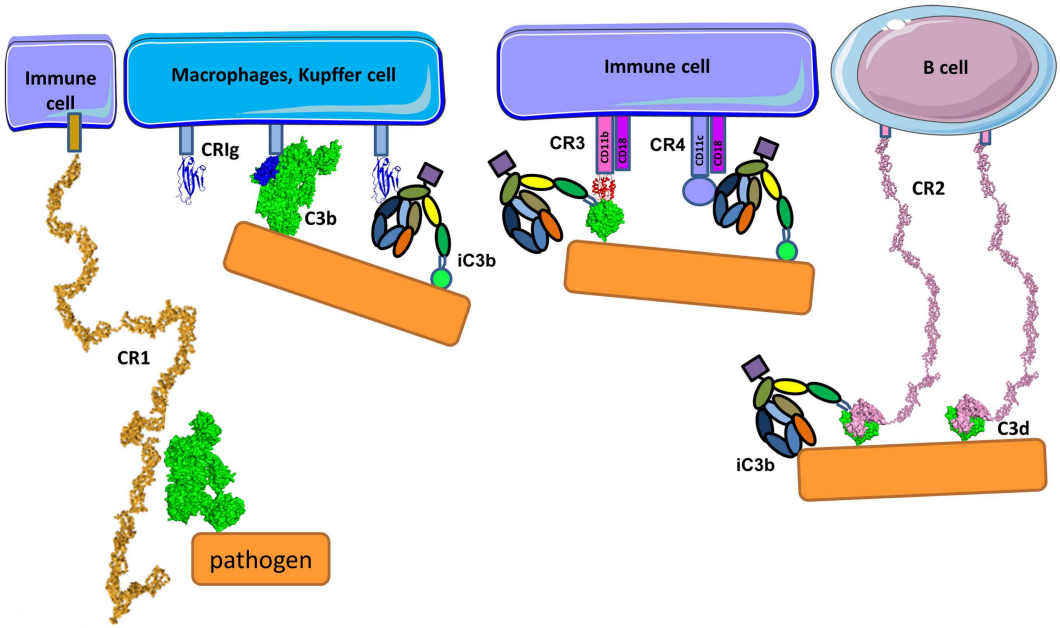

FIGURE 14 | Complement receptors. CR1 is composed of CCP domains and is expressed primarily by immune cells and erythrocytes. Apart from being cofactor of FI, CR1 is also a complement receptor facilitating immune complex clearance and phagocytosis. CR1 interacts with C3b. CRIg has immunoglobulin-like structure in its C3b recognition domain. CRIg binds to C3b and iC3b and is expressed on macrophages and Kupffer cells. Immune cells also express CR3 and CR4 containing integrin domains that bind to iC3b (and C3d for CR3) on different binding sites on iC3b molecule. CRIg, CR3, and CR4 facilitate phagocytosis and modulate the activation state of cells. CR2 is expressed primarily on B-cells and recognizes C3d using the first two CCP domains. It serves as a co-stimulatory molecule for the B-cell receptor upon binding C3d-opsonized pathogen.

a CR4 binding site at the interface between MG3 and MG4 (306). The flexibility of the CUB domain after cleavage by FI allows re-orientation of the C3c part of iC3b leading to better exposure of the MG3–MG4 interface (9, 308). Initial studies of the structure and molecular details of the complex conformational changes in CR4 are starting to be elucidated (309) showing that CR4 binds iC3b through the αI domain on the face known to bear the metal ion-dependent adhesion sites similar to CR3 (306).

Immunoglobulin Superfamily Receptor CRIg

Immunoglobulin superfamily receptor CRIg is a CR expressed on macrophages and Kupffer cells in the liver that binds to C3b and iC3b (**Figure 14**) and mediates the phagocytosis of opsonized particles and pathogens (310, 311). CRIg acts as an inhibitor of the AP preventing the entry of the substrate molecule C3 and C5 into the C3 convertase (312). The CRIg-binding site on C3b is located in the MG ring of the β chain, engaging MG3 and MG6 domains. A conformational change in MG3 during the transition of C3 to C3b contributes to the formation of the CRIg-binding site and explains why CRIg binds to C3b and iC3b, but not to intact C3.

Understanding Complement-Related Diseases Using Structure–Function Relationships

The importance of the complement system in physiology is illustrated by the severe and life threatening diseases occurring due to inefficient or excessive complement activity. Abnormal complement activity is associated with many inflammatory, autoimmune, neurodegenerative, and age-related diseases. Here, we will describe the role of complement dysfunction in aHUS.

Atypical Hemolytic Uremic Syndrome

The aHUS is a rare thrombotic microangiopathy that predominates in the kidney. Without appropriate treatment, it leads to end stage renal disease in approximately 60% of patients (313, 314). This thrombotic microangiopathy is different than typical HUS and thrombotic thrombocytopenic purpura because it is not associated with infection by Shiga toxin-producing bacteria or ADAMST13 deficiency, respectively. aHUS occurs at any age and has a poor prognosis (prior to the development of Eculizumab). In contrast, typical HUS is predominantly a pediatric disease and has a favorable renal outcome. aHUS is characterized by a triad of hemolytic anemia due to fragmented erythrocytes, thrombocytopenia, and acute renal failure. Renal failure is a result of platelets rich microthrombi, formed in the small vessels (capillaries and arterioles) of the kidney resulting in a prothrombotic state. The hallmark of the aHUS is the association with alternative complement pathway mutations (**Figure 15A**). Endothelial damage is known to be related to complement dysregulation. Screening for and characterization of mutations in the components of the C3 convertase (C3 and FB), its regulators (FH, FI, MCP, CFHR1, and thrombomodulin), or anti-FH antibodies has become an indispensable part of the diagnostic of the disease (315).

The importance of screening for mutations in complement factors can be observed in the examples of FH and C3. The majority of the mutations in FH found in aHUS patients does not induce protein quantitative deficiency and are located in the C-terminus of the protein (CCP19–20). These mutations affect either the interaction with C3b, GAG, or both ligands leading to impaired cell surface protection against complement attack [(175, 177, 316); summarized in Ref. (191, 315, 317, 318)]. Mapping of these disease-associated mutations on the complex of C3d with FH19–20 (130, 131, 174) revealed that the residues which decreased C3b-binding mapped to the C3b/C3d-binding site of FH in CCP19. The residues that affected both C3b and GAG interaction were located in the FH CCP20 interface (**Figure 15B**). These structural analyses help to explain the mechanism by which genetic abnormalities in FH induce impairment of C3b and/or GAG binding and hence a predisposition to develop aHUS. A similar phenomenon is observed with C3 mutations found in aHUS. These genetic changes are not randomly distributed but cluster alongside FH CCP1–4 and CCP19–20 binding sites (**Figure 15C**) (142, 315, 319, 320). Functional analysis revealed that mutations located in the FH-binding sites resulted in decreased FH binding, thus showing the link with aHUS. Currently, there is a debate as to whether or not in physiological conditions FH CCP20 can interact with an adjacent C3d molecule (130, 131, 174). It is possible the observed interaction between the second molecule C3d and FH CCP20 may be a crystallization artifact and in turn the functional consequences of certain aHUS associated FH and C3 mutations will be more difficult to explain. However, these mutations fall within this suggested C3b/C3d binding site and indeed decrease FH/C3b interaction. This case exemplifies how structural analyses can aid in understanding disease physiopathology and how disease physiopathology improves our understanding of complement.

Disease-associated mutations also has resulted in the mapping of the MCP binding site on C3b. C3 mutations that decreased MCP binding mapped in an area overlapping with the FH CCP-3–4-binding site (142). However, these mutations did not affect CR1 binding suggesting that the CR1 binding site is distinct and that CR1 is not involved in aHUS (142). Experimental and structural analysis revealed that for FH and C3 almost all studied genetic changes mapped to the ligand binding site and had clear functional consequences. aHUS mutations in FB seem to be exception of this rule. Mutations in FB were located in multiple

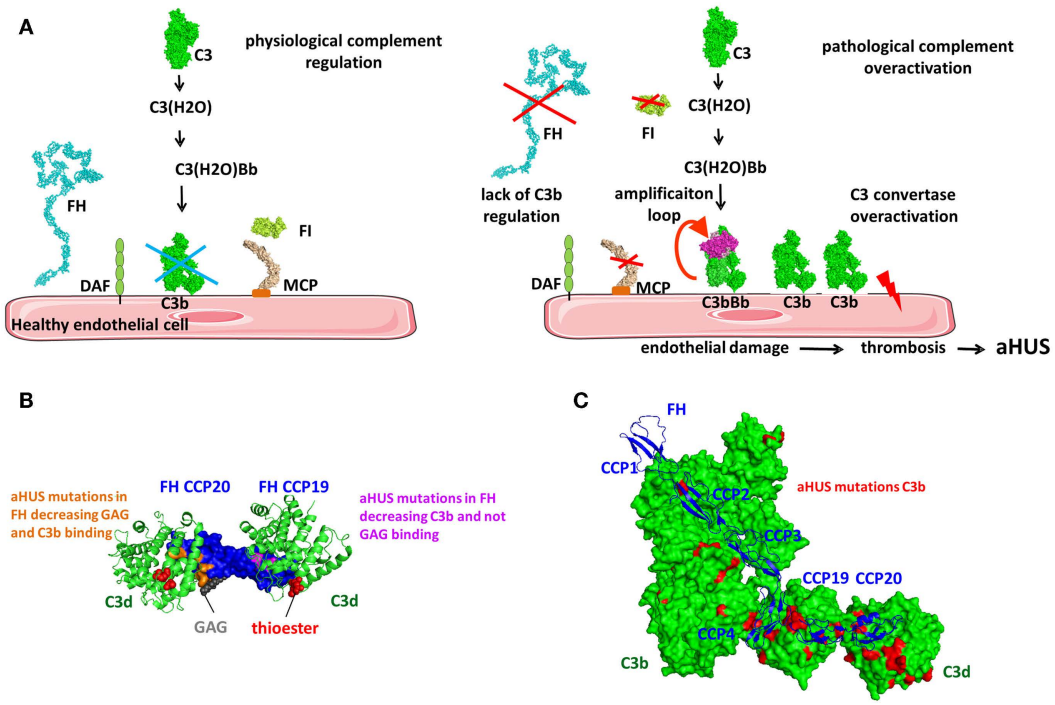

FIGURE 15 | Understanding aHUS using structure–function relationships. (A) The role of complement alternative pathway in the physiopathology of aHUS. On healthy endothelial cells, deposited C3b is rapidly inactivated by regulatory molecules including FH, MCP, and FI. For FB binding and C3 convertase formation, FB is dissociated by FH and DAF preventing excessive host tissue damage. Mutations in the complement regulators FH, MCP, and FI can result in inefficient complement regulation. Mutations in the components of the C3 convertase (C3 and FB) induce the formation of overactive C3 convertase or a convertase that is resistant to regulation. In both cases, the complement cascade is activated on glomerular endothelial cell surface leading to endothelial damage, thrombosis, erythrocyte lysis, and aHUS. **(B)** FH mutations in the CCP19–20 region are mapped on the structure of the C3d–C3d–FH19–20 complex. A model GAG bound to FH CCP20 is indicated in gray. FH disease-associated mutations that decrease only C3b-binding are indicated in orange and mutations decreasing both C3b and GAG binding are in magenta. Reduced C3b and/or GAG binding will cause inefficient endothelial cell protection and complement overactivation. **(C)** C3 mutations found in aHUS patients. The majority of the mutations (in red) are not randomly distributed, but mapped to the FH binding sites on C3b. These mutations correlated with decreased FH and MCP binding allowing characterization of the MCP binding site, which overlaps with the FH binding site in CCP-3–4.

domains of the protein and in more than half of the cases they were far from any known binding sites (321). Mutations located within the C3b-binding site did induced formation of an overactive C3 convertase or a convertase resistant to regulation (321–323). The mutations far from this binding interface showed no functional defect as observed by FB functional tests (321). As shown for the majority of complement mutations in aHUS, mapping of disease-associated mutations together with detailed functional analysis should be performed to understand the mechanism of complement dysregulation associated with a disease.

Structural Basis of Therapeutic Complement Intervention

We have described in depth the known molecular mechanisms of the complement system and this unleashes many possibilities for rational design of complement inhibitors for treatment of disease (324, 325). Here, we will give a few examples that illustrate this concept. Blockade of the late effector functions of complement can be obtained if the cleavage of C5 by the two C5 convertases is prevented. The therapeutic monoclonal antibody, Eculizumab, targets human C5 and blocks cleavage by C5 convertases (326). The Eculizumab binding epitope on C5 interacts with the contact interface between C5 and the C5 convertase (211) preventing the entry of C5 into the C5 convertase and blocking further cleavage and generation of the bioactive fragments C5a and C5b-9. Eculizumab blocks the terminal complement pathway but leaves the C3 convertases unaffected. Eculizumab showed significant improvement in clinical outcome and has been accepted for treatment of complement-mediated diseases including paroxysmal nocturnal hemoglobinuria (PNH) (327) and aHUS (328, 329) and clinical trials are ongoing for other diseases.

In order to control complement at an earlier step, inhibitors acting at the level of C3 have also been designed. Compstatin is a 13-residue peptide that is being tested pre-clinically. It binds to C3 and blocks its cleavage (330, 331). Compstatin binds to MG4 and MG5 of C3c and C3b where it undergoes a large conformational change upon interaction (332) causing steric hindrance of the substrate C3 to the convertase complexes and blocking complement activation and amplification. Compstatin has shown efficacy in complement blocking *in vitro* and in animal models including extracorporeal circulation (333), sepsis (334), and PNH (335). Both Compstatin and Eculizumab are species-specific and act only in humans and monkeys, but not in mice or rats, reflecting subtle differences in the structure of human and murine complement components.

Another strategy of rational design of complement inhibitors is to target the regulatory domains of FH or CR1 to the cell surface via potent C3b, iC3b, C3d, or membrane recognition domains, derived from FH or CR2. Mini FH molecules, containing CCP1–4 and CCP19–20 bind C3b and C3d with high affinity and show better efficacy compared to native FH in *in vitro* models of aHUS (336) and PNH (337). A hybrid molecule TT30, containing FH CCP1–5 and CR2 CCP1–4 is designed to accumulate preferentially at sites already under complement-mediated attack (338). TT30 interacts simultaneously with C3b and C3d merging the functionality of fluid phase FH binding to C3b with CR2 interaction to C3d on the surface of host cells (339). TT30 and its murine analog showed significant improvement in models of AMD, ischemia/reperfusion injury, and PNH (340–342).

Conclusion

Currently, we know that complement is not only a simple lytic system, but rather a powerful innate immune surveillance tool, serving as a sentinel against pathogens, modulator of the adaptive immune response, and as a regulator of host homeostasis. This cascade of enzymatic reactions is driven by conformational changes induced after a recognition event assuring that complement will be activated only when and where needed. This special and temporal control of complement activation is guaranteed also by the high specificity and selectivity of the enzymatic reactions, where the involved enzymes cleave only a single substrate and have a single ligand-binding site. In contrast to this high specificity of the propagation of the chain reaction, complement activation relies on target patterns binding by versatile recognition molecules, such as C1q, MBL, ficolins, and properdin. The activation of the three complement pathways leads to the generation of C3b, the Swiss army knife of complement, which interacts with a large variety of ligands and receptors with multiple distinct binding sites. The balance of these interactions determines whether full-blown activation will occur by the amplification loop of the complement pathways, with a generation of one of the most potent inflammatory mediators C5a or the effect will be attenuated by the C3b breakdown cycle. Again, the attenuation relies in large part on the capacity of a versatile recognition molecule FH to discriminate between self and non-self and to stop the amplification loop. C3b and C3(H_2O) may bind to the cell surface via proteins-platforms, like properdin and P-selectin. Expression/binding of these molecules accelerates the complement activation and anaphylatoxin generation. The anaphylatoxins increase the expression/binding of P-selectin and properdin contributing to a vicious circle of complement activation, enhancing local inflammation, thrombosis, and tissue damage.

The knowledge of these molecular mechanisms that has been accumulated during recent years has allowed for better understanding of complement-related diseases. It also opens up the possibility for a rational design of novel molecules with therapeutic potential to control steps in the complement cascade. Clinical application of the anti-C5 blocking antibody Eculizumab has already demonstrated that controlling complement can revolutionize the treatment of patients with overactive complement-mediated diseases.

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Complement system part II: role in immunity

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The complement system has been considered for a long time as a simple lytic cascade, aimed to kill bacteria infecting the host organism. Nowadays, this vision has changed and it is well accepted that complement is a complex innate immune surveillance system, playing a key role in host homeostasis, inflammation, and in the defense against pathogens. This review discusses recent advances in the understanding of the role of complement in physiology and pathology. It starts with a description of complement contribution to the normal physiology (homeostasis) of a healthy organism, including the silent clearance of apoptotic cells and maintenance of cell survival. In pathology, complement can be a friend or a foe. It acts as a friend in the defense against pathogens, by inducing opsonization and a direct killing by C5b–9 membrane attack complex and by triggering inflammatory responses with the anaphylatoxins C3a and C5a. Opsonization plays also a major role in the mounting of an adaptive immune response, involving antigen presenting cells, T-, and B-lymphocytes. Nevertheless, it can be also an enemy, when pathogens hijack complement regulators to protect themselves from the immune system. Inadequate complement activation becomes a disease cause, as in atypical hemolytic uremic syndrome, C3 glomerulopathies, and systemic lupus erythematosus. Age-related macular degeneration and cancer will be described as examples showing that complement contributes to a large variety of conditions, far exceeding the classical examples of diseases associated with complement deficiencies. Finally, we discuss complement as a therapeutic target.

Keywords: complement system, adaptive immunity, crosstalk TLR-complement, pathogen strategies for immune evasion, complement in cancer, complement and innate immunity, complement-related diseases, anaphylatoxins

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The Complement System

Complement system represents a major part of the innate immunity. It is a cascade of soluble proteins and membrane expressed receptors and regulators (**Figure 1**), which operates in plasma, in tissues, on cell surface, and even within the cell. It is composed of more than 40 proteins, the soluble ones being produced mainly by the liver. Complement was discovered at the end of the nineteenth century and described as a "factor" or "principle" capable to induce bacterial lysis.

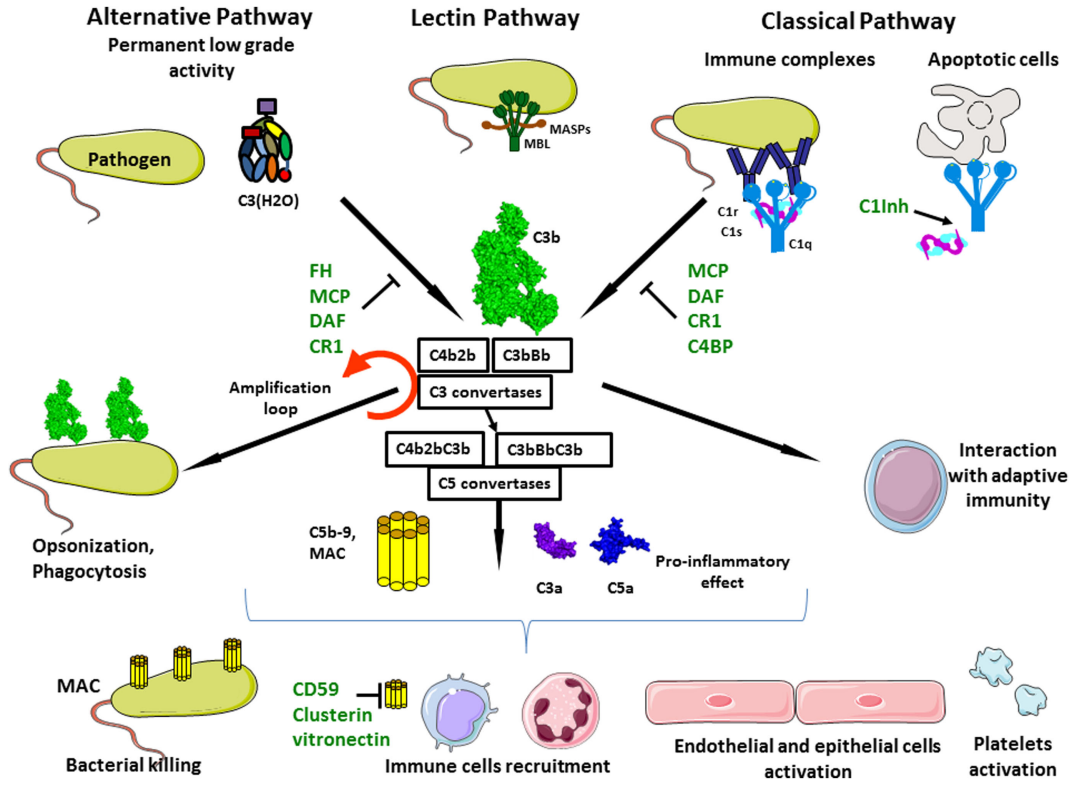

FIGURE 1 | Complement activation. Complement system is composed of three different pathways. CP is activated by immune complex formation on pathogen surface and by calreticulin expressed on apoptotic cells, leading to C1 complex association. LP recognizes mannose-terminating glycan on pathogens leading to MBL MASP complex activation. Both induce formation of the classical C3 convertase C4b2a. AP is permanently activated at low level by spontaneous hydrolysis of C3 into C3(H_2O). Lack of complement inhibitor on pathogens induces alternative C3 convertase activation C3bBb. Complement activation leads to opsonization and phagocytosis by C3b deposition, bacterial lysis by C5b–9 complex formation and inflammation by recruitment of immune cells, endothelial and epithelial cells activation, and platelets activation.

After that, for a long time, complement system has been considered as a supportive part of the innate immunity and received relatively little attention from the immunologists. Over the years, it became clear that complement has versatile functions and that its action extends far beyond the simple bactericidal activity. In a healthy individual, it orchestrates the immunologically silent clearance of host cells after their programed cell death. Complement cascade is activated immediately after encountering the pathogen. Hence, complement participates in pathogen opsonization, tagging it for engulfment by antigen presenting cells (APC); it plays a central role in the inflammatory process and modulates the activity of T- and B-cells. After generation of pathogen-specific antibodies, complement contributes in the clearance of immune complexes and pathogen elimination. Studies over the years demonstrated that complement takes part in nearly every step of the immune reaction and that it deserves a central position in the immunological research. Unfortunately, the lack of coherence in complement proteins nomenclature and the complexity of the enzymatic cascade render complement one of the "most complicated and incomprehensible" parts of immunology and is frequently avoided by students and scientists. With this review, we aim to underline the crucial importance of complement in physiology and pathology.

Complement in Physiology

Sampling for Foreign and Altered Cells

The main complement rule is that everything that is not specifically protected has to be attacked. Host cells carry an armamentarium of "don't attack me" molecules, which are either expressed by the cell or recruited to the cell membrane from the plasma. Therefore, any cell, debris, microorganism, or artificial material lacking these molecules (and carrying $-OH$ or $-NH_2$ chemical groups, which is the case of all biological and most synthetic materials) will represent an "activating surface" and will support complement deposition, i.e., covalent binding of C3b, an activated form of the central complement component C3. This action is provided by the so-called alternative complement pathway, which is permanently active and constantly probes the environment for the presence of activating surfaces (**Figure 2A**). In addition, several pattern recognition molecules recognize material that has to be eliminated, such as apoptotic cells, debris, pathogens, immune complexes, and activate the classical and lectin complement pathways (CP and LP) (**Figure 2B**). All pathways converge at the point of C3 cleavage, which results in generation of bioactive fragments C3a and C3b. C3b binds covalently to any surface and, if it is not specifically protected. The level of C3 deposition is increased by the so-called

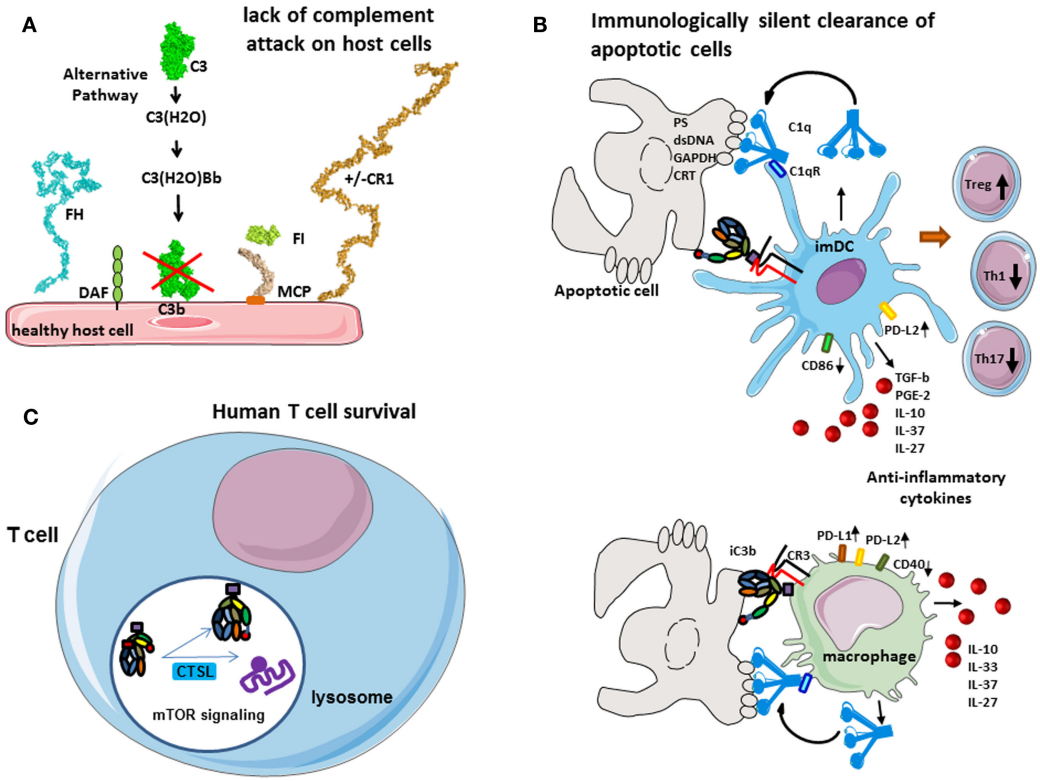

FIGURE 2 | Role of complement in physiology. **(A)** Protection of host cells against complement. Complement AP is permanently activated and deposits C3b molecules to any surface. On host cells, these C3b molecules are rapidly inactivated by different membrane expressed or plasma complement regulators. **(B)** Immunologically silent clearance of apoptotic cells. Limited complement activation occurs on apoptotic cells. C1q recognizes "eat-me" signals on the surface of dying cells. It serves as a bridging molecule, facilitating the phagocytosis by dendritic cells or macrophages. Uptake of C1q-opsinized cargo induces an anti-inflammatory program, increased expression of immunological checkpoint molecules, and prevents up-regulation of maturation markers. iC3b on apoptotic cells interacts with CR3 on phagocytes and induces anti-inflammatory response. **(C)** Complement in human T cell homeostasis. Cathepsin L cleaves C3 intracellularly, generating C3a and C3b. C3a binds on C3aR, expressed in the lysosomes, and stimulates mTOR signaling pathway. This signaling is important for the cell survival in resting state.

"amplification loop" of the alternative pathway (AP). Detailed description of the mechanisms of activation and regulation of the complement system are described in "Complement system part I – molecular mechanisms of activation and regulation" (1), from the same research topic of Frontiers in Immunology.

Briefly, in a healthy organism, the sentinel role is assured by the permanent activity of the AP (1 and 2). A number of complement components are produced in biologically inactive form, which allows them to co-exist in plasma, or to be produced by the same cells, without interacting with one another and inducing unnecessary and undesired complement activation. Complement components are activated in a cascade fashion after a triggering event, each step of the chain reaction, resulting in a conformational change or a cleavage of the downstream component, which becomes activated and gains the capacity to activate the subsequent component in the cascade. Spontaneous hydrolysis, called tick-over, of C3 plays an important role in the immune surveillance and AP activation (2). C3 is present in plasma at high concentrations (~1 mg/ml) and a small portion of it undergoes spontaneous hydrolysis of a particular thioester bond between the side chains of two aminoacids, located in the thioester domain (TED). This hydrolysis induces a dramatic conformational change in C3 and renders it biologically active due to exposure of novel binding sites. In this new form – the C3(H_2O), it recruits two other plasma molecules – factor B (FB) and factor D (FD). FD cleaves C3(H_2O)-bound FB to generate an enzymatic complex C3(H_2O)Bb, called fluid phase C3 convertase. The name convertase indicates that this enzymatic complex can cleave (convert) a native C3 molecule into bioactive fragments C3a (the small fragment) and C3b (the big fragment). C3a is an anaphylatoxin – a pro-inflammatory molecule, which activates the surrounding cells when reaching a threshold concentration. Upon releasing the C3 ANA domain, which becomes the C3a molecule, the remaining part C3b undergoes a dramatic conformational change, similar to that of C3(H_2O). In the newly generated C3b, contrary to C3(H_2O), the thioester bond is not hydrolyzed but becomes transiently exposed, allowing, for a very short time, a covalent reaction with OH^- or $NH2^-$ groups on any molecule or cell in its immediate surroundings. If this bond is not formed, the very short-lived nascent form is hydrolyzed and inactivated in few milliseconds, leaving the inactivated C3b molecule in the fluid phase. When covalently bound to a cell, C3b has different fates depending if it is on a host cell or on a foreign surface.

Protection of the Host Cells Against Complement Attack

The deposition of C3b is highly controlled on host cells. Host cells express complement regulatory molecules on their surface (membrane bound such as membrane cofactor protein, MCP, CD46 or complement receptor 1, CR1, CD35), or recruit plasma regulators, like factor H (FH), which bind to C3b. These proteins serve as cofactors, allowing interaction with a plasma serine protease factor I (FI), which cleaves C3b into iC3b (**Figure 2A**). This results in a conformational change, which suppresses the ability to interact with FB but exposes of novel binding sites in the iC3b molecule, allowing it to interact with other complement molecules. FH also blocks the binding of FB and FD to C3b, thus preventing the formation of C3 convertases. The overall action of all these regulators results in the prevention of complement activation on host cells. In the absence of surface regulators, such as in the case of pathogens or other foreign surface, C3b interacts with FB and FD, forms C3 convertases, which cleave more molecules of C3 to C3a and C3b, thus fueling the amplification loop and allowing full-blown complement activation and, finally, pathogen elimination.

Immunologically Silent Phagocytosis of Apoptotic Cells

Between these two extremes (healthy cell and pathogen) remains the case of stressed and apoptotic host cells. The human body is composed to a myriad of different cells, forming a highly organized system. The proliferation, differentiation, activity, and also the death of cells in this system are tightly controlled. Programed cell death induces major cellular modifications. During a classical cell death, the cell surface undergoes many structural and molecular modification, leading to "eat-me" signals expression. Phagocytes recognize these signals and execute the degradation of apoptotic cell without mounting of an immunologic response. Among these modifications, a major one is the expression of phosphatidylserine (PS) on the external side of the cell membrane, which is normally sequestered in the inner surface of the cell membrane (3). Also, the expression level of some complement regulators (such as MCP) can be reduced. Clearance of apoptotic cells is critical for many physiological processes, including development, tissue remodeling, and maintenance of homeostasis.

The complement system plays a major role in the tolerogenic perception of apoptosis, which is in part mediated by opsonization with C1q and iC3b and subsequent clearance of dying cells (4, 5) (**Figure 2B**). C1q, which is the recognition molecule of the CP, is produced by macrophages and dendritic cells (DCs) and binds to a variety of ligands that can be expressed on the surface of apoptotic cells such as PS, double stranded DNA, GAPDH, or calreticulin (6–9). C1q has complex immune-modulatory effects and a failure of C1q to opsonize apoptotic cells results in defective phagocytosis by monocytes (10) and activation of the DCs (11). Consistently, a quantitative or functional deficiency in C1q may be related to improper apoptotic cell clearance and autoimmunity (12, 13). Similar functions are described for mannose binding lectin (MBL), one of the recognition molecules of the LP (14).

C1q coated apoptotic cells suppress macrophage inflammation through induction of interleukine 10 (IL-10), IL-27, IL-33, and IL-37, inhibit inflammasome activation and increase the expression of negative regulators ASC2 and NLRP12 (15). It has recently been demonstrated that opsonization of apoptotic cells by C1q induced an increase of the expression of PD-L1 and PD-L2 and a diminution of CD40 at the surface of macrophages (16). Similar effects are observed also on dendritic cells. Presentation of self-antigens by DC in the presence of C1q promotes the development of regulatory T cells (Treg) and the production of anti-inflammatory cytokines such as TGF-b, IL-10, PGE2, IL-37, and IL-27 and thus confers tolerance. In addition, opsonization of the apoptotic cells with C1q induced a high expression of PD-L2 and less CD86 on dendritic cells surface after phagocytosis. This "polarization" by C1q-induced decrease of T helper 1 (Th1) and Th17 and proliferation. The non-maturation of the phagocytes, which is showed by the expression of CD40 and CD86 on macrophages and dendritic cells, respectively, and by the secretion of anti-inflammatory cytokines, makes the phagocytosis immunologically silent (16). Therefore, C1q is of critical importance for the silent, non-immunogenic clearance of apoptotic cells (17) (**Figure 2B**).

The inactivated fragment of the central complement component C3–iC3b participates in the clearance of apoptotic cells via interaction with CR3 on monocytes, macrophages, DC, and microglial cells (18–20). iC3b opsonization and CR3-dependent phagocytosis is accompanied by a down-regulation of the pro-inflammatory mediator IL-12 and a lack of oxidative burst in macrophages (21, 22) or by a reduction in the expression of costimulatory molecules and impaired maturation of DC (23, 24). This confers anti-inflammatory properties and supports tolerogenic apoptotic cell clearance.

Cell Homeostasis

In the native state, different cell types secrete complement components and generate C3a and C5a in their microenvironment, an important fact for their viability and function. Liszewski et al. demonstrated that C3 activation can occur continuously within human T cells, mediated by cathepsin L (CTSL) and in a C3 convertase-independent manner (25) (**Figure 2C**). In resting T cells, this C3a binds to intracellular C3aR, expressed in lysosomes, but not on the cell surface. Intracellular C3aR signaling sustains basal mTOR activation (25), required for homeostatic cell survival (26). Of note, C3a generated outside the cell cannot restore the mTOR signaling in cells with inhibited CTSL, suggesting the importance of intracellular generated C3a (25). Upon T cell receptor (TCR) activation, C3aR is expressed on the cell surface and contributes to the mounting of Th1 response in concert with CD46 signaling.

Of note, the phenomenon of intracellular C3 cleavage seems to be species specific, because it does not operate in mice. In mice, resting T cells synthesize complement components constitutively, complement activation occurs in their microenvironment, and the resultant C5a and C3a signaling, through the C5aR and C3aR, participates in cell viability by maintaining the level of phosphorylated AKT (PKB), a T cell activation intermediate that suppresses apoptosis (27). In the absence or after blockade of C5aR and C3aR, the expression of MHC class II and costimulatory-molecules is decreased on dendritic cells (27, 28). The exact contribution to

T cells survival of the intracellular and extracellular C3a generation and C3aR signaling is still not well defined. It is important to note that AKT (PKB) and mTOR belong to the same signaling cascade, which has anti-apoptotic, survival, and proliferation effects (29). The intracellular complement activation is not restricted to T cells (25), and may have an important role in the physiology of other human cell types.

Complement as a First Line of Defense Against Pathogens

Direct Killing

Pathogens are attacked by all complement pathways, but the prevalence of one or another pathway depends on the exact membrane composition. C3b generated by the spontaneous activation of the AP tag all pathogens and, when no regulator is present, the cascade and the deposition of C3b are accelerated (**Figure 3A**). In addition, the pathogen-associated molecular patterns can be recognized by the recognition molecules of the CP and LP C1q and MBL or ficolins. C1q recognizes mostly charged patterns and can bind to more than 100 different target molecules (30), including pathogen-associated molecular patterns such as lipopolysaccharide (LPS) (31) or bacterial porins (32). MBL binds to a wide range of repeating sugar arrays normally presented by many microorganisms, including mannose structures on fungal and micrococcal surfaces, and *N*-acetylglucosamine residues in cell walls of bacteria, in order to initiate neutralization of these organisms (33). C1q may bind also on natural antibodies, which due to their polyreactivity can recognize the pathogens (34). Upon target recognition, C1q undergoes conformational change and activates the two serine proteases C1r and C1s, associated with it in the context of the C1 complex (35, 36). Despite the similarity between C1 and MBL/MASP complex architectures, the mechanism of activation of the CP and LP differ (37). In particular, the serine proteases of the LP, MASP-1, and 2, are associated with different MBL (or ficolin) molecules, thus requiring a juxtaposition to allow MASP-1 from one complex to activate MASP-2 from the adjacent complex (38, 39). However, MASP-2 alone provides about 10% to cleave its natural substrate C4 by auto-activation (40). Activated serine proteases of the CP and LP cleave C4 and C2 to allow formation of the CP C3 convertase C4b2a, which cleaves C3. If this convertase is not regulated, C3 deposition will be accelerated and

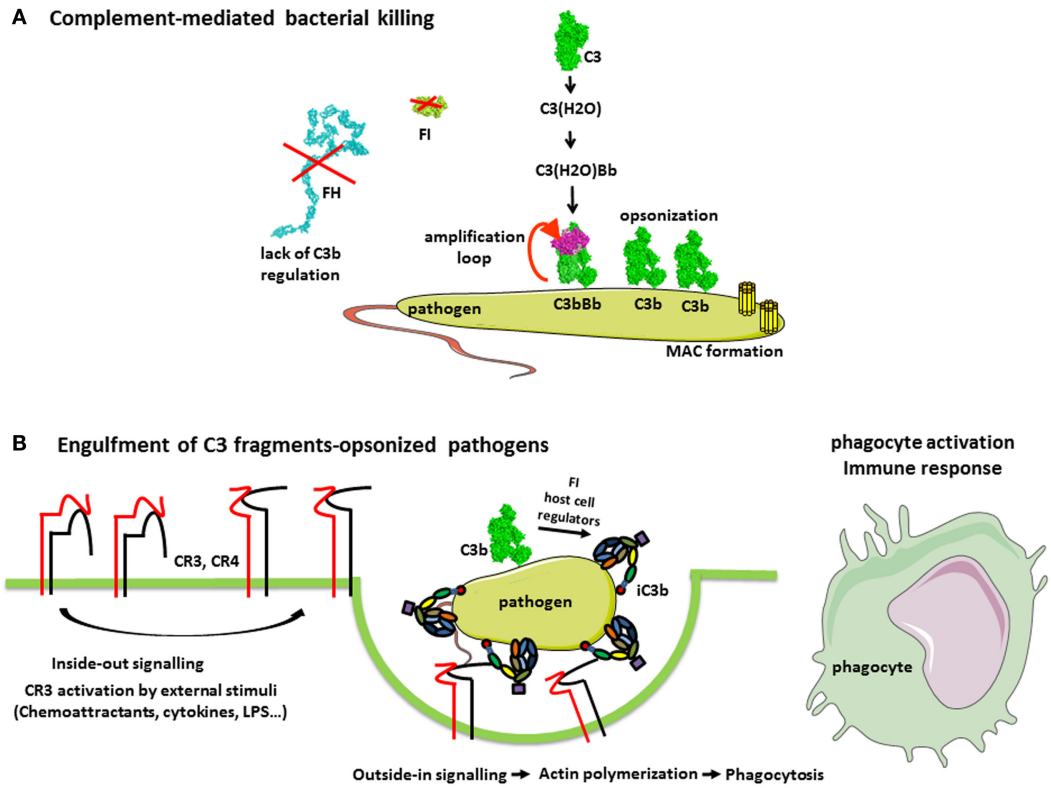

FIGURE 3 | Complement in the defense against pathogens.
(A) Complement-mediated bacterial killing. C3b is deposited on any pathogen surfaces due to the constant activity of the AP. Since most pathogens do not have complement regulatory molecules, C3b is not inactivated and interacts with FB and FD to form a C3 convertase C3bBb. This enzymatic complex cleaves more C3 molecules, resulting in pathogen opsonization with C3b. Further, the cascade proceeds to a C5 convertase and MAC formation, contributing to bacterial killing. **(B)** Complement receptors-mediated phagocytosis of C3b and iC3b-opsonized pathogens. Extracellular stimulatory signals, which are necessary for the CR3-mediated phagocytosis, include chemoattractants (not only chemokines but also bacterial formylpeptides and C5a for neutrophils), cytokines (e.g., TNF-α), and bacterial products (e.g., lipopolysaccharide). External stimuli activate the integrin CR3, (i.e., change to a conformation with high affinity for iC3b) by a Rap-1-mediated signaling. Stabilization of CR3 high-affinity conformation by its engagement with iC3b triggers a RhoA-mediated signaling, which drives the actin polymerization to engulf iC3b-coated target. Complex actin movements are then involved in the intracellular movement of the phagosome during its maturation to the phagolysosome.

the amplification loop will be turned on. Binding of an additional C3b molecule in the immediate proximity, or most probably on the C3 convertase itself, modifies the specificity of the enzyme (41). It then starts to cleave C5, thus turning on a C5 convertase of the classical (C4b2aC3b) or alternative (C3bBbC3b) pathway. Cleavage of C5 gives rise to a powerful anaphylatoxin C5a and to a C5b fragment, which initiates the terminal part of the cascade, common for all activation pathways.

Activation of the terminal complement pathway results in the formation of the membrane-attack complexes (MAC), which forms large, 10 nm wide, pores in the target membrane (42). In fact, most pathogens are able to repair MAC-induced lesions and are resistant to complement lysis by MAC, as Gram-positive bacteria (43). Nevertheless, some Gram-negative bacteria are relatively sensitive to complement killing, particularly the meningitis causing *Neisseria* species (44, 45). This is illustrated by the susceptibility of individuals, deficient in terminal complement components as well as in properdin, to recurrent meningitis. Gram-positive bacteria have a very thick cell wall, which MAC cannot penetrate, therefore being resistant to complement-mediated lysis. Metabolically active nucleated cells are also resistant to lysis by complement (46, 47). However, increase of Ca flux and signal transduction have been described as the result of the insertion of multiple MAC in the membrane, inducing either apoptosis and cell killing or leading to cell proliferation depending on the cell type (48, 49). The molecular mechanisms of complement activation on pathogens are reviewed in detail and illustrated in Merle et al. (1).

Opsonization and Phagocytosis

The main role of complement in pathogen elimination is indirect, namely, the deposition of complement fragments on the surface of pathogen targets, so-called opsonization that allows their recognition, ingestion, and destruction by phagocytic cells, neutrophils, monocytes, and macrophages (**Figure 3B**). Both IgG antibodies and C3 fragments are the classical opsonins. But complement opsonization, resulting from the direct activation of the AP on pathogens surface allows their elimination by phagocytes before the mounting of an adaptive immune response and the appearance of antibodies (**Figure 3**). Phagocytes express specific receptors for C3 fragments, described in "Complement system part I – molecular mechanisms of activation and regulation" (1).

CR1 is a complement component molecule (CCP) domain containing molecule, involved in the control of C3 convertases. It is present on erythrocytes, on phagocytes, and on kidney glomerular podocytes and binds C3b and C4b. CR1 on erythrocytes plays a major role in the clearance of soluble immune complexes, by transporting them to the liver and spleen, where they are cleared by macrophages. The binding of C3b-coated targets to phagocyte CR1 is not sufficient to trigger phagocytosis, but C3b–CR1 interaction enhances the FcγR-mediated phagocytosis of targets bearing both IgG and C3b. Moreover, immune mediators that activate phagocytes, such as fibronectin (50) or LPS (51), induce the phagocytosis of targets opsonized with C3b only. However, this is probably partially mediated by CR1 in that case, since elastase, a major protease released by activated phagocytes, is able both to degrade CR1 and to cleave C3b into iC3b, allowing then iC3b-coated targets to be recognized by the efficient phagocytic receptor CR3 (52).

CR3 and CR4 are specific receptors for iC3b, among C3 fragments, able to induce the phagocytosis of iC3b-coated targets (53, 54). CR3 and CR4 belong to the integrin family, involved in cell adhesion processes, due to their ability to interact, in particular, with intercellular molecule-1 (ICAM-1), present on many cells, including endothelial cells. Integrins are formed from two chains, alpha and beta bearing magnesium ions necessary for their function. CR3 (also called MAC-1, αMβ2 or CD11bCD18) and CR4 (p150,95, αxβ2 or CD11cCD18) form, with LFA-1 and αDβ2, the leukocyte-specific integrin subfamily sharing the β2 chain (CD18). The CR3 also bear a lectin site, different from the iC3b and ICAM-1 binding site, and able to recognize microorganism-derived sugar ligands. If both the lectin and the iC3b-binding sites of CR3 are engaged, the strength of the CR3-mediated phagocytic response is enhanced (55).

CR3 is present on monocytes and its expression is up-regulated upon monocyte to macrophage differentiation. Resting neutrophils express low levels of CR3 on their surface but these levels increase dramatically following cell activation, due to the externalization of large intra-granular pools of receptors. CR4 is expressed poorly on neutrophils and monocytes, but its expression increases upon monocyte differentiation to macrophage or to dendritic cells.

The cellular processes involved in the phagocytosis of IgG- or C3-opsonized targets are different. When phagocytosis is mediated primarily via CR3, the reaction is slow and pathogens gently sink into the phagocyte, by a process involving actin polymerization dependent on the small GTPase Rho (53). By contrast, the Fcγ receptors provide a very vigorous engulfment of IgG-coated pathogens, with membrane extensions driven by small GTPases Rac and Cdc42. However, the CR3-mediated phagocytic response to iC3b-coated targets is boosted by the participation of additional receptors such as toll-like receptors (TLRs) recognizing pathogen patterns.

Indeed, it is worth noting that CR3 and CR4 induce phagocytosis mainly in conjunction with stimuli, such as pro-inflammatory cytokines, which activate phagocytic cells (56). These stimuli induce an inside-out signaling, which switches integrins to an active conformation with a higher affinity for iC3b and ICAM-1 (53). This may represent a control mechanism to avoid unwanted responses to host cells passively coated with a few iC3b molecules resulting from the continuous low-grade activation of the AP.

Apart from CR3 and CR4, some resident macrophages such as liver Kupffer cells express CRIg, a receptor for iC3b belonging to the immunoglobulin family and able to mediate the phagocytosis of iC3b-coated pathogens (57).

The main consequence of phagocytosis is the elimination of pathogens. Internalized microorganisms are killed both by toxic reactive oxygen compounds, generated through a NADPH oxidase complex assembled at the phagosomal membrane, and by microbicidal components, such as lysozyme and proteases, present in phagocyte granules fused with the phagosome to form the phagolysosome. Finally, CR3-mediated phagocytosis results in the apoptosis of phagocytic cells, an important step of the resolution of infection and inflammation (58).

Complement in Inflammation

Anaphylatoxins and Their Receptors

Complement anaphylatoxins C3a and C5a play a critical role in the modulation of immune system activity by complement. C3a and C5a are the small fragments released after cleavage of C3 and C5 by the C3 and C5 convertases of the classical and APs. They contribute to the inflammation and activate immune cells and non-myeloid cells, which express G-protein coupled anaphylatoxin receptors C3aR and C5aR (59, 60) (**Figure 4**). Anaphylatoxins stimulate inflammation by inducing an oxidative burst in macrophages (61), eosinophils (62), and neutrophils (63). Moreover, C3a and C5a induce histamine production by basophils (64) and mast cells (65), resulting in vasodilatation. Even if pro-inflammatory effects of C3a are not in question, studies highlight the anti-inflammatory role of C3a in different contexts (66). Neutrophil migration and degranulation are prevented by the presence of C3a, whether others granulocytes are activated by this anaphylatoxin (63, 67, 68). Thus, this suggests an anti-inflammatory role in acute phase of inflammation, and in cases of ischemia–reperfusion injury and in sepsis mouse models (69, 70).

The activation product of C4, C4a, seems also to have a functional activity on macrophages and monocytes (71, 72). Nevertheless, no C4a receptor has been reported, making it difficult to ascertain the physiological role of C4a (73). More studies are needed to understand whether C4a is an anapylatoxin and what is its mode of action.

In plasma, C3a and C5a are quickly converted by carboxypeptidase N and carboxypeptidase B into C3a-desArg and C5a-desArg, by cleavage of the C-terminal arginine (74–76). Recent study determined a central role of C3a in carboxypeptidase B2 (CBP2) negative mice, which are unable to convert C3a and C5a to C3a-desArg and C5a desArg (77). Using a treatment with blocking antibody against C3aR or C5aR, mice with only a functional C3a/C3aR axis present better survival after sepsis induction, whereas mice with only a functional C5a/C5aR axis present a less survival compare to wild-type mice. These data demonstrate two opposite effects between C3a and C5a, highlighting the complex role of C3a depending on the context. Another particularity of C3a and C5a is that C3a-desArg loses its ability to bind to C3aR and C5a-desArg has 90% weaker pro-inflammatory activity compared to C5a, as shown in human astrocyte model (78). By contrast, murine C5a-desArg is as potent as murine C5a upon binding to C5aR on murine cells (79). These inter-species differences have to be taken into account when *in vivo* experiments are analyzed.

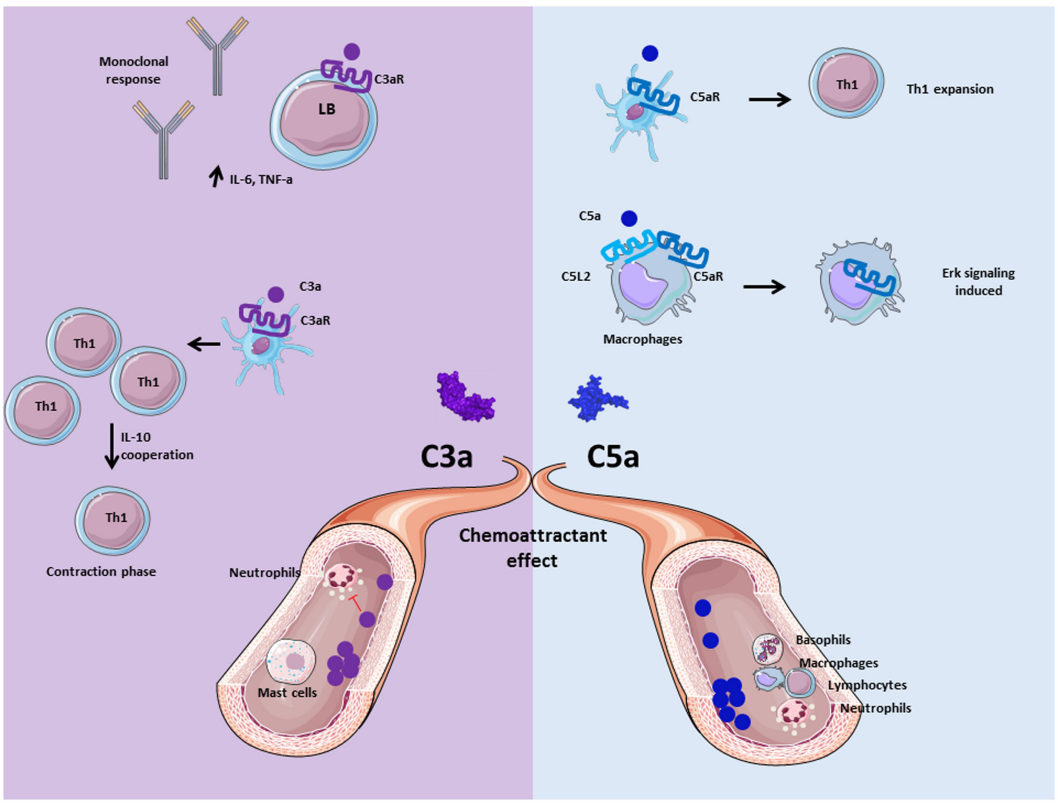

FIGURE 4 | Role of anaphylatoxins C3a and C5a. Anaphylatoxins C3a and C5a participate in inflammation by interacting and activating immune cells via C3aR and C5aR, respectively. C3a is implicated in the adaptive immunity by inducing monoclonal response from B cells and up-regulation of pro-inflammatory cytokines. Moreover, C3a facilitates the contraction phase of T cells by increasing IL-10 synthesis. C5a is implicated in Th1 expansion to improve adaptive immunity response, and allows C5aR internalization in presence of C5L2 to induce ERK signalization and pro-inflammatory effect of macrophages. Both are chemoattractant molecule, and allow mast cells migration for C3a, basophils, macrophages, neutrophils, and lymphocytes recruitment for C5a at the inflammatory site. Nevertheless, C3a has an anti-inflammatory effect on neutrophils by inhibiting their degranulation and recruitment.

Human *C3a* specifically binds its receptor *C3aR* (80), whereas C3a-desArg and C5a cannot bind to this receptor (81). C3aR belongs to the G-protein coupled receptor (GPCR) family with seven transmembrane domains. C3a binding leads to transduction of intracellular signals via heterotrimeric G-proteins and phosphorylation of PI3K, Akt, and mitogen-activated protein kinase (MAPK), leading to chemokine synthesis in human (82). Moreover, in human mast cells, C3a plays a role of chemoattractant molecule and can play an important role in hypersensitivity and inflammatory process (83). In case of chronic inflammation, C3a has pro-inflammatory activity and contributes to disease progression (60). In human monocytes and monocyte-derived macrophages, C3aR and TLR-4 costimulation induce the production of pro-inflammatory mediators, such as IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, and PGE2 (84, 85). Contrary to these pro-inflammatory functions, in acute inflammation conditions C3a prevents mobilization and degranulation of neutrophils (66, 68). The difference in the response of inflamed tissues to C3a, between the acute and chronic phases of inflammation, may well be due to the differing cell types involved (e.g., neutrophils versus monocyte/macrophages) (66).

C3a modulates also the responses of the cells of the adaptive immunity. Human C3a has been described to regulate B cell function by suppressing the polyclonal immune response, IL-6 and TNFα release, in a dose-dependent manner (86). Mice C3aR signaling on DCs is important for the generation of Th1 cells (28, 87). A lack of C3aR activation on DC induces a Th2 polarization and favors the emergence of Treg. C3aR is also expressed on adaptive immune cells, such as T lymphocytes. Indeed, the importance of C3aR and C5aR expression for Th1 induction has been demonstrated in mice (27, 88, 89). Moreover, C3 deficiency induced a decreased expression, on T cells, of the α and β chains of the IL2 receptor. This may explain the aberrant Th1 response observed in patients with C3 deficiency. C3a also participates in the contraction phase of Th1 by regulating IL-10 expression (88).

C3aR is expressed not only by the immune cells but also by endothelial and epithelial cells. Stimulation of C3aR on endothelial cells induces a rapid mobilization of intracellular granules, Weibel–Palade bodies, containing von Willebrand factor and P-selectin. Hence, the cell acquire a pro-inflammatory and prothrombotic phenotype, since P-selectin helps the recruitment of leukocytes via binding to PSGL-1 (90) and von Willebrand factor mediates platelet adhesion (91). Moreover, human and mouse P-selectin binds to C3b on cell surfaces and serves as a platform for the formation of C3 convertases and the activation of the AP (92, 93). C3a activation of endothelial cells thus forms an amplification loop of complement activation, implicated in microvascular thrombosis, including in a mouse model of Shiga-toxin (Stx2)/LPS induced hemolytic uremic syndrome (HUS) (93).

Human and mouse *C5a* bind to *C5aR*, which also belongs also to the GPCR family (94). C5aR stimulation induces downstream effect such as activation of PI3K-γ (95, 96), phospholipase Cβ2 (97), phospholipase D (98), and Raf-1/B-Raf-mediated activation of MEK-1 (99). C5a is known to be a powerful chemoattractant molecule and plays a critical role in the inflammatory response by recruiting immune cells such as macrophages (100), neutrophils (101), basophils (102), and myeloid-derived suppressor cells (MDSCs) (103). Human C5a also recruits adaptive immune cells, such as T cells, which express constitutively C5aR on their surface (104). Human B cells express C5aR but respond to C5a only in case of activation (105). In a $CD55^{-/-}$ mouse model, it has been demonstrated that C5a production by APC is essential for differentiation of T cell in Th1 effector cells (106). Indeed, addition of C5a on $CD80^{-/-}$, $CD86^{-/-}$, and $CD40^{-/-}$ APC restored T cell activation, providing a link between GPCR, costimulation signals via CD28 and T cell survival (27). The axis C5a/C5aR appears to have a crucial role in sepsis, based on the observation that the inhibition of C5a/C5aR interaction decreased the mortality in a rat model (107). Another function of C5a is to induce vascular endothelial growth factor (VEGF) expression and to promote angiogenesis in a human retinal model (108). In a model of enteric infection, C5a is generated thanks to the stabilization of the AP C3 convertase by properdin and leads to IL-6 release from colonic epithelial cells (109). Then, IL-6 regulates inflammation induced by bacteria. In properdin deficient mice, the C5a-dependent IL-6 production is impaired, aggravating the infection. This provides evidences of the implication of C5a in the defense against bacteria-triggered epithelial injury.

C3a, C5a, and C5a desArg are also able to bind *C5L2*. In human, C3a-desArg is able to bind C5L2 and regulates triglyceride synthesis rate (110). C5a has a lower affinity for C5L2, as compared to C5a desArg in human basophil cell lineage (111). As C5a- and C3a-receptors, C5L2 is composed by seven transmembrane domains but it is not coupled with G protein (112). Previous work on human neutrophil determined a role of C5L2 as a negative regulator of anaphylatoxin receptor activity after activation and interaction with β-arrestin, this was confirmed in mouse model (113, 114). However, its role remains unclear. C5L2 has been described as an anti-inflammatory receptor, because $C5L2^{-/-}$ mice have increased production of pro-inflammatory cytokines IL-6 and TNF-α (115). Nevertheless, deficiency of C5L2 on macrophages, neutrophils, and fibroblasts decreased their proinflammatory capacity *in vitro*. C5L2 is also essential for the C5a-mediated cell infiltration in *in vivo*, in a mouse model, suggesting a role of positive modulator of C5a-induced responses (67).

C5L2 expression in human atherosclerosis lesions is correlated with an increase of IL-1β and TNF-α release, supporting the pro-inflammatory effect of C5L2 (116). It has recently been reported that human C5L2 and C5aR are able to form a heterodimer (117). This complex induces internalization of C5aR upon C5a binding and promotes ERK and MEK signaling in a mouse model of acute experimental colitis (118). This internalization of GPCR has already been demonstrated in *in vitro* assays as essential for the induction of the late stage of ERK signaling (119, 120).

Crosstalk Between C3aR and C5aR Signaling Pathways with Toll-Like Receptor Signaling

C3a and C5a are able to induce potent inflammatory pathways via their receptors C3aR and C5aR. The implication of intermediates such as NF-kB, MAPK, and c-Jun N-terminal kinase (JNK) in their transduction pathways suggests a potential crosstalk with other pathways, such as those of TLRs. Indeed, complement is involved in TLR-induced inflammation (121) (**Figure 5**).

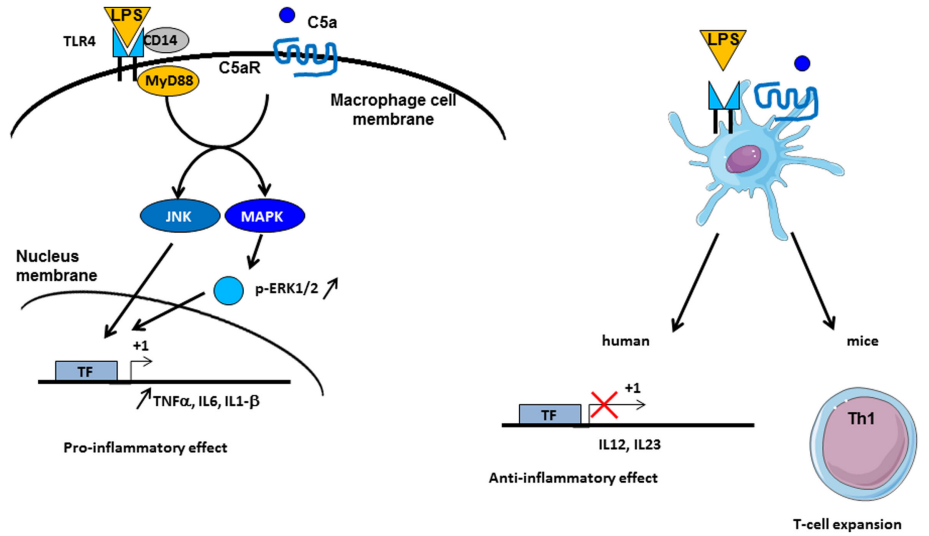

FIGURE 5 | Crosstalk between complement and TLR pathways. C5a/C5aR signaling pathway can cooperate with TLR-4 activation by LPS on macrophages. Intermediate signaling pathways JNK and MAPK are activated and thus lead to pro-inflammatory effect by TNF-α, IL6, and IL1-β synthesis. On dendritic cells (DCs), TLR-4 and C5aR cooperate in different manner between mice and human. *In vivo* experiments have demonstrated an implication in Th1 cells expansion, whereas in human, an anti-inflammatory role of TLR-4/C5aR collaboration has been described by an antagonized effect on IL-12 and IL-23 synthesis by DC.

Co-activation of MyD88-dependent TLRs (TLR-2, TLR-4, and TLR-9) and complement in $CD55^{-/-}$ knockout mice increased plasma inflammatory cytokines such as IL-6, TNF-α, and IL-1beta. Moreover, complement activation by the fluid phase cobra venom factor (CVF) synergizes with LPS to cause a dramatic increase of IL-6 production. These results suggest a strong interaction between TLRs and complement signaling *in vivo* to promote inflammation and modulate adaptive immunity (121). Complement may interact with TLR signaling by C3aR and C5aR, because of the involvement of MAPK, extracellular signal-regulated kinase (ERK1/2), and the JNK, but not p38 MAPK (121). TLR2 also crosstalks with CR3, since TLR2 can transactivate CR3 via PI3K by an inside-out signal (122). In turn, CR3 can regulate TLR2 signalization by recruiting TIRAP and facilitating the recruitment of MyD88 signaling adaptor to initiate TLR signaling (123). Several studies have shown a crosstalk between complement and TLR-4. It is known that TLR-4 activation by LPS can induce C5aR up-regulation on hepatocytes mediated by IL-6 (124). Another, immune-modulatory function of C5a is to reduce the production of IL-12 family cytokines by mouse inflammatory macrophages stimulated by TLR-4 ligands (125). In turn, this resulted in regulation of Th1 cells polarization and a limitation of their expansion. A synergy between C5a and TLR ligands on mouse DC stimulation was found upon stimulation of these cells with a fusion protein composed of C5a and an endogenous ligand for TLR-4 (extra domain A) and an antigen (126). This induced strong antigen-specific T cell responses *in vivo*, without production of immunosuppressive molecules. In humans, the C5a-mediated immature DC stimulation appears more complex. C5a increases the cytokines production in immature DCs, but upon TLR-4 stimulation, C5a inhibits the production of IL-12, IL-23, and TNFα, thus having an anti-inflammatory role (127). These results emphasize that the effects of anaphylatoxins on immune response depend on the crosstalk not only with TLRs but also with other receptors.

Complement and Adaptive Immunity

Discoveries over five decades have shown that the complement system plays an important role not only in the inflammation but also in the adaptive immunity.

The Complement System and Its Interplay with B Cells

The relationship between complement and B cells has already been demonstrated 40 years ago *in vitro* and using *in vivo* models. C3 plays important role in antibody generation by B cells (**Figure 6**). In case of C3 depletion [by a structural analog of C3 from snake venom (CVF), which is capable to bind to FB and to activate complement in fluid phase], the humoral immunity toward certain thymus-dependent antigens and the lymphocyte cooperation was impaired (128, 129). These and other early studies demonstrated that complement binds and localizes foreign antigens within sites where the lymphocytes response takes place. B cells express complement receptor CR2 (CD21), which interacts with C3d and iC3b on the surface of the antigen and forms also a co-receptor complex with CD19 and CD81 (130). Thus, the complex C3d:CR2 induces an increase of B cell receptor (BCR) signaling in the presence of C3d-opsonized antigen on B cell surface (131). *In vivo*, C3 is required for the induction and maintenance of memory cells of the B cell lineage within the microenvironment of germinal centers (GCs), where B cells encounter antigen–antibody–C3 complexes on the surfaces of follicular dendritic cells (FDCs) (132). When C3d-opsonized antigen

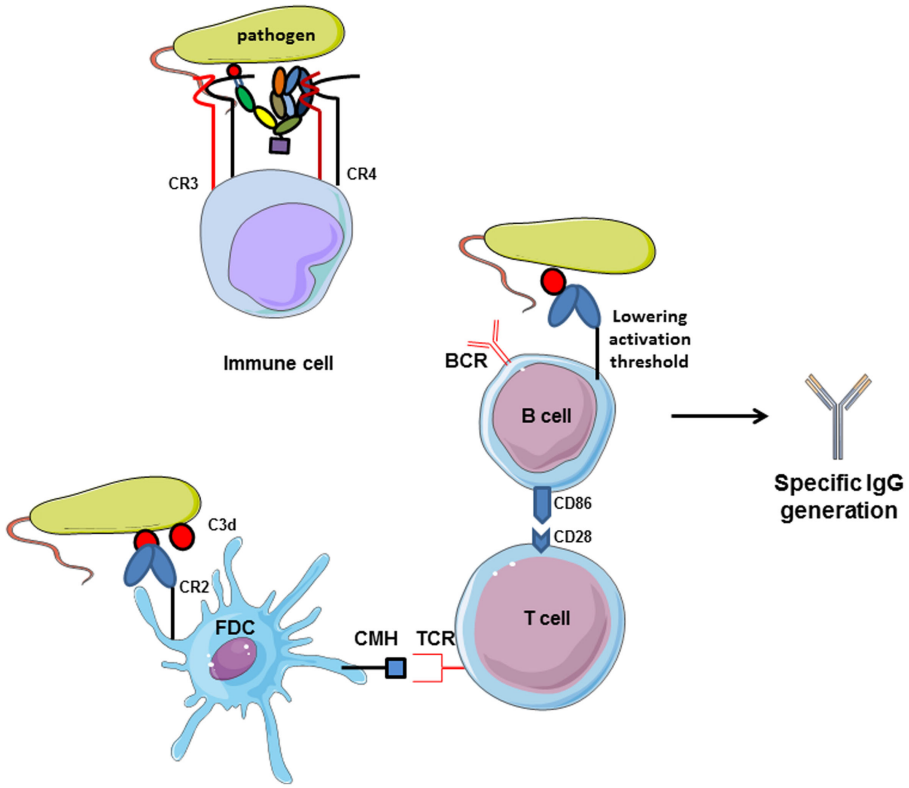

FIGURE 6 | Complement receptors implication in adaptive immunity. CR2 activation by interaction with C3d-opsonized antigen on follicular dendritic cells increases CMH expression and allows antigen presentation to TCR. Then, costimulatory molecules are expressed and T lymphocytes help in memory B cells maturation in germinal centers. Moreover, C3d/CR2 interaction lowers the activation threshold of B cells and increases BCR signaling activity. Cumulated, C3d/CR2 interaction induces specific IgG generation by B cells, and C3d works as a natural adjuvant. CR3 and CR4, expressed on immune cells.

binds to CR2 on FDCs, they can present the antigen in the GC and induce effector and memory B cells (133). This underlines the importance of CR2 expression on FDCs and B cell surface for the generation of antigen-specific IgG. C3d serves as a molecular adjuvant by lowering the threshold for B cell activation by 1000 to 10,000 times (134).

The human complement regulator of the CP C4b binding protein (C4BP) has functions extending beyond the dissociation of the classical C3 convertase and serving as cofactor for FI for C4b inactivation. It binds directly to the costimulatory protein CD40 on human B cells at a site that differs from that used by the CD40 ligand. C4BP induces proliferation, the up-regulation of CD54 and CD86 expressions, and IL4-dependent IgE isotype switching in normal B cells. These observations suggest that C4BP is an activating ligand for CD40 and establish another interface between complement and B cell activation (135).

The auto-reactivity of B cell is also tightly regulated by complement. C4 has been demonstrated to be essential to maintain peripheral B cell tolerance (136). Deficiency in C4 promotes the emergence of autoreactive B cells during the maturation in the GCs. These results could be explained by a lack in the clearance of apoptotic cells in GCs that leads to an impairment in host antigen presentation by APC, essential for the education and the peripheral B cell tolerance.

The complement system can contribute to autoimmune diseases by decreasing the threshold for B cell activation. For example, in a collagen-induced arthritis (CIA) mouse model, C3 depletion delays and decreases severity of the disease (137). Complement via C3d plays a key role in B cell function, and C3d antigen can break anergy in autoreactive B cells (138). Complement can modify the antigen-specific B cell response in experimental autoimmune encephalomyelitis (EAE) and possibly in multiple sclerosis (MS). In EAE mouse model, consumption of complement, using CVF, significantly attenuates clinical and histological EAE (139). The authors suggest that complement breaks the anergy of autoreactive B cells, leading to autoantigen-specific IgG production, while the total IgG response remained unaffected.

The Complement System and Its Interplay with T Cells

The importance of complement for the survival of resting T cells has been described above. Upon infection with a pathogen, T cell proliferation and differentiation are controlled by APC and their microenvironment. Complement is able to polarize T cells and participates to the induction and effector phase, as well as to the contraction phase of the T cell response (140–142). In fact, during inflammation, anaphylatoxins C3a and C5a are able to bind their corresponding receptors expressed on the T cells and APC surface, leading to cytokine production by these cells (27)

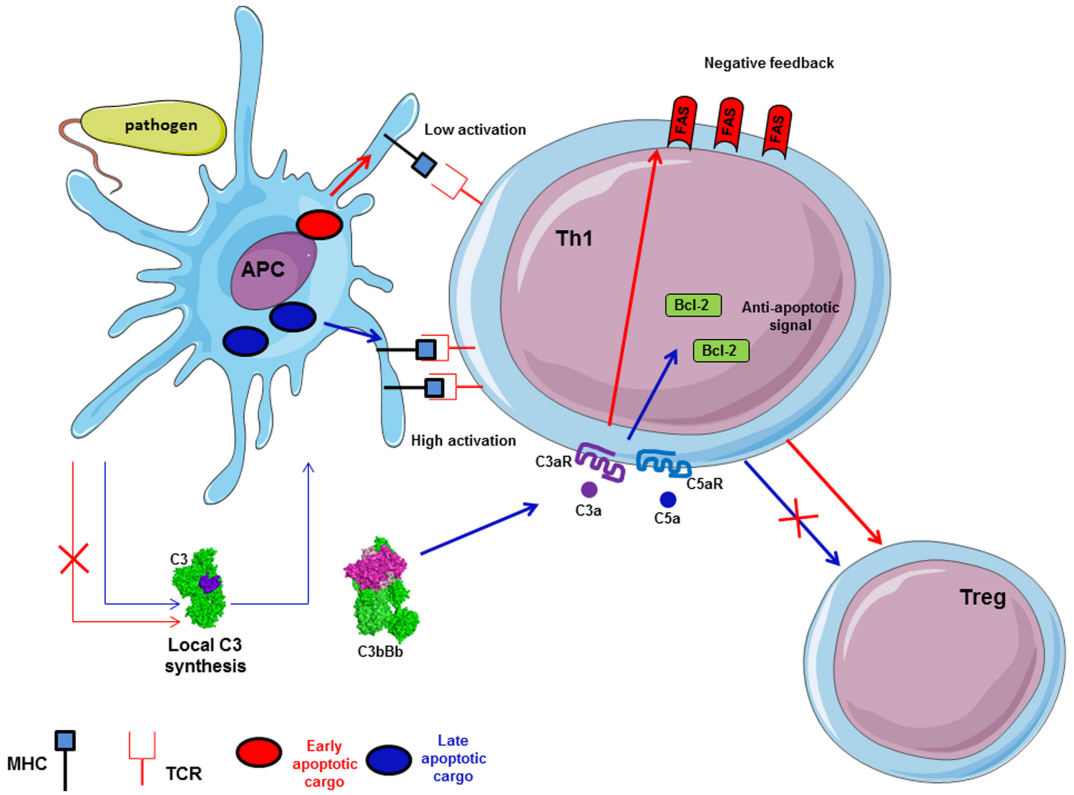

FIGURE 7 | Local synthesis of C3 by antigen presenting cells (APC) is implicated in antigen-specific T cell response. C3 synthesis by APC in case of pathogen internalization induces late apoptotic cargo and allows high expression of MHC on APC surface. Thus, it participates in antigen-specific T cell proliferation and Th1 generation. Local C3 and C5 generate C3a and C5a, which induce up-regulation of Bcl-2 and down-regulation of FAS expression via C3aR and C5aR to facilitate T cell proliferation. Moreover, C3a/C3aR and C5a/C5aR signaling pathways activation promotes Th1 generation and avoid Treg differentiation.

(**Figure 7**). Local C3 synthesis by DC is necessary to induce T cell activation and Th1 response (28). C3 deficiency was shown to accelerate the fusion of the apoptotic cargo with lysosomes and led to impaired antigen-specific T cell proliferation *in vitro* and *in vivo* (143). Moreover, C3a/C3aR is responsible for up-regulation of the anti-apoptotic Bcl2 and down-regulation of the pro-apoptotic molecule FAS during infection on myeloid and lymphoid cells, inducing immune cells survival and proliferation (144). The absence of C3aR and C5aR stimulation during T cell activation induces Treg development (145, 146). Thus, C3-deficient patients, who cannot produce C3a and C3b, present a lack of Th1 response whereas Th2 response remains normal (147, 148).

The complement regulator CD46 plays an important role in the regulation of T cells (149) (**Figure 8**). CD46 has different isoforms affecting its cytoplasmic tail and resulting from alternative splicing. Different isoforms are expressed in different organs. CD46 engagement on $CD4^+$ T cells promotes the effector potential of Th1 cells (150). As IL-2 accumulates, it switches cells toward a regulatory phenotype, attenuating IL-2 production and upregulating IL-10. The interaction of the CD46 cytoplasmic tail with the serine–threonine kinase SPAK plays an important role in this process. The γδ T cells express an alternative CD46 isoform and thus are unable to switch from IL2 to IL10 production. The Treg express different CD46 cytoplasmic tails, as compared to Th1 cells. Therefore, CD46 uses distinct mechanisms to regulate different T cell subsets during an immune response. Recently, the Notch family member Jagged1, which is expressed on T cells, has been identified as a new natural ligand of CD46 (147). Jagged1 binds Notch-1 and this interaction is responsible for cellular activation and proliferation. The *cis* interaction (on the same cell) of CD46 and Jagged1 leads to a competition with Notch, thus controlling the homeostasis of naïve T cells and inhibiting their activation. In the case of TCR stimulation, CD46 is down-regulated, which allows Notch-1 and Jagged1 to bind in *cis* and in *trans* (from two different cells). This induces Th1 proliferation and polarization, leading to IFN-γ and IL2 induction. Interestingly, CD46-deficient patients are unable to produce IFN-γ and have a lack of Th1 response, whereas Th2 response remains normal. Taken together, these results suggest that the expression of CD46 is necessary to induce a Th1 response. Then, CD46 plays a negative feedback when proliferation leads to T cell/T cell contact limiting the expansion of Th1 cells and allows a contraction phase. The binding site for Jagged1 on CD46 has been mapped to the first two CCP domains (147). There is no overlap between this binding site and the C3b binding site, which is located in the CCP3 and 4.

CD55 (decay accelerating factor, DAF) also plays a role in the establishment of the adaptive immune response. In addition to control complement activation, experiments on CD55 deficient

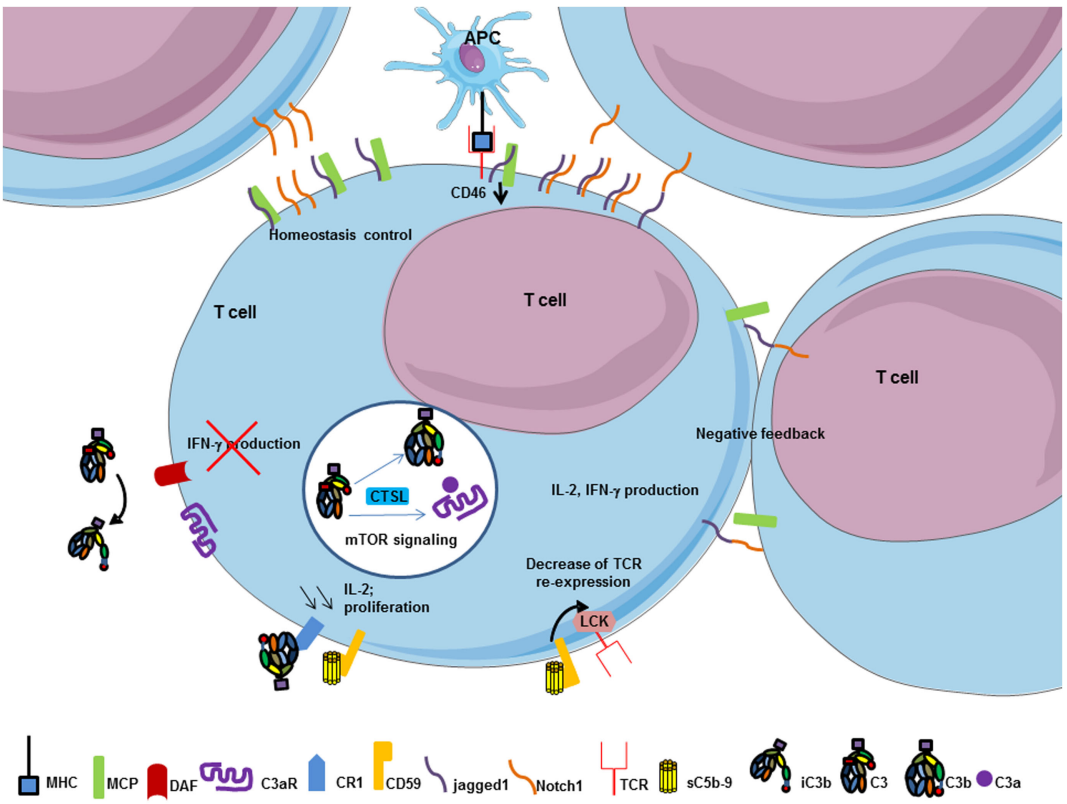

FIGURE 8 | T cell response is modulated by complement components. MHC/TCR interaction between APC and T cell decrease CD46 expression on T cell and allows *cis* interaction between jagged1 and Notch-1 on T cell surface to promote T cell proliferation, IL-2, and IFN-γ production. Thereafter, *trans* interaction between jagged1 and Notch-1, and CD46 work as negative feedback to control T cell homeostasis. Soluble C5b–9 and CR1 regulate T cell activation. Interaction between soluble form of C5b–9 and its specific inhibitor CD59 on T cells decrease TCR re-expression after its internalization to limit T cell activation by transmitting a signal via Lck. CR1 activation by iC3b decreases IL-2 synthesis and proliferation of T cell to promote a negative feedback of T cell activation. CD55 engagement on T cells negatively regulates Th1 induction cells by inhibiting IFN-γ production. Intracellular C3 in T cell is cleaved by CTSL and promotes C3b and C3a intracellular generation. Interaction between C3a and C3aR induces mTOR signaling and survival signal of the immune cell.

mice showed an enhanced Th1 response with a hypersecretion of IFN-γ (151, 152). This may be explained by the overactivation of complement leading to strong local anaphylatoxin production (153).

CD35 and CD59 also participate in T cell regulation. Recent study showed that CD59 is able to modulate T cell activation by transmitting a signal via Lck to TCR/CD3ζ. A knock-down of Lck accelerated the re-expression of CD3 at the cell surface (154). Engagement of CD35 (CR1) on T cells reduces their rate of proliferation and IL-2 secretion (155). Reduction of the expression of CD46 on activated T cells may lead to local complement overactivation, thus generating larger amount of C3b, iC3b, and C5b–9. Therefore, binding of C3b and iC3b to CD35 and C5b–9 to CD59 could contribute to the negative feedback controlling Th1 expansion (149).

In addition, intracellular mechanisms of sensing C3b-opsonized pathogens have recently been described (156). Pathogens that cross the cell membrane, with covalently attached complement C3 on their surface, activate mitochondrial antiviral signaling. This mechanism would represent an autonomous immunity of the cells against non-enveloped viruses and cytosolic bacteria, by inducing signaling pathways, such as NF-kB, IRF3/5/7, and AP-1, leading to pro-inflammatory cytokines production (156).

All these examples illustrate the importance of complement for the mounting of a successful immune response. Therefore, this cascade should not only be considered simply as a humoral factor, mediating innate immunity and inflammation, but also as a potent regulator of cells functions in the adaptive immunity.

Strategies of the Pathogens to Evade the Complement System

Complement system works as a first line of defense and allows in many cases to avoid infections. Nevertheless, the evolution of the pathogens resulted in elaboration of evasion strategies against complement attack. These mechanisms of complement evasion can be classified in different groups (157) (**Figure 9**).

Binding of Host Complement Regulators

Binding of host complement regulators on their membrane allows pathogens to inactivate complement. Bacteria, viruses, fungi, and parasites have been shown to bind high levels of efficient complement regulators such as FH, factor H-like 1 (CFHL-1), and C4

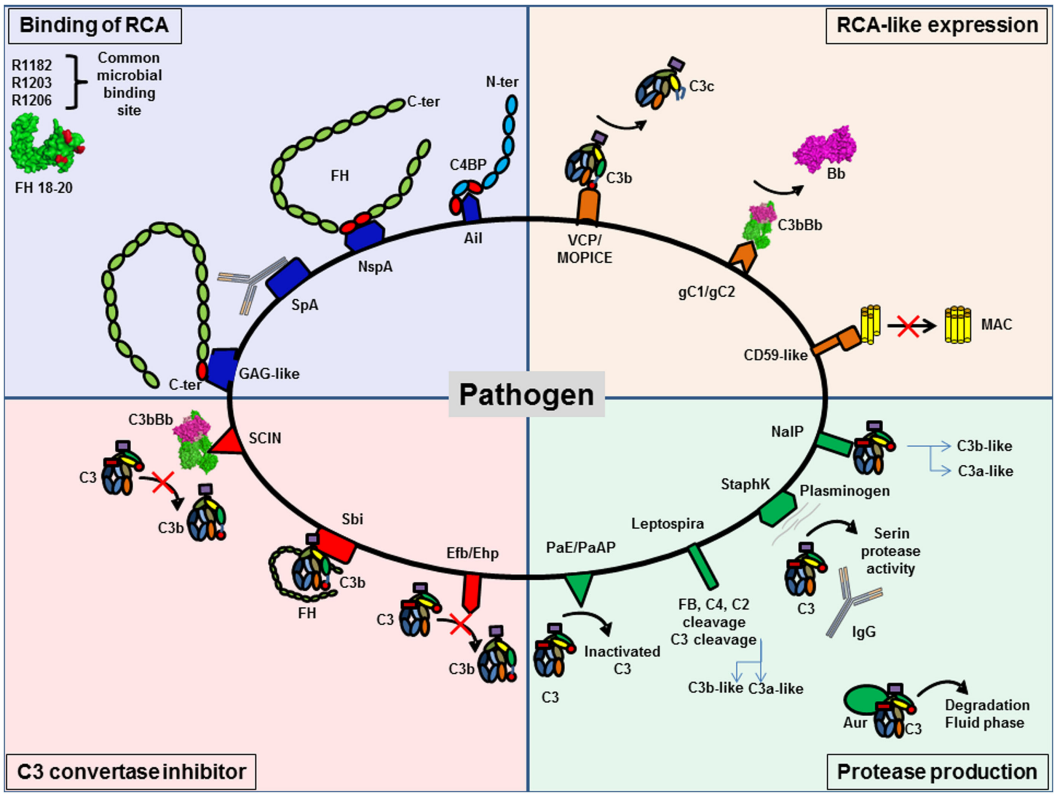

FIGURE 9 | Pathogens are able to protect themselves from complement activation. Pathogens have developed different strategies to inhibit complement activation. They can be classified in four different groups. Several pathogens are able to bind regulators of complement activation (RCA), such as FH and C4BP, to decrease C3 deposition. RCA-like expression allows pathogens to block complement activation without the need to recruit complement regulator. Synthesis of proteases specifically against complement proteins degrades complement components. The last group is pathogens able to express C3 convertase inhibitors.

binding protein (C4BP) (158). Recruitment of these regulators can accelerate the decay of the C3 convertase and provide cofactors for FI, which cleaves C4b and C3b, thus protecting the pathogen against complement attack.

To bind fluid phase complement regulators, pathogens express specific molecular platforms with high affinity, such as PorA in *Neisseria meningitidis* (159), filamentous hemagglutinin in *Bordetella pertussis* (160), Ail in *Yersinia pseudotuberculosis* (161), factor H binding protein (fHbp) and neisserial surface protein A (NspA) in *N. meningitidis* (162), and M protein family in group A streptococci (163).

Factor H binds to pathogens by a "common microbial binding site," located in its C-terminal domain CCP20. This domain binds to heparin and other GAGs on the host cell surface, but the binding sites for GAG and for pathogens surface proteins are not identical (164, 165). Three key amino acids (R1182, R1203, R1206) are critical for this "common microbial binding site" for at least seven Gram-negative and Gram-positive pathogens (165). In addition, the microbial proteins enhanced binding of FH19–20 to C3b, forming a triple complex, and leading to a more efficient complement inactivation on the pathogen surface. This is a unique example of convergent evolution, resulting in enhanced immune evasion of important pathogens via utilization of a "superevasion site" (165).

In addition to CCP 19–20, pathogens trap FH by binding to CCP6–8. *Neisseria* species bind this region of FH. Por1A from *Neisseria gonorrhoeae*, interacts with CCP 6, in addition to CCP 19–20, to escape from complement attack (166). Also, fHbp and NspA from *N. meningitidis* can recruit FH by binding to CCP 6–7 (162, 167). Study of the structure of fHbp by mutagenesis revealed that fHbp binding site for CCP 6–7 overlaps with sucrose octasulfate, a GAG analog. FhbB from *Treponema denticola* binds FH by a similar mechanism. Study of FH/FhbB interaction by site-directed mutagenesis revealed a binding site localized in CCP7 of FH (168).

Staphylococcus aureus has developed a different strategy to prevent complement initiation. Staphylococcal protein A (SpA) recognizes the Fc portion of Igs with high affinity and hide the binding site for C1q, thus blocking the initiation of the CP (169). The outer membrane protein SdrE binds FH and C4BP to *S. aureus* (170, 171). Together, these findings suggest that *S. aureus* is able to inhibit the three complement pathways.

Expression of Complement Regulators-Like Proteins

Expression of complement regulators-like proteins can contribute to pathogens camouflage. Some viruses, such as poxvirus, have found ways to produce soluble proteins that closely mimic the

structure and function of host regulators, such as vaccinia virus, which produce VCP, a protein similar to CD55 and MCP. Thus, these viruses inhibit both CP and AP, dissociating C3 convertases formed on C3b and C4b. Monkeypox virus produces a complement regulator-like protein, MOPICE, which is able to bind human C3b and C4b and serves as cofactor for FI (172). MOPICE is considered as a virulence factor of this virus, since it is expressed in the more virulent strains from Central Africa and absent in the less virulent pathogens from West Africa. Another example of complement regulators-like activity comes from Herpes viruses, which express transmembrane gC1 and gC2 glycoproteins able to bind C3b and to specifically accelerate the decay of the AP C3 convertase (173). *Borrelia burgdorferi* produces a CD59-like protein, which has affinity for C8b and C9 and avoids MAC formation (157).

Production of Proteases that Degrade Complement

Production of proteases that degrade complement proteins is frequently observed in bacteria. Indeed, *Pseudomonas* produce *Pseudomonas* elastase (PaE) and alkaline protease (PaAP) that cleaves immunoglobulins and C1q, thus preventing activation of the CP (174). In addition, these proteases are able to inactivate C3 into a non-functional fragment and inhibit complement activation (174). *Leptospira* strains are able to cleave directly complement proteins and inhibit the three complement pathways. *Leptospira* produced metalloproteases may not only cleave C3 but also FB, C4, and C2 (175). Generated C3 degradation products differ from those obtained by FI, suggesting that they would not result in opsonization and phagocytosis. *S. aureus* is also able to produce distinct proteolytic enzymes against complement proteins. Staphylokinase can form a complex with plasminogen, resulting in a serine protease activity efficient against C3 and IgG. Moreover, plasmin is able to bind the surface of *S. aureus* and inhibits the binding of IgG, C3b, and iC3b and blocks the effect of opsonization (176). This enzyme activity leads to the CP inhibition. *S. aureus* produces four important proteases: cysteine proteases staphopain A and B, serine protease V8, and metalloproteinase Aur. These proteases lead to a drastic decrease in the hemolytic activity of serum and are efficient against the three pathways (177). Aur is responsible for the consumption of C3 in the fluid phase (178). *N. meningitidis* is able to produce a protease NalP, which cleaves C3. This degradation produces two fragments, a shorter C3a-like and a longer C3b-like, degraded by host serum and leading to a decreased C3b deposition on the bacterial surface (179). *N. meningitidis* is able to inhibit the CP by capsular oligosaccharides, which represents a virulence factor of meningococcal infections (180). Capsular oligosaccharides interfere with engagement of C1q by IgG Fc, and lead to decreased C4b deposition and inhibition of CP activation.

Viruses can also produce C3b cleaving enzymes. Nipah virus particles carry a FI-like protease activity able to cleave and inactivate C3b, using FH and CR1, but not CD46, as cofactors (181). These data help to explain how an enveloped virus such as Nipah virus can infect and disseminate through body fluids that are rich in complement.

Production of Inhibitors of C3 Convertase

Production of inhibitors of C3 convertase has been observed in a few cases. *S. aureus* produces extracellular fibrinogen-binding protein (Efb), which inhibits the conversion of C3 to C3b by its affinity for the thioesther domain (TED). It binds to the TED domain on an area, shared by FH and CR2 binding sites (182–185). Efb acts as a C3 convertase inhibitor by blocking C3b formation and inhibits the opsonophagocytosis by granulocytes (186). Ehp (also known as Ecb), a homologous protein of Efb, binds two molecules of C3b and works as an efficient inhibitor of the alternative C3 convertase (187). Thus, it leads to a decrease of the activation of the C5 convertase and of the resulting C5a level. Surface immunoglobulin-binding protein (Sbi) forms a tripartite complex with C3b and functional FH that potentiates inhibition of complement activation (188). Efb and Sbi are able to recruit human plasminogen after binding to C3/C3b. Plasminogen is converted to plasmin by bacterial staphylokinase or by host-specific urokinase-type plasminogen activator and degrades C3 and C3b in the local microenvironment, thus protecting *S. aureus* (184, 189). Of note, the tripartite complex formed by C3, plasminogen, and Efb-C is more efficient than the one with Sbi, probably due to the higher affinity of C3d to Efb than Sbi, (184). The action of these molecules prevents the activity of the C3 and C5, as shown by a decreased C5a generation (190).

Contrary to Efb, staphylococcal complement inhibitor family (SCIN-A, SCIN-B, SCIN-C), can bind C3b in two distinct regions with a primary site at domain MG8 (191–194). By competing with FB and FH, SCIN is able to block the formation of alternative C3 convertase on one side, and to block the generation of iC3b that could induce phagocytosis on the other side. Moreover, SCIN binds and stabilizes the C3 convertase C3bBb, blocks the decay acceleration, and inhibits the cleavage of C3 into C3b. Thus, it avoids opsonization and complement activation in the same time (193).

Targeting binding sites for complement inhibitors on bacterial surfaces and complement-degrading proteases with vaccine-induced antibodies may be used as a strategy for novel vaccines designed to prevent complement escape. On the other hand, some bacterial proteins with anti-complement activity, such as Efb and SCIN, represent potential novel therapeutics, able to control undesired complement activation and tissue injury in multiple non-infectious diseases (195, 196). It is important to consider that by blocking C3 convertase formation, these inhibitors will avoid opsonophagocytosis and, by such, increase the risk of infection in treated patients (197). The increasing knowledge of the complement evasion strategies of pathogens provides novel insights for more efficient vaccine development and for designing novel therapeutic complement inhibitors.

Complement and Non-Infectious Diseases

The importance of the complement system in physiology is illustrated by severe and life threatening diseases, occurring in case of inefficient or exuberant complement activity (**Figure 10**). Abnormal complement activity is associated with a large number of inflammatory, autoimmune, thrombotic, and age-related diseases. The examples of systemic lupus erythematosus (SLE), atypical

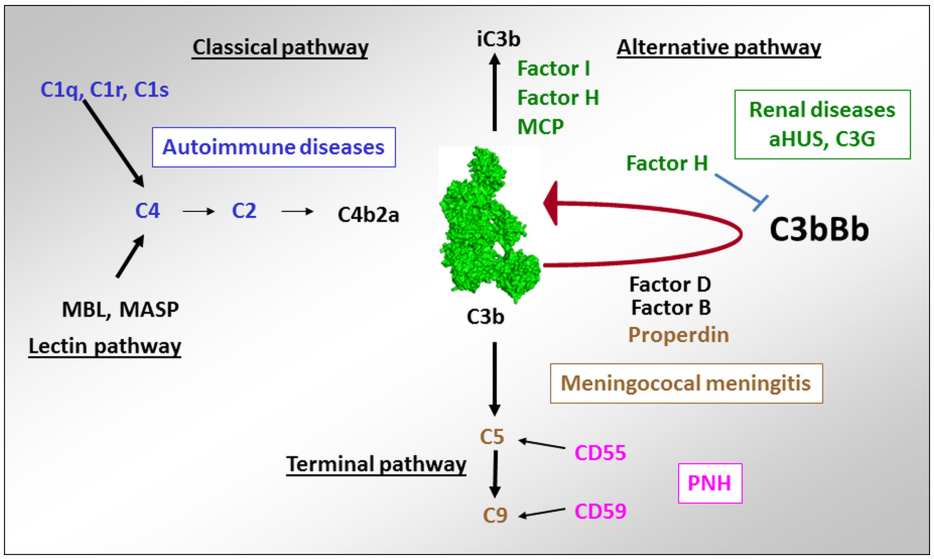

FIGURE 10 | Implication of complement deficiencies in pathologies. Deficiencies of the components of the CP C1q, C1r, C1s, C2, and C4 are associated with autoimmunity. Lack of regulators of the AP FH, CD46, and FI (as well as overactivation of the components of the C3 convertase C3 and FB) is linked to aHUS and C3G. Deficiencies of the terminal complement components C5, C6, C7, C8, and C9 as well as of the only positive regulator of complement – properdin is susceptibility factors to development of meningococcal meningitis. Lack of expression of the regulators of the C3/C5 convertase CD55 and the terminal pathway CD59 on erythrocytes are a cause of red cell lysis in paroxysmal nocturnal hemoglobinuria (PNH).

hemolytic uremic syndrome (aHUS), C3 glomerulopathies (C3G), age-related macular degeneration (AMD), and cancer are treated here in more details, but these are just few among a large list of diseases, including also the paroxysmal nocturnal hemoglobinuria (PNH), graft rejection after transplantation, ischemia/reperfusion injury, Alzheimer and Parkinson diseases, etc.

Diseases Associated with Deficient Complement Activation

Systemic Lupus Erythematosus

Deficiency of components of the classical complement pathway C1q, C1r, C1s, and C4 are very rare but are strongly associated with autoimmune manifestations. The autoimmune disease SLE is characterized by symptoms ranging from skin rashes, chronic fatigue, and arthritis to the more severe glomerulonephritis, serositis, and neurological involvement (198). C1q is the strongest genetic susceptibility factor for SLE, since over 90% of the individuals homozygous for mutations in this protein develop lupus-like symptoms (199). The mutations can cause quantitative deficiency (199) or functional abnormalities (13). A functional abnormality of C1q, due to a mutation in the C1r2C1s2 binding site, resulted in an inability of C1 complex formation and presence of free C1q in the patient serum. The strong association of the mutations of the CP proteins with autoimmunity can be explained by the "waste disposal" hypothesis of Walport (17), suggesting that lack of C1q opsonization and CP activity perturbs the immunologically silent uptake of apoptotic cells and debris, thus resulting in an immune response against self-antigens.

Alteration of the function of C1q can be also induced by presence of autoantibodies against C1q, which are frequently found in SLE, especially in patients with lupus nephritis (200). These antibodies bind to the collagenous tail of C1q (201) or the globular domain (202). These antibodies amplify the effect of C1q-containing immune complexes in the kidneys (203, 204), bind to C1q on early-apoptotic cells, and enhance complement activation (205).

Impaired clearance of apoptotic cells, induced by lack of C1q or inefficient complement activation due to lack of C1r, C1s, C2, and C4 are associated with SLE. At the same time, complement overactivation, due to the presence of anti-C1q antibodies or complement activating immune complexes in the circulation and in kidney glomeruli, is also leading to the same disease, albeit by a different mechanism. Therefore, both lack of activation and a too strong activation of the CP can be linked to autoimmunity.

Diseases Associated with Complement Overactivation

Inherited and acquired quantitative and functional deficiency of the regulators of the alternative complement pathway FH, FI, and CD46 as well as abnormalities inducing overactivation of the AP C3 convertase are associated with rare renal diseases like aHUS (206, 207) and C3G (208, 209).

Atypical Hemolytic Uremic Syndrome

Atypical hemolytic uremic syndrome is a renal thrombotic microangiopathy disease, characterized by glomerular microvascular endothelial cells activation and damage, leading to microthrombi formation and mechanical hemolysis (206). Within the last decade, complement AP dysregulation has emerged as the cause of aHUS (207, 210). In this disease, complement induces glomerular endothelium damage, secondary to unrestricted complement activation with C5b–9 deposits on the endothelial

cells surface. A loss of regulation is a result of mutations in FH, FI, or CD46 genes, found in ~50% of the cases. FH mutations are frequently located in the C-terminal domains and result in reduced capacity of FH to bind C3b and the GAG of the cell membrane (211–213). Mutations in FI impair its catalytic activity (214, 215). Complement overactivation in 10% of the cases is due to mutations in the components of the C3 convertase C3 and FB, forming a more potent convertase and/or a converatase resistant to decay by the regulators (216–221). In addition to mutations in complement genes, autoantibodies against FH, leading to an acquired FH functional deficiency, have been reported in aHUS patients (222–224). The binding epitopes of these autoantibodies are localized to the C-terminal recognition region of FH, which represents a hot spot for aHUS mutations (225).

Altogether complement-associated abnormalities are found in about 60% of the aHUS patients (226, 227). At present, over 200 distinct mutations have been reported in aHUS patients (207). All reported mutations were heterozygous, except in 15 patients (mostly from consanguineous families) with homozygous FH or CD46 deficiency (228). In a particular form of aHUS, mutations were found in DGKE – a gene outside of complement, involved in the protein kinase C signaling pathway (229). Its deficiency induces permanent pro-coagulant phenotype of the endothelial cells (230). aHUS has an incomplete penetrance among the mutation carriers and a triggering event (infection, pregnancy, etc.) and additional genetic predisposing factors (particular at risk haplotypes in FH and CD46) are needed to induce the disease (220, 231, 232). The hemolysis, which accompanies the disease onset, can serve as a secondary hit for the aHUS development, since released heme activates the alternative complement pathway in the fluid phase and on the endothelial cells surface (233, 234). The availability of the first effective anti-complement therapeutic drug, eculizumab, has changed dramatically the outcome of this rare kidney disease.

C3 Glomerulopathies

C3 glomerulopathies are rare chronic kidney diseases, characterized by predominant C3 deposits in glomeruli, in particular, in the mesangium and along the glomerular basement membrane, frequently associated with mesangial cells proliferation. According to the pattern of C3 deposits observed by immunofluorescence and electronic microscopy, two subtypes of C3G are described – dense deposit disease (DDD) and C3 glomerulonephritis (C3GN) (208). The pathogenic process in C3G is presumed to be due to uncontrolled C3 and/or C5 convertases activation, leading to C3 deposits and intra-glomerular inflammation. Autoantibodies targeting the AP C3 convertase, named C3 nephritic factor, are present in more than 50% of C3G patients (235–237). Few genetic abnormalities have been identified. These include mutations of complement genes, coding for C3 (1 case) (238) and the regulator FH (<20 mutations) (237, 239). Mutations affecting *FH* gene result more frequently a protein deficiency in the plasma. More recently, mutations in the *CFHR5* gene were reported, as well as rearrangements and copy number variations in the *CFHR* gene cluster (240–242). A particular form of C3GN is the CFHR5 nephropathy, characterized by a genetic defect in CFHR5, rendering it a strong competitor of FH. It is found in patients from of Cypriot descent (241, 243).

In C3G, genetic abnormalities affect the same genes as in aHUS, with a lesser frequency compared to aHUS, affecting only about 20% of the patients (209, 237). An interesting observation is that the mutations in FH and in FI, which are associated with C3G, are frequently the same, as the ones found in aHUS. They induce either complete or partial FH deficiency or functional defects in the N-terminal part of the protein (237). Mutations in the membrane anchoring C-terminal part of FH are found frequently in aHUS and nearly not in C3G. Therefore, the mutation itself (particularly the deficiencies of FH and FI and the mutations in the N-terminal part of FH) may not be sufficient to determine the type of the renal injury. The triggers of the AP dysregulation remain undetermined.

C3 nephritic factor and FH or FI mutations, inducing an overactivation of the AP are found also in patients with another type of membranoproliferative glomerulonephritis, associated with immunoglobulin deposits in the kidney – MPGN type I (237). Therefore, the role of the AP has to be considered even in diseases, for which CP activation is expected.

Contribution of Complement in Diseases, not Associated with Complement Defects

Age-Related Macular Degeneration

A particular polymorphism of FH (Y402H) is strongly associated with development of AMD, which is the first cause of blindness in the developed countries (244–247). The loss of central vision is associated with loss of photoreceptors and drusen formation in the retina (248). This is due to the constant exposure to light, smoking, the high-metabolic rate in the eye, and its particular sensibility to oxidative stress. Oxidized lipids and malondialdehyde are generated and if not properly handled by FH, they induce complement activation (249, 250) (**Figure 11**). FH binding to oxidized epitopes on altered or dying cells leads to inactivation of C3b to an anti-inflammatory fragment iC3b. Moreover, FH attenuates malondialdehyde-induced IL-8 production by macrophages and retinal pigment epithelial cells and decreases the expression of genes involved in macrophage infiltration, inflammation, and neovascularization in the eye. At risk, FH variant H402 has a weaker capacity to interact with oxidized lipids and malondialdehyde in the drusen, thus allowing constant background complement activation, leading to retinal epithelial cells damage and macrophages activation (249, 250). These results explain the strong genetic association of the H402 with the risk of AMD. Increased risk for AMD is conferred also by polymorphisms in several other genes of the alternative complement pathway, including FI, C3, C2/FB, and C9 (251).

Genetic analyses revealed that the deletion of CFHR1 and CFHR3, which is a polymorphism in the normal population with varying frequency depending on the ethnicity between 2 and 20% (252), is a protective factor against development of AMD (253). CFHR1 and CFHR3 are natural deregulators of FH, competing with it for cell surface binding (241, 254, 255). Therefore, their absence will increase the local binding of FH to oxidized surfaces, leading to a better protection, thus explaining the protective effect of this deletion in AMD.

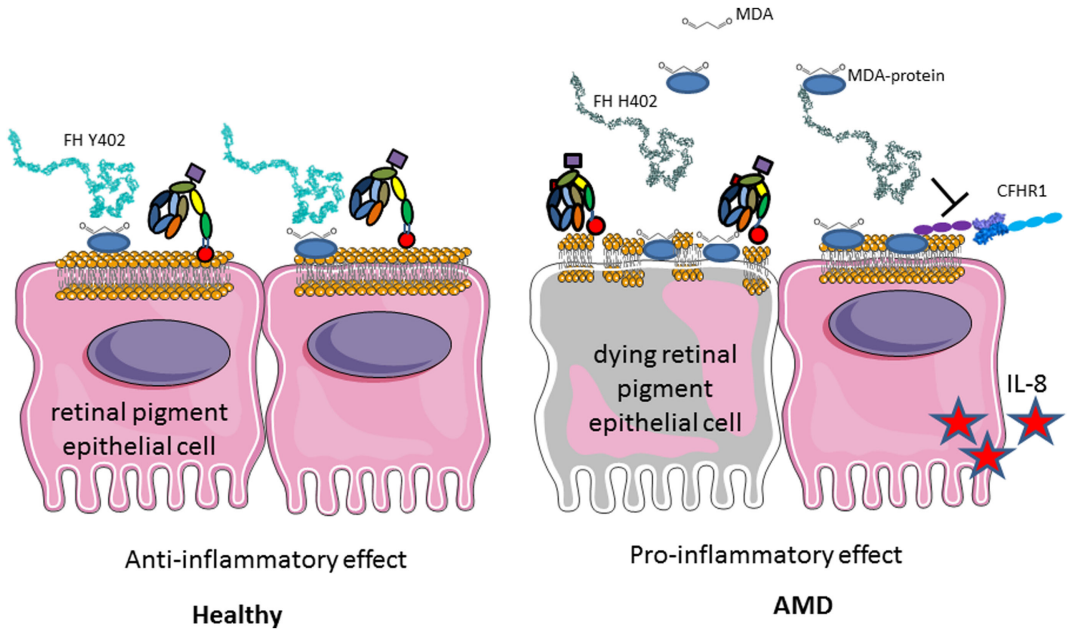

FIGURE 11 | Role of factor H Y402H polymorphism in age-related macular degeneration. FH binds to GAG, oxidized lipids, including malondialdehyde via CCP7 on the membrane of injured retinal epithelial (RPE) cells and protects them from complement attack. H402 variant has a weaker affinity to these products of the oxidative stress and protects less well the cells surface. Deposited C3b is not inactivated to iC3b and promotes complement activation. Less iC3b is generated, which contributes to the silent clearance of the injured cells. RPE cells are activated, secrete pro-inflammatory cytokines, and activate macrophages in their microenvironment. This chronic inflammation predisposes to AMD development with aging.

Cancer

Complement has been considered since a long time as an immune surveillance system against cancer, because complement is activated on the surface of tumor cells. Nevertheless, tumor cells develop inhibitory mechanisms for the terminal steps of the complement cascade, thus preventing complement-mediated cytotoxicity. Surprisingly, recent studies demonstrated that complement activation within the tumor microenvironment can promote tumor growth. Complement activation may support chronic inflammation, promote an immunosuppressive microenvironment, induce angiogenesis, and activate cancer-related signaling pathways. The mechanisms of these phenomena are not fully understood. Prolonged complement activation supports chronic inflammation, promotes an immunosuppressive microenvironment, induces angiogenesis, and activates cancer-related signaling pathways.

Several lines of evidence indicate a role for molecules of the complement system in tumor growth and metastasis, (**Figure 12**). C3, C4, or C5aR deficiencies prevent tumor growth in mice, potentially via inhibition of the CP and the generation of C5a, which has a potent inflammatory potential. In mouse models, the presence of C5a in the tumor microenvironment enhances tumor growth by recruitment of MDSC and increasing T cell-directed suppressive abilities (103, 256, 257). In a breast cancer model, C5aR facilitated metastasis in the lungs through different immune mechanisms in the metastatic niche, including the suppression of effector CD8(+) and CD4(+) T cell responses, the recruitment of immature myeloid cells and the generation of Tregs and a Th2-oriented response (258).

Cancer cells also secrete complement proteins that stimulate tumor growth upon activation via a direct autocrine effect through C3aR and C5aR signaling (256). In patients with ovarian or lung cancer, higher tumoral C3 or C5aR mRNA levels were associated with decreased overall survival. In addition, patients with non-small cell lung cancer have elevated C5a plasma levels (257).

C3a and C5a seem to have opposing effects during tumor development and in case of anti-tumor radiotherapy. While C3a and especially C5a promote tumor growth, radiotherapy-induced tumor cell death and transient local complement activation with production of C3a and C5a (259). The latter appeared crucial to the tumor response to radiotherapy and concomitant stimulation of tumor-specific immunity.

Overexpression of FH has been described in non-small cell lung cancer cell lines and on non-small cell lung cancer biopsies (but not in small cell lung carcinoma and carcinoid cell lines) (260, 261), in bladder tumor cells (262), in cutaneous squamous cell carcinoma (cSCC) and cell lines (263), and in hepatocellular carcinoma tumors (264). Low titer anti-FH antibodies were also found in sera from patients with non-small cell lung cancer (265). Recent studies demonstrated that FH binds to pentraxin 3 (PTX3) in the tumor microenvironment, thus preventing local complement overactivation and generation of pro-tumorigenic C5a (266). Hence, deficiency of PTX3 accelerated tumor development in mouse models of drug-induced cancerogenesis. FI was also suggested to be associated with tumor development of cSCC (267). These results provided evidence for a potential role of FH and FI in the cancer development, but the mechanism of action are still unknown.

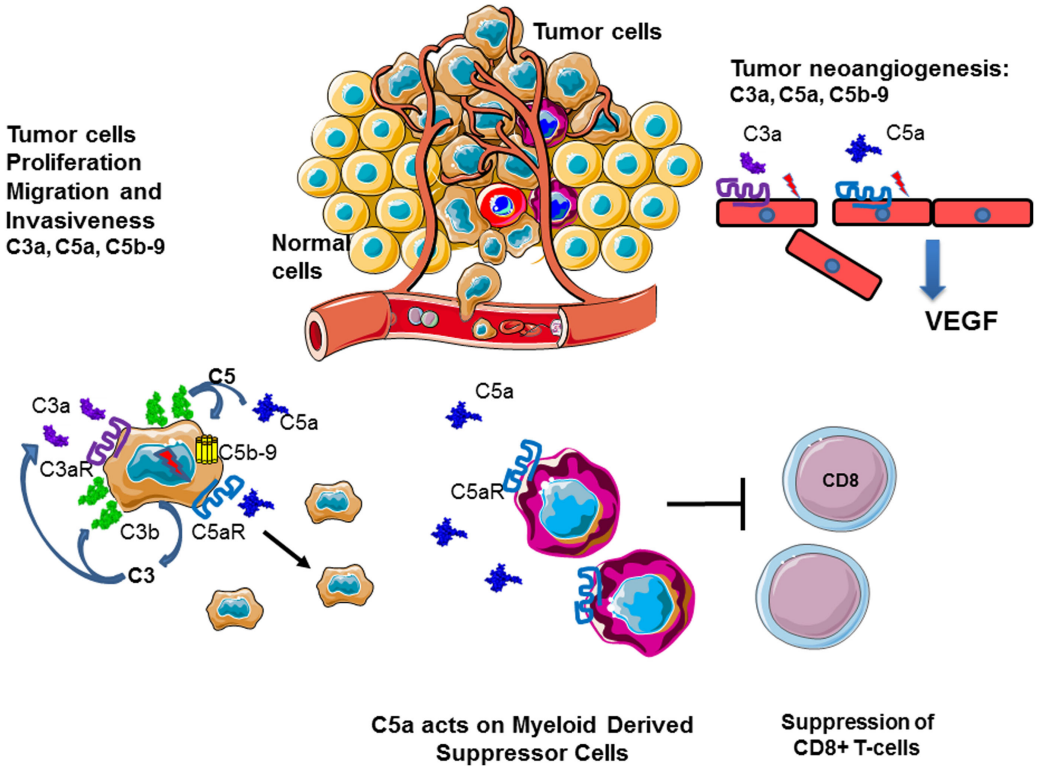

FIGURE 12 | Role of complement in cancer. Complement plays an important role in the chronic inflammation and tumor development. Tumor cells produce complement components and generate C3a, C5a, and C5b–9 in their microenvironment. This result in autocrine tumor cells stimulation, leading to proliferation, migration, and invasiveness. C5a stimulates MDSC, which dampen the immune response, suppressing cytotoxic T cells, and stimulate Treg. C3a, C5a, and C5b–9 promote angiogenesis, helping in tumor nutrient support and dissemination.

These examples clearly indicate that complement is indispensable immunosurveillance system, which needs to function with the right force when and where is needed. Therefore, therapeutic strategies are needed to adjust the level of complement activation in pathological conditions.

Complement as a Therapeutic Target

Since C1q is produced by the myeloid cells, a bone marrow transplantation can overcome this deficiency. Such therapy was efficiently applied to a patient with SLE, resulting in normalization of C1q levels and improvement of the clinical status (268). Deficiency of C1INH in hereditary and autoimmune angioedema (which is not a complement-mediated disease, but induced by excessive production of bradykinin, which is a potent vasodilator) is efficiently treated by plasma derived or recombinant C1INH (269). Alternatively, missing soluble complement components can be introduced to the body by plasma exchange. This was successfully achieved for C1q (270) as well as for FH and FI deficiency in aHUS. Plasma therapy was the first line treatment for aHUS before the development of anti-C5 blocking antibody eculizumab (271, 272). Eculizumab binds to C5 and prevents its entry into the C5 convertase, thus blocking completely the generation of C5a and the formation of MAC (273). This therapeutic is approved for use in PNH and aHUS and gives excellent results (274, 275). Currently, clinical trials are ongoing for many different diseases, where complement is overactivated. Novel inhibitors are under development to block complement at different levels. Particular interest is focused on the blockers of complement at the level of C3 and the C3 convertase. C3b binding peptide compstatin and its derivatives prevent the entry of C3 into the C3 convertase (276, 277). Plasma derived or recombinant FH and recombinant soluble CR1 dissociate the alternative C3 convertase and serve as cofactors for FI (278). Targeted inhibitors like TT30 (FH CCP1–5:CR2) or mini FH (CCP1–4:CCP19–20) bring the regulatory N-terminal domains to the cell membrane, where they are needed to control complement activation (279–282).

Conclusion

Complement plays a central role in the homeostasis and the installation of the adaptive innate immune response. By its supporting role in the clearance of apoptotic and necrotic cells, and its importance in the polarization of T lymphocytes and the humoral response by the cooperation with the B-lymphocytes, complement represents a real keystone of the immune system. This crucial role for the correct functioning of the organism is illustrated by the fact that both complement deficiencies and complement overactivation are associated with severe and life threatening diseases. The improvement of our understanding of the role of complement in health and disease opens up the possibility to use complement modulating drugs in the clinical practice.

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