Von Willebrand factor binds surface-bound C1q and induces platelet rolling

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Deutschland

Basel, 2015

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von
Prof. Ed Palmer, PD Dr. Marten Trendelenburg und Prof. Gennaro De Libero

Basel, den 19.05.2015

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“Science may set limits to knowledge, but should not set limits to imagination.”

Bertrand Russell (1872 - 1970)

Abstract

Pre-mature atherosclerosis and thrombotic complications are major causes of morbidity and mortality in patients with systemic lupus erythematosus (SLE). However, the high incidence of these complications cannot be explained by traditional risk factors alone suggesting direct effects of an activated immune system on hemostasis.

The sequence homology between SLE patient-derived autoantibodies against complement C1q (Fab anti-C1q) and von Willebrand factor (vWF) suggested an interaction between the complement and hemostatic system on the level of initiating molecules.

vWF was found to bind to surface-bound C1q under static conditions. The binding was specifically inhibited by Fab anti-C1q and C1q-derived peptides. Under shear stress the C1q-vWF interaction was enhanced, resembling the binding of vWF to collagen I. In addition, I could show that C1q-vWF complexes induced platelet rolling and firm adhesion. Furthermore, I observed vWF binding to C1q-positive apoptotic micro particles and increased vWF deposition in C1q-positive glomeruli of SLE patients compared to control nephropathy.

I show for the first time binding of vWF to C1q and thus a direct interaction between starter molecules of hemostasis and the complement system. This direct interaction might contribute to the pathogenic mechanisms in complement-mediated, inflammatory diseases.

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1 Introduction

1.1 Complement and Hemostasis

The complement and the hemostatic systems are archetypes of complex, multifunctional cascades of molecules. These two cascades fulfill not only anti-infectious and hemostatic tasks respectively as described initially but gradually were shown to have multiple additional functions requiring tight regulatory mechanisms. The complement and the hemostatic systems are two unique and distinct systems, but with several functional similarities. Both systems belong to the innate protections against external factors. They are initiated through foreign or altered cellular surfaces. Furthermore, both fulfill their function by a cascade of serine proteinases, which is organized by an initiation, amplification and a propagation phase. Each of these two cascades contains regulatory molecules, like inhibitors or cofactors, which are present at the same time. In addition, both cascades consist of certain components interacting with cell surface receptors to mediate downstream effects. These common characteristics provide an explanation for the association of the complement system and hemostasis with several clinical thrombotic and inflammatory conditions.

1.1.1 Complement system

The Complement system is part of the innate immunity and was discovered in the 1890s by Jules Bordet. It consists of a large number of different proteins found in plasma and on cell surfaces, which are presented as inactive pro-enzymes, also called zymogens. The inactive forms become active after proteolytic cleavage usually by other complement proteins. After activation they have at least three physiological functions: Defense against infections, bridging innate and adaptive immunity, and clearing apoptotic cells and immune complexes. The complement system achieves that by the opsonization of pathogens for phagocytes, the release of inflammatory mediators and the induction of cell lysis through the formation of a pore in a membrane. Three pathways, the classical, the lectin and the alternative pathway can induce and execute these effects (Figure 1.1). All these pathways cumulate in the formation of a C3 convertase.

1.1.1.1 Complement pathways

The classical pathway is activated through binding of the C1 complex to the Fc region of bound IgG and/or IgM or directly to certain surfaces. The C1 complex comprises of a recognition protein (C1q) and two associated proteases (C1s and C1r). Immobilization of the C1 complex leads to a conformational change, which induces the enzymatic activity in C1r. C1r cleaves C1s to generate an active serine protease, which in turn splits C4 into C4a and C4b. C4b binds covalently to the surface, whereas C4a is released to induce inflammation. In addition to that, C1s cleaves C4b-bound C2 to generate the serine protease C2a. C4b and C2a form the C3 convertase (C4b2a) of the classical pathway.
The initiation of the lectin pathway starts with the binding of mannose-binding lectin (MBL) to carbohydrate residues on the surface of e.g. microorganisms. The plasmatic form of MBL forms a complex with the serine proteases MASP-1 and MASP-2. After a conformational change in MASP-2, it cleaves, in analogy to C1s, C4 and C2 to form the C3 convertase.
The third pathway of the complement system is called alternative pathway and is able to spontaneously initiate the complement cascade. This occurs through hydrolysis of soluble C3 to form C3(H_2O), which binds factor B. This complex allows factor D to cleave factor B into the fragments Ba and Bb. Consequently, Bb binds to C3(H_2O) and forms the short-living fluid phase C3 convertase C3(H_2O)Bb. This convertase has the capacity to split C3 into C3a and C3b. Most of the fluid phase C3b is immediately inactivated by hydrolysis, but some bind covalently to surfaces. Bound C3b is now able to bind factor B, which is in a next step cleaved by factor D to form the C3 convertase of the alternative pathway (C3bBb). This convertase has a special place in complement activation. By producing C3b, it can generate more of itself. That means that once C3b has been formed through any of the three complement pathways, the alternative pathway acts as an amplification loop by rapidly increasing the C3b production.

1.1.1.2 Immune response of complement

As outlined before, each pathway induces the immune response through the formation of a C3 convertase. The function of this convertase is the proteolytic cleavage of C3 into C3a and C3b. C3a is an important inflammatory mediator recruiting immune cells, whereas C3b alone is a potent opsonin for phagocytes and,

bound to the C3 convertase, part of the C5 convertase (C4b2a3b of the classical and lectin pathway or $C3b_2Bb$ of the alternative pathway). The C5 convertase splits C5 into C5a, like C3a a strong inflammatory mediator, and C5b, important for the assembly of the terminal complement complex in membranes. For this, C5b forms a complex with C6, which leads to the binding of C7. That induces a conformational change in C7 exposing a hydrophobic site, which is inserted into the lipid bilayer. Afterwards the C5b67 complex captures and inserts C8 and C9 into the membrane. C8 induces the polymerization of 10 to 16 C9 molecules to form a pore in the membrane, which is called membrane-attack complex (MAC or C5b-9). Finally, the pore can induce cell lysis.

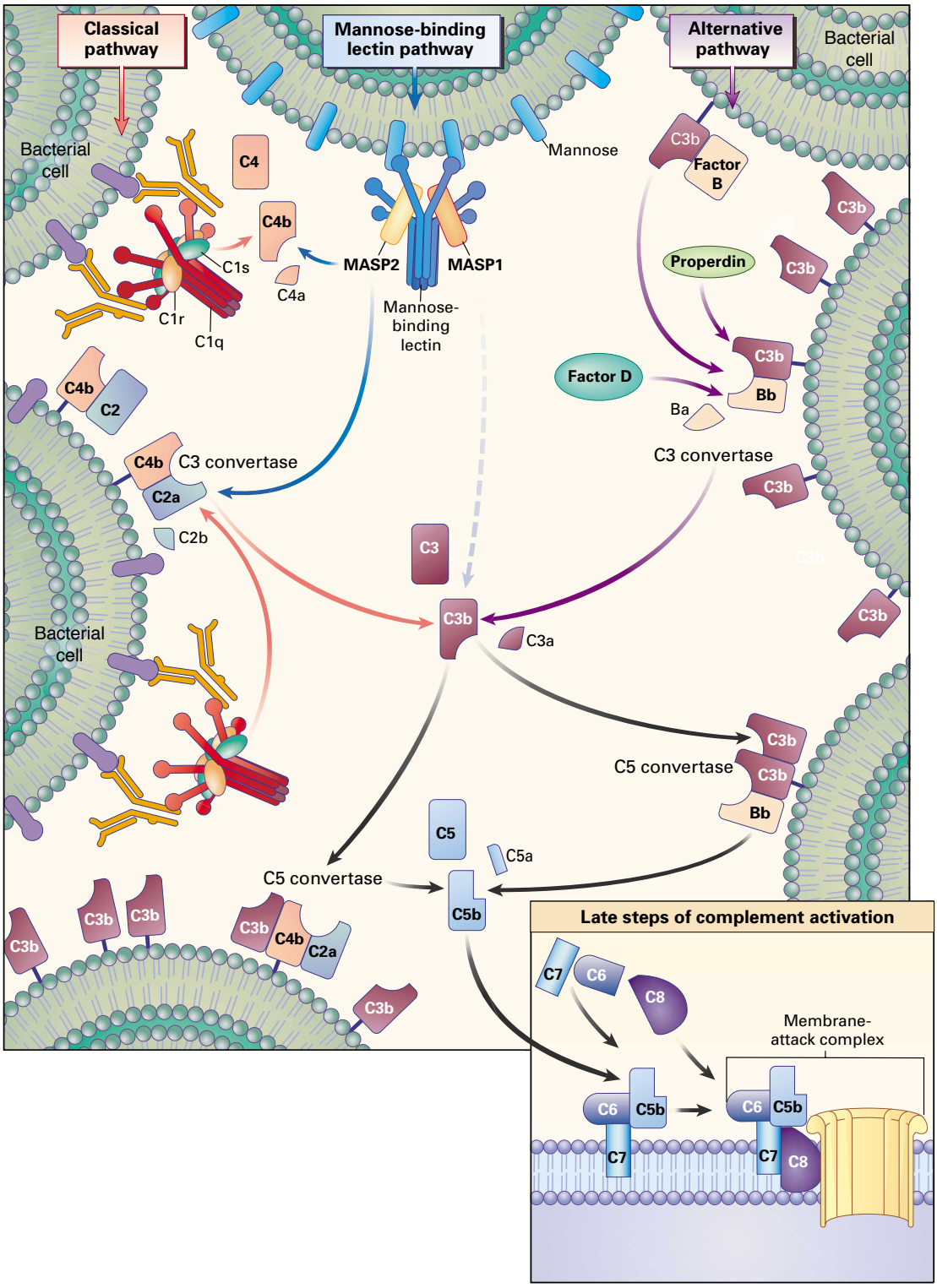

Figure 1.1: Scheme of the complement system. N Engl J Med, Vol. 344, No. 14, 2001

1.1.1.3 Regulation of complement

To prevent uncontrolled activation of the complement system, which could lead to tissue damage, several regulatory mechanisms are of crucial importance. One of

these is the inhibition of the classical pathway by the C1 inhibitor (C1INH). This protein dissociates C1r and C1s from the C1 complex and blocks therefore the proteolysis of C4 and C2. Another level where complement activity is regulated is during the formation of the C3 convertase. Here, C2a can be displaced from the C4b2a complex by the decay-accelerating factor (DAF or CD55), the C4 binding protein (C4BP) and the complement receptor 1 (CR1). C2a assembled with the membrane cofactor protein (MCP), C4BP and CR1 can be inactivated by factor I. The C3 convertase of the alternative pathway is similarly regulated through CR1, DAF, MCP and factor H. Inhibition of MAC formation on host cells is achieved by the membrane bound protectin (CD59), which prevents C9 binding to the C5b678 complex.
On the other hand there are also proteins, which support complement activity such as properdin (factor P), which enhance the stability of C3bBb.

1.1.1.4 C1q

The 460kDa C1q protein circulates in blood as part of the C1 complex, the initiator of the classical complement pathway, and interacts directly with immunoglobulin. Nevertheless, its function is not restricted to the initiation of the complement system alone but also comprises a wide spectrum of functions such as cell processes (e. g. migration, adhesion and differentiation of fibroblasts, granulocytes and mast cells), immune cell modulation and clearance of apoptotic cells[1]. In addition, C1q seems to be able to activate platelets, which are key players in hemostasis[2,3]. Furthermore C1q plays also a role in pregnancy[4], in the formation of eye-specific synapses[5] and in angiogenesis[6].
The major sites of C1q biosynthesis are macrophages, but also dendritic cells, endothelial cells, microglial cells, neurons and trophoblasts can express C1q. The C1q molecule is a multimeric glycoprotein composed of 18 polypeptide chains of 3 different types: C1q-A, -B and -C. Each chain consists of a N-terminal collagen-like region (CLR) and a C-terminal globular head domain recognizing the Fc part of aggregated immunoglobulins[7,8](Figure 1.2). Through covalent and non-covalent interactions in the collagen-like region C1q obtains its typical ‘bunch of a tulip-like’ structure.

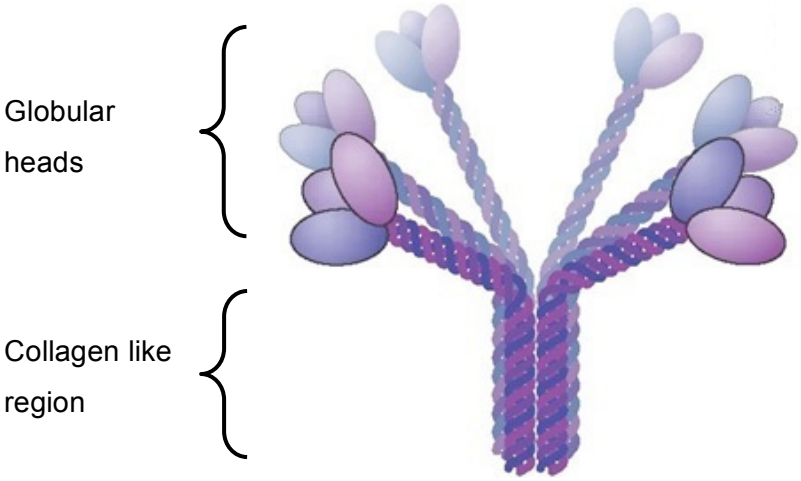

Figure 1.2: Scheme of C1q representing its typical 'bunch of a tulip-like' structure

1.1.2 Hemostatic system

The hemostatic system is a complex mechanism, which prevents bleeding after trauma, invasion of microorganisms through a ruptured surface and their spreading in the circulation by engulfment. Therefore it is also considered to be part of the innate immune system.

Although taking place in parallel the hemostatic system can be divided into primary and secondary hemostasis [Bettina Kemkes-Matthes, Gerd Oehler: "Blutgerinnung und Thrombose", 3rd edition]. The primary hemostasis acts immediately after injury of a blood vessel to stop bleeding within approximately 5 min by the formation of a first instable platelet clot, whereas the delayed (> 5 min) secondary hemostasis, which is also called coagulation cascade, is used to stabilize the thrombus.

1.1.2.1 Primary hemostasis

After cell damage components of the extracellular matrix are exposed to the blood circulation. Collagen is the most abundant component of the extracellular matrix and is recognized by the von Willebrand factor (vWF). Thrombocytes start rolling through interaction of their glycoprotein (GP) Ibα with the A1-domain of vWF. Finally, firm adhesion of platelets to vWF is induced by the $\alpha_{IIb}\beta_3$-vWF C1-domain interaction leading to platelet activation (Figure 1.3). An additional stimulus for platelet activation is the interaction of platelet GP VI with collagen. In a following step circulating vWF binds to adherent platelets via the GPIbα-A1 and $\alpha_{IIb}\beta_3$-C1 interaction and allows

additional platelets to adhere to the construct, which leads finally to the formation of a thrombus consistent of platelets and vWF.

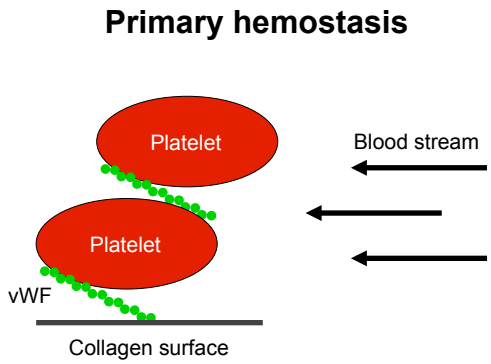

Figure 1.3: Scheme of primary hemostasis

1.1.2.2 Secondary hemostasis

To stabilize the platelet clot, fibrin, which is a product of the coagulation cascade, is essential. Two pathways can lead to fibrin formation, the extrinsic and intrinsic pathways (Figure 1.4). In the extrinsic pathway tissue factor (TF) is released by damaged endothelium and activates factor VII (FVIIa) in the presence of calcium. The TF-FVIIa-complex activates FX and FIX through consumption of calcium on the phospholipid surface of thrombocytes. In the intrinsic pathway FXII is activated through direct contact with damaged vessels, also called Kinin-Kallikrein-pathway. FXIIa activates FVII of the extrinsic pathway and FXI, which activates FIX. FIXa in turn activates FX in an amplification loop, which is responsible for the cleavage of prothrombin (FII) into thrombin. The major function of thrombin is the cleavage of fibrinogen into fibrin, which stabilizes the vWF-induced platelet aggregate. Thrombin-activated FXIII induces cross-links between single fibrin molecules and induces therefore the formation of a fibrin meshwork. Another function of thrombin is the activation of FV (cofactor for FXa) and FVIII (cofactor for FIXa) to accelerate the generation of thrombin in a positive feedback loop.

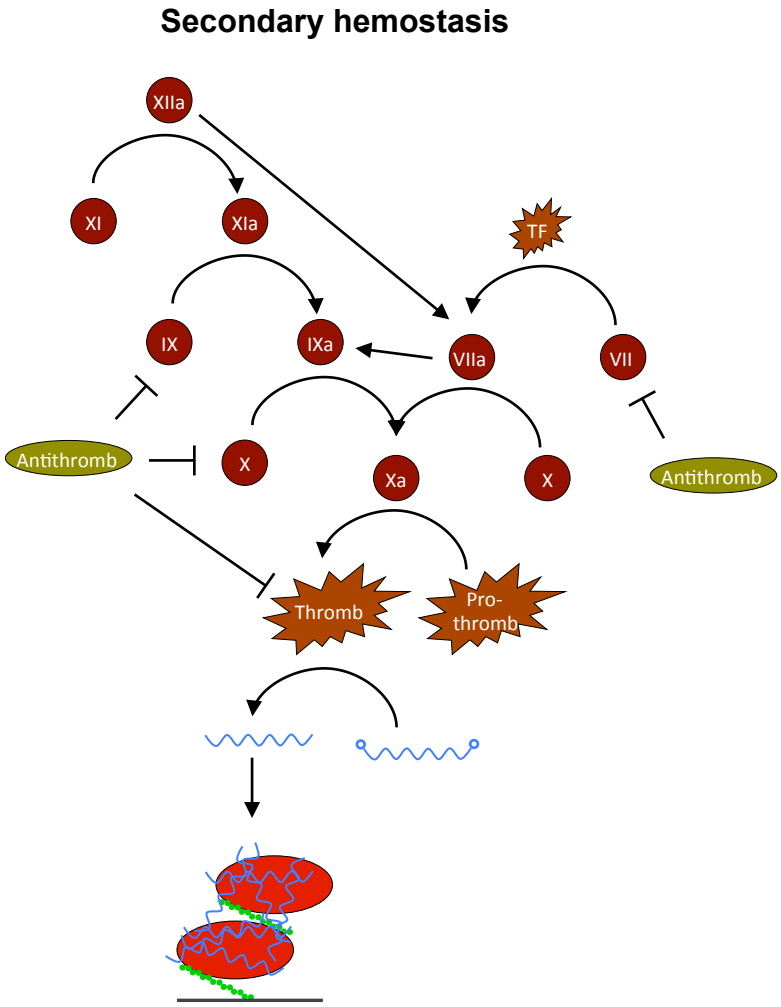

Figure 1.4: Schema of secondary hemostasis and the following stabilization of the primary thrombus. TF, tissue factor; Thromb, thrombin.

1.1.2.3 Regulation of hemostasis

Thrombin induces also a negative feedback loop to regulate the coagulation cascade, which is necessary to avoid uncontrolled thrombus growing and to limit the reaction to the point of injury. For this, it binds to the membrane protein thrombomodulin (TM) on the endothelium and looses by that the ability to cleave fibrinogen and to activate platelets. TM bound thrombin is now able to activate protein C (APC), which forms a complex with its cofactor protein S on the platelet surface. APC is responsible for the inactivation of the coagulation enhancers FVIIIa and FVa through specific cleavage. The inactivation of the other coagulation enzymes (FVIIa, FIXa, FXa, FIIa) occurs through serine protease inhibitors (serpins). The most important serpin in the coagulation system is antithrombin, which needs heparin expressed on the endothelium as cofactor for its response.

The primary hemostasis, induced by the binding of vWF to extracellular matrix components, needs to be regulated, too. This is achieved by the cleavage of multimeric vWF into small fragments through a metalloproteinase called ADAMTS13 decreasing the avidity for thrombocytes.

1.1.2.4 Von Willebrand factor

The von Willebrand factor (vWF) is a glycoprotein, which is released by endothelial cells and megakaryocytes/platelets into circulation. Before release vWF is synthesized as a pre-pro-vWF in the endoplasmic reticulum in megakaryocytes and endothelial cells. Through further processing and the generation of disulfide bridges vWF dimers are formed. In a last step vWF multimers are formed through additional disulfide bridges, and are stored in the Weibel Palade bodies of endothelial cells and in α-granules of platelets.

Whereas the classical functions of vWF are considered to be the initiation of thrombus formation on ruptured surfaces and the stabilization of coagulation factor VIII, a major enhancer of the coagulation cascade, more versatile functions were elucidated in the last years, such as intima thickening, tumor cell apoptosis and inflammatory processes[9]. For most of these functions particular structural characteristics of vWF are crucial. In circulation the vWF molecules occur as a heterologous series of covalently linked subunits ranging from dimers to multimers with more than 40 subunits. The primary structure of vWF reveals different discrete functional domains, such as the A1-domain recruiting platelets and the C1-domain initiating firm adhesion and activation of platelets via the integrin $\alpha_{IIb}\beta_3$ (Figure 1.5). For the correct structure-target interaction shear stress is essential to turn the globular structure of vWF into an elongated chain decrypting multiple functional binding sites[10-12].

After damage of a vessel wall vWF binds to exposed collagen fibers via its A3-domain. Through blood stream induced shear the vWF-A1 domain is exposed and enables platelets to role on vWF through the interaction with glycoprotein (GP) Ibα, a component of the GPIbα-IX-V receptor complex. Platelet activation is induced by the following interaction of the integrin $\alpha_{IIb}\beta_3$ with the C1-domain of the vWF. Activated platelets spread out and release pro coagulant mediators like thrombin, serotonin and ADP. Additional vWF can adhere on immobilized platelets via the A1 domain and capture additional thrombocytes by the A1- and C1-domains. To avoid

uncontrolled platelet aggregation induced by large vWF multimers in the circulation, ADAMTS13 cleaves the vWF in the A2-domain into less active small vWF fragments.

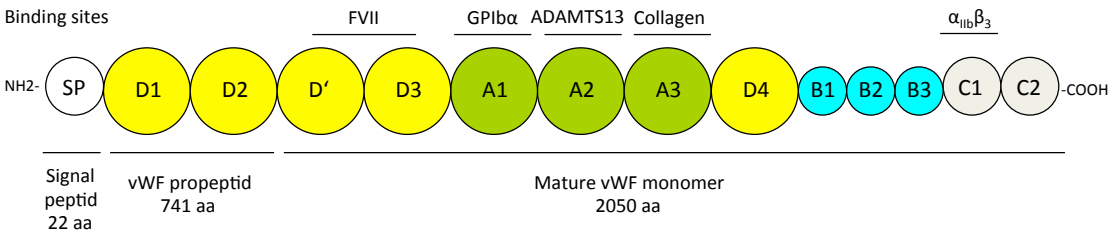

Figure 1.5: Schema of vWF

1.1.3 Crosstalk between Complement and Hemostasis

A first hint for the extensive cross-talk between complement and coagulation was observed in 1988 where the levels of complement activation products were found to be significantly higher in human serum than in anti-coagulated blood, suggesting complement activation during blood clotting[13]. Additional work demonstrated the generation of active C3 fragments by human plasma kallikrein, either directly or through activation of factor B[14,15]. A more recent study demonstrated the proteolytic activation of C5 by thrombin in C3-defiecient mice[16]. Plasma kallikrein and thrombin are just two examples of complement activation through coagulation factors. Other examples are the activation of C3 and C5 by FIXa, FXa, FXIa and plasmin[17]. Furthermore, the C1 complex can be activated through activated FXII[18]. Moreover, other studies could show that the complement system can also be activated by thrombocytes[19].

In addition to the complement activating capacity of the coagulation system, vice versa the complement system has stimulating effects on the coagulation. One effect is the ability of C3a and C1q to activate thrombocytes[2,20]. Regulation of TF expression on the endothelium via the complement system is an additional way of affecting the hemostasis. Ikeda et al. could demonstrate the induction of TF expression by endothelial cells through C5a[21]. There are more overlaps reported supporting the tight interaction between complement and coagulation, but the basic mechanisms are less well understood.

However, understanding the cross talk between complement and hemostasis has fundamental clinical implications in the context of inflammatory diseases in which the complement-coagulation interplay contributes to the development of life-threatening complications. For example, these complications can be found in the systemic

inflammatory response syndrome (SIRS)[22], in the primary anti-phospholipid syndrome (APS)[23,24] and in systemic lupus erythematosus (SLE)[25,26]. In SLE, as well as in APS, autoantibody-mediated complement activation leads to thrombotic disorders, representing one of the major causes of morbidity and mortality[27].

1.2 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease with clinical manifestations mainly involving the skin, joints, kidneys, nervous system, serosal surfaces and blood components[28]. Disease flares and autoimmune dysregulation characterize the process of SLE. In addition to that, hypocomplementemia can be frequently found in SLE patients, which is due to consumption of C1q and other components of the classical complement pathway. Furthermore, SLE is characterized by the presence of autoantibodies with various specificities. Anti-nuclear, anti-Ro, anti-phospholipid, anti-double strand DNA and anti-C1q antibodies (anti-C1q) belong to the repertoire of these antibodies forming immune complexes and causing tissue damages.

However, nowadays the major cause of increased morbidity and mortality in patients affected by SLE for more than 5 years is cardiovascular disease (CVD)[29]. Atherosclerosis is the main cause for most of the CVD[30]. Clinical studies suggest that not only classical risk factors, such as age, hypertension, smoking and diabetes mellitus, but also other non-classical, disease-specific factors contribute to accelerated atherosclerosis in inflammatory diseases like SLE[31,32].

Today the prognosis for SLE is optimistic, but depends on the involvement of organs, the response to treatment and the extent of drug-induced adverse effects.

1.2.1 Clearance deficiency in SLE

Apoptosis, the programmed cell death, is an essential mechanism in homeostasis and is usually completed by an anti-inflammatory and anti-immunogenic clearance through phagocytes. Altered mechanisms in the clearance of dying cells might play a central pathogenic role in the development and acceleration of SLE. This hypothesis is supported by several observations. Mice with a defect in the clearance of apoptotic cells were shown to develop severe autoimmunity with the occurrence of autoantibodies directed against intracellular components, as observed in patients

with SLE[33]. Vice versa, studies on lupus-prone mice demonstrated an impairment of apoptotic cell uptake[34]. Results from experiments with macrophages derived from SLE patients could also demonstrate a defective uptake of apoptotic cells[35]. Decelerated clearance of apoptotic cells leads to necrosis and consequently to the release of intracellular, pro-inflammatory and immunogenic material. That could be the leading cause for the occurrence of autoantibodies[36,37] including those mentioned above. This hypothesis has been called the "waste disposal" hypothesis. In parallel, the complement activation is upregulated. A reduction in early complement protein levels can be observed in patients with SLE, probably because of activating mechanisms leading to secondary depletion[38].

In addition to its clearance deficiency in SLE, apoptosis is also implicated in the progression of atherosclerosis[39-41]. Apoptosis seems to be a strong thrombogenic trigger of atherosclerotic plaques, e. g. by the expression of tissue factor (TF), an initiator of the extrinsic pathway of coagulation, and phosphatidylserine, an enhancer of TF activity[42,43]. Since atherosclerosis is developing in a pro-inflammatory environment, these findings point to a link between the immune system and the coagulation system in lupus.

1.2.2 Anti-C1q in SLE

Besides the elimination of pathogens, the complement system plays also an important role in the clearance of apoptotic cells[44]. Especially C1q seems to be important for the uptake of apoptotic material by opsonization for phagocytes[45,46]. C1q exposes cryptic epitopes in the collagen like region (CLR) when it is bound to apoptotic surfaces[47], which might become immunogenic due to an inefficient phagocytosis. That might be the basis for the occurrence of anti-C1q, recognizing bound C1q but not fluid phase C1q. One of these cryptic epitopes is the peptide sequence A08 on the N-terminal C1q A-chain, which has been shown to be a major linear epitope for anti-C1q in SLE patients[48].

The unexpected observation of a sequence homology between bone marrow-derived autoantibodies against complement C1q of a patient with systemic lupus erythematosus (SLE) and vWF suggested that vWF might also be able to bind to surface-bound C1q[49]. This homology was unusual with only a few proteins being known to have such a homology with vWF (e.g. mucins, thrombospondin 1 and 2). However, in the context of a study on the binding of vWF to collagens, no vWF

binding to bound C1q could be detected[50]. The discovered homology and the lack of more detailed analyses brought us to resume this study.

1.3 Hypothesis

Based on the observations outlined before, we assumed an interaction between C1q (recognition domain of the C1 complex being the starter complex of the classical pathway of complement) and the vWF, the starter molecule within the primary hemostasis.

Binding of vWF to bound C1q would for the first time establish a direct link between starter molecules of primary hemostasis and the complement cascade. In fact, C1q could then be considered as an initiator of hemostasis. Furthermore, the direct interaction of complement C1q with vWF might be a major pathogenic mechanism in the context of complement-mediated inflammation, e.g. for the occurrence of thrombotic and/or atherosclerotic events as frequently observed in patients with SLE[51].

2 Material and Methods

2.1 Reagents and antibodies

In the experiments outlined below we used PPACK (Enzo Life Science; USA), Quinacrine (Sigma; USA), NeutrAvidin (Pierce Biotechnology; USA), peptides (GenScript; USA), C1q (Complement Technology; USA), collagen I (Santa Cruz Biotechnology; USA), recombinant (r)vWF (kind gift of Baxter; Austria), streptavidin-alkalin phosphatase conjugate (Invitrogen; USA), alkaline phosphatase substrate (Sigma; USA), pepsin from porcine gastric mucosa (Sigma; USA), collagenase from Clostridium histolyticum (Sigma; USA), staurosporine (Enzo Life Science; USA) and Sheep-derived red blood cells 10% (Innovative Research; USA). Furthermore we used the following antibodies. A monoclonal mouse anti-human C1q (Bigler et al., 2009[52]), human bone marrow derived anti-C1q Fabs (Schaller et al., 2009[49]), a polyclonal rabbit anti-vWF (Abcam; UK), a polyclonal goat anti-vWF and FITC-labeled polyclonal rabbit anti-C1q antibody (both from Dako; USA), a monoclonal mouse anti-vWF (AbD Serotec; USA), a biotinylated horse anti-mouse (Vector; USA), an alkaline phosphatase-conjugated $F(ab)_2$ donkey anti-rabbit (Jackson; USA), an alkaline phosphatase-conjugated mouse anti-goat antibody (Sigma; USA), a polyclonal donkey anti-mouse Alexa Fluor 488 and a polyclonal goat anti-rabbit Alexa Fluor 647 (both from Invitrogen; USA), a Cy3-labeled goat anti-mouse antibody (Dako; Denmark), polyclonal goat anti-human C4b antibody (Complement Technology; USA).

2.2 Methodes

2.2.1 vWF ELISA

The incubations in all performed ELISAs were done at room temperature on a plate shaker, if not stated differently. Coating was done in carbonate buffer (0.1M sodium carbonate, pH 9.6) over night at 4°C and blocking was performed with PBS 1% BSA for 1h. PBS 0.05% Tween 20 was used for washing.

96-well MaxiSorp microplates (Nalgene Nunc International; USA) were coated with 5µg/ml C1q or C1 complex. Wells coated with 20µg/ml collagen I and uncoated wells served as positive and negative control respectively. After washing 3 times all wells were blocked and incubated with different concentrations of rvWF (0.1-10µg/ml diluted in PBS 0.05% Tween 20) for 1h. This step was also done using 1M NaCl

high-salt buffer including 0.05% Tween 20 as diluent. Alternatively, whole human blood collected from healthy volunteers in 50µM PPACK, as source of native vWF, was diluted (1:100, 1:10, undiluted) in Hepes-Tyrode buffer (130mM NaCl, 1mM $MgCl_2$, 12mM $NaHCO_3$, 2.9mM KCl, 0.34mM NaH_2PO_2, 5mM Glucose and 5mM Hepes, pH 7.4) and incubated for 1h. Unbound vWF was removed by 3 wash steps and bound vWF detected by incubation with a polyclonal rabbit anti-vWF antibody (diluted 1:10000 in PBS) for 1h. Wells were washed and binding was detected by addition of an alkaline phosphatase-conjugated secondary $F(ab)_2$ donkey anti-rabbit antibody (diluted 1:1000 in PBS) for 45min. Thereafter wells were washed 4 times. To quantify the amount of enzyme linked secondary antibody, alkaline phosphatase (AP) substrate was added to the wells and incubated for 30min in the dark. A plate reader at a wavelength of 405nm analyzed the AP-induced color change of the substrate.

2.2.2 C1q-vWF complex capturing ELISA

Soluble C1q (10µg/ml; 20µM) and rvWF (2µg/ml; 8µM) were preincubated for 2.5h. This mixture was then added to 96-well microplates that had been coated with either a monoclonal mouse anti-vWF or a polyclonal goat anti-C1q antibody (diluted 1:1000 in carbonate buffer) prior blocking. After a 5-minute incubation the plates were washed. vWF-binding was measured as outlined before. In parallel C1q was detected using a monoclonal mouse anti-human C1q antibody (diluted 1:500 in PBS) for 1h. Following a washing step an AP-conjugated secondary goat anti-mouse antibody (diluted 1:1000 in PBS) was added for 1h and afterwards quantified by AP-induced color change of the AP substrate as outlined before.
In parallel soluble C1q (10µg/ml) was added to a 96-well microplate coated with 5µg/ml rvWF and blocked with PBS 1% BSA. After 1h of incubation C1q was detected by a polyclonal goat anti-human C1q (diluted 1:5000 in PBS) and an AP-conjugated secondary mouse anti-goat antibody (diluted 1:20000 in PBS), each time incubated for 1h.

2.2.3 Peptide ELISA

C1q peptides: Biotinylated and non-biotinylated peptides with >95% purity were synthesized by GenScript (USA). Peptide A08 (GRPGRRGRPGLKG) is derived from

the C1q-A chain. The control peptide (GAPGKDGYDGLPG), derived from the N-terminal region of C1q-C chain, is the counterpart of peptide A08 and was used as a negative control.
A 96-well microplate coated with 5µg/ml NeutrAvidin was used to immobilize a biotinylated A08 peptide at 5µg/ml diluted in PBS. In parallel, mutant A08 peptides (scrambled A08: RRGPRLRGPKGGG; A08 [R->K]: GRPGKKGKPGLKG; A08 [R->A]: GRPGAAGAPGLKG) were used as controls. After 2h of incubation the wells were washed 3 times and incubated with the rvWF like in the experiments with coated C1q before. vWF-binding was measured as outlined before.

2.2.4 Competition/Blocking ELISA

Purified bone marrow derived Fabs from a SLE patient (6, 32, 160µg/ml diluted in PBS 0.05% Tween 20) were added to a C1q-coated 96-well microplate blocked with PBS 1% BSA. Equimolar amounts of IgG from a healthy individual added to the plate were used as a control. After 1h incubation 2µg/ml rvWF was added for 1h without prior washing of the plate. vWF-binding was measured as outlined before.
Additionally, in other experiments rvWF was preincubated with soluble A08 peptide or control peptide diluted in PBS 0.05% Tween 20 for 2.5h. In a next step the mixtures were added for 1h to a 96-well microplate coated with C1q and blocked with 1% BSA. vWF-binding was measured as outlined before.

2.2.5 vWF binding to different C1q regions after enzymatic digestion of C1q

Digestion of the collagen-like region (CLR): C1q (75µg) was digested with 187U collagenase (100µg, diluted in 0.2M NaCl, 0.005M $CaCl_2$, 0.05M Tris) for 16h at 37°C. After 1h incubation at 4°C the mixture was centrifuged at 14000x*g* for 10 min.
Digestion of the globular heads (GH): C1q was dialyzed against 0.1M sodium acetate anhydrate (pH 4.45) over night at 4°C. Afterwards C1q was digested with pepsin (dissolved in 10mM hydrochloric acid) at a ratio of 30/1 for 20h at 37°C.
After the digestions, CLR and GH were isolated and fractionized by FPLC using a Superdex^{TM} 200 (10/300 GL; GE Healthcare; England) column and fractions were directly coated on a 96-well plate over night at 4°C. The vWF binding procedure to different C1q fractions was performed as outlined before. In parallel, SLE patient-derived anti-C1q autoantibodies recognizing CLR but not GH, and heat aggregated IgG (HAGG) binding to GH but not to CLR were used as controls.

2.2.6 vWF binding to C1q under flow conditions

Flow chambers (µ-Slide VI$^{0.1}$, Ibidi, Germany) were coated with 100µg/ml C1q and blocked. Recombinant vWF (2µg/ml diluted in PBS) was perfused with a syringe pump (Graseby; USA) for 10, 30, 60, 120 and 300s at different shear rates (250, 1000, 2500, 10000/s). Subsequently, the flow chambers were washed for 1min at a shear rate of 1000/s with PBS 0.05% Tween 20 and incubated with a polyclonal rabbit anti-vWF antibody (diluted 1:10000 in PBS) for 1h, at room temperature. Afterwards, flow chambers were incubated with a secondary F(ab)$_2$ donkey anti-rabbit alkaline phosphatase labeled detection antibody (diluted 1:1000 in PBS) to capture the primary antibody after a washing step. Forty-five min after the addition of the secondary antibody the flow chambers were washed for 1.5min at a shear rate of 1000/s with PBS 0.05% Tween 20 and covered with AP substrate for 1h at room temperature in the dark. The color change was detected by scanning (Epson Perfection V700 Photo) the flow chambers, and analyzed using the public domain software "ImageJ" (Rasband, W.S., National Institutes of Health, Bethesda, Maryland, USA).

2.2.7 Determination of the dissociation rate between bound C1q and vWF

To obtain information on the binding affinity, studies with surface plasmon resonance (ProteOn^{TM} XPR36, Biorad, USA) were done. C1q and collagen I were immobilized on two different channels of a GLC chip for ProteOn^{TM} XPR36 protein interaction array system at a concentration of 3µg/ml and 25µg/ml respectively, diluted in acetate buffer pH 5, according to manufacturer's instruction. Different concentrations of rvWF (6.25, 12.5, 25, 50µg/ml) diluted in 10mM Hepes 140mM NaCl (alternatively 40mM NaCl), 0.05% Tween 20 were injected and binding was recorded at a flow rate of 30µl/min. The association and dissociation phases of the interaction of rvWF with C1q were monitored for 300s and 600s, respectively. The chip was regenerated using 4M NaCl. The off-rate was calculated from the dissociation phase, using ProtOn Manager software.

2.2.8 vWF binding to mircoparticles (MPs) in the presence of C1q

Jurkat cell line: Jurkat cells were cultured in RPMI 1640 medium (Gibco, USA) conditioned with 10% heat inactivated FCS and 1% penicillin/streptomycin. Cells were splitted every second day and diluted 1:6 using fresh culture medium.
To generate MPs, $5*10^5$ Jurkat cells/sample were centrifuged at 650x*g* and 4°C for 6min followed by resuspension in 100µl RPMI media/sample containing 1µM staurosporine to induce apoptosis and 50µg/ml C1q. The cell suspension was incubated for 24h at 37°C, 5% CO_2. Next day apoptotic Jurkat cells were removed by centrifugation at 400x*g* and 4°C for 7min. An additional centrifugation step of the MPs-containing supernatant followed. rvWF was added to the MPs with a final concentration of 10µg/ml, vortexed for 30s at 3200rpm and afterwards incubated at room temperature for 1h on a plate shaker (500rpm). Subsequently the suspensions were centrifuged at 4°C for 20min and 1600x*g* to obtain a MP pellet. Purified MPs were resuspended in 100µl PBS 1% BSA/sample and incubated for 1h at room temperature on a plate shaker (500rpm) with a monoclonal mouse anti-human C1q (diluted 1:20;[52]) and/or a polyclonal rabbit anti-human vWF antibody (diluted 1:500 in PBS). After a centrifugation step at 1600x*g* at 4°C for 20min a polyclonal donkey anti-mouse Alexa Fluor 488 labeled antibody and/or a polyclonal goat anti-rabbit Alexa Fluor 647 labeled antibody (both diluted 1:200 in PBS 1% BSA) captured the primary antibodies. An incubation time on ice for 30min in the dark was followed by the addition of 400µl PBS 1% BSA/sample. The fluorescence signal was quantified by flow cytometry (CyAn^{TM} ADP Analyser, Beckman Coulter, USA) and analyzed using FlowJo software (version 9.5.2).

2.2.9 Detection of glomerular vWF

For the analysis of glomerular vWF deposition, kidney sections of 25 SLE patients with proliferative lupus nephritis fulfilling at least 4 out of 11 American College of Rheumatology (ACR) criteria for the classification of SLE were used. All SLE biopsies stained positive for glomerular C1q. As controls, C1q-negative kidney sections of 24 unselected patients with IgA nephropathy were used. This study was approved by the ethical committee Northwest Switzerland (6th of December 2014) and blinded for the experimenter.
Cryosections with 4µm thickness were washed 5min in PBS 0.05% Tween 20 and afterwards incubated at room temperature with a monoclonal mouse anti-vWF antibody (diluted 1:500 in PBS 1% BSA) for 1h. Following a 2 times washing step, each 5min in PBS, a secondary Cy3-labeled goat anti-mouse antibody (diluted 1:600

in PBS) was added for 1h at room temperature. After washing 2 times 5min with PBS a FITC-labeled polyclonal rabbit anti-C1q antibody (diluted 1:40 in PBS) was added to the cryosections and incubated for 1h at room temperature. Finally, after 2 more washing steps of 5min in PBS, the sections were mounted in Celvol-Gallat-Hoechst Blue mounting medium and stored at 4°C. Confocal imaging was done at a 40x magnitude (Nikon A1R Nala; Japan) and images were analyzed by using "ImageJ" software.

2.2.10 Platelet binding to C1q in the presence of vWF under flow conditions

Platelet treatment: Human whole blood (from healthy volunteers) was collected into PPACK (final concentration 25µM diluted in 1x PBS) and centrifuged at 250x*g* for 15min (room temperature) to obtain platelet-rich plasma (PRP). PRP was stained with Quinacrine (final 10µM, diluted in Hepes-Tyrode buffer) in the dark (room temperature) for 1h prior to reinfusion into whole blood. To separate stained platelet-poor plasma (PPP) from whole blood, whole blood was centrifuged at 500x*g* for 15min (room temperature). The blood was supplemented with the equivalent volume of unstained PPP.

Platelet rolling and adhesion: We measured platelet rolling and adhesion on C1q in a parallel-flow chamber (µ-Slide VI$^{0.1}$, Ibidi, Germany) in real time. Briefly, C1q coated flow chambers were blocked with PBS 1% BSA for 1h at room temperature. Blood was perfused through the chambers at a shear rate of 1000/s for 2min (room temperature) following perfusion with 0, 1 and 5µg/ml rvWF diluted in PBS at the same shear rate for 5min (room temperature). 150 frames were recorded with a confocal microscope (Nikon A1R Nala; Japan) corresponding to 5s microscopic observation time within the last seconds of the blood perfusion. The acquired records were analyzed offline using the software "ImageJ", which generated stacks of the records. For estimating the rolling of platelets on C1q the average intensity of all pixels was determined. With increasing dwell time the average intensity per pixel is increasing. To determine the adhesion of platelets all moving particles were erased as described by Meyer dos Santos et al.[53]. Briefly, a particle was considered as "moving" when the intensity of a pixel reached a low value at locations assigned to a moving particle in at least one frame of the stack.

Following this platelet-binding assay the vWF amount was detected as described above, but using a monoclonal mouse anti-vWF detection antibody (diluted 1:1000 in PBS, 1h incubation), a biotinylated secondary horse anti-mouse antibody (diluted

1:1000 in PBS, 1h incubation) and a streptavidin-alkalin phosphatase conjugate (diluted 1:10000 in PBS, 45min incubation).

2.2.11 C1q-induced complement activation assay

One percent normal human serum (NHS), diluted in Veronal buffer, was added to a C1q coated 96-well microplate preincubated with 0, 2 and 8 µg/ml rvWF respectively (see 2.2.1). After 1h incubation and a washing step, a primary anti-C4b antibody (diluted 1:50000 in PBS) and a secondary AP labeled mouse anti-goat antibody (diluted 1:20000 in PBS) measured C4b deposition. The quantification of the bound secondary antibody was performed as outlined before.

2.2.12 Statistics

If not indicated differently, data were presented as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism software, version 6.0b. Comparisons between multiple groups were performed using One-Way ANOVA. P values of below 0.05 were considered to be significant.

2.2.13 List of Supplementary Material

Supplemental movies can be found on the attached CD
Movie S1. Human platelet rolling on a BSA-coated surface using a time-lapse epifluorescence microscope.
Movie S2. Human platelet rolling on a C1q-coated surface in the presence of rVWF using a time-lapse epifluorescence microscope.
Movie S3. Human platelet rolling on a C1q-coated surface using a time-lapse epifluorescence microscope.
Movie S4. Human platelet rolling on a collagen I-coated surface using a time-lapse epifluorescence microscope.

3 Results

3.1 Von Willebrand factor (vWF) binds to surface-bound C1q

To analyze our hypothesized novel interaction between hemostasis and the complement cascade we first examined the binding of vWF to C1q under static conditions using ELISA. We could observe a dose-dependent binding of recombinant (r)vWF to plate-bound C1q (Figure 3.1A). Whole human blood derived vWF also recognized surface-bound C1q comparable to rvWF binding and seemed to bind C1q in higher dilutions more efficiently than collagen I (Figure 3.1B). However, this trend was not confirmed when using undiluted blood, estimated to contain 10µg/ml vWF, for which collagen I was a better binding partner than C1q.

In order to investigate a possible interaction between unbound C1q not exposing cryptic epitopes and vWF, a capture ELISA was performed with anti-vWF and anti-C1q antibodies serving as capture antibodies respectively (Figure 3.1C). In both cases only the capture antibody-specific ligand of a C1q-vWF mixture was detectable, whereas the assumed binding partner was not. Furthermore, we also could not observe significant binding of C1q to immobilized vWF.

In a next step, to reduce unspecific charge-dependent binding effects on this interaction, vWF was incubated in 1M NaCl high-salt buffer on bound C1q (Figure 3.1D). Whereas the binding to C1q under these conditions was halved compared to physiological salt conditions (0.15M NaCl), vWF binding to collagen I was blocked almost completely.

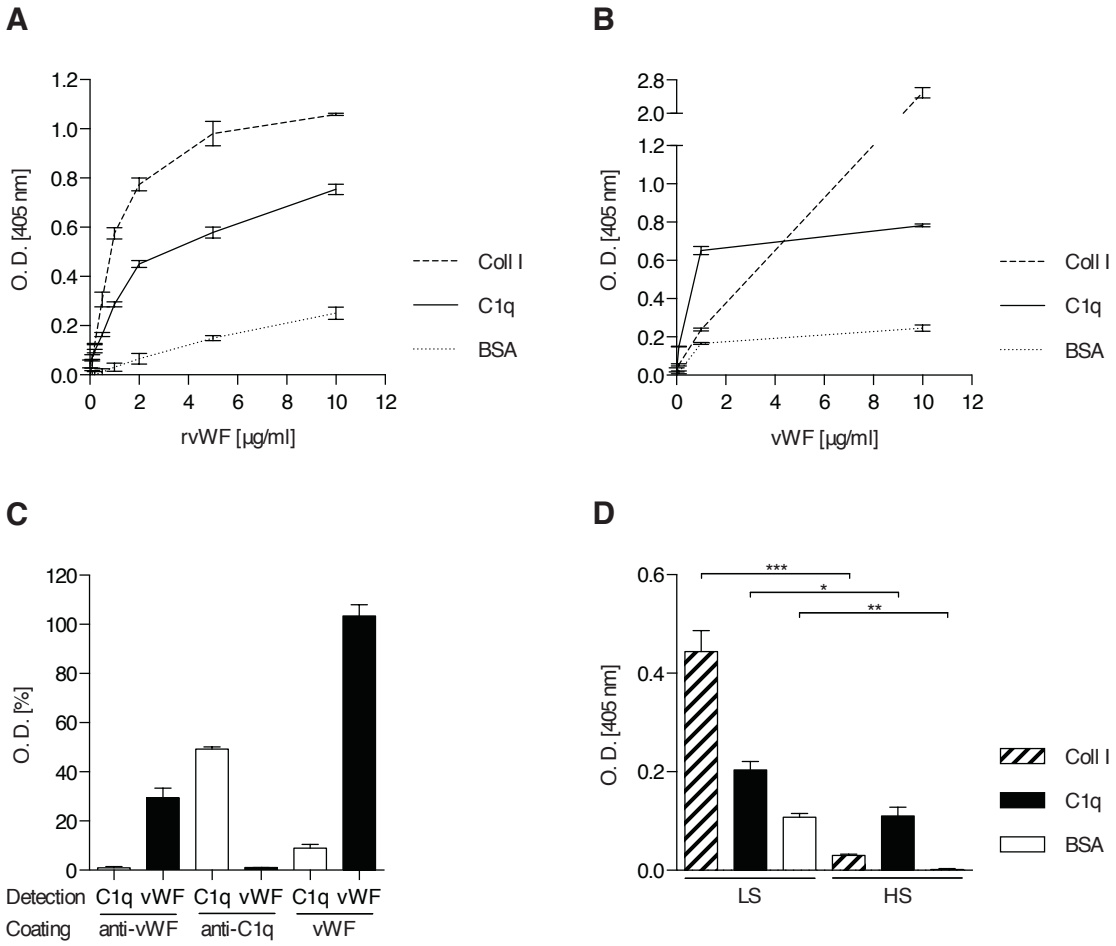

Figure 3.1: vWF binding to plate-bound C1q. Dose-dependent binding of recombinant (r) vWF (A) and human blood-derived vWF (B) to bound C1q compared to collagen I- and BSA-coating as controls. (C) C1q and vWF binding to anti-vWF and anti-C1q capturing antibodies, respectively, were studied in order to detect C1q-vWF complexes formed in the fluid phase. A mixture of C1q and vWF was added. Detection antibodies indirectly visualized the specific interactions. Furthermore, C1q binding on plate bound rvWF was analyzed. These data are expressed as percentage of the amount of C1q and vWF, respectively, directly coated on the surface. (D) vWF binding to C1q dependent on the salt concentration, i.e. physiological salt conditions (i.e. 0.15M NaCl - LS) versus high salt conditions (1M NaCl - HS). Decrease of vWF binding to C1q in the presence of high salt concentration was less pronounced than binding to collagen I that was nearly completely abolished. Representative diagrams are shown as mean of duplicates with standard deviation (One Way ANOVA test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In addition to these C1q-vWF binding assays, the binding of vWF to the surface-bound C1 complex (consisting of C1q, C1r and C1s) was studied. No significant binding could be observed (Figure 3.2).

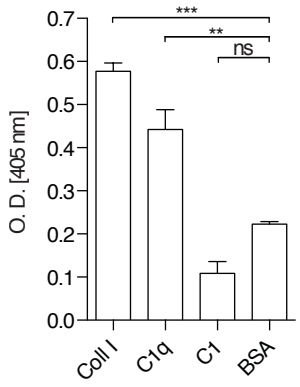

Figure 3.2: vWF binding to the surface-bound C1 complex. Binding of 4µg/ml rvWF to surface-bound C1 in comparison to collagen I and C1q. A representative diagram is shown as mean of duplicates with standard deviation (n = 3, One Way ANOVA test, ns not significant, ** $p < 0.01$, *** $p < 0.001$).

3.2 Shear stress enhances vWF binding to C1q

An important factor for the physiological function of vWF is shear stress, which induces a conformational change in the vWF protein. Therefore, we analyzed the binding of vWF to surface-bound C1q under dynamic conditions and compared this interaction to static conditions (Figure 3.3). Addition of vWF to C1q-coated surfaces under static conditions, for short incubation times (up to 300s), only led to a weak binding of vWF to C1q as well as to collagen I, when compared to background binding (Figure 3.3A). However, the analysis of the C1q-vWF interaction under flow conditions showed a strong and fast binding of vWF to surface-bound C1q, at least equivalent to the level of vWF binding to collagen I (Figure 3.3B). An interaction between vWF and bound C1q could be demonstrated already within 10s of flow at a shear rate of 1000/s (8.33 ± 0.03fold), and this interaction further increased within the following 300s (45 ± 0.41fold).

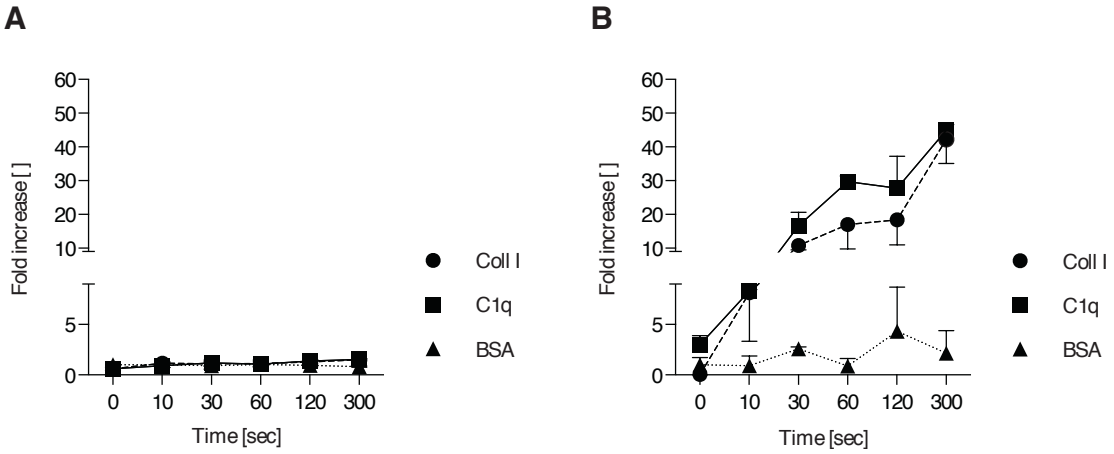

Figure 3.3: Time-dependent binding of vWF to C1q under (A) static and (B) dynamic conditions. Recombinant (r) vWF was added to surfaces coated with C1q and compared to collagen I and BSA as positive and negative controls respectively. The binding of rvWF is expressed as fold-increase compared to binding to BSA at the start (0s). (A) Static conditions were analyzed in a 96-well plate, (B) dynamic conditions in a flow chamber at a shear rate of 1000/s and compared to vWF binding to BSA at the same time point. Representative diagrams are shown as mean with standard deviation (n = 3).

Examining the binding between vWF and bound C1q under physiological flow conditions, different shear rates were used. The binding of vWF to C1q within low shear rates (250/s), mimicking venous flow conditions, high arterial shear rates (2500/s) and pathological shear rates (10000/s), such as shear in narrowed atherosclerotic vessels, were comparable to each other (Data not shown).

3.3 Complex stability of the C1q-vWF interaction

In order to get information about the affinity between bound C1q and vWF, a surface plasmon resonance-based technology (SPR) was used. As expected, rvWF bound to both immobilized collagen I and C1q (Figure 3.4). The off-rate, measuring the speed of dissociation of the rvWF-C1q complex ($4.7x10^{-5}$/s) was found to be more slowly than for the rvWF-collagen I complex ($2.4x10^{-3}$/s). In addition, reducing the ionic strength by a reduction of the salt concentration from 140mM to 40mM, the vWF-C1q interaction was further enhanced suggesting a primarily charge-dependent interaction as also observed in the ELISA-based experiments (Data not shown). However, due to the complex mixture of rvWF multimers and the complex, hexameric structure of C1q we were not able to correctly calculate the binding affinity.

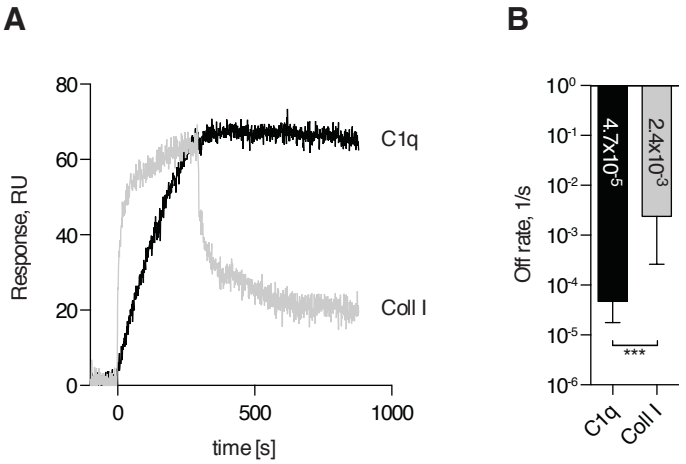

Figure 3.4: Complex stability of the C1q-vWF interaction. (A) A representative diagram of vWF (100nM) binding to immobilized C1q (black) and immobilized collagen I (Coll I, grey) from 5 surface plasmon resonance (SPR) assays is shown. (B) A representative comparison of the off rate between vWF binding to C1q and vWF binding to collagen I, calculated from the SPR assay (A). Data were shown as mean of duplicates with standard deviation (t-test, *** $p < 0.001$). Data were generated by Lubka Roumenina from the INSERM in Paris, France.

3.4 Defining a potent binding site for vWF

The sequence homology between vWF and anti-C1q Fab antibodies suggested not only binding of vWF to bound C1q but also to the same epitope that is recognized by anti-C1q antibodies. Therefore, we wanted to know whether anti-C1q Fabs are able to block vWF binding to C1q (Figure 3.5A). Through the addition of different amounts of anti-C1q Fab antibodies, recognizing a cryptic epitope of C1q, the binding of vWF indeed could be inhibited by about 50 ± 3%. In comparison, irrelevant IgG from a healthy donor showed no inhibitory effect.

Furthermore, we could previously identify a potent target peptide (A08) as a cryptic epitope of C1q for anti-C1q autoantibodies[48]. Coincubation of vWF and A08 peptide inhibited the binding of vWF to surface-bound C1q in a dose-dependent manner (Figure 3.5B). A 15000-fold molar excess of the A08 peptide reduced the binding of vWF to C1q by about 59 ± 12%, whereas no inhibition was observed by the control peptide. Thus, we suggest a specific interaction of vWF to the A08 peptide of C1q.

In a next step, we analyzed direct binding of vWF to A08. Figure 3.5C illustrates binding of rvWF to the A08 peptide and A08 peptides with modified sequences. rvWF binding to the wild type A08 peptide of the C1q-A chain was found to be almost two-fold higher than the binding to collagen I. A clear vWF signal could also be observed on a scrambled amino acid sequence form of A08 (scA08), comparable with the vWF level on collagen I, as well as to an A08 mutant with Lysine replacing Arginine

keeping all positive charges. No binding could be detected on an A08 variant with Alanine replacing Arginine - lacking all positive charges. Thus, vWF binding to A08 of C1q seems to be primarily charge-dependent.

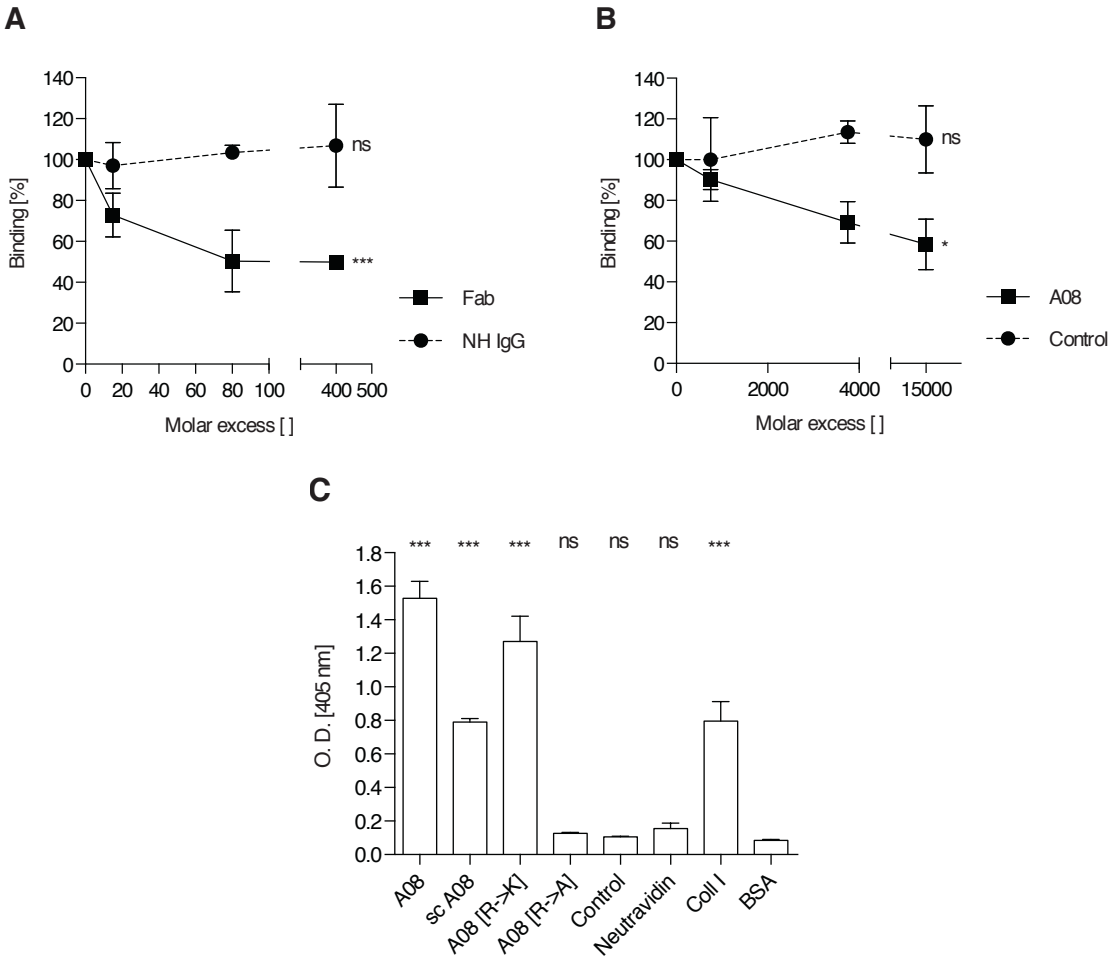

Figure 3.5: vWF interaction with a cryptic C1q epitope. (A) Inhibition of recombinant (r) vWF binding to C1q by increasing amounts of anti-C1q Fab antibodies compared to total IgG from a healthy donor as a negative control. (B) Inhibition of rvWF binding to C1q by increasing amounts of fluid-phase C1q-A chain-derived peptide A08 was compared to increasing amounts of C1q-C chain peptide (control peptide) as negative control. Pooled data are shown as mean with standard deviation. Significant differences were observed between vWF binding in the presence of excess of soluble peptide A08 versus the absence of the peptide (n = 3, One Way ANOVA test, *** $p < 0.001$, * $p < 0.05$). (C) Binding of rvWF to biotinylated peptides immobilized on neutravidin coated surfaces (A08 being part of the C1q A chain, a scrambled (sc) form of A08, A08 with Arginines being replaced by either Lysines ([R->K]) or Alanines ([R->A]) and control peptide, the C1q-C chain region being complementary to A08). Representative columns are shown as mean of duplicates with standard deviation comparing them with BSA (n = 3, One Way ANOVA test, ns not significant, *** $p < 0.001$).

Since A08 is located at the N-terminal end of the collagen-like region (CLR) of the C1q A chain, we investigated then the binding of vWF to the two different regions of C1q, the globular heads (GH) and the CLR (Figure 3.6). GH (Figure 3.6A) and CLR (Figure 3.6B) where obtained by limited digestion of C1q using collagenase and

pepsin respectively. As shown in Figure 3.6C, there was only very little binding of vWF to GH corresponding to about 2% of binding to intact C1q. In contrast, binding to CLR of C1q consisted of about 57% of the binding to undigested C1q. Although the enzymatic digestion procedures apparently affected the overall vWF-binding capacity, the data confirm that the CLR of C1q is the major binding site of vWF.

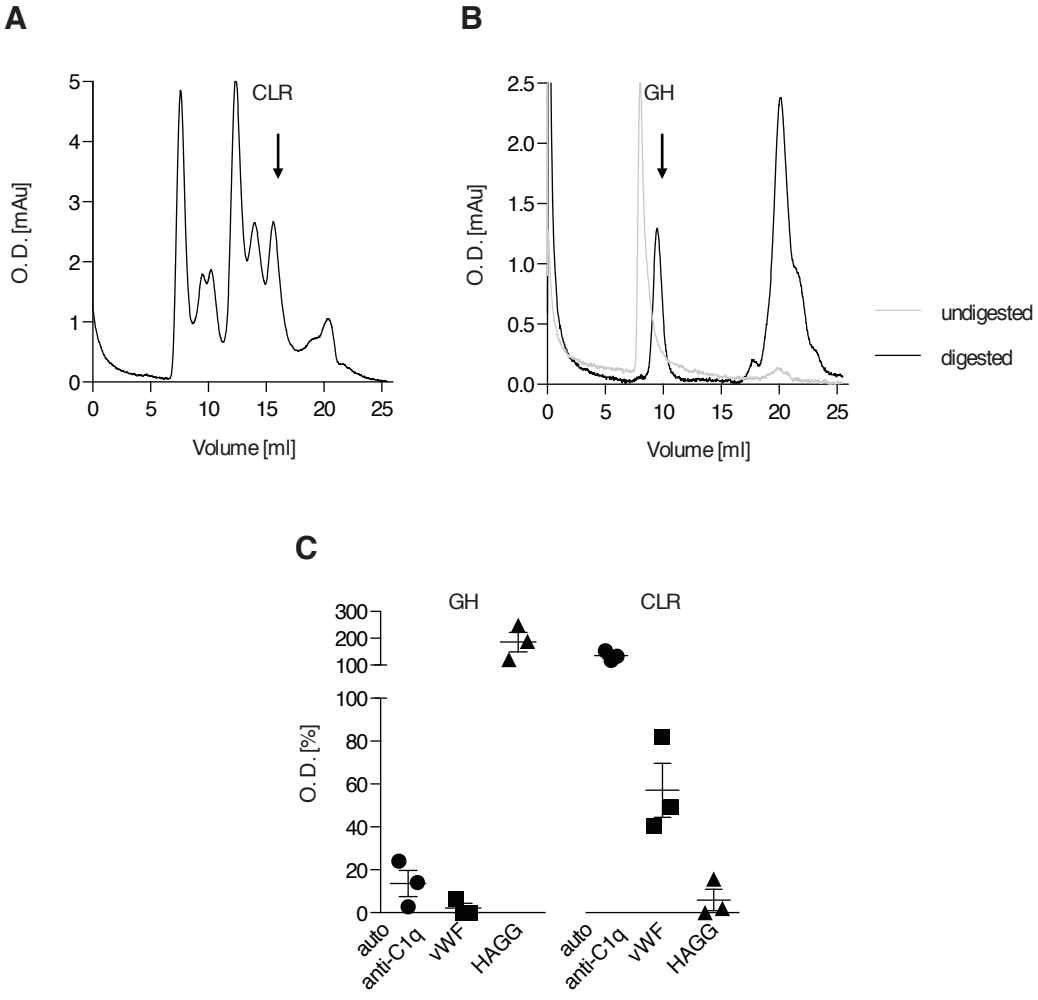

Figure 3.6: Binding of vWF to globular heads (GH) and collagen-like region (CLR) of C1q respectively after partial enzymatic digestion of C1q followed by FPLC purification. (A) shows a representative FPLC diagram of C1q digested with collagenase (removal of the CLR). The arrow indicates the GH. (B) illustrates a representative FPLC diagram of C1q digest with pepsin (removal of GH). The arrow indicates the CLR. (C) Pooled data from 3 independent experiments are shown as mean with standard deviation. The GH were identified through binding of heat aggregated IgG (HAGG) and the lack of anti-C1q autoantibody (auto anti-C1q) binding, whereas the CLR was identified the other way around. Binding of auto anti-C1q and HAGG (controls) as well as rvWF was expressed as percent binding to intact C1q.

3.5 vWF-C1q interaction occurs on an apoptotic surface

To investigate whether the vWF-C1q interaction also occurs on a biological surface, we studied the binding of vWF to C1q immobilized on apoptotic Jurkat cell-derived micro particles (MPs) (Figure 3.7). Figure 3.7A illustrates a representative flow cytometry diagram of MPs in the absence of C1q and vWF. Incubating MPs with complement C1q and no additional vWF led to 48.3% C1q-positive MPs (Figure 3.7B). In contrast vWF binding to MPs lacking C1q could not be detected in this system (Figure 3.7C). However, treating MPs with vWF following incubation with C1q resulted in binding of vWF to about 70% of all C1q-positive particles (Figure 3.7D). Figure 3.7E represents pooled data from 3 experiments showing C1q-dependent vWF binding to MPs expressed as mean fluorescence intensity.

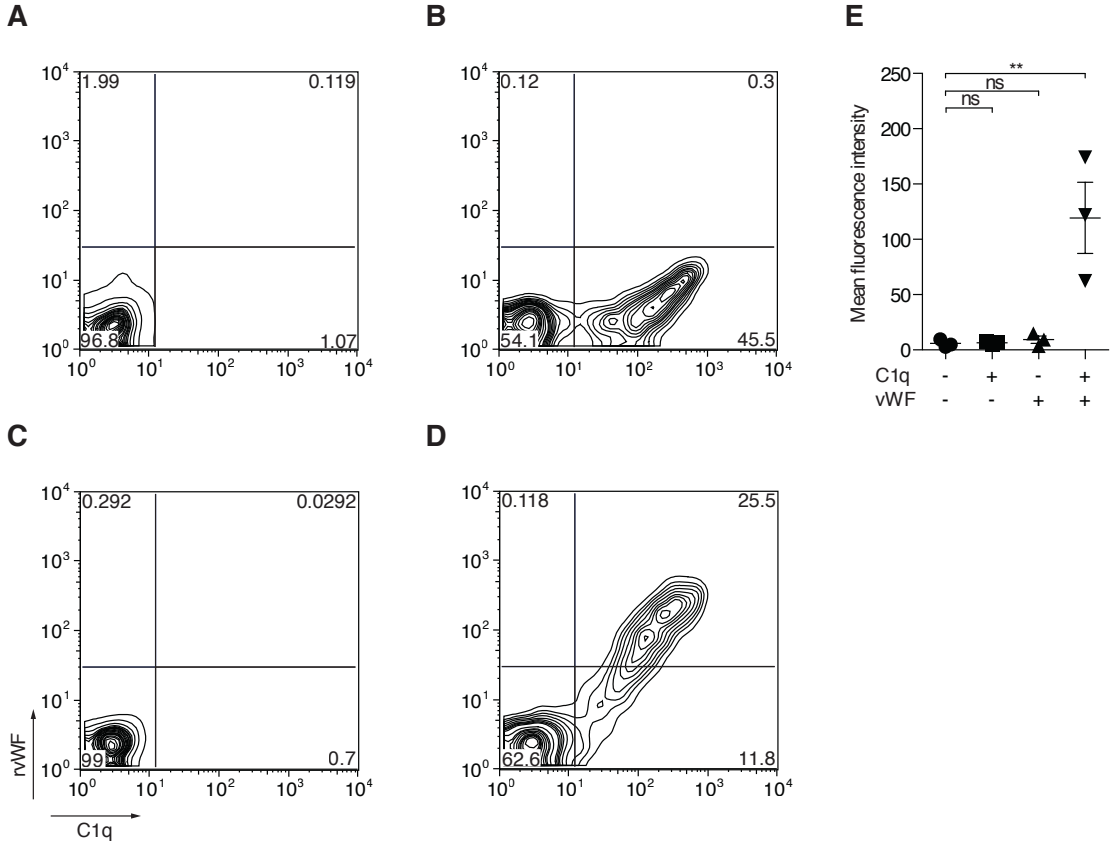

Figure 3.7: vWF-C1q interaction on apoptotic Jurkat cell-derived micro particles (MPs). (A-D) Representative FACS diagrams showing (A) untreated MPs, (B) MPs incubated with C1q alone, (C) MPs incubated with rvWF alone and (D) MPs incubated with both C1q and vWF. (E) Pooled FACS data are shown as mean fluorescence intensity with standard deviation (n = 3, One Way ANOVA test, ns not significant, ** $p < 0.01$).

3.6 The vWF deposition is increased in C1q-positive human glomeruli

Patients with active lupus nephritis typically show glomerular C1q deposition. Therefore we wanted to know whether vWF binding to C1q can be detected in this setting (Figure 3.8). In Figure 3.8A the typical pattern of C1q deposition in SLE glomeruli outlining the mesangial areas can be seen. In contrast to C1q deposition in SLE, vWF normally is more continuously expressed on the endothelium and is used as marker for histological examination of the glomerulus. Therefore the goal of this analysis was to determine a C1q-dependent change of vWF deposition. The co-localization analysis of the signal intensities of both, C1q and vWF, illustrated an overlap of about 36% of the vWF signal intensity over the C1q signal intensity in this representative glomerulus (Figure 3.8C, white areas).

To estimate the C1q-dependent increase of deposited vWF, the signal intensities of vWF in C1q-positve glomeruli were compared with C1q-negative glomeruli of patients with IgA nephropathy (IgAN). Figure 3.8D shows the median of the vWF deposition in a group of 25 SLE patients compared to a group of 24 IgAN patients. The signal of each patient was calculated as mean signal achieved from 5 glomeruli. The signal intensity of vWF was 34% higher in SLE glomeruli when compared to IgAN glomeruli, with this increase being in the same range as the C1q-vWF co-localization as described above.

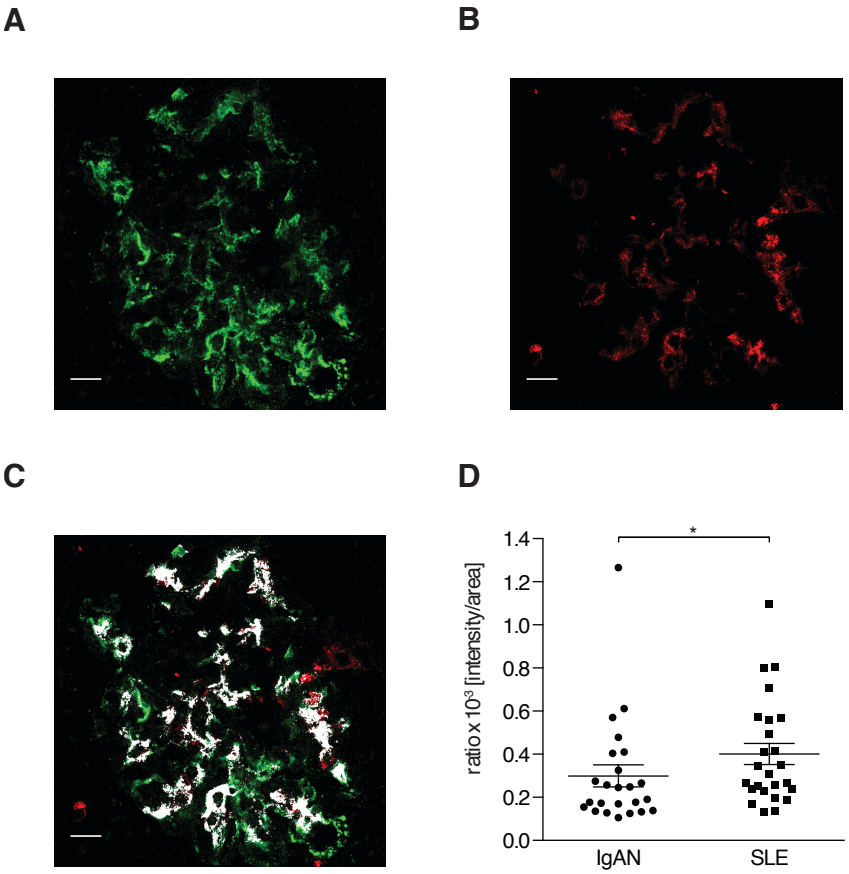

Figure 3.8: Co localization of C1q and vWF in human SLE glomeruli. (A-C) Representative fluorescent image of a glomerulus of a patient with SLE showing (A) C1q deposition in green, (B) vWF accumulation in red and (C) co localizing signal intensities illustrated in white (scale bar = 20µm). (D) Relative signal intensity for vWF comparing glomeruli from patients with IgAN (n = 24) with glomeruli from patients with SLE (n = 25). The ratio is demonstrated as median with interquartile range (Mann Whitney test, * $p < 0.05$).

3.7 vWF immobilized on C1q recruits thrombocytes

One major function of vWF in the hemostasis is the recruitment and adhesion of thrombocytes at the site of vascular injury. Therefore we examined the thrombocyte-recruiting capacity of vWF, immobilized on surface-bound C1q. Figure 3.9A depicts the presence of vWF on differently coated surfaces, after 2min of blood flow, at 1000/s shear rate, following perfusion with different amounts of rvWF. The amount of deposited vWF could be dose-dependently increased on C1q-coated surfaces and by coating different amounts of rvWF, whereas no such dose-dependent increase of vWF on surfaces coated with anti-vWF antibodies could be seen, suggesting saturation of the binding sites by blood-derived vWF. The vWF levels bound to the C1q-coated surfaces were lower when compared to surfaces coated with anti-vWF and rvWF, but higher than on collagen I-coated surfaces.

Rolling of platelets on C1q in the presence of rvWF was strongly enhanced (around 500-fold) when compared to the BSA-coated control (Figure 3.9B, Movies S1 and S2). In the absence of rvWF no platelet rolling on C1q-coated surfaces could be observed (Movie S3). Furthermore, adhesion of platelets on C1q-vWF complexes was observed as well (Figure 3.9C). Platelet rolling and adhesion capacity on surfaces coated with C1q that captured rvWF was comparable to surfaces having been either directly coated with rvWF alone or indirectly by the primary coating with a capturing anti-vWF in spite of the fact that the latter led to higher vWF levels as mentioned before. This suggests that vWF bound to C1q is capable of exposing its A1 domain in a platelet-binding conformation. In direct comparison, adhesion of platelets on a surface coated with collagen I was more efficient than all other tested conditions (Movie S4). However, we observed immediate recruitment of platelets, within a few seconds, on C1q-coated surfaces following perfusion with rvWF, whereas platelet recruitment and adhesion on collagen I-coated surfaces was a relative slow and ever-expanding process (data not shown).

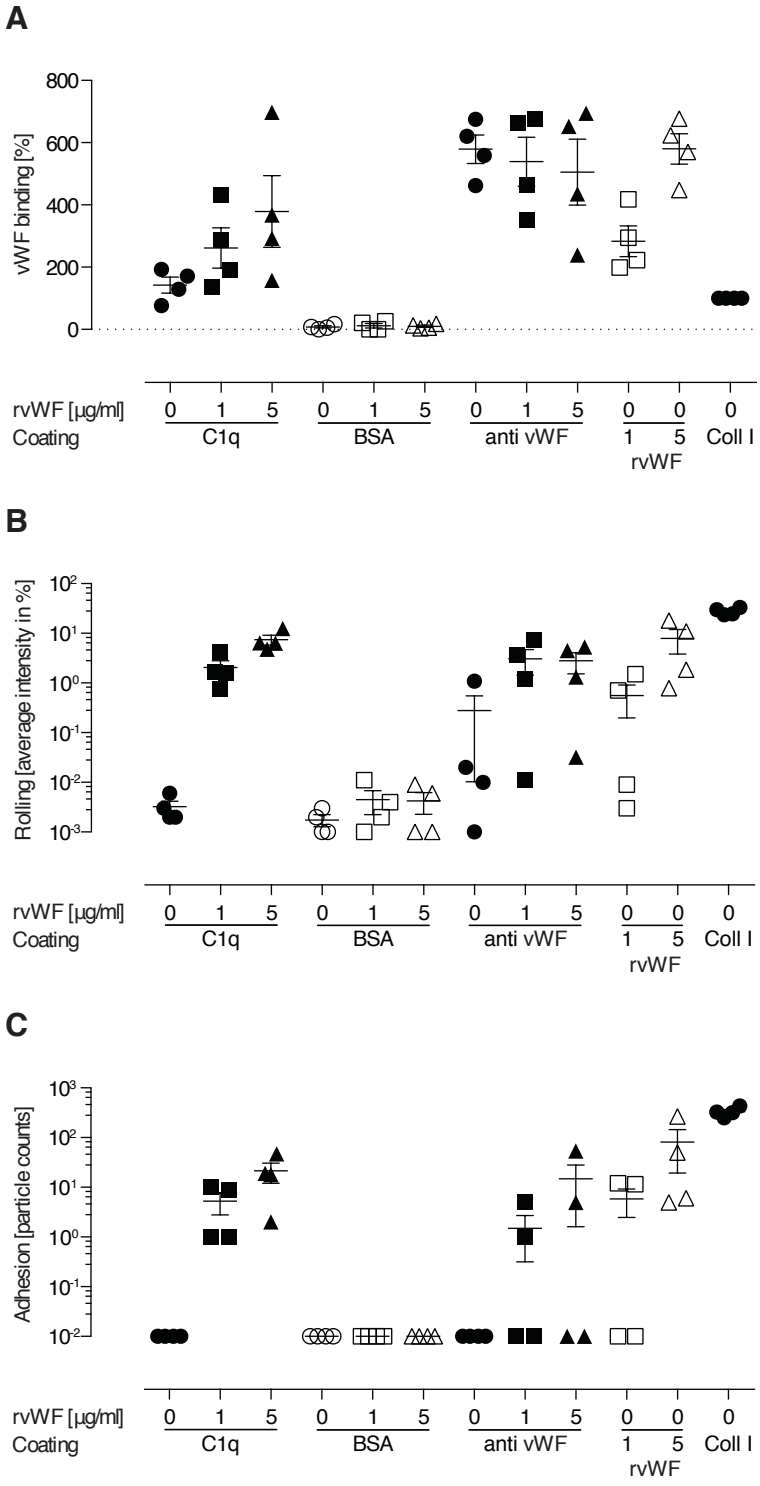

Figure 3.9: vWF dependent rolling and adhesion of thrombocytes on C1q. Whole human blood containing fluorescence-labeled thrombocytes was perfused through different coated flow chambers for 2min at a shear rate of 1000/s following perfusion with 0, 1 and 5µg/ml of recombinant (r) vWF for 5min at 1000/s. (A) vWF binding was detected after blood perfusion. The measured levels of vWF were comparable on surfaces coated with polyclonal rabbit anti-vWF and rvWF to levels on C1q following perfusion with 5µg/ml of rvWF. vWF levels were expressed as percent related to collagen I-coated surfaces. Video records of flowing platelets were made (150 frames) at a 40x magnification, and platelet rolling (B) and adhesion (C) were analyzed with the software “ImageJ”. Rolling and adhesion of thrombocytes on a C1q-coated surface with additional rvWF were comparable to coated rvWF alone. Pooled data are shown as mean with standard error of mean (n = 4). See also supplemental movies S1-4.

3.8 vWF-independent complement activation

Taking into account that vWF binds to C1q, we wondered whether this interaction alters the complement activating capacity of C1q, the starter molecule of the classical pathway. Thus, C1q-induced classical complement activation was measured in the presence of vWF bound to C1q. More precisely, the deposition of C4b, a cleaving product of the classical complement pathway, was detected by ELISA (Figure 3.10). We observed C4b deposition independently of varying amounts of C1q-bound vWF.

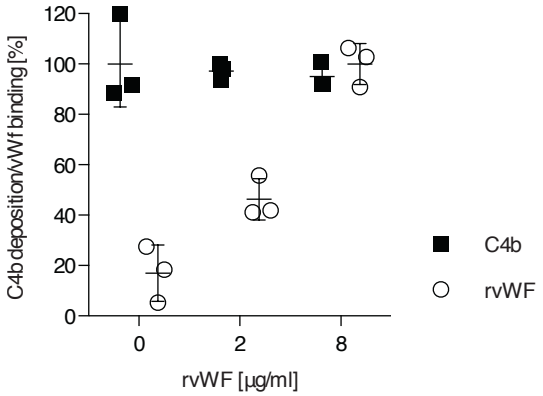

Figure 3.10: C4b deposition on a C1q-coated surface dependent on the presence of vWF bound to C1q. Normal human serum (NHS) was added to a surface coated with C1q following preincubation with recombinant (r)vWF. C4b deposition is expressed relative to 1% NHS (source for C4) in the absence of rvWF. RvWF binding to C1q is expressed relative to 8µg/ml rvWF in the presence of 1% NHS. Pooled data are shown as mean with standard deviation (n = 3, One Way ANOVA test, ns not significant, ** $p < 0.01$).

4 Discussion

In this study we identified a novel interaction between complement component C1q, the first molecule of the classical complement pathway, and the hemostatic molecule vWF expanding the perspective of immune-mediated platelet aggregation. We observed vWF binding to surface-bound C1q *in vitro*, whereas no binding occurred to fluid phase C1q and only weak binding to the C1 complex, suggesting a conformation-dependent effect induced by the binding of C1q. In this context we identified a cryptic epitope in the collagen-like region of C1q as, at least, one binding site for vWF. Interestingly, the C1q-vWF interaction occurred immediately under shear stress, mimicking blood flow conditions and was stable also under pathological flow conditions. Furthermore, we demonstrated by the analysis of SLE patient-derived kidney sections that the interaction of vWF with C1q might also occur in human disease. Finally, our findings clearly illustrate a vWF-dependent recruitment of platelets to a C1q-coated surface and the maintenance of the classical complement function of C1q in the presence of bound vWF.

The observation of vWF interacting with C1q raised several questions. One was the affinity of vWF to bound C1q. In the initial ELISA-based experiments we observed a more efficient binding of plasma-derived vWF to C1q than to collagen I at lower concentrations. The effect was inverted in undiluted blood. This observation might be explained by a higher affinity of vWF to C1q than to collagen. Whereas collagen provides numerous binding sites for vWF by its fibrous structure, allowing binding of higher vWF amounts, C1q exposes most likely only a limited number of binding sites leading to earlier saturation. Consequently, with increasing amounts of vWF binding to C1q could be more efficient than binding to collagen until all C1q binding sites were saturated. This assumption is supported by SPR-based data showing a higher complex stability of the C1q-vWF interaction when compared to the collagen-vWF interaction. Taken together, vWF bound not only under static conditions to C1q but also under shear stress representing physiological blood flow conditions.

Since vWF is a glycoprotein circulating in blood, this proof of interaction to surface-bound C1q under physiological shear stress was of particular interest. Data derived from the perfusion assays demonstrated a faster and enhanced binding of vWF to C1q when compared to static conditions resembling vWF binding to collagen I. These findings are well in line with studies demonstrating characteristic changes in the vWF structure due to shear forces, which were shown to be of functional importance for hemostasis[10]. Through shear stress the number of exposed functional

vWF domains is strongly enhanced and increases the avidity to collagen, platelets and, as we demonstrated, also to C1q. Furthermore, the observation of binding under pathological shear stress, e.g. as occurring in narrowed atherosclerotic vessels, illustrated a highly stable interaction between C1q and captured vWF.

In addition to conformational changes in the vWF molecule, changes in the conformation of C1q seem to be important as well. Experiments with fluid phase C1q and vWF showed no interaction between the two molecules pointing to a certain conformation induced through the immobilization of C1q that is necessary for the binding of vWF. This assumption is supported by the observation of a strongly reduced vWF binding to C1q within the C1 complex exposing a different conformation than bound C1q alone. Indeed, C1q is well known to expose cryptic epitopes in the collagen-like region (CLR) upon binding to apoptotic cells and immune complexes[47,52]. This uncovering of epitopes can also be mimicked by the binding to polystyrene surfaces[54]. As a consequence, we assumed the CLR of C1q being the major binding site for vWF. This assumption is supported by our observation that vWF recognized the arginine-rich domain of the N-terminal end of the C1q A-chain (A08), which had been shown to be part of a cryptic C1q epitope in the CLR and a potent target for anti-C1q autoantibodies (anti-C1q)[48]. The competition of A08 on the C1q-vWF interaction and the strong inhibition of this interaction through Fab anti-C1q targeting the A08 sequence in C1q emphasize the importance of the CLR of C1q for the binding of vWF. However, the inhibition of the formation of C1q-vWF complexes was incomplete and data derived from the enzymatic digestion of C1q do not fully exclude that also the globular head regions of C1q might be recognized by vWF. Taken together, binding of vWF to C1q shows a striking analogy to the binding of anti-C1q to C1q. Both anti-C1q and vWF showed binding under high salt conditions. In addition, both interact with a cryptic epitope on the CLR with the N-terminal Arginine-rich region of the C1q A-chain (A08) being a potent binding site. In this context however it is of importance to note that vWF binding to A08 seems to be mostly charge-dependent and therefore differs from the more specific interactions of anti-C1q[55]. The analogy had been expected due to the striking sequence homology between anti-C1q and the vWF C1 domain uncovered through the analysis of 6 bone-marrow derived Fab anti-C1q from a patient with SLE[49].

Anti-C1q antibodies are known to form complexes with C1q bound to apoptotic cells. Therefore, considering the striking similarities between the binding of anti-C1q and the binding of vWF to C1q we further assumed that apoptotic surfaces might be of relevance for the vWF-C1q interaction. Indeed, we found a C1q-dependent binding of

vWF to apoptotic microparticles. This observation is of particular interest as the classical function of vWF is the recruitment of platelets to damaged blood vessels exposing extracellular matrix proteins such as collagen. A milieu of apoptotic surfaces opsonized through C1q might be a new area of operation for vWF.

Following the analogy between anti-C1q and vWF, we tried to confirm our *in vitro* data in human pathology, by studying sections from renal biopsies of SLE patients. Kidney biopsies from patients with lupus nephritis (LN) showed a characteristic glomerular deposition of C1q[56]. We compared the amount of deposited vWF in C1q-positive glomeruli from LN patients with C1q-negative control glomeruli from IgA nephropathy (IgAN) patients. Although increased levels of deposited vWF caused by inflammation is common for both diseases[57-59], the vWF amount was even more pronounced in C1q-positive glomeruli from LN patients when compared to patients with IgAN. This increase points to the complex formation of vWF and C1q, a hypothesis that is strongly supported by the colocalization analysis between these two proteins.

To analyze whether binding of vWF to C1q indeed triggers primary hemostasis, we established a perfusion assay where platelet rolling and adhesion was investigated. In fact, we demonstrated that platelets were decelerated and frequently tethered on a surface coated with C1q depending on the presence of vWF *ex vivo*. This observation indicates that C1q-bound vWF was exposed in its active conformation presenting a surface for platelet activation. Interestingly, the platelet recruitment on C1q-vWF complexes occurred immediately after flow induction, whereas the recruitment on a collagen I coated surface was more delayed. This finding is in line with our data suggesting a higher avidity of vWF to bound C1q than to collagen I. Nevertheless, platelet adhesion capacity of a surface covered with C1q-vWF complexes was less pronounced when compared to a surface exposing vWF bound to collagen I. One reason for this might be the lack of interaction between platelet glycoprotein VI and collagen that is required for platelet activation and firm adhesion. Independently, the involvement of the C1q receptors gC1qR, targeting the globular heads, and cC1qR, targeting the CLR, on platelets is unclear and requires further studies.

Interestingly the functional capacity of C1q to induce classical complement activation is maintained in the presence of C1q-bound vWF. Since vWF is a relatively large molecule a steric blockage of important functional C1q-domains might be expected. Nevertheless, our data point to a less randomized but specific interaction between C1q and vWF

Although we could show binding of vWF to C1q-positive glomeruli of patients with lupus nephritis, the pathogenic relevance of this observation remains to be further dissected. We hypothesize a role of this interaction in cardiovascular disease of SLE patients, since these patients show an altered clearance of apoptotic cells and, consequently, generate the basis for a C1q-vWF interaction. In addition vWF binding to C1q could contribute to microangiopathies, which are frequently observed in lupus nephritis. Besides this, lupus nephritis is well known for a high prevalence of glomerular microthrombosis[60,61] where a direct relation between glomerular microthrombosis and C1q deposition had been demonstrated[62].

In conclusion, we have demonstrated a novel link between the complement system and the hemostatic system on the level of starter molecules of the two cascades. The data emphasize the tight connection between hemostasis and the immune systems. Our findings provide new insights into pathological mechanisms of the occurrence of thrombotic and/or atherosclerotic complications in complement-mediated inflammation, as observed in SLE.

5 Acknowledgement

I would like to thank

Marten Trendelenburg for providing me the opportunity to do my PhD in his lab at the Department of Biomedicine at the University Hospital Basel. I'm especially grateful for your support, your guidance and your never-ending patients and optimism during the track of my thesis.

Ed Palmer for giving me the chance to do my PhD at the Faculty of Science in Basel, and Gennaro De Libero for being my co-referee. Thank you also for the fruitful discussions and your inspiring feedback.

The members of my research group for their assistance, their occasional distractions and for ensuring a pleasant atmosphere in the lab.

Further thanks go to research groups of Jürg Schifferli and of Christoph Hess for their engagement during long discussions and their support. Especially I would like to thank Arun Cumpelik for his encouragement und his altruistic help throughout my thesis.

Many thanks to all others in the DBM who helped me out with material, knowledge and feedback within these years.

Special thanks go to my family and to Steffi for their love, patients and support without any reward.

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7 Supplemental Material

Supplemental Material can be found on the attached CD.

Movie S1. Human platelet rolling on a BSA-coated surface using a time-lapse epifluorescence microscope.
For this, human blood was collected in PPACK, and platelets were isolated and stained with Quinacrine. After reinfusion into whole blood, platelets were perfused for 2min at a shear rate of 1000/s over a BSA-coated surface. Frames were collected within the last seconds of the blood perfusion (150 frames/5s).

Movie S2. Human platelet rolling on a C1q-coated surface in the presence of rvWF using a time-lapse epifluorescence microscope.
For this, human blood was collected in PPACK, and platelets were isolated and stained with Quinacrine. After reinfusion into whole blood, platelets were perfused for 2min at a shear rate of 1000/s over a C1q-coated surface pretreated with rvWF. Frames were collected within the last seconds of the blood perfusion (150 frames/5s).

Movie S3. Human platelet rolling on a C1q-coated surface using a time-lapse epifluorescence microscope.
For this, human blood was collected in PPACK, and platelets were isolated and stained with Quinacrine. After reinfusion into whole blood, platelets were perfused for 2min at a shear rate of 1000/s over a C1q-coated surface. Frames were collected within the last seconds of the blood perfusion (150 frames/5s).

Movie S4. Human platelet rolling on a collagen I-coated surface using a time-lapse epifluorescence microscope.
For this, human blood was collected in PPACK, and platelets were isolated and stained with Quinacrine. After reinfusion into whole blood, platelets were perfused for 2min at a shear rate of 1000/s over a collagen I-coated surface. Frames were collected within the last seconds of the blood perfusion (150 frames/5s).

8 Curriculum Vitae

Personal Data:

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Since 07/11:	PhD-Student in the Research Group "Clinical Immunology", Department of Biomedicine at the University Hospital Basel

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11/10 until 03/11:	Research assistant in the Research Group "Signalling and Motility" (Head Prof. Dr. Stradal), Institute of Molecular Cell Biology at the University of Münster (Germany)

Academic studies and degrees:

10/10	Master's degree in Biotechnology
04/10 until 10/10	Master's thesis at the German Diabetes Centre in Düsseldorf, Institute for Clinical Diabetology: "Investigation on the effect of the inflammatory mediator Lipopolysaccharid on Insulin dependent activities of murine adipocytes"
10/08 until 10/10	Master's studies of Biotechnology at the Technical University of Braunschweig (Germany)
09/08	Bachelor's degree in Biotechnology
10/05 until 09/08	Bachelor's studies of Biotechnology at the Technical University of Braunschweig (Germany) Bachelor's thesis: "*Examination of in-vivo interaction between Lis1 und RanBP#9 and its deletion mutants RanBP#9-N and RanBP#9-C*"

Civilian service:

07/04 until 03/04	Community worker in Jonsdorf (Germany)

School education:

2001 until 2004	German school of the Borromäerinnen in Alexandria (Egypt)
1995 until 2001	Richard-von-Schlieben Grammar School in Zittau (Germany)

Internship abroad:

10/09 until 11/09	Internship at the University of Namibia, Faculty of Agriculture and Natural Resources, Department of Animal Science

Studies-related positions:

01/09 until 12/09	Molecule-drawer at the Braunschweiger Enzyme Database (BRENDA)
10/08 until 06/09	Graduated assistant in the Research Group "Signalling and Motility" (Head Dr. Stradal) and the Research Group "Cytoskeleton Dynamics" (Head Dr. Rottner), Department of Cell and Immunobiology at the Helmholtz Centre for Infection Research in Braunschweig (Germany)
06/07 until 01/08	Graduated assistant in the Department of Cell and Molecular Biology at the Biocenter of the University of Braunschweig

Personal skills and competences:

First language	German
Foreign language	English (fluently) Russian (basics) Arabic (basics in speaking and hearing)
Certificates	Certificate in conflict management Certificate in presentation Certificate in human resources management
Social competence	sense of responsibility, ability of teamwork, self-employment, insistence, flexibility
Publication:	J. Hänisch, **R. Kölm**, M. Wozinczka, D. Bumann, K. Rottner, TE. Stradal: Activation of a RhoA/Myosin II-Dependent but Arp2/3 Complex-Independent Pathway Facilitates Salmonella Invasion. Cell Host Microbe. 2011 Apr 21:9(4):273-85