General introduction
Thrombotic thrombocytopenic purpura
Thrombotic thrombocytopenic purpura (TTP) is a rare and life-threatening disorder of the blood coagulation system that mainly affects adult patients; the annual incidence is approximately 4 cases per million people. TTP is characterized by systemic aggregation of platelets within the vasculature (generally arteries and arterioles) causing microvascular thrombosis, hemolytic anemia and thrombocytopenia. The systemic clumping of platelets in brain, kidney and other organs is due to lack of von Willebrand factor (VWF) cleaving protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). The first case of TTP was described by Moschowitz in 1924. In 1982 Moake and co-workers observed the presence of unusually large VWF multimers in plasma of 4 patients and postulated that TTP was due to a deficiency of a VWF cleaving protease. Furlan and co-workers and Tsai developed an assay for measuring the activity of VWF cleaving protease which confirmed the absence of the protease in patients with TTP. The identity of the VWF cleaving protease was disclosed following its purification from plasma. Levy et al. used genome-wide linkage analysis to identify ADAMTS13 as the VWF cleaving protease involved in the pathogenesis of congenital TTP. Similar to the phenotype observed in patients with congenital TTP development of autoantibodies targeting ADAMTS13 in patients with acquired TTP results in sustained VWF-dependent accumulation of platelets in small vessels that eventually results in microvascular thrombosis. Congenital TTP, also know as Upshaw-Schulman Syndrome (USS), has an autosomal recessive inheritance and is caused by mutations in the ADAMTS13 gene (located on chromosome 9q34, spans 29 exons and is about 37Kb). It represents 5% of all TTP cases associated with ADAMTS13 deficiency. More than 140 different mutations have been identified so far and have been shown to cause reduced secretion and/or reduced catalytic activity of ADAMTS13. Half of the patients with USS remain asymptomatic until early adulthood while others manifest the disease within their first years of life. The high variability of the phenotype of congenital TTP has been associated with disease modifiers that can influence the clinical manifestations, such as defects in other genes (complement factor H), pregnancy, infections, traumas and/or heavy alcohol intake. Acquired TTP is caused by development of autoantibodies that bind and neutralize the proteolytic activity of ADAMTS13 and/or accelerate clearance of ADAMTS13 in vivo. A severe deficiency of plasma ADAMTS13 activity (less than 5%) and the presence of autoantibodies are considered to be highly specific for the diagnosis of acquired TTP. To date, plasma infusion and/or plasma exchange remains the first-line therapy regardless of the etiology and pathophysiology of TTP.
patients because of a high frequency of relapse especially for patients affected by acquired TTP presenting high titer inhibitors.\textsuperscript{32,33} Adjunctive immunosuppressive therapies such as cyclophosphamide, cyclosporine and rituximab are found to be effective in attaining remission and reducing relapse rates.\textsuperscript{17,21,22}

**ADAMTS13: structure and function**

ADAMTS13 is synthesized in both hepatic stellate cells in the liver\textsuperscript{6,34} and endothelial cells.\textsuperscript{35,36} It is present in plasma of normal individuals at a concentration of about 1 μg/ml (approximately 10 nM).\textsuperscript{37} The half-life of ADAMTS13 is relatively long; approximately 2-3 days.\textsuperscript{38} ADAMTS13 is a multidomain protein belonging to the ADAMTS family (a disintegrin and metalloproteinase with thrombospondin type 1 repeats).\textsuperscript{39} As the other members of ADAMTS family, it consists of a signal peptide (S), propeptide (P), metalloprotease domain, disintegrin-like domain (Dis), several thrombospondin type 1 repeats (TSRs), a cysteine-rich domain (Cys) and a spacer domain (Figure 1).\textsuperscript{7,39}

**Figure 1. Domain organization and three-dimensional structure of ADAMTS13.** (A) Schematic representation of the domain structure of ADAMTS13: signal peptide (S), propeptide (P), metalloprotease, disintegrin domain (Dis), cysteine-rich domain (Cys), spacer domain, thrombospondin type-1 repeats (1-8) and CUB domains (CUB). Interactive sites on ADAMTS13 involved in binding to VWF are indicated in the model. (B) Crystal structure of ADAMTS13 DTCS domains (PDB ID code 3GHM; ADAMTS13 disintegrin, thrombospondin type 1 repeat, cysteine-rich and spacer domain) highlighting the RYY motif, crucial for antibody and VWF binding.
Unlike other ADAMTS family members ADAMTS13 contains two additional CUB domains that are highly homologous to complement components C1r and C1s, embryonic sea urchin epidermal growth factor (UEGF) and the bone morphogenic protein 1 (BMP-1). These domains have been found in a different metalloproteinase family that includes procollagen C-proteinase and the mammalian tolloid-like proteins 1 and 2 where they have been shown to be involved in substrate recognition. Also the propeptide of ADAMTS13 differs from that of the other ADAMTS proteases. It consists of only 41 amino acids and does not contain a cysteine-switch motif. Although the propeptide is cleaved off by furin, cleavage is not required for VWF proteolysis while propeptide cleavage is required for development of full activity of most ADAMTS family members.

Several reports have shown that N-linked and O-linked glycans modulate secretion of ADAMTS13.

Cleavage of von Willebrand factor (VWF) by ADAMTS13

VWF is a large glycoprotein that is produced by endothelial cells and megakaryocytes as ultra large multimers (UL-VWF). Most of the synthesized VWF is constitutively secreted from endothelial cells; however, part of it is stored in cell-specific organelles designated Weibel-Palade bodies and released after stimulation with agonists such as thrombin or histamine. Under normal conditions, VWF circulates in plasma as a multimeric molecule (ranging from 20- to 40- but even 100- or 200- covalently linked VWF subunits) adopting a quiescent globular conformation, which hides its platelet binding site. When vascular damage occurs UL-VWF binds to collagen through its A3 domain. Under high shear stress the newly released UL-VWF multimers are able to unfold and assemble into string-like structures and bind platelets, leading to the formation of a platelet plug. Newly released UL-VWF strings are rapidly processed by ADAMTS13 (Figure 2). In the absence of ADAMTS13 UL-VWF strings are retained on the surface of endothelial cells promoting platelet adhesion which eventually can result in microvascular thrombosis (Figure 2).

Unlike the majority of the hemostatic proteases, ADAMTS13 activity is not regulated by a specific inhibitor. Thrombin, plasmin, hemoglobin and IL-6 have been shown to inhibit ADAMTS13 activity, although the physiological relevance of these findings is presently unclear. Under normal conditions ADAMTS13 is unable to cleave the Tyr1605-Met1606 scissile bond in the A2 domain of VWF, but is able to bind VWF through its C-terminal TSR 5-8 and CUB domains. This interaction allows the formation of VWF and ADAMTS13 complexes which have been shown to circulate in plasma. Even if only a small percentage of ADAMTS13 (3%) circulates bound to VWF, it is considered to be particularly effective at colocalizing VWF and ADAMTS13 to the site of vascular injury.
stress, which can occur upon secretion, collagen binding and/or passage through microcirculation, the VWF A2 domain is unfolded and ADAMTS13 can bind and cleave VWF.\textsuperscript{64,65} Unfolding of VWF results in exposure of additional binding sites for ADAMTS13. The exosite region Arg660, Tyr661 and Try665\textsuperscript{66-68} within the spacer domain of ADAMTS13 can interact with the partially unfolded VWF A2 domain (Glu1660-Arg1668).\textsuperscript{69-71} Thereafter, a critical low affinity interaction between VWF (Asp1614) and the disintegrin domain of ADAMTS13 (Arg349) helps to orientate the scissile bond toward the active site of ADAMTS13.\textsuperscript{72-74} This enables and facilitates the interaction between the metalloproteinase domain (Leu198, Leu232 and Leu274) with the A2 domain (Leu1603).\textsuperscript{53} Together these interactions precisely position the Tyr1605-Met1606 scissile bond for cleavage by the catalytic metalloprotease domain of ADAMTS13.\textsuperscript{54,75}

**Figure 2. Processing of von Willebrand factor in healthy individuals and in patients with TTP.**
Upon stimulation endothelial cells release UL-VWF multimers from intracellular storage pools. Due to shear stress UL-VWF multimers unfold thereby exposing the VWF A2 domain for cleavage by the metalloprotease ADAMTS13. Upper panel shows ADAMTS13 cleavage of UL-VWF in healthy individuals. UL-VWF is cleaved into smaller VWF multimers that circulate in plasma and are less efficient in binding platelets. Lower panel represents patients with TTP that lack ADAMTS13. Deficiency of the enzyme leads to accumulation of UL-VWF on the surface of endothelial cells. Platelets are able to bind to these UL-VWF multimers forming platelet aggregates occluding small arteries and capillaries.
Autoantibodies in patients with acquired TTP

Anti-ADAMTS13 antibodies are found in the majority of the patients (94-97%) suffering from acquired TTP and are considered to be strongly involved in the pathogenesis of the disease.\textsuperscript{16} Although most of these antibodies inhibit the proteolytic activity of ADAMTS13 towards VWF, 11.5-17% of the patients suffering from acute TTP have non-neutralizing antibodies and severe ADAMTS13 deficiency.\textsuperscript{24,76,77} Such antibodies are considered to enhance the clearance of ADAMTS13 from the circulation or interfere with ADAMTS13 interaction with cells and/or other plasma proteins.\textsuperscript{24} Anti-ADAMTS13 antibodies are comprised mainly of the immunoglobulin class G isotype (IgG), predominantly of subclass IgG\textsubscript{4} that is present in 90% of the patients, followed by IgG\textsubscript{1}, IgG\textsubscript{2} and IgG\textsubscript{3} that is observed in 30-50% of the patients.\textsuperscript{76-78} High levels of IgG\textsubscript{4} are found in relapsed cases of TTP and therefore are considered to be a risk factor for recurrence of TTP.\textsuperscript{76} Moreover, a limited number of patients also have circulating anti-ADAMTS13 IgM and/or IgA antibodies.\textsuperscript{78} The clinical relevance of these subclasses, which are found in 17-20% of TTP patients, is at present unclear.\textsuperscript{76,77} Recently it has been observed that significant amounts of ADAMTS13-specific immune complexes (ICs) can be detected in plasma of patients with acquired TTP. ICs can perpetuate a pro-inflammatory state promoting thrombosis and predisposing to relapse.\textsuperscript{79}

The majority of the antibodies in patients affected by acquired TTP target an antigenic site in the ADAMTS13 spacer domain. Fine epitope mapping of the ADAMTS13 spacer domain revealed Arg660, Tyr661 and Tyr665 as the primary antigenic target of these inhibitory antibodies.\textsuperscript{66} These residues contribute to the binding of ADAMTS13 to the A2 domain of VWF\textsuperscript{66} and therefore such antibodies are likely to interfere with the binding and limit processing of VWF.\textsuperscript{66,68} Although 90% of the antibodies found in plasma of TTP patients bind the spacer domain,\textsuperscript{80-82} patients suffering from acquired TTP can also have antibodies directed towards the C-terminal half of ADAMTS13 including the TSR 2-8 repeats and/or the CUB1-2 domains.\textsuperscript{80,81,83} Only very few TTP patients harbor anti-ADAMTS13 antibodies that specifically target these domains without having antibodies binding to the spacer domain of ADAMTS13.\textsuperscript{80} It has been shown that the inhibitory antibodies directed towards ADAMTS13 preferentially incorporate the heavy chain (VH) gene segment VH1-69 during assembly.\textsuperscript{83-85} Diversity of antibodies is created by several rearrangements that lead to joining of a variable heavy gene segment VH to a diversity segment DH and a joining segment JH and joining of a variable light chain segment VL to a joining segment JL. Although the clinical relevance of the restricted VH1-69 gene usage in patients with acquired TTP remains unclear, usage of the VH1-69 germline has been observed in neutralizing antibodies directed towards a
highly conserved region in the hemagglutinin ectodomain of influenza virus. This suggests that anti-ADAMTS13 spacer domain antibodies might originate from pre-existing cross reactive antibodies that target viral antigens during an infection.

**Initiation of development of anti-ADAMTS13 antibodies**

The mechanisms involved in loss of tolerance and development of autoantibodies in patients affected by acquired TTP are still unknown. As in other autoimmune disorders both genetic and environmental factors contribute to the development of autoimmune responses. Several observations provide evidence for a genetic predisposition related to the development of acquired TTP. First, the MHC class II allele HLA-DRB1*11 was found to be overrepresented in patients suffering from acute TTP when compared with a control population. In addition HLA-DRB1*04 was underrepresented in patients suffering from acquired TTP. Second, a case report described the development of inhibitory anti-ADAMTS13 antibodies in identical twin sisters not carrying the HLA-DRB1*11 allele. This indicates that additional, not yet identified, genetic risk factors are involved. The observation of a link between the MHC class II allele and development of acquired TTP implies a role for helper T cells in the initiation of the autoimmune reactivity against ADAMTS13. Moreover, clonal and subclass analysis revealed that anti-ADAMTS13 antibodies are composed of subclasses IgG1 and IgG4 and that the variable domains are highly modified by somatic hypermutation. Both isotype switching and somatic hypermutation of antibodies depends on activation of specific CD4+ T cells. Activation of antigen-specific T cells requires endocytosis and processing of the antigen by antigen presenting cells (APCs) such as dendritic cells. Antigen derived peptides are then presented on the surface of APCs in complex with MHC class II molecules. This allows the appropriate activation of specific T cells and subsequent secretion of cytokines that stimulate B cells and initiate isotype switching (Figure 3). Further evidence for a role of T cells in the immune response in acquired TTP comes from the clinical observation during plasma exchange treatment. About 30-50% of TTP patients with severe acquired ADAMTS13 deficiency experience a clinical exacerbation often associated with an increase of ADAMTS13 inhibitor titers. This phenomenon, however, is not observed in patients treated with cyclosporin A and bortezomib, potent T and B cell immunosuppressants. Microbial agents have been implicated in the onset of a variety of autoimmune disorders. Microbial stimuli can induce up-regulation of MHC class II proteins on the cell surface of antigen presenting cells (APCs). This results in an enhanced presentation of self-derived peptides which may overcome the activation threshold of T cells that have escaped negative selection in the thymus and have
an intermediate/low affinity for MHC-class II/peptide complexes (Figure 3).\textsuperscript{96,97} Moreover, molecular mimicry between foreign and self-antigens can lead to self-activation of these T cells.\textsuperscript{93} A large number of case reports have suggested a role for viral or bacterial infections in the etiology of acquired TTP.\textsuperscript{23} Influenza A,\textsuperscript{98} HIV,\textsuperscript{99} parvovirus,\textsuperscript{100} Helicobacter pylori,\textsuperscript{101} Hepatitis C,\textsuperscript{102} Brucella\textsuperscript{103} and Legionella,\textsuperscript{104} infections have been suggested as a priming event for the development of acquired TTP. This suggests that triggering of the innate system plays a role in the onset and perhaps in the recurrence of TTP by lowering the threshold for activation of ADAMTS13-specific T cells. In addition to microbial agents high levels of steroids, for instance during pregnancy, have also been suggested to contribute to the onset of acquired TTP by an as yet undefined mechanisms. Indeed, it is well established that pregnant women develop inhibitory anti-ADAMTS13 antibodies and typical features of the disease.\textsuperscript{20,105-107} Moreover, other malignancies, collagen diseases and use of drugs have been related to the development of acquired TTP, in particular the anti-platelet agents ticlopidine and clopidogrel.\textsuperscript{108-110}
Figure 3. Generation of self-reactive CD4+ T and B cell responses. Left panel indicates the negative selection of CD4+ T cells. Self-antigens are endocytosed by antigen presenting cells (APCs) processed into peptides and presented on the cell surface in complex with MHC class II molecules. T cells binding with low affinity to self-antigens survive and become naive T cells while those that bind with high affinity to self-antigens undergo apoptosis. Intermediate affinity CD4+ T cells may escape from negative selection in the thymus and therefore become potentially auto-reactive. Right panel shows how auto-reactive T cells can contribute to the formation of auto-antibodies. Once primed, T cells can activate B cells in an antigen-specific manner, which leads to formation of long-living plasma cells, producing high-affinity auto-antibodies. Co-stimulatory molecules and the different receptors are indicated in the inset of the figure.
Scope of the thesis

At present the mechanisms involved in development of autoantibodies targeting ADAMTS13 in previously healthy individuals is unclear. In this thesis we explored how ADAMTS13 interacts with the immune system and how this may lead to the initiation of auto-immune responses in acquired TTP. First, we analyzed the epitope specificity of anti-ADAMTS13 antibodies in a large cohort of patients with acquired TTP (Chapter 2). We observed that the majority of anti-ADAMTS13 antibodies are directed towards a specific epitope within the spacer domain. Subsequently we analyzed how ADAMTS13 is recognized by antigen-presenting cells. In Chapter 3 we identified the mechanism of endocytosis of ADAMTS13 by human dendritic cells. ADAMTS13 is efficiently endocytosed by monocyte-derived dendritic cells in a mannose receptor-dependent manner. In Chapter 4 we identified, for the first time, the repertoire of naturally MHC class II presented ADAMTS13-derived peptides. Recently the MHC class II allele HLA-DRB1*11 has been recognized as a risk factor for the development of acquired TTP, indicating a role for CD4+ T cells in the onset of the disease. Interstingly our findings demonstrate that HLA-DRB1*11 positive donors exclusively present a CUB2 domain derived peptide. In Chapter 5 we analyzed N- and O-glycosylation profile of plasma derived ADAMTS13. Nine N-linked glycosylated, six O-fucosylated and two C-mannosylated sites were identified. In Chapter 6 we investigated the endocytic mechanisms contributing to the uptake of ADAMTS13 by macrophages and their possible role in clearance of ADAMTS13 from the circulation. Taken together, our findings provide novel insights into the immune recognition and processing of ADAMTS13 by antigen presenting cells thereby contributing to a better understanding of the development of anti-ADAMTS13 antibodies in patients with acquired TTP.
References

18. Lotta LA, Wu HM, Mackie IJ, et al. Residual plasmatic activity of ADAMTS13 is correlated with
42. Takahara K, Lyons GE, Greenspan DS. Bone morphogenetic protein-1 and a mammalian tolloid homologue (mtld) are encoded by alternatively spliced transcripts which are differentially expressed in some tissues. *J. Biol. Chem.* 1994;269(51):32572–32578.
52. Schneider SW, Nuschele S, Wixforth A, et al. Shear-induced unfolding triggers adhesion of von


