Acquired TTP: ADAMTS13 meets the immune system

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General Discussion
The majority of the patients affected by acquired thrombotic thrombocytopenic purpura (TTP) develop autoantibodies directed towards ADAMTS13 that interfere with the processing of newly released high molecular weight von Willebrand factor (VWF) multimers. In the absence of ADAMTS13, high molecular weight VWF multimers persist and promote platelet adhesion in the microvasculature. Systemic platelet aggregation in the microcirculation of patients suffering from TTP results in thrombocytopenia and hemolytic anemia. So far the majority of the studies have been performed in order to characterize the heterogeneity of B cell responses in acquired TTP. As yet, our knowledge on the mechanisms involved in the onset of the autoimmune response to ADAMTS13 in previously healthy individuals is limited. In this thesis we studied the immune recognition and processing of ADAMTS13 by antigen-presenting cells (APCs). In this chapter we discuss our findings in relation to studies performed by other investigators. We also address the potential relevance of our studies for the pathogenesis and treatment of patients with acquired TTP.

**Triggering the immune response in TTP patients: a complex mechanism**

It is well established that anti-ADAMTS13 antibodies undergo both isotype switching and somatic hypermutation; two mechanisms that require help from CD4+ T cells. These observations strongly suggest that development of anti-ADAMTS13 antibodies in patients affected by acquired TTP requires activation of self-reactive T cells. An important step in activation of self-reactive T cells is presentation of antigen-derived peptides on MHC class I or II molecules. Upon endocytosis, internalized antigens, such as ADAMTS13, can be targeted to endosomal compartments, degraded or processed into peptides and presented on MHC class II or cross-presented on MHC class I molecules. In Chapter 3 we demonstrated that dendritic cells, professional APCs, endocytose ADAMTS13 via the macrophage mannose receptor (MR). Upon endocytosis, ADAMTS13 is processed into peptides and loaded on MHC class II molecules (Chapter 4). In Chapter 4 we identified ADAMTS13 derived peptides that are loaded on MHC class II in the late endosomal MHC class II compartment and presented on the cell surface of human dendritic cells. Dendritic cells from 17 healthy donors, expanded and pulsed with different concentrations of ADAMTS13, presented peptides derived from different domains of ADAMTS13. In particular peptides derived from the C-terminal CUB2 domain were presented with higher efficiency compared to peptides derived from other domains of ADAMTS13, indicating that this domain contains a number of potential immuno-dominant T cell epitopes. Although the spacer domain is the major target of antibodies in patients with acquired TTP, it is not surprising to find potential T cell epitopes elsewhere in the molecule. T cells recognize linear peptides, at least 9 to 16 residues in length,
that are loaded on MHC class I or II following proteolytic degradation of antigens by APCs. B cells instead recognize surface exposed conformational epitopes that are often comprised of noncontiguous amino acid sequences. Interestingly, the findings reported in Chapter 4 show that HLA-DRB1*11 positive donors present only a single CUB2 derived peptide: FINVAPHAR (Figure 1). HLA DRB1*11 has been identified as a risk factor for the development of acquired TTP.\textsuperscript{11-13} The increased frequency of DRB1*11 in patients with acquired TTP together with the results of our study, suggests that preferential presentation of FINVAPHAR by HLA DRB1*11 positive individuals contributes to the onset of acquired TTP. We speculate that appropriate presentation of the predicted HLA DRB1*11 binding sequence FINVAPHAR by APCs leads to proliferation of self-reactive CD4\textsuperscript{+} T cells that have escaped negative selection in the thymus (Figure 1).

\textit{Figure 1. Model of FINVAPHAR-peptide bound to HLA-DRB1*11 containing MHC class II complex.} FINVAPHAR-peptide indicated in yellow and red modeled into a binding groove of a MHC class II complex composed of HLA-DRB1*1101 (dark blue) and HLA-DRA*0101 (grey). Anchor residues of the FINVAPHAR core sequence are indicated in red: P1: Phe, P4: Val, P6: Pro and P9: Arg. The peptide-MHC class II complex was built using the MHCsim webserver (http://igrid-ext.cryst.bbk.ac.uk/MHCsim) and displayed using Chimera imaging software.
In the thymus, developing T cells which bind strongly to self peptide-MHC complexes are deleted from the repertoire. However, not all self-reactive thymocytes are eliminated, some of those with low/intermediate affinity escape the thymus and persist in the periphery becoming part of the normal repertoire in healthy individuals and have the potential to cause autoimmunity.\textsuperscript{14-16} Activation of T cells not only requires interaction between MHC peptide complex and the T cell receptor (TCR) but also requires co-stimulatory signals and cytokine stimulation. This indicates that additional triggering events, like infection and inflammation, are necessary to induce expression of the co-stimulatory molecules on APCs and activate self-reactive T cells in TTP patients (Figure 2). Viral and bacterial infection have been implicated in the etiology of TTP.\textsuperscript{1} As yet no single pathogen has been linked with the onset of TTP. However, cross-reactive CD4\textsuperscript{+} T cells arising during bacterial or viral challenges may be able to recognize complexes of MHC class II and the FINVAPHAR-peptide. It should be noted that TTP can also develop in HLA-DRB1\textsuperscript{*11} negative individuals. In Chapter 4 we have shown that other ADAMTS13 peptides can also be presented on cells derived from non-HLA-DRB1\textsuperscript{*11} donors although higher concentrations of ADAMTS13 are needed to allow sufficient presentation. It has been shown in other autoimmune disorders, like multiple sclerosis, that although the immuno-dominant peptide binds with low affinity to MHC class II it is still able to activate self-reactive T cells when high levels of antigen are present.\textsuperscript{17} This suggests that high densities of peptide MHC class II complexes are necessary in order to allow activation of self-reactive low affinity CD4\textsuperscript{+} T cells in DRB1\textsuperscript{*11} negative individuals (Figure 2). We hypothesize that increased endocytosis of ADAMTS13 by APCs would overcome the threshold required for recognition of unstable ligands and activation of ADAMTS13-specific T cells. Modification of the glycosylation profile can potentially result in an increased endocytosis thereby promoting antigen specific T cell proliferation.\textsuperscript{18} A recent study showed that modifying carbohydrates in different allergens influenced the immune recognition by epithelial and dendritic cells.\textsuperscript{18} As shown in Chapter 5, plasma derived ADAMTS13 is a highly glycosylated protein containing complex N-linked and O-linked glycans as well as two C-mannosylated tryptophans in TSR1 and TSR5. Glycosylation of ADAMTS13 is required for appropriate endocytosis by immature dendritic cells that occurs through the MR (Chapter 3). As reported in Chapter 3, deglycosylation of ADAMTS13 results in a significant reduction of its uptake by immature dendritic cells. Several studies have shown that pathogens and/or inflammatory conditions affect host glycosylation by up-regulating glycosyltransferase activities that modify the glycan structure of glycoproteins.\textsuperscript{19} This raises the possibility that inflammatory conditions, resulting from viral or bacterial infections, may result in hyperglycosylation or deglycosylation of ADAMTS13 that
might contribute to the onset of acquired TTP. Enhanced endocytosis and processing of ADAMTS13 by APCs would result in an increased MHC class II/peptide density required to overcome the activation threshold of intermediate/low affinity T cells that have escaped negative selection in the thymus (Figure 2; panel B).

**Glycosylation and presentation of ADAMTS13 derived peptides**

Protein glycosylation not only plays an important role in immune recognition of antigens by APCs, but is also important for appropriate T cell recognition. Several studies have suggested that glycosylated self-derived peptides are often not efficiently presented on MHC class II molecules during negative selection in the thymus. Therefore, no tolerance is established against these peptides derived from endogenous antigens. Inflammatory conditions may promote partial deglycosylation of self proteins, like ADAMTS13. This may result in presentation of so-called “naked peptides” on MHC class II and activation of autoreactive CD4+ T cells. Interestingly, we have observed (Chapter 5) that two ADAMTS13 derived peptides presented on MHC class II molecules, GCRLFINVAPHARIA (residues 1327-1338) and ASYILRDTHSLRTTA (residues 1355-1370) are located in close proximity to the N-linked glycosylation site N1354. Furthermore, evidence was obtained for the partial addition of N-glycans at this position although we were unable to quantify the extent of glycosylation at this site (Chapter 4). Our findings raise the possibility that modulation of the efficiency of N-linked glycosylation at N1354 may affect the efficiency of MHC class II presentation of peptides that are in proximity to this N-linked glycosylation site. Removal of the N-linked glycan at N1354 by site-directed mutagenesis and subsequent pulsing of the resulting ADAMTS13 variant to dendritic cells will provide information on whether the presentation of the two CUB2 domains derived peptides identified in Chapter 4 is indeed affected by the glycan at N1354.

**Macrophages and anti-ADAMTS13 antibodies a dual role in acquired TTP**

In Chapter 6 we show that ADAMTS13 is rapidly internalized by human monocyte-derived macrophages. Macrophages are a large heterogeneous group of immune cells that can induce both anti- and pro-inflammatory responses. Depending on the microenvironment and stimuli, activated macrophages can be divided in two major groups: classical activated macrophages (M1) and alternative activated macrophages (M2). M1 cells have increased antigen presentation capacity and increased synthesis of pro-inflammatory and toxic mediators involved in the defense of the host from a variety of bacteria, protozoa and viruses.
Figure 2. Model for the stimulation of self reactive CD4+ T cells in acquired TTP. In both DRB1*11 positive and DRB1*11 negative individuals ADAMTS13 is endocytosed via the mannose receptor (MR). Following its MR-dependent internalization, ADAMTS13 is processed into peptides and loaded on MHC class II. (A) Patients carrying the DRB1*11 allele preferentially present the CUB2 domain peptide FINVAPHAR. In order to activate naive T cells co-stimulatory signals are needed. These can be upregulated by intercation of pathogens with pattern recognition receptors on antigen presenting cells. (B) In Chapter 4 we reported that ADAMTS13 peptides are less efficiently presented by APCs derived from non-DRB1*11 positive donors. In order to obtain a sufficient density of MHC class II peptide complex and allow CD4+ T cell activation enhanced endocytosis of ADAMTS13 is needed. In DRB1*11 negative individuals ADAMTS13 endocytosis may be enhanced by alteration of the glycans on ADAMTS13. Alternatively, more severe viral or bacterial challenges may promote enhanced upregulation of MHC class II and co-stimulatory molecules eventually resulting in the activation of self-reactive ADAMTS13 specific CD4+ T cells.
Activation of M1 cells is due to stimulation by Interferon (IFN)-γ, produced by activated CD4+ T helper 1 cells, CD8+ T cells and natural killer cells. Conversely M2 cells are characterized by an increased expression of the macrophage mannose receptor and MHC II molecules, which stimulate endocytosis and antigen presentation and also express high levels of scavenger receptors that are known to play a role in clearance, tissue remodeling, immune modulation and tumor progression. In contrast to M1 macrophages, M2 cells are mainly induced by cytokines such as IL-4 and IL-13 that are produced by activated CD4+ T helper 2 cells. Although M2 macrophages exhibit potent anti-inflammatory activity and have important roles in wound healing and fibrosis, it has been shown that they are also involved in the development of T helper 2-dependent immunity to some extracellular parasites and fungi. This suggests that depending on the microenvironment M2 macrophages can have different roles in the immune response. M2 macrophages might therefore play a dual role in the pathogenesis of acquired TTP by not only participating in the immune response against ADAMTS13 by enhancing presentation of ADAMTS13 derived peptides and stimulating activation of T helper cells, but can also contribute to the clearance of ADAMTS13 in normal and pathological conditions. It is well established that in addition to inhibitory antibodies also non-neutralizing antibodies can develop in patients with acquired TTP. These antibodies are known to enhance clearance of ADAMTS13 from the circulation. In Chapter 6 we analyzed the mechanism of internalization of ADAMTS13 by human macrophages. Surprisingly, our data show that in contrast to dendritic cells MR is not involved in the uptake of ADAMTS13 by macrophages. Our experimental results indicate a role for scavenger receptors in internalization of ADAMTS13 by macrophages. These observations implicate distinctive roles for dendritic cells and macrophages in the pathogenesis of acquired TTP. Dendritic cells by virtue of their ability to stimulate naive T cells are primarily involved in the onset of TTP whereas macrophages contribute to the clearance of ADAMTS13 from the circulation. This suggests that in normal physiological conditions ADAMTS13 is removed from the circulation by recognition and binding of scavenger receptors. In plasma of TTP patients ADAMTS13-specific immune-complexes (ICs) have been detected. Current data show that ADAMTS13-ICs are rapidly cleared from the circulation. We propose that macrophages, in addition to clearance of ADAMTS13 via scavenger receptors, also participate in the clearance of ADAMTS13-ICs through rapid internalization via Fc receptors. This mechanism most likely contributes to the low ADAMTS13 antigen levels in patients with acquired TTP.
Final remarks and future directions

Plasma exchange is the current treatment in acquired TTP and is considered to remove circulating inhibitory antibodies and to replenish the deficient enzyme. In addition to plasma exchange immunosuppressive methods, such as treatment with corticosteroids or other agents like rituximab and cyclosporine, are required in order to reduce early (exacerbation) and late (relapse) recurrences of TTP. Therapeutic administration of recombinant ADAMTS13 has been suggested as an alternative treatment for plasma exchange. In vitro experiments have shown that high dosages of ADAMTS13 can overcome neutralizing inhibitors in plasma from acquired TTP patients. In Chapter 2 we describe critical amino acid residues that contribute to an immuno-dominant antigenic site in the spacer domain. Follow-up studies by Jian and collaborators have shown that modification of the antigenic epitope identified in Chapter 2 results in an antibody resistant variant with increased VWF processing activity.

This gain-of-function variant that is resistant to inhibition by anti-spacer domain antibodies can potentially be used for treatment of patients with acquired TTP. However, a subset of TTP patients also develop antibodies targeting other domains besides the spacer domain prohibiting broad clinical application of gain-of-function spacer domain variants of ADAMTS13. During the last decade many studies have attempted to use tolerogenic dendritic cells (tDCs) to treat autoimmunity. It is well established that dendritic cells can present a tolerogenic phenotype and selectively promote formation of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) while induce apoptosis of effector T cells. It has been shown that loading tolerogenic DCs with CD4⁺ T cell specific epitopes induces apoptosis of auto-reactive CD4⁺ T cells and converts naive CD4⁺ T cells into antigen-specific Tregs. This in turn endows DCs with regulatory capacity. In Chapter 4 we identified a number of potential ADAMTS13 specific T cell epitopes. In particular, HLA-DRB1*11 positive donors presented a unique CUB2 domain derived peptide, FINVAPHAR. It will be interesting to test whether loading tDCs with the CUB2 domain derived peptide might promote the formation of Tregs thereby restoring tolerance to ADAMTS13 in patients with acquired TTP.

Together our findings have provided detailed information on the immune recognition and processing of ADAMTS13 by antigen presenting cells. Follow-up studies will need to address whether the identified peptides are recognized by ADAMTS13 specific T cells. We also do not know which events trigger the development of autoantibodies directed towards ADAMTS13 in previously healthy individuals. Detailed analysis of the T cell repertoire in patients with acquired TTP is needed to answer these questions. The outcome of these studies is expected to greatly
increase our understanding of the mechanisms underlying the development of autoantibodies directed towards ADAMTS13 in patients with acquired TTP.
References


