Acquired TTP: ADAMTS13 meets the immune system
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Endocytic mechanisms contributing to the uptake of ADAMTS13 by macrophages

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Abstract

Acquired thrombotic thrombocytopenic purpura (TTP) is a severe disorder characterized by the production of autoantibodies directed against ADAMTS13, a metalloprotease that regulates platelet adhesion and aggregation through cleavage of ultra-large von Willebrand factor (UL-VWF) multimers. At present, the cause of antibody formation is still unknown. We have previously shown that ADAMTS13 is efficiently internalized and presented on MHC class II by dendritic cells suggesting a possible role of CD4+ T cells in the initiation of the autoimmune reactivity towards ADAMTS13. Internalization of ADAMTS13 by macrophages may contribute to its clearance from the circulation. Here we investigated endocytic mechanisms contributing to the uptake of ADAMTS13 by macrophages. Human monocyte-derived macrophages (MDMs) were used to monitor the uptake of fluorescently labelled recombinant ADAMTS13 by flow cytometry. Internalization of ADAMTS13 was partially blocked upon addition of mannan and EDTA suggesting a possible role of C-type lectin receptors (CLRs). However, uptake of ADAMTS13 by MDMs was not affected by a blocking antibody directed towards the macrophage mannose receptor. Interestingly, a robust inhibition of ADAMTS13 endocytosis was observed upon incubation with polyanions such as dextran sulphate and fucoidan suggesting a role for class A scavenger receptors. Taken together our data suggest that internalization of ADAMTS13 by macrophages proceeds via a mechanism that is dissimilar from that previously described in dendritic cells. We anticipate that endocytic uptake by macrophages might participate in the clearance of ADAMTS13 from the circulation.
Introduction

Patients affected by the autoimmune disorder acquired thrombotic thrombocytopenic purpura (TTP) develop autoantibodies against ADAMTS13, a metalloproteinase that regulates proteolysis of ultra large von Willebrand factor multimers (UL-VWF).\(^1\) Lack of cleavage of platelet-reactive UL-VWF multimers promotes the formation of platelet-rich clots resulting in microvascular obstruction, low platelet counts (thrombocytopenia) and fragmentation of red blood cells (hemolytic anemia).\(^2\) Anti-ADAMTS13 antibodies are composed predominantly of subclasses IgG\(_1\) and IgG\(_4\)\(^{1,3,5}\) and the variable domains are modified by somatic hypermutation.\(^1,3,6\) Both isotype switching and somatic hypermutation of antibodies depends on the presence of antigen specific CD4\(^+\) T cells. Three independent studies have shown that HLA-DRB1*11 comprises a risk factor for acquired TTP further implicating CD4\(^+\) T cells in the pathogenesis of this disorder.\(^7,9\) We have previously shown that ADAMTS13 is endocytosed by antigen presenting cells (APCs), such as dendritic cells, by the macrophage mannose receptor.\(^10\) Following its intracellular processing a restricted set of ADAMTS13 derived peptides is presented on MHC class II for presentation to CD4\(^+\) T cells.\(^11\) These findings reveal a critical role for dendritic cells in the initiation of CD4\(^+\) T cell responses to ADAMTS13 in patients with acquired TTP. As yet cellular mechanisms contributing to the clearance of ADAMTS13 from the circulation have not been defined. The circulatory half-life of ADAMTS13 following plasma exchange using fresh frozen plasma ranged from 2 to 3 days.\(^12\) Patients suffering from acute TTP can develop non-neutralizing antibodies\(^4,5,13\) that are considered to enhance clearance of ADAMTS13 from the circulation.\(^13,14\) It is well established that macrophages can directly recognize both microbial structures as well as endogenous ligands through the expression of a large repertoire of pattern recognition receptors.\(^15,17\) In this study we therefore analyzed the mechanism underlying the immune recognition of ADAMTS13 by human monocyte derived macrophages. Our results indicate that endocytosis of ADAMTS13 by human monocyte-derived macrophages does not depend on the macrophage mannose receptor as previously shown for dendritic cells. Using a number of pharmacological approaches our findings suggest that class A scavenger receptors mediate the uptake of ADAMTS13 by macrophages.
Materials and methods

Materials
The following antibodies were used in this study: APC-conjugated mouse monoclonal anti-CD206 (mannose receptor, MR, BD Biosciences, San Jose, CA, USA), APC conjugated mouse IgG isotype control, mouse monoclonal anti-early endosome antigen (EEA1, BD Biosciences), mouse IgG isotype control conjugated with FITC and PE (Dako, Glostrup, Denmark), anti-CD36 (Abcam, Cambridge, UK), 488-conjugated secondary antibody anti-mouse (Invitrogen, Carlsbad, CA, USA). The monoclonal anti-MR antibody clone 15.2 (BioLegend, San Diego, CA, USA), the monoclonal blocking antibody anti-CD36 (Abcam, Cambridge, UK) and the monoclonal control antibody (BioLegend) were used for blocking experiments.

Generation of immature monocyte derived macrophages, uptake of ADAMTS13
Blood of healthy individuals was drawn in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the declaration of Helsinki. Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy individuals using anti-CD14+ magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were differentiated into macrophages (MDMs) by culturing monocytes in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) for 6 days in the presence of 50 ng/ml of M-CSF (Peprotech, London, UK). For uptake experiments 0.2 x 10^6 MDMs were incubated with 25 nM of recombinant ADAMTS13-488 (rADAMTS13-488) at 37°C for 1 hour in serum-free RPMI medium (Lonza, Breda, The Netherlands). Recombinant ADAMTS13 was purified and labeled as described previously. Uptake was analyzed by flow cytometry (Fortessa flow cytometer, BD Biosciences). In all uptake experiments fluorescently labeled isotype controls were used. Uptake of rADAMTS13 was also analyzed by confocal microscopy. rADAMTS13-488 (100 nM) was added to 0.4 x 10^6 MDMs that were allowed to adhere to fibronectin coated glass slides for 2 hours in serum-free RPMI medium and uptake was performed for 1 hour at 37°C. Cells were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes and subsequently stained in PBS supplemented with 0.5% human serum albumin (HSA) with or without 0.1% saponin with antibodies against early endosome antigen (EEA1) and subsequently with Alexa568-conjugated secondary antibodies (Molecular Probes, Breda, The Netherlands). Next coverslips were mounted with Prolong gold DAPI (Invitrogen) and viewed by confocal microscopy using a Zeiss LSM 510 microscope (Carl Zeiss, Heidelberg, Germany).
Blocking experiments

In order to analyze the mechanism of binding and uptake of ADAMTS13-488, MDMs were first pre-incubated for 20 minutes at 37°C with sucrose (0.75 M), dynasore (80 μM; Sigma Aldrich, Zwijndrecht, The Netherlands) and with EDTA (5 mM). Uptake of rADAMTS13-488 was also monitored after pre-incubation of the cells with mannan (1 mg/ml; Sigma Aldrich), D-mannose (10 mM; Sigma Aldrich), GlcNac (10 mM; Sigma Aldrich), D-galactose (10 mM; Sigma Aldrich) or with monoclonal antibody clone 15.2 directed against MR and with a monoclonal antibody directed against CD36. Blockage of uptake and/or binding was also analyzed after pre-incubation of the cells with different molecular weights of dextran sulphate (1 mg/ml and 100 μg/ml; 500 kDa, 9-20 kDa, 6-10 kDa and 5 kDa; Sigma Aldrich), heparin (1 mg/ml and 100 μg/ml; Sigma Aldrich), fucoidan (500 μg/ml; Sigma Aldrich), polyinosinic acid (poly (I); 200 μg/ml; Sigma Aldrich) and polycytidylic acid (poly (C); 200 μg/ml, Sigma Aldrich).
Results

ADAMTS13 is internalized by monocyte-derived macrophages
To examine the uptake of ADAMTS13 by human macrophages we isolated monocytes from healthy donors and differentiated them into MDMs. To determine the ratio of endocytosed and cell surface bound ADAMTS13, MDMs were incubated with 25 nM of ADAMTS13-488 for 1 hour at both 37°C and 4°C. As observed in figure 1A an increase in the mean fluorescent intensity (MFI) was obtained when cells were incubated at 37°C. Only limited uptake of ADAMTS13-488 was detected at 4°C (Figure 1A). Incubation with various concentrations of ADAMTS13-488 as well as a prolonged time of incubation with MDMs revealed that uptake of ADAMTS13 was concentration and time-dependent (Figure 1B). Endocytosis of ADAMTS13-488 was confirmed by confocal microscopy. MDMs were incubated with 100 nM of ADAMTS13-488 for 30 minutes at 37°C. Subsequently cells were fixed and stained with an antibody against the early endosome marker EEA-1 (Figure 1C). Confocal microscopy revealed that ADAMTS13-488 was present within endocytic vesicles. Taken together, our data show that ADAMTS13 is efficiently internalized by MDMs.
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Figure 1. ADAMTS13 endocytosis by MDMs. (A) ADAMTS13-488 (25 nM) was incubated with MDMs for 1 hour at 37°C and 4°C. Cells were then analyzed by FACS analysis. (A) Left panel represents histogram of ADAMTS13 uptake at 37°C. The gray histogram represents control cells incubated without ADAMTS13-488. Right panel displays reduced uptake of ADAMTS13 at 4°C. (B) Uptake was performed at different time intervals (0-120 minutes; left panel) or with increasing concentrations of ADAMTS13-488 (0-100 nM; right panel). Graphs represent data of 3 independent experiments (mean ± SD). Data are expressed as percentage of mean fluorescent intensity (MFI) at 37°C, where 100% corresponds to the highest MFI observed for individual experiments. (C) ADAMTS13-488 was added to MDMs for 30 minutes at 37°C. MDMs were stained with anti-EEA1 antibody, followed by Alexa 568 labeled secondary antibody (EEA1 staining in red).

Uptake of ADAMTS13 by MDMs is clathrin-dependent
Previously we have shown that ADAMTS13 is efficiently endocytosed by monocyte-derived dendritic cells through a receptor mediated pathway.\textsuperscript{10} In order to analyze the mechanism that mediates uptake of ADAMTS13, MDMs were pre-incubated for 20 minutes at 37 °C with sucrose (0.35 M) or dynasore (80 μM) prior to the addition of ADAMTS13-488. Sucrose forms clathrin microcages on the inner surface of the plasma membrane thereby blocking receptor recycling\textsuperscript{19,20} while dynasore, a potent cell permeable inhibitor of dynamin blocks pinching-off of endocytic vesicles from the plasma membrane.\textsuperscript{21-23} Uptake of ADAMTS13-488 was completely inhibited when MDMs were pre-incubated with either dynasore or sucrose (Figure 2). These data indicate that uptake of ADAMTS13 by MDMs proceeds via a clathrin-dependent pathway.

ADAMTS13 uptake by MDMs proceeds via a mannose receptor-independent pathway
MDMs express several C-type lectin receptors (CLRs) on the cell surface that act as pattern recognition receptors and are able to bind and internalize both foreign and self antigens. These include the classical calcium-dependent sugar binding proteins like MR and Endo180, but also non classical CLRs that display structural homology with the classical CLRs but bind sugar and non sugar ligands in a calcium-independent manner.\textsuperscript{24} To study whether CLRs might contribute to the uptake of
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ADAMTS13, we pre-incubated MDMs with either 5 mM EDTA or with 1 mg/ml or 100 μg/ml of mannan, 10 mM D-mannose, N-acetyl-glucosamine (GlcNAc) or D-galactose. We found that endocytosis of ADAMTS13-488 by MDMs was significantly inhibited by EDTA and mannan (Figure 3A and 3B). Only 26 and 30% of residual uptake of ADAMTS13-488 was observed when cells were incubated respectively with EDTA or mannan (Figure 3A and 3B). Pre-incubation of MDMs with D-mannose, GlcNAc and D-galactose did not affect uptake of ADAMTS13 (Figure 3C). Taken together, these results indicate that ADAMTS13 endocytosis by MDMs is mannan sensitive. We have shown previously that the macrophage mannose receptor (MR) is responsible for ADAMTS13 uptake by human monocyte-derived dendritic cells. To assess whether the MR also contributes to the uptake of ADAMTS13 by MDMs, we pre-incubated MDMs with a specific blocking antibody against the MR, clone 15.2 (50 μg/ml). Endocytosis of 25 nM of ADAMTS13-488 was not reduced by the addition of the anti-MR antibody (Figure 4A). Interestingly, confocal microscopy revealed that part of endocytosed ADAMTS13-488 co-localized with MR (Figure 4B) suggesting that ADAMTS13 and MR co-localize in the same endocytic compartment. MR in fact is predominantly internalized into early endosomes and subsequently, after release of its ligand, is recycled to the plasma membrane. Taken together, our data indicate that ADAMTS13 endocytosis by MDMs proceeds via a MR-independent pathway.

Figure 2. MDMs endocytose ADAMTS13 through a receptor mediated pathway. MDMs were pre-incubated with sucrose (0.35 M) or dynasore (80 μM) for 20 minutes prior to the addition of 25 nM of ADAMTS13-488. Cells were then analyzed by FACS. Grey histograms represent control cells incubated without ADAMTS13-488. Dotted histograms indicate cells pre-incubated with sucrose or dynasore while opened histograms represent cells incubated with only ADAMTS13-488. Data of 3 independent experiments (mean ± SD) are represented in the right panel and are expressed as percentage of MFI, where 100% corresponds to the mean fluorescence signal obtained for ADAMTS13-488 in the absence of sucrose and dynasore.
Figure 3. ADAMTS13 uptake is mannan sensitive. ADAMTS13-488 uptake by MDMs was analyzed by FACS. (A, B) Endocytosis of ADAMTS13 was inhibited by pre-incubation with EDTA and mannan. (C) Different monosaccharides did not affect ADAMTS13 uptake by MDMs. The grey histograms (right panel) represent controls incubated in the absence of ADAMTS13. Cells incubated with ADAMTS13 are indicated by a solid line; cells incubated with ADAMTS13 and different sugar components are indicated by a dotted line. Left panels express ADAMTS13 uptake as percentage of MFI, where 100% corresponds to the mean fluorescence signal obtained for ADAMTS13-488 in the absence of EDTA or different sugars.

Scavenger receptors participate in ADAMTS13 endocytosis

The scavenger receptors are a large family of immunosurveillance receptors highly expressed by MDMs.26 These receptors are known to bind and internalize...
several self and non self molecules and participate in several cellular functions such as antigen presentation, inflammation and clearance.\textsuperscript{26,27} We evaluated the possible role of scavenger receptors in ADAMTS13 uptake by pre-treating MDMs with various polymers of dextran sulphate (100 μg/ml and 1 mg/ml) and heparin (100 μg/ml and 1 mg/ml). ADAMTS13 uptake was significantly reduced by both compounds (Figure 5A and B). To evaluate which class of scavenger receptors might be involved in uptake of ADAMTS13, we performed additional endocytosis assays using a specific monoclonal antibody against the scavenger receptor CD36, a class B scavenger receptor, and (CASR)-blocking agents, directed against standard non-selective class A scavenger receptors.\textsuperscript{28-31} It is well established that the class B scavenger receptor CD36 is able to bind thrombospondin repeat domains and it has been show to be able to bind ADAMTS13 in vitro.\textsuperscript{32} A blocking antibody directed towards CD36 did not prominently affect the uptake of ADAMTS13 by MDMs (Figure 6C). MDMs were pre-incubated with fucoidan (500 μg/ml), poly (I) (200 μg/ml) and the control compound poly (C) (200 μg/ml) for 20 minutes at 37°C prior to the addition of ADAMTS13-488. Both fucoidan and poly (I) blocked endocytosis of ADAMTS13 whereas the control compound poly (C) did not affect internalization of ADAMTS13 (Figure 6A and 6B). Taken together, our data show that polyanionic ligands block the internalization of ADAMTS13 by MDMs implicating a role for class A scavenger receptors in this process.

\textbf{Figure 4. Uptake of ADAMTS13 is mannose receptor independent.} \textit{(A)} MDMs were pre-incubated with 50 μg/ml of anti-MR antibody (clone 15.2) for 20 minutes. Subsequently ADAMTS13-488 was added to the cells and uptake was analyzed by FACS. Graphs represent data of 3 independent experiments (mean ± SD). Uptake is expressed as percentage of MFI, where 100% corresponds to the mean fluorescence signal obtained for ADAMTS13-488 in the absence of the monoclonal antibody. As control, cells were incubated with a control antibody prior addition of ADAMTS13-488. \textit{(B)} 100 nM of ADAMTS13-488 was added to MDMs for 1 hour at 37°C. MDMs were stained with anti-MR antibody, followed by Alexa 568 labeled secondary antibody.
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**Figure 5. Heparin and dextran sulfate reduce ADAMTS13 uptake by MDMs.** MDMs were treated with heparin (A) or different molecular weights of dextran sulfate (B; 1 mg/ml and 100 μg/ml) for 20 minutes at 37°C. ADAMTS13-488 was subsequently added to the cells and endocytosis was analyzed by FACS. Data are expressed as percentage of MFI at 37°C, where 100% corresponds to the highest mean fluorescence signal observed in the absence of heparin and dextran sulfate. Data of 3 independent experiments ± SD are represented.

**Figure 6. Class A scavenger receptors participate in ADAMTS13 uptake by MDMs.** (A, B, C) MDMs were incubated with fucoidan, poly (I), poly (C) and anti-CD36 blocking antibody prior addition of ADAMTS13-488. Cells were analyzed by FACS. (A) Grey histograms represent controls incubated in the absence of ADAMTS13. Cells incubated with ADAMTS13 are indicated by a solid line; cells pre-incubated with CASR-inhibitors are indicated by a dotted line. (B,C) Data of 3 independent experiments ± SD are represented and uptake is expressed as percentage of MFI at 37°C, where 100% corresponds to the highest mean fluorescence signal observed in absence of inhibitors.
Discussion

In this study we demonstrate that ADAMTS13 is endocytosed by monocyte derived macrophages (MDMs). Blocking experiments with mannan and EDTA suggested that CLRs participate in the endocytosis of ADAMTS13 by macrophages. These receptors share at least one Ca\(^{2+}\)/carbohydrate recognition domain (CRD), and bind sugars in a calcium dependent manner.\(^{24}\) Previously we have shown that the macrophage mannose receptor (MR), a mannan sensitive CLR, mediates internalization of ADAMTS13 by dendritic cells.\(^{10}\) Although mannan and EDTA significantly reduced ADAMTS13 uptake by macrophages, ADAMTS13 endocytosis appears to be independent from this C-type lectin, as its uptake by MDMs is not influenced neither by the presence of the blocking anti-MR antibody clone 15.2 nor by the addition of D-mannose or GlcNAc, that interfere with binding of ligands to mannan-sensitive CLRs.\(^{33}\) Our experiments therefore indicate that ADAMTS13 is internalized by macrophages through a different mechanism then that previously described for human monocyte-derived dendritic cells.\(^{10}\)

Macrophages express several pattern recognition receptors which include CLRs, Toll-like receptors and scavenger receptors (SR).\(^{15}\) SR comprise a large group of proteins that are divided in eight different classes of membrane and soluble proteins (Class A, B, C, D, E, F, G and H) according to shared structural domains.\(^{26,27,34}\) All SRs are able to bind modified lipoproteins and a range of different polyanionic ligands of both host and exogenous origin.\(^{30,34}\) Incubation of MDMs with polyanionic ligands, such as heparin, dextran sulphate, fucoidan and polyinosinic acid (pol (I)), resulted in a significant inhibition of ADAMTS13 uptake. This suggests a possible role for scavenger receptors in ADAMTS13 internalization by MDMs. Interestingly, not all SRs are able to bind heparin. Stabilin-1 and stabilin 2 represent a class of receptors known to be involved in heparin binding and clearance.\(^{35,36}\) Both receptors are expressed on macrophages and different subtypes of endothelial cells \(^{35,37}\) and mediate endocytosis of several ligands including hyaluronic acid, heparin, acetylated LDL and advanced glycation end products.\(^{35,37}\) Lectin-like ox-LDL receptor, also known as LOX-1, is expressed on macrophages, endothelial cells and platelets.\(^{38}\) LOX-1 is a structurally distinct SR with an extracellular C-type lectin domain (CRD) that mediates endocytosis of its ligands in a Ca\(^{2+}\)-dependent manner. Although the specific ligands binding to the CRD domain of LOX-1 are still unknown, experiments have shown that even high concentrations of mannan, do not block transcytosis of the pancreatic bile salt-dependent lipase, suggesting that mannan does not bind the CRD region of the LOX-1 receptor.\(^{39}\) So far, not many scavenger receptors with both polyanion
and sugar binding properties have been identified. Recently, the scavenger receptor C-type lectin (SRCL), a unique component of the class A scavenger receptors, has been identified on the surface of endothelial cells.\(^{40}\) Like other SR it binds modified low density lipoproteins, but the additional CRD domain potentially endows SRCL with glycan binding properties. Functional analysis have shown that SRCL recognizes and binds LewisX-containing glycoproteins and that it shares several characteristics with the dendritic cell surface receptor DC-SIGN.\(^{40,41}\) Further studies are needed to address whether SRCL, or other SRs, participate in the binding and uptake of ADAMTS13 from MDMs. Taken together, our findings indicate that macrophages are able to endocytose ADAMTS13 through a mechanism that is distinct from that previously described in monocyte-derived dendritic cells. Our findings document that polyanionic ligands block the uptake of ADAMTS13 thereby implicating class A scavenger receptors in internalization of ADAMTS13 by macrophages.
References


