Acquired TTP: ADAMTS13 meets the immune system

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The macrophage mannose receptor promotes uptake of ADAMTS13 by dendritic cells

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Abstract

ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation by cleaving ultra-large von Willebrand factor (VWF) multimers on the surface of endothelial cells. Autoantibodies directed against ADAMTS13 prohibit the processing of VWF multimers initiating a rare and life-threatening disorder called acquired thrombotic thrombocytopenic purpura (TTP). Formation of autoantibodies depends on the activation of CD4⁺ T cells. This process requires immune recognition, endocytosis and subsequent processing of ADAMTS13 into peptides that are presented on MHC class II molecules to CD4⁺ T cells by dendritic cells. Here, we investigate endocytosis of recombinant ADAMTS13 by immature monocyte-derived dendritic cells (iDCs) using flow cytometry and confocal microscopy. Upon incubation of fluorescently labeled-rADAMTS13 with DCs, significant uptake of ADAMTS13 was observed. Endocytosis of ADAMTS13 was completely blocked upon addition of EGTA and mannan. ADAMTS13 endocytosis was decreased in presence of a blocking monoclonal antibody directed towards macrophage mannose receptor (MR). Furthermore, siRNA silencing of MR reduced the uptake of ADAMTS13 by dendritic cells. Additionally, in vitro binding studies confirmed the interaction of ADAMTS13 with the carbohydrate recognition domains of MR. Together, our data indicate that sugar moieties on ADAMTS13 interact with MR thereby promoting its endocytosis by antigen presenting cells.
Introduction

Antigen presenting cells (APCs) continuously sample antigens from their environment and following their processing load antigen-derived peptides on MHC class I or II molecules for presentation to CD8⁺ or CD4⁺ T cells, respectively. In order to appropriately respond to incoming pathogens, APCs are equipped with a large diversity of cell surface receptors, so-called pattern recognition receptors (PRRs) that recognize specific pathogen-associated molecular patterns (PAMPs).1 This provides APCs like dendritic cells with the capacity to recognize a wide diversity of foreign pathogens. Upon encountering a pathogen, dendritic cells undergo maturation and migrate to the draining lymph nodes where they encounter and prime naive T cells. This mechanism ultimately generates a humoral response that is capable of rapidly neutralizing re-entering pathogens. It is well established that besides recognizing PAMPs, PRRs can also interact with molecular patterns present on endogenous proteins.2 This results in the continuous presentation of “self-derived” peptides to CD4⁺ T cells.3 The efficient removal of potentially self-reactive T cells in the thymus prevents the stimulation of CD4⁺ T cells by self-antigens presented on MHC class II molecules. Nevertheless, autoimmunity can develop as a result of activation of low affinity CD4⁺ T cells that have not been efficiently eliminated from the repertoire in the thymus.4 Also, shared T cell epitopes between pathogen encoded and self-antigens are a potential source for cross-reactive T cells.5 The autoimmune disorder thrombotic thrombocytopenic purpura results from the development of autoantibodies directed towards the metalloprotease ADAMTS13.6 A number of studies have shown that these antibodies target an exposed surface in the spacer domain which mediates binding of ADAMTS13 to its substrate von Willebrand factor (VWF).6-13 Lack of cleavage of ultra-large VWF multimers by ADAMTS13 results in accumulation of blood platelets at sites of vascular perturbation.14 This promotes microvascular obstruction resulting in low platelet counts (thrombocytopenia) and fragmentation of red blood cells (hemolytic anemia).15,16 Our current knowledge on the etiology of acquired TTP is limited. A large number of case reports suggests that microbial infections are linked to the onset or recurrence of this autoimmune disease.6 This has led us to hypothesize that triggering of the innate immune system associates with the initiation of acquired TTP.6 Recently, it has been established that HLA-DRB1*11 is more frequent, whereas HLA-DRB1*04 is underrepresented in patients with acquired TTP when compared to controls.17,18 This observation implies that ADAMTS13 derived peptides presented on HLA-DRB1*11 on the surface of antigen presenting cells promote the activation of ADAMTS13-
specific CD4+ T cells. 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.11,19,20 Both isotype switching and somatic hypermutation of antibodies depend on the presence of antigen specific CD4+ T cells. Immune recognition, endocytosis and subsequent processing of ADAMTS13 into peptides that are presented on MHC class II molecules by dendritic cells are needed for the generation of ADAMTS13 specific CD4+ T cells. In this study, we explored the requirements for immune recognition of ADAMTS13 by human monocyte-derived dendritic cells. Our results indicate that ADAMTS13 is efficiently internalized by immature dendritic cells (iDCs) in a mannose receptor dependent manner.
Materials and methods

Materials
The following antibodies were used in this study: phycoerythrin (PE) conjugated CD14 (Sanquin Reagents Amsterdam, The Netherlands), allophycocyanin (APC) conjugated CD83, fluorescein isothiocyanate (FITC) conjugated CD80, APC-conjugated mouse monoclonal anti-CD206 (Mannose receptor, MR, BD Biosciences, CA, USA), APC conjugated mouse IgG isotype control, mouse monoclonal anti-early endosome antigen (EEA1, BD Biosciences, San Jose, CA, USA), PE conjugated mouse anti-human CD209 (DC-SIGN, AbD Serotec, Düsseldorf, Germany), mouse IgG isotype control conjugated with FITC and PE (Dako, Glostrup, Denmark), anti-human CD209 (R&D systems, Minneapolis, MN, USA), anti-human CD206 (Santa Cruz Biotechnology, Heidelberg, Germany) and monoclonal blocking antibody anti-MR clone 15.2 (BioLegend, San Diego, CA, USA). Monoclonal antibody AZN-D1 directed towards CD209 has been described previously.21,22 Mannan, D-mannose, N-acetyl-D-glucosamine (GlcNac) and D-galactose were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fluorescent labeling of recombinant ADAMTS13
Wild type full-length ADAMTS13 was produced in stable HEK293 cells and purified as described previously.7 Purified recombinant ADAMTS13 was labeled using the Microscale Alexa Fluor 488 protein Labeling kit (Invitrogen, Breda, The Netherlands). Protein concentration and efficiency of labeling was spectrophotometrically determined at 280 nm and at 494 nm. The integrity of Alexa 488 labeled ADAMTS13 (ADAMTS13-488) was confirmed by SDS-PAGE (data not shown).

Generation of immature dendritic cells, uptake of ADAMTS13
Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) using anti-CD14+ magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). 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. Differentiation of monocytes into immature dendritic cells (iDCs) was obtained by culturing them in Cellgro DC Medium for 5 days in the presence of 800 U/ml IL-4 and 1000 U/ml GM-CSF (CellGenix, Freiburg, Germany). In uptake experiments 0.2 x 10^6 iDCs were incubated with 50 nM of ADAMTS13-488 at 37°C for 45 minutes in serum-free IMDM medium (Lonza, Breda, The Netherlands). Uptake was analyzed by flow cytometry (LSRII flow cytometer, BD Biosciences). Histograms were processed using Flowjo software (Tree Star Inc., Ashland, OR,
USA). Data are expressed as percentage of mean fluorescent intensity (MFI) at 37°C where 100% of uptake corresponds to the maximal fluorescent signal obtained. In all uptake experiments we used fluorescently labeled isotype controls. In our data analysis we have subtracted from each sample the background staining (mean fluorescent intensity obtained with a control IgG-FITC).

To monitor uptake by confocal microscopy, 0.5 x 10^6 iDCs were allowed to adhere to fibronectin coated glass slides for 4 to 5 hours. Next, ADAMTS13-488 (100 nM) was added to the cells in serum-free IMDM medium and uptake was performed for 45 minutes at 37°C. Cells were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes. Cells were stained with antibodies against DC-SIGN (CD209), early endosome antigen (EEA1) or mannose receptor (MR, CD206) and subsequently with Alexa 568-conjugated secondary antibodies (Molecular Probes, Breda, The Netherlands) in PBS supplemented with 0.5% human serum albumin (HSA) with or without 0.1% saponin. Stained coverslips were mounted with Mowiol (Polysciences, Warrington, PA, USA) and viewed by confocal microscopy using a Zeiss LSM 510 microscope (Carl Zeiss, Heidelberg, Germany).

**Blocking experiments and siRNA gene silencing**

To analyze the involvement of C-type lectin receptors in the uptake and binding of ADAMTS13-488, iDCs were first pre-incubated for 20 minutes at 37°C with mannan (1 mg/ml), D-mannose (10 mM), GlcNac (10 mM) or D-galactose (10 mM). Subsequently, 50 nM of ADAMTS13-488 was added to the cells for 45 minutes at 37°C and uptake was analyzed by flow cytometry. Blockage of antigen uptake was also monitored after preincubation of the cells with sucrose (0.75 M) and with EGTA (5 mM) or with monoclonal antibody clone AZN-D1 directed against DC-SIGN and monoclonal antibody clone 15.2 directed against MR.

For gene silencing experiments, 6 µl of 100 µM MR, DC-SIGN specific or non-targeting control siRNA pools (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA) were added to 4 x 10^6 iDCs on day 2 in antibiotics and serum-free medium (Cellgro, Cellgenix, Freiburg, Germany). Cells were then pulsed at 250 V, 150 µF and ∞ Ω in a Bio-Rad Genepulser (Bio-Rad, Hercules, CA, USA). After 78 hours cells were analyzed for MR and DC-SIGN expression using APC-conjugated mouse monoclonal anti-MR (BD Biosciences, CA, USA) and PE-conjugated mouse anti-DC-SIGN (AbD Serotec, Germany). Subsequently, uptake experiments were performed as described previously with ADAMTS13-488.
In vitro binding of ADAMTS13 to MR and DC-SIGN-Fc chimeras

Binding of ADAMTS13 to soluble DC-SIGN-Fc chimera (R&D systems, Minneapolis, MN, USA) and to MR-Fc chimera containing the major ligand binding C-type lectin domains CTLD 4-7, prepared as described previously\textsuperscript{23} was performed as follows. ADAMTS13 (5 µg/ml) was immobilized onto a Maxisorp plate (Nunc, Roskilde, Denmark) in 50 mM NaHCO\textsubscript{3} (pH 9.5) overnight at 4°C. Wells were blocked for 1 hour in TSM buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM CaCl\textsubscript{2} and 2 mM MgCl\textsubscript{2}) containing 1% Bovine Serum Albumin (BSA). Subsequently, 10 µg/ml of the soluble receptor was added for 1 hour at 37°C. Unbound fragments were washed away and Fc-chimera binding to ADAMTS13 was determined by incubation with a peroxidase-labeled murine anti-human IgG\textsubscript{1} antibody (Sanquin Reagents). Binding specificity was verified by the preincubation of the recombinant receptors with 1 mg/ml of mannan.
Results

ADAMTS13 is endocytosed by immature monocyte-derived dendritic cells
To study ADAMTS13 endocytosis by APCs, we used monocyte-derived immature dendritic cells (iDCs). To determine the ratio of internalized and cell surface bound ADAMTS13-488, incubations of 50 nM ADAMTS13-488 were performed at both 37°C (uptake) and 4°C (binding). A strong increase in mean fluorescent intensity was observed upon incubation with ADAMTS13-488 at 37°C (Figure 1A and B), however, there was only 6% of ADAMTS13-488 detected when the incubation was performed at 4°C (Figure 1B). This observation suggests that ADAMTS13-488 is mainly endocytosed by iDCs.

Figure 1. ADAMTS13 uptake by iDCs. (A) 50 nM of ADAMTS13-488 was incubated with iDCs for 45 minutes at 37 °C. Cells were analyzed by FACS. The gray histogram represents control cells without ADAMTS13-488. (B) Cell-surface bound versus internalized ADAMTS13 was determined by incubating iDCs with ADAMTS13-488 at 4°C or 37°C for 45 minutes. (C) Uptake was performed with increasing concentrations (0-200 nM) of ADAMTS13-488 (45 minutes) or at prolonged time intervals (0-50 minutes with 50 nM of ADAMTS13-488). Graphs represent data of 2-3 independent experiments mean ± SD. Data are expressed as percentage of mean fluorescent intensity (MFI) at 37°C, where 100% corresponds to the highest mean fluorescence signal observed for individual experiments such as 50 nM (panel B) or 200 nM of ADAMTS13-488 and 50 minutes of ADAMTS13-488 uptake (panel C). (D) ADAMTS13-488 was added to iDCs for 45 minutes at 37 °C. DCs were fixed, permeabilized and stained with anti-EEA1 antibody, followed by Alexa 568 labeled secondary antibody (EEA1 staining in red).
Increased concentrations of ADAMTS13 and prolonged incubation time resulted in an increase of the fluorescent intensity demonstrating that ADAMTS13 endocytosis is dose- and time-dependent (Figure 1C). We subsequently investigated the intracellular targeting of ADAMTS13 following its uptake by iDCs. Cells were first incubated with ADAMTS13-488 for 45 minutes at 37°C, fixed and then stained for the early endosome marker EEA-1. Confocal microscopy revealed that a significant part of endocytosed ADAMTS13-488 was present within early endosomes (Figure 1D). Inspection of these cells with Image Pro Plus 6.1 revealed that about 55% ± 6 of the endocytosed ADAMTS13 is present within the endosomal compartment (Supplemental Table I). Our data show effective endocytosis of ADAMTS13 by iDCs.

Receptor-mediated endocytosis of ADAMTS13

Uptake of antigens by iDCs can occur through two main pathways: receptor-mediated endocytosis and macropinocytosis.24 In order to identify the mechanism that mediates ADAMTS13 endocytosis, we evaluated the binding and uptake of ADAMTS13-488 after pre-incubation of iDCs with sucrose. Sucrose blocks receptor recycling through the formation of clathrin microcages on the inner surface of the plasma membrane and therefore prevents clathrin-mediated endocytosis.25,26 Uptake of ADAMTS13 was completely abolished by pre-incubation of iDCs with 0.75 M sucrose (Figure 2A and B). In contrast, uptake of Lucifer Yellow (LY) by iDCs, which proceeds via macropinocytosis, was not inhibited by sucrose24 (Figure 2B). These findings indicate that ADAMTS13 is internalized by a cell surface receptor on iDCs.

Figure 2. Uptake of ADAMTS13 by iDCs is mediated by a clathrin dependent cell surface receptor.

Immature DCs were pre-incubated with 0.75 M of sucrose prior to the addition of 50 nM of ADAMTS13-488. (A) Cells were analyzed by FACS. Gray histogram represents control cells without ADAMTS13-488. (B) Data of 3 independent experiments (mean ± SD) are represented and expressed as percentage of MFI, where 100% corresponds to the mean fluorescence signal obtained for ADAMTS13-488 in the absence of sucrose. Lucifer Yellow (LY) which is internalized by macropinocytosis was used as a control.
C-Type lectins are involved in uptake of ADAMTS13

Immature DCs express several endocytic receptors, such as C-type lectin receptors (CLRs), which are abundantly expressed on iDCs. These receptors share at least one Ca$^{2+}$/carbohydrate recognition domain (CRD), and bind sugars in a calcium dependent manner. To study whether CLRs contribute to the uptake of ADAMTS13, we pre-incubated iDCs with either 5 mM EGTA or 1 mg/ml of mannan, 10 mM D-mannose, N-acetyl-glucosamine (GlcNAc) or D-galactose. These blocking reagents did not effect the viability of the cells (Figure 3S; supplemental data). We found that endocytosis of ADAMTS13-488 by iDCs was significantly inhibited by EGTA and mannan (Figure 3A, B, C and D). Only 18% of residual uptake of ADAMTS13-488 was observed when cells were incubated with EGTA or mannan (Figure 3B and D). Pre-incubation of iDCs with D-mannose or GlcNAc resulted in a reduction of uptake of approximately 33 to 50% while as expected pre-incubation of the cells with D-galactose did not affect uptake of ADAMTS13 (Figure 3D). Mannan, D-mannose and GlcNAc bind with different affinity to C-type lectin receptors. We observed a more pronounced inhibition of ADAMTS13 uptake when cells were pre-incubated with mannan (Figure 3D). Mannan is a polysaccharide consisting of multiple mannose-residues and therefore has over 100-fold higher affinity for mannose sensitive CLRs when compared to monomeric D-mannose. The inhibitory effect of mannan on the endocytosis of ADAMTS13-488 was further confirmed by confocal microscopy (Figure 3E). Pre-incubation of iDCs with 1 mg/ml mannan resulted in significantly reduced amount of intracellular ADAMT13-488. To demonstrate the specificty of ADAMTS13 uptake by iDCs we analyzed the uptake of α2-macroglobulin which is mediated by low density lipoprotein related protein LRP (CD91). Endocytosis of α2-macroglobulin was not reduced by mannan, D-mannose, GlcNAc, D-galactose, EGTA and monoclonal antibody 15.2 directed towards the mannose-receptor (Supplemental Figure 2). Together, these results show specific involvement of a mannose sensitive C-type lectin receptor in the uptake of ADAMTS13 by iDCs. We also analyzed whether ADAMTS13 N- or O-linked glycans moieties were important in mediating uptake by iDCs. ADAMTS13 was untreated or treated overnight at 37°C with PNGaseF or O-glycosidase together with neuraminidase in order to remove respectively N-linked and O-linked sugars (Supplemental Figure 4). We found that treatment of ADAMTS13 with PNGaseF resulted in a 70% of reduction of uptake when compared to untreated ADAMTS13. O-glycosidase treatment did not have any effect on ADAMTS13 endocytosis by iDCs (Supplemental Figure 4B). These data indicate that N-linked sugar moieties present on ADAMTS13 are involved in its uptake by iDCs.
Figure 3. C-type lectin receptors mediate ADAMTS13 uptake by iDCs. FACS analysis of ADAMTS13-488 uptake by iDCs was performed. (A, B) Uptake of ADAMTS13 (expressed in percentage) was prevented by pre-incubation of the cells with 5 mM EGTA. (C) Inhibition of ADAMTS13-488 endocytosis by different monosaccharides. The gray histograms 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. (D) Blocking of ADAMTS13 uptake by different sugars is expressed in percentages. (E) Confocal analysis of the effect of mannan on ADAMTS13 endocytosis. iDCs were incubated with or without mannan (E) prior addition of ADAMTS13-488. Cells were stained with anti-DC-SIGN antibody (red).
In vitro binding of ADAMTS13 to mannose receptor and DC-SIGN

It has been previously reported that the two major C-type lectin receptors expressed on iDCs displaying mannan binding specificity are the dendritic cell specific ICAM3 grabbing non integrin receptor (DC-SIGN) and the mannose receptor.\textsuperscript{2,22} We tested \textit{in vitro} binding of ADAMTS13 to MR and DC-SIGN. Increasing concentrations of recombinant DC-SIGN or MR CTLD 4-7 Fc-chimera were added to immobilized ADAMTS13 for 2 hours. A dose-dependent binding to both receptors was observed (Figure 4A and B). Following pre-incubation of the receptor chimeras with 1 mg/ml mannan, binding to ADAMTS13 was completely abolished (Figure 4A and B). These data demonstrate that ADAMTS13 can bind to both MR and DC-SIGN \textit{in vitro} in a mannan-sensitive manner.

Figure 4. Binding of ADAMTS13 to mannose receptor and DC-SIGN. ADAMTS13 was immobilized on microtiter plates. Increasing concentrations (0-10 µg/ml) of recombinant MR CTLD 4-7-Fc (A) or DC-SIGN-Fc (B) chimera were added to the wells. To study whether ADAMTS13 binding to these receptors is specific the Fc-chimera receptors were pre-incubated with mannan prior addition to the wells (A, B). Data represents values of 2 independent experiments. (C, D) iDCs were pre-incubated at 37°C in normal medium or in medium containing different concentrations of anti-MR antibody (C) or anti-DC-SIGN blocking antibody AZND1 (D) for 20 minutes. Then 50 nM of ADAMTS13-488 was added to the cells. Uptake was measured after 45 minutes by FACS and expressed as percentage of uptake. Graphs represent data of 3 independent experiments (mean ± SD).
Mannose receptor contributes to the uptake of ADAMTS13 by human iDCs

To further explore the involvement of MR and DC-SIGN in the uptake of ADAMTS13 by iDCs, we first investigated whether we could interfere with the uptake of ADAMTS13-488 by pre-incubating iDCs with increasing concentrations of blocking antibodies directed towards DC-SIGN and MR.\textsuperscript{21,22,29,30} Endocytosis of 50 nM of ADAMTS13-488 was reduced by the addition of anti-MR antibody clone 15.2 (Figure 4C). In contrast, AZN-D1 directed towards DC-SIGN did not affect uptake of ADAMTS13 (Figure 4D). To confirm the involvement of the MR in the uptake of ADAMTS13, we used small interfering RNA pools to knock down the expression of MR or DC-SIGN on iDCs. Strong reduction of MR and DC-SIGN expression was observed (Figure 5A and C). ADAMTS13-488 uptake in iDCs treated with siRNA targeting MR was reduced compared to cells receiving non-targeting siRNA (Figure 5D). No reduction in ADAMTS13-488 uptake was observed in cells transfected with DC-SIGN siRNAs (Figure 5B) indicating that this receptor is not involved in the uptake of ADAMTS13 by iDCs.

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\caption{Gene silencing of MR reduces uptake of ADAMTS13 by iDCs. Silencing of MR in iDCs reduces uptake of ADAMTS13. iDCs were transfected with non-targeting siRNA or targeting MR- and DC-SIGN siRNA. After 72 hours, expression of MR and DC-SIGN was measured by FACS (A, C). After siRNA transfection, cells were incubated with 50 nM of ADAMTS13-488 and its uptake was monitored by FACS (B, D). Dotted line histograms represent cells transfected with siRNA targeting MR and DC-SIGN (KD). Solid line histograms represent cells transfected with non-targeting siRNA (NT). Grey histograms represent cells stained with isotype control antibodies. Graphs represent data of 3 independent experiments (mean ± SD). Results are expressed as percentage of MFI, where 100% corresponds to the highest mean fluorescence intensity obtained with ADAMTS13-488.}
\end{figure}
Confocal analysis was performed to verify the involvement of MR in ADAMTS13 uptake. iDCs were incubated with ADAMTS13-488, fixed, permeabilized and stained with specific antibodies against DC-SIGN and MR. Endocytosed ADAMTS13 was co-localizing with MR but not with DC-SIGN (Figure 6). Taken together, our data suggest the involvement of MR in ADAMTS13 endocytosis. Once endocytosed both ADAMTS13 and MR co-localize within early endosomes (Figure 1, Supplemental Figure 1). Additional \textit{in vitro} binding studies between CTLD 4-7-Fc and ADAMTS13 were performed using SPR analysis and revealed an apparent equilibrium dissociation constant (KD) value of 474 nM (Supplemental Figure 5). Although our \textit{in vitro} data suggest a role for DC-SIGN in binding to ADAMTS13, the lack of inhibition of the prototypic blocking antibody AZN-D1 together with the absence of a reduction in ADAMTS13 uptake after knockdown of the receptor implies that DC-SIGN is not involved in binding and uptake of ADAMTS13 by iDCs.

\textbf{Figure 6. Colocalization of intracellular ADAMTS13 and mannose receptor in iDCs.} MR mediates endocytosis of ADAMTS13. iDCs were incubated with ADAMTS13-488 for 45 minutes at 37°C. Subsequently, samples were fixed and labeled for MR (A) or DC-SIGN (B) and analyzed by confocal microscopy. (A) Colocalization of ADAMTS13 (green) with MR (red) was observed (merge). (B) Labeling of DC-SIGN (red) showed no colocalization with ADAMTS13 (green).
Discussion

In this study, we demonstrate that ADAMTS13 is effectively internalized by human immature monocyte-derived dendritic cells. Internalization of ADAMTS13 can be blocked by mannan and EGTA suggesting a prominent role of C-type lectin receptors. In agreement with these findings, we show in vitro interaction of ADAMTS13 with two mannan sensitive CLR s, DC-SIGN and MR. Despite the capacity of ADAMTS13 to interact in vitro with DC-SIGN, ADAMTS13 endocytosis appears to be independent from this C-type lectin as its uptake by iDCs is not influenced neither by the presence of the blocking anti-DC-SIGN antibody AZN-D1 nor by siRNA-mediated knockdown of DC-SIGN. In contrast, our experiments suggest MR as a candidate receptor for ADAMTS13 uptake by iDCs. Both MR knockdown and pre-incubation of anti-MR antibody clone 15.2 with iDCs resulted in significantly reduced amounts of internalized ADAMTS13. Mannose receptor is a 175 kDa transmembrane glycoprotein belonging to the type I C-type lectin family. The extracellular portion of this receptor consists of a N-terminal cysteine-rich domain (CR-domain) that binds sulphated sugars in a calcium-independent manner and 8 carbohydrate recognition domains (CTLD 1-8) that bind in a calcium dependent manner to glycoconjugates or glycoproteins terminating in D-mannose, L-fucose or N-acetylglucosamine. Our SPR studies revealed that ADAMTS13 binds to the 4-7 CTLD repeats of the MR. Eight complex N-linked glycans associated to ADAMTS13 have been described together with O-linked disaccharide glucose-β(1,3)-fucose residues in the thrombospondin repeats. Lectin binding assays have provided evidence for the presence of high mannose residues also on plasma derived ADAMTS13. We demonstrate that removal of N-linked sugar moieties from ADAMTS13 reduces its binding and uptake by iDCs. Taken together, these findings support a model in which exposed mannosylated or fucosylated sugar-moieties mediate binding of ADAMTS13 to the MR thereby promoting its internalization by iDCs. MR internalization is dependent on the interaction of tyrosine-based and dihydrophobic motifs in its cytoplasmic tail with the clathrin adaptor complex AP-2 thereby recruiting MR to clathrin-coated pits. Following its recruitment into clathrin-coated pits MR is rapidly internalized. MR is predominantly internalized into early endosomes (Figure 1S). The low pH in early endosomes disrupts one of the Ca²⁺ binding sites in CRD4 of MR which destabilizes the sugar binding properties of this domain. This results in release of ligands from MR which subsequently recycles to the plasma membrane. A number of recent studies in murine dendritic cells have shown that antigens internalized via the MR are targeted to a “stable” population of early endosomes that have been implicated in cross-presentation of internalized antigen to CD8⁺ T cells.
However in cytokine-treated cells, MR can also be detected in late endosomes.\textsuperscript{31,39,40} Under these conditions MR, together with its ligand, is transported to late endocytic compartments where antigen derived peptides can be loaded on MHC class II molecules.\textsuperscript{31,39,40} This suggests that depending on the nature of the ligand, the amount of antigen internalized, the maturation level of DCs or the cytokine environment, MR internalization can lead to cross-presentation to CD8\textsuperscript{+} T cells or presentation of antigen-derived peptides to CD4\textsuperscript{+} T cells. The described association between HLA-DRB1*11 strongly suggests that ADAMTS13 specific CD4\textsuperscript{+} T cells contribute to the pathogenesis of acquired TTP.\textsuperscript{17,18} Based on these findings, we suggest a model in which MR-mediated internalization of ADAMTS13 by dendritic cells results in priming of naive CD4\textsuperscript{+} T cells, thereby initiating autoimmune responses to ADAMTS13 eventually resulting in acquired TTP.

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**Author Contributions**
N.S., W.P. and E.H. performed experiments. N.S., R.F. and J.V. conceived the study. T.B.G., L.M.vdB. and L.M.P. provided expertise and crucial reagents. N.S. and J.V. wrote the manuscript. All authors provided input for the manuscript.

**Disclosures of Conflict of Interest**
The authors declare no competing financial interests.
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Supplemental material

Materials and methods

Confocal microscopy
To analyze endocytosis of the macrophage mannose receptor (CD206) by confocal microscopy, 0.5x10^6 iDCs were allowed to adhere to fibronectin coated glass slides for 4 to 5 hours. Next, cells were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes. Cells were stained with antibody against early endosome antigen (EEA1) and mannose receptor (MR, CD206). Subsequently cells were washed with PBS 1% and stained respectively with Alexa 488- and 568-conjugated secondary antibody (Molecular Probes, Breda, The Netherlands). Stained coverslips were mounted with Mowiol (Polysciences, Warrington, PA, USA) and viewed by confocal microscopy using a Zeiss LSM 510 microscope (Carl Zeiss, Heidelberg, Germany).

Quantification of ADAMTS13 in EEA1
Quantification of the amount of ADAMTS13 vesicles co-localizing within early endosomes was performed using Image Pro Plus 6.1 (Media Cybernetics, Breda, the Netherlands). We analyzed in total 8 different cells obtained from 3 different experiments. Using the 3D constructor module, that automatically recognize vesicles, we counted the number of early endosomes, the number of ADAMTS13 containing vesicles and the number of early endosomes containing ADAMTS13. From these data the percentage of ADAMTS13 containing early endosomes was calculated.

Uptake of human FITC-α2-macroglobulin-MA
In uptake experiments, 0.2 x 10^6 iDCs were incubated with 50 nM of FITC-α2-macroglobulin-MA (BioMac, Leipzig, Germany) at 37°C for 45 minutes in serum-free IMDM medium (Lonza, Breda, The Netherlands). In blocking experiments iDCs were first pre-incubated for 20 minutes at 37°C with mannan (1 mg/ml), D-mannose (10 mM), GlcNac (10 mM) or D-galactose (10 mM). Subsequently, FITC-α2-macroglobulin-MA was added to the cells and analyzed by flow cytometry. Blockage of antigen uptake was also monitored after preincubation of the cells with EGTA (5 mM) or with monoclonal antibody clone 15.2 directed against MR. Uptake of FITC-α2-macroglobulin-MA is expressed as percentage of uptake where 100% corresponds to the highest number of FITC-positive cells observed in FACS analysis.
Macrophage mannose receptor promotes uptake of ADAMTS13 by DCs

**Lactadherin staining of iDCs**
In order to exclude that treatment of iDCs with the panel of sugars, EGTA or monoclonal antibody clone 15.2 killed or damaged the cells we stained the iDCs with the phosphatidyl-serine-binding reagent lactadherin-FITC (Haematologic Technologies Inc., Vermont, USA) after treating the cells for 20 minutes at 37°C with mannan (1 mg/ml), D-mannose (10 mM), GlcNac (10 mM), D-galactose (10 mM), EGTA (5 mM), monoclonal antibody clone 15.2 directed against MR (100 μg/ml) or calcium ionophore A23187 (20 μM). Stained cells were analyzed by flow cytometry (LSRII flow cytometer, BD Biosciences). Histograms were processed using Flowjo software (Tree Star Inc., Ashland, OR, USA).

**Treatment of ADAMTS13 with PNGaseF and O-Glycosidase**
Purified rADAMTS13 (50 nM) was incubated with or without PNGaseF (2 U; Roche Diagnostics, Almere, the Netherlands) or O-glycosidase (1 mU; Roche Diagnostics, Almere, the Netherlands) together with neuraminidase (1 mU; Merck, Darmstadt, Germany) overnight at 37°C in order to remove respectively N-linked and O-linked sugar moieties from the protein. Removal of the glycans was assessed by SDS-PAGE on NuPAGE Bis-Tris Gel System 4-12% gel (Invitrogen, Breda, the Netherlands) followed by silver staining.

**Uptake and quantification of PNGaseF and O-glycosidase treated ADAMTS13 by iDCs**
In uptake experiments, 0.4 x 10^6 iDCs were incubated with 50 nM of untreated ADAMTS13, PNGaseF treated or O-glycosidase/neuraminidase treated ADAMTS13 at 37°C for 45 minutes in serum-free IMDM medium. Unbound ADAMTS13 was washed away with PBS. Subsequently cell pellets were resuspended in lysis buffer (150 mM of NaCl, 2.5 mM of CaCl_2, 0.1% Tween-20, 1% Nonidet P40, 2% human serum albumin, 10 mM of benzamidine, 5 mM of N-ethylmaleimide and 50 mM of Tris-HCl pH 7.4) and incubated end-over-end at 4°C for 30 minutes. ADAMTS13 levels in cell lysates were then measured by enzyme-linked immunosorbent assay (ELISA). In brief, a polyclonal antibody directed against ADAMTS13 DO53 (2.5 μg/ml) was immobilized on 96 well microtiter plates (Nunc, Roskilde, Denmark) in 50 mM NaHCO_3 (pH 9.5). Plates were blocked for 1 hour with blocking buffer (0.5% casein and 0.1% Tween-20). Plasma and sample dilutions were prepared in lysis buffer. Plasma ADAMTS13 was used as standard. Bound ADAMTS13 was detected with HRP-labeled monoclonal antibody II-1. Uptake is expressed in percentage relative to the uptake of untreated ADAMTS13.
Surface plasmon resonance analysis of the ADAMTS13 and mannose receptor interaction

Binding of ADAMTS13 to recombinant MR-Fc chimera (CTLD 4-7) was determined by surface plasmon resonance (SPR) analysis on a BIAcore 3000 biosensor (Biacore, AB, Uppsala, Sweden). ADAMTS13 was coupled to a CM5-sensor chip (~2700 RU) using the amino-coupling kit (Biacore, Breda, the Netherlands). Different concentrations of the recombinant MR CTLD 4-7-Fc (10-1500 nM) in binding buffer (20 mM HEPES, 150 mM NaCl, 5 mM CaCl₂, 2 mM MgCl₂, 0.005% Tween 20, pH 7.5) were then passed over the sensor chip at a flow rate of 20 µl/min for 2 minutes at 25°C. After correction for background binding to the chip, the data was analyzed with one-phase exponential association equation with Graphpad Prism 4.03 software (San Diego, CA, USA). The responses at equilibrium (Ymax) of each concentration of MR CTLD 4-7-Fc were then fitted by non-linear regression using a one-site binding hyperbola to calculate the apparent KD values.
Results

Percentage of endocytosed ADAMTS13 in early endosomes.
iDCs were incubated with ADAMTS13-488 for 45 minutes at 37°C, fixed and then stained for the early endosome marker EEA-1. Confocal microscopy revealed that a significant part of endocytosed ADAMTS13-488 was present within early endosomes (Figure 1). In order to quantify the amount of ADAMTS13 vesicles co-localizing with early endosomes we analysed the cells using Image Pro Plus (Supplemental Table I). On average 55 ± 6% ADAMTS13 containing vesicles co-stained for the early endosomal marker EEA-1 (Supplemental Table I). Quantitative analysis revealed that 45 ± 6% of the vesicles containing ADAMTS13 did not correspond to early endosomes. These vesicles most likely correspond to later stages of the endocytic pathway such as late endosomes and lysosomes.

Supplemental Table I: Intracellular distribution of ADAMTS13.
Intracellular distribution of ADAMTS13 within 8 different cells from 3 independent experiments was analyzed by Image Pro Plus 6.1 Total number of early endosomes, ADAMTS13-positive vesicles and ADAMTS13-positive early endosomes was determined. The percentage of ADAMTS13 present in the early endosomes was calculated.

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<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>13</td>
<td>26</td>
<td>ADAMTS13</td>
</tr>
<tr>
<td>43</td>
<td>24</td>
<td>41</td>
<td>EEA1</td>
</tr>
<tr>
<td>19</td>
<td>9</td>
<td>13</td>
<td>Colocalization</td>
</tr>
</tbody>
</table>

In figure 6 we show co-localization of ADAMTS13 with mannose receptor. To investigate whether mannose receptor is also targeted to early endosomes we analyzed the subcellular localization of mannose receptor by confocal microscopy. Cells were fixed and then stained for both the early endosome marker EEA-1 and MR. As shown in supplemental figure 1 endocytosed MR is primarily present within early endosomes. Together, these data shown that 55 ± 6% of ADAMTS13 is contained within early endosomes; co-stainings reveal that MR is also targeted to this compartment which is consistent with the proposed role of MR in endocytosis of ADAMTS13.

ADAMTS13 is specifically endocytosed by immature dendritic cells
To demonstrate the specificity of ADAMTS13-488 uptake by iDCs we used FITC labeled α2-macroglobulin as a control. Alpha-2 macroglobulin interacts with the surface receptor LRP/CD91 which is expressed on immature dendritic
Supplemental Figure 1. Colocalization of mannose receptor and early endosomes in iDCs. We analyzed the subcellular localization of the mannose receptor by confocal microscopy. Cells were fixed, permeabilized and stained with anti-EEA1 and anti-MR antibody, followed by respectively Alexa 488 (green) and 568 (red) labeled secondary antibodies.

We analyzed the uptake of 50 nM of α2-macroglobulin-FITC by iDCs. Uptake was performed at 37°C for 45 minutes and monitored by FACS analysis in the absence or presence of mannan, D-mannose, GlcNAc, D-galactose, EGTA and monoclonal antibody 15.2 directed towards the macrophage mannose receptor. Uptake of α2-macroglobulin-FITC was observed but was not reduced by the blocking reagents mentioned above (Supplemental Figure 2). These findings show that treatment with mannan, D-mannose, GlcNAc, D-galactose, EGTA and monoclonal antibody 15.2 do not affect receptor-mediated endocytosis of α2-macroglobulin by iDCs. In order to further assess if the panel of sugars, EGTA and monoclonal antibody 15.2 could damage or kill the cells we stained the iDCs with phosphatidyl-serine-binding reagent lactadherin-FITC after treating the cells for 20 minutes at 37°C with the blocking reagents. As observed in supplemental figure 3 the cells did not expose phosphatidyl-serine indicating that they do not undergo apoptosis when treated with the above mentioned compounds. In contrast, treatment of iDCs with calcium ionophore A23187, which induces exposure of phosphatidyl-serine on the cell surface, did show lactadherin-FITC staining (Supplemental Figure 3). Taken together these findings provide evidence that the blocking reagents used in the endocytosis experiments do not affect the viability of the cells and that there is a selective effect of these compounds on the mannose receptor mediated uptake of ADAMTS13 by iDCs.

ADAMTS13 N-linked sugars play a role in endocytosis by iDCs
To further analyze the involvement of C-type lectins in the endocytosis of ADAMTS13
Macrophage mannose receptor promotes uptake of ADAMTS13 by DCs

Supplemental Figure 2. Uptake of α2-macroglobulin-FITC by iDCs. Immature DCs were pre-incubated at 37°C for 20 minutes in normal medium or medium containing different monosaccharides, 5 mM EGTA or anti-MR antibody. Then 50 nM of α2-macroglobulin-FITC was added to the cells. Uptake was measured after 45 minutes by FACS and expressed as percentage of uptake relative to the amount of α2-macroglobulin-FITC observed in the absence of inhibitors. Graphs represent data of 3 independent experiments (mean ± SD).

Supplemental Figure 3. Blocking reagents do not affect the viability of iDCs. FACS analysis of lactadherin-FITC stained iDCs was performed. Immature DCs were stained with lactadherin-FITC after treatment at 37°C for 20 minutes with normal medium or medium containing different monosaccharides, 5 mM EGTA or anti-MR antibody. Cells were analyzed by FACS. Gray histogram represents untreated cells. Treatment of iDCs with calcium ionophore A23187 (20 μM) was used as a positive control.

we analyzed whether the N- or O-linked glycans present on ADAMTS13 played a role in uptake by iDCs. ADAMTS13 was treated with PNGaseF or O-glycosidase together with neuraminidase in order to remove respectively N-linked and O-linked sugar moieties from the protein (Supplemental Figure 4A). Uptake of untreated and treated ADAMTS13 was then analyzed and quantified by ELISA. As shown in supplemental figure 4B, PNGase F treatment of ADAMTS13 resulted
in a reduction of uptake of approximately 70% when compared to untreated ADAMTS13. O-glycosidase treatment of ADAMTS13 did not have any effect on ADAMTS13 endocytosis by iDCs. These results indicate that N-linked sugar moieties present on ADAMTS13 are involved in its binding and uptake by iDCs.

**Supplemental Figure 4.** N-linked glycans on ADAMTS13 are important for its endocytosis by iDCs. (A) ADAMTS13 was untreated (-) or treated with PNGaseF or O-glycosidase together with neuraminidase (+) overnight at 37°C. Decrease in the molecular weight of treated ADAMTS13 can be observed by SDS-PAGE (NuPAGE Bis-Tris Gel System 4-12% gel) followed by silver staining. (B) Immature dendritic cells were incubated with untreated (-) or treated (+) ADAMTS13 at 37°C for 45 minutes. ADAMTS13 was quantified in cell lysates by ELISA. Data are expressed as percentage of uptake where 100% corresponds to the amount of unmodified ADAMTS13 internalized by iDCs. Graphs represent data of 4 independent experiments (mean ± SD).

**ADAMTS13 binds to the CTLD 4-7 domains of the mannose receptor**

To further investigate ADAMTS13 interaction with MR we performed binding experiments using SPR analysis. For this, we used recombinant MR-Fc chimera fragment comprising the CTLD 4-7 regions of MR. The CTLD domains of MR are involved in Ca^{2+}-dependent recognition of carbohydrates, while the NH_{2}-terminal CR-domain interacts with sulphated glycans in a Ca^{2+}-independent manner. MR fragment CTLD 4-7-Fc at the concentration range of 50-1500 nM was passed over ADAMTS13 that was previously immobilized to the sensor chip. A dose dependent increase in binding of MR to ADAMTS13 was observed (Supplemental Figure 5A). The affinity of binding between CTLD 4-7-Fc and ADAMTS13 was determined using SPR analysis (Supplemental Figure 5B) and revealed an apparent equilibrium dissociation constant (KD) value of 474 nM.
Supplemental Figure 5. SPR analysis of the interaction between ADAMTS13 and MR CTLD 4-7 domains. (A) SPR analysis of the interaction between ADAMTS13 and MR. ADAMTS13 was first immobilized to a CM5 sensor chip. Then, different concentration (50-1500 nM) of recombinant MR CTLD 4-7-Fc were perfused over immobilized ADAMTS13 at flow rate of 20 µl/min for 2 minutes. Graph represents binding curves using 50, 100, 250, 500, 750, 1000, 1500 nM of MR CTLD 4-7-Fc. (B) Analysis of binding was done fitting the responses at equilibrium (Ymax) of each concentration of MR CTLD 4-7 to a one-site binding hyperbola. Binding curves were used to calculate apparent KD values.

References
