Anti-ADAMTS13 Autoantibodies against Cryptic Epitopes in Immune-Mediated Thrombotic Thrombocytopenic Purpura

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Thromb Haemost 2018;118:1729–1742.

Abstract

Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is characterized by severe ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13) deficiency, the presence of anti-ADAMTS13 autoantibodies and an open ADAMTS13 conformation with a cryptic epitope in the spacer domain exposed. A detailed knowledge of anti-ADAMTS13 autoantibodies will help identifying pathogenic antibodies and elucidating the cause of ADAMTS13 deficiency. We aimed at cloning anti-ADAMTS13 autoantibodies from iTTP patients to study their epitopes and inhibitory characteristics. We sorted anti-ADAMTS13 autoantibody expressing B cells from peripheral blood mononuclear cells of 13 iTTP patients to isolate anti-ADAMTS13 autoantibody sequences. Ninety-six B cell clones producing anti-ADAMTS13 autoantibodies were identified from which 30 immunoglobulin M (IgM) and 5 IgG sequences were obtained. For this study, we only cloned, expressed and purified the five IgG antibodies. In vitro characterization revealed that three of the five cloned IgG antibodies, TTP73–1, ELH2–1 and TR8C11, indeed recognize ADAMTS13. Epitope mapping showed that antibodies TTP73–1 and TR8C11 bind to the cysteine–spacer

Keywords
- thrombotic thrombocytopenic purpura
- ADAMTS13
- anti-ADAMTS13 autoantibodies
- cloning
- cryptic epitopes

received
May 11, 2018
accepted after revision
July 18, 2018

DOI https://doi.org/10.1055/s-0038-1669459.
ISSN 0340-6245.

domains, while the antibody ELH2–1 recognizes the T2–T3 domains in ADAMTS13. None of the antibodies inhibited ADAMTS13 activity. Given the recent findings regarding the open ADAMTS13 conformation during acute iTTP, we studied if the cloned antibodies could recognize cryptic epitopes in ADAMTS13. Interestingly, all three antibodies recognize cryptic epitopes. In conclusion, we cloned three anti-ADAMTS13 autoantibodies from iTTP patients that recognize cryptic epitopes. Hence, these data nicely fit our recent finding that the conformation of ADAMTS13 is open during acute iTTP.

Introduction

A disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13 (ADAMTS13) is an enzyme comprising a metalloprotease domain (M), a disintegrin-like domain (D), 8 thrombospondin type 1 repeats (T1–T8), a cysteine-rich domain (C), a spacer domain (S) and 2 CUB domains.[1] A severe ADAMTS13 deficiency leads to the rare and life-threatening disease, thrombotic thrombocytopenic purpura (TTP).[2,3] In TTP, ultra-large von Willebrand factor (UL-VWF) multimers are not processed due to the absence of a functional ADAMTS13. UL-VWF multimers spontaneously bind platelets and the resulting micro-thrombi obstruct the capillaries in several organs. ADAMTS13 deficiency is in 5% of the cases caused by mutations in the *ADAMTS13* gene. However, the majority of TTP patients (95%) have immune-mediated TTP (iTTP), due to the presence of anti-ADAMTS13 autoantibodies.[2,3]

A detailed insight into the repertoire of anti-ADAMTS13 autoantibodies in iTTP patients is crucial to identify which anti-ADAMTS13 autoantibodies are pathogenic and cause ADAMTS13 deficiency. Such insights might be helpful in the development of specific immune therapeutic approaches. Many groups have used plasma of iTTP patients and cloned anti-ADAMTS13 autoantibodies to study the immune response in iTTP patients. Currently, more than 90 individual anti-ADAMTS13 autoantibodies have been cloned via phage display libraries or Epstein–Barr virus immortalization of peripheral B cells or spleen cells.[4–10]

Epitope mapping studies have shown that the immune response in iTTP patients is polyclonal, as antibodies against all domains of ADAMTS13 have been found.[11,12] Remarkably, almost all iTTP patients have antibodies targeting the spacer domain, showing that the spacer domain harbours a dominant immunogenic region.[13–16] Mainly autoantibodies targeting the spacer domain inhibit ADAMTS13 function, and this by targeting an exosite in the spacer domain known to play a role in VWF binding.[17] Based on these findings, inhibition of ADAMTS13 function has long been thought to be the major cause of ADAMTS13 deficiency in iTTP. However, many iTTP plasma samples do not contain inhibitory anti-ADAMTS13 autoantibodies.[18] In addition, recently it was shown that ADAMTS13 antigen levels are often severely decreased in iTTP patients even in the presence of inhibitory anti-ADAMTS13 autoantibodies. Hence, it was concluded that ADAMTS13 clearance rather than ADAMTS13 inhibition is probably the major pathogenic cause of ADAMTS13 deficiency in iTTP.[18]

A possible explanation for the presence of an immunodominant epitope in the spacer domain evolved from structure-function studies on ADAMTS13 indicating that the spacer and CUB domains interact and by this keep ADAMTS13 in a folded conformation.[19–21] We recently showed that this spacer–CUB interaction is abrogated during acute iTTP, where ADAMTS13 is in an 'open' conformation with a cryptic epitope in the spacer domain exposed.[22] Exposure of cryptic epitopes could either induce an immune response and/or could allow anti-ADAMTS13 autoantibodies recognizing cryptic epitopes to bind. However, it remains unclear whether anti-ADAMTS13 autoantibodies that recognize cryptic epitopes are present in iTTP patients and how they contribute to ADAMTS13 deficiency.

In this article, we isolated, cloned and expressed individual anti-ADAMTS13 autoantibodies from iTTP patients. To further unravel the contribution of individual anti-ADAMTS13 autoantibodies causing ADAMTS13 deficiency or iTTP, we determined the epitope and the inhibitory potential of the cloned antibodies and investigated if they recognize cryptic epitopes.

Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) from whole blood of 13 iTTP patients (►**Table 1**) were isolated using Lymphoprep (Progen Biotechnik GmbH, Heidelberg, Germany). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the committees of all involved institutions (Medical Research Ethics Committee [08/H0716/72], Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Hospital Pitié-Salpêtrière, KU Leuven).

Selection of Anti-ADAMTS13-Specific Single B Cells

Specific anti-ADAMTS13 B cells were selected through fluorescence-activated cell sorting (FACS) as previously described for anti-RhD-specific B cells.[23] Isolated PBMCs from the iTTP patients were labelled with CD19-PerCy7 antibodies, and with fluorescein (F) and phycoerythrin (PE) fluorescently labelled (Bio-Rad, Hercules, California, United States) recombinant human ADAMTS13 (rhADAMTS13) to mark anti-ADAMTS13-specific B cells. Cells positive for CD19-PerCy7, rhADAMTS13-F and rhADAMTS13-PE were selected using a BD FACS Aria II (BD Biosciences, San Jose, California, United States) and seeded

Table 1 Sorting and identifying anti-ADAMTS13 autoantibody producing B cells from iTTP patients

Patient no.	ID	Acute or remission	Immune suppression	Sorts (*n*)	Sorted B cells (*n*)	Anti-ADAMTS13 positive B cells (*n*)	IgG positive B cells (*n*)	IgM positive B cells (*n*)	IgM and IgG positive B cells (*n*)	Viability (%)
1	BJ	Acute	Yes (prednisolone)	1	480	1	1	0	0	ND
2	BM	Remission	No	2	864	9	1	4	4	47–64
3	CJ	Acute	No	1	480	4	0	0	4	ND
4	ELH	Remission	No	2	1113	11	3	0	8	39-ND
5	HA	Acute	No	2	840	14	1	7	6	22–61
6	LC	Acute	Yes (steroids, rituximab)	1	384	2	0	2	0	ND
7	P	Acute	No	1	480	19	12	0	7	ND
8	PI	Acute	Yes (steroids)	1	576	1	0	1	0	6
9	SZ	Acute	Yes (prednisolone, IVIG)	1	192	6	5	0	1	ND
10	TR	Acute	Yes (steroids)	1	960	18	3	11	4	45
11	TTP436	Remission	No	1	768	1	0	1	0	14
12	TTP73	Remission	Yes (azathioprine)	2	904	9	1	5	3	32–48
13	TTP783	Remission	Yes (steroids)	1	480	1	0	1	0	13
	Total			17	8521	96	27	32	37	

Abbreviations: ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13; ELISA, enzyme-linked immunosorbent assay; ID, identity; IgG, immunoglobulin G; IgM, immunoglobulin M; iTTP, immune-mediated thrombotic thrombocytopenic purpura; IVIG, intravenous immunoglobulin; ND, not determined; PBMC, peripheral blood mononuclear cell.

Note: PBMCs were isolated from 13 iTTP patients who were either in acute phase or remission (absence of clinical signs: normal platelet count and lactate dehydrogenase [LDH] levels) and who did or did not receive immune suppressive therapy. PBMCs of the iTTP patients were sorted during 1 or 2 sorts into 96-well plates. From a total of 17 sorts, 8,521 wells containing individual sorted B cells were obtained. Of those wells, 96 wells contained anti-ADAMTS13-specific autoantibodies as determined by an ADAMTS13-specific ELISA on the cell supernatant. An IgM- or IgG-specific ELISA showed that these 96 wells contained either only IgG antibodies, only IgM antibodies or both IgM and IgG antibodies. Presence of both IgM and IgG in one well was due to class switching of IgM to IgG antibodies during the 9-day incubation with the irradiated EL4.B5 cells. The percentage of B cells that still produced IgM or IgG antibodies after the 9-day incubation with the irradiated EL4.B5 cells and hence were still viable, was determined for 11 of the 17 sorted samples by making the ratio of the number of cells producing IgG and/or IgM antibodies over the total number of sorted cells (% viability). When 2 sorts were performed, viability is indicated twice.

at 1 cell/well in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark), which contained 10^5 irradiated (50 Gy) EL4.B5 cells per well in Iscove's Modified Dulbecco's Media (IMDM) medium (Lonza, Basel, Switzerland). The IMDM medium was supplemented with 10% foetal calf serum (Bodinco, Alkmaar, The Netherlands), 1% L-glutamine (Invitrogen), 1% penicillin–streptomycin (Invitrogen), 2.5% T cell supernatant, 20 µg/mL transferrin (Sigma Aldrich, Saint Louis, Missouri, United States) and 0.5 µg/mL R848 (Enzo Life Sciences, Antwerp, Belgium). Cells were incubated for 9 days at 37°C, 5% CO_2 to allow B cells to proliferate and produce antibodies.

Anti-ADAMTS13 Antibodies

The mouse anti-human ADAMTS13 antibodies 3H9,[21,24] 20A5,[21,25] 5C11[21,25] and 1C4[22] are in-house developed murine monoclonal antibodies. Anti-ADAMTS13 antibody 3H9 has an epitope in the metalloprotease domain, inhibits ADAMTS13 activity in vitro and in vivo[21,24,26] and is an antibody that is ideally suited to capture ADAMTS13 from plasma or expression medium in enzyme-linked immunosorbent assay (ELISA).[21,22] Antibody 20A5 targets the T8 domain, increases ADAMTS13 activity and is able to capture ADAMTS13.[21,25–27] Anti-ADAMTS13 antibody 5C11 has an epitope in the T2 domain and is a non-inhibitory anti-ADAMTS13 antibody that is ideally suited to capture ADAMTS13 in ELISA.[21,25,26,28,29] Anti-ADAMTS13 antibody 1C4 recognizes a cryptic epitope in the spacer domain (R568, R660, Y661 and Y665) in ADAMTS13. Only when the conformation of ADAMTS13 is changed by direct coating of ADAMTS13 or by addition of, for example, the activating anti-CUB1 antibody 17G2,[22] the cryptic epitope of antibody 1C4 becomes exposed. Antibody 1C4 is a non-inhibitory antibody.

Human anti-human ADAMTS13 autoantibodies II-1 and I-9 are two autoantibodies isolated from iTTP patients that both target the residues R568, R660, Y661 and Y665 in the spacer domain, while antibody II-1 also targets residue F592.[6,7,14,16] Antibody II-1 is a strong inhibitor while antibody I-9 is a weak inhibitor of ADAMTS13 function in vitro.[6]

Selection of Specific Anti-ADAMTS13 B Cell Clones and Determination of Antibody Isotype

B cell supernatant of the sorted B cells was tested in ELISA to identify B cells producing anti-ADAMTS13 autoantibodies. The murine anti-ADAMTS13 monoclonal antibody 3H9 (5 µg/mL) was coated[21,24] and expression medium containing rhADAMTS13[22] was added. Next, 50 µL of supernatant from the cultured B cells was added. Human anti-ADAMTS13 autoantibodies binding to the captured rhADAMTS13 were detected using biotin-labelled rabbit anti-human immunoglobulin G (IgG) + IgM (1/10,000; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, United States) followed by horseradish peroxidase (HRP)-labelled Pierce High Sensitivity Streptavidin (1/10,000; Invitrogen). Colorimetric development was performed by using o-phenylenediamine and hydrogen peroxide (H_2O_2). The reaction was stopped using 4 M H_2SO_4 and absorbance was measured at 490 nm. Cells from which the supernatant showed specific binding to ADAMTS13 were harvested and 15 µL of Trizol (Thermo Fisher Scientific, Merelbeke, Belgium) was added. In parallel, also the IgG or IgM isotype of the anti-ADAMTS13-specific autoantibodies produced by the B cells was determined using ELISA as described.[30] The viability of the B cells was determined by making the ratio of the number of cells producing IgG or IgM antibodies over the total number of sorted cells that were tested in ELISA.

Determination of the Variable Sequences of Heavy and Light Chains

To isolate and determine the variable sequences of both heavy (VH) and light chains (VL) of the antibodies, sequences were amplified as described previously.[23,31] Messenger ribonucleic acid from the Trizol-treated cells was isolated and was transcribed into complementary deoxyribonucleic acid (cDNA) by a 5′ rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) using a SMARTER RACE cDNA amplification and Advantage 2 polymerase kit (Clontech, Palo Alto, California, United States). To increase the amount and specificity of the amplified cDNA, a nested PCR was performed. Depending on the isotype(s) that was/were determined through ELISA, 3 or 4 PCR reactions were performed per B cell clone, one or two reactions (γ and/or µ) to amplify the VH and always two reactions (κ and λ) to amplify the VL. As forward primers, a mixture present in the SMARTER kit was used, while primers complementary to the constant region of either chain were used as reverse primers. PCR products were sent to GATC (GATC Biotech AG, Constance, Germany) for sequencing. Sequences were searched for homologous germline genes using the ImMunoGeneTics (IMGT) database (http://www.imgt.org).

Cloning, Expression and Purification of the Anti-ADAMTS13 Autoantibodies

The five antibody sequences derived from an IgG antibody were selected for cloning. The variable sequences including the leader sequence of both heavy and light chain were codon optimized using the GeneArt Algorithm (Invitrogen),[32–35] a Kozak sequence (CGCCACC) and restriction cleavage sites (*Hind*III and *Nhe*I or *Xho*I for the heavy and κ light chain, respectively) were added and plasmids were ordered. After restriction digestion, the variable antibody sequences were ligated in plasmids containing the γ1 or κ constant region with an *EcoR*I restriction site at the end. The complete λ light chain (variable and constant region) was ordered as such flanked by *Hind*III and *EcoR*I restriction sites. The complete antibody heavy and light chains were eventually ligated into the pcDNA3.1 (+) expression plasmid by restriction digestion with *Hind*III and *EcoR*I. Next, HEK293 F cells (Invitrogen) were transiently transfected with the plasmids as previously described.[36] Antibodies were purified using Sepharose Protein G 4 Fast Flow column chromatography (GE Healthcare, Waukesha, Wisconsin, United States). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Instant Blue Protein Gel staining (Westburg, Leusden, The Netherlands) was used to evaluate purity.

In Vitro Characterization of Cloned Anti-ADAMTS13 Autoantibodies

Binding of Cloned Autoantibodies to ADAMTS13

To check whether the cloned autoantibodies bind to ADAMTS13, the anti-ADAMTS13-specific ELISA as described above was used with some modifications. After coating a 96-well microtiter plate with the antibody 3H9 (5 µg/mL),[21,24] pure plasma from a normal human plasma pool (NHP) was added in a 1 in 2 dilution series, as a source of ADAMTS13 (1 µg/mL). Next, biotinylated cloned human autoantibodies (5 µg/mL) were added. The anti-ADAMTS13 autoantibodies II-1 and I-9 were also biotinylated and were added as a positive control.[7,14,16] Next, HRP-labelled streptavidin (1/10,000; Sigma Aldrich) was added. Colorimetric development and absorbance measurement was performed as described above. The optical density (OD) value corresponding to the binding of II-1 to captured ADAMTS13 was set as 1 and was used to calculate relative OD values.

Epitope Mapping

The domains to which the cloned anti-ADAMTS13 autoantibodies bind were determined by ELISA by using previously described ADAMTS13 truncation (M, MD, MDT, MDTCS, T5, T6, T7, T8, T2C2) and deletion mutants (delT2, delT3, delT4, delT5, delT8, delCUB1).[21] First, the cloned anti-ADAMTS13 autoantibodies were mapped against MDTCS or T2C2. Here, a 96-well plate was coated with the antibodies 3H9 (anti-M)[21,24] and 5C11 (anti-T2)[21,25] to test TTP73–1 and TR8C11 or 20A5 (anti-T8)[21,25] to test ELH2–1 (5 µg/mL) to which MDTCS or T2C2 (15 nM) was added, respectively, in a 1 in 2 dilution series. Next, the cloned anti-ADAMTS13 autoantibodies were added and bound antibodies were detected using HRP-labelled rabbit anti-human IgG and IgM antibodies. To further refine the epitope of the cloned anti-ADAMTS13 autoantibodies, microtiter plates were coated with the antibody 3H9 (5 µg/mL)[21,24] and expression medium or 15 nM of the different truncation and deletion mutants was added in a 1 in 2 dilution series followed by incubation with the cloned anti-ADAMTS13 autoantibodies (1.5 µg/mL). Bound antibodies were detected using

HRP-labelled rabbit anti-human IgG and IgM antibodies. Colorimetric development and absorbance measurement was performed as described above. Relative OD values were used to express the binding of antibody ELH2–1: binding to wild-type ADAMTS13 was used as a reference and set as a relative OD of 1.

Western Blot

To identify which of the cloned anti-ADAMTS13 autoantibodies recognize a denatured or linear epitope in ADAMTS13, SDS-PAGE and western blot was performed. A 7.5% SDS-PAGE gel was run with 50 nM of rhADAMTS13 under non-reducing and reducing conditions. After transferring the samples to a Roti-polyvinylidene fluoride membrane (Carl Roth GmbH, Karlsruhe, Germany), the membrane was blocked with 3% milk in phosphate-buffered saline. Next, the membrane was incubated with the cloned anti-ADAMTS13 autoantibodies (1.5 µg/mL) and bound antibodies were detected using HRP-labelled rabbit anti-human IgG and IgM antibodies. For visualization, the membrane was incubated for 5 minutes with 2 mL of the Supersignal West Pico Chemiluminescent Substrate kit (Acros Organics, Geel, Belgium) and illuminated in a Fujifilm LAS-4000 device (Fujifilm, Tokyo, Japan). Incubation with only secondary antibody HRP-labelled rabbit anti-human IgG and IgM was used as negative control. As a positive control, the anti-ADAMTS13 autoantibodies II-1 and I-9 were used.[6,7,14,16]

Influence of Cloned Anti-ADAMTS13 Autoantibodies on ADAMTS13 Activity

To test the inhibitory potential of the cloned anti-ADAMTS13 autoantibodies, the FRETS-VWF73 (Peptides International, Louisville, Kentucky, United States) assay was used.[21,37] The cloned anti-ADAMTS13 autoantibodies (10 µg/mL) were incubated with 0.03 µg/mL of plasma ADAMTS13 (from NHP). A standard curve containing 0.005, 0.01, 0.02, 0.025 and 0.03 µg/mL plasma ADAMTS13 was used. The anti-ADAMTS13 autoantibodies II-1 and I-9 were used as controls.[6,7,14,16]

Capturing ADAMTS13 from NHP by Anti-ADAMTS13 Autoantibodies

To investigate whether the cloned anti-ADAMTS13 autoantibodies recognize cryptic epitopes in ADAMTS13, we investigated whether they could capture ADAMTS13 from NHP. First, a 96-well microtiter plate was coated with the cloned human anti-ADAMTS13 autoantibodies (5 µg/mL). The autoantibodies II-1 and I-9 were included as a control.[6,7,14,16] Next, pure plasma from NHP was added in a 1 in 2 dilution series. Bound ADAMTS13 was detected with biotinylated antibody 3H9 (5 µg/mL). Finally, HRP-labelled streptavidin (1/10,000; Sigma Aldrich) was added. Colorimetric development and absorbance measurement was performed as described above. Relative OD values were calculated using the OD value measured for antibody II-1 that was set as 1.

Binding of Cloned Anti-ADAMTS13 Autoantibodies to Coated rhADAMTS13

To investigate whether the cryptic epitopes of the cloned anti-ADAMTS13 autoantibodies can be exposed by coating of ADAMTS13, a 96-well microtiter plate was directly coated with rhADAMTS13 (15 nM). Next, the autoantibodies (100 µg/mL) were added in a 1 in 2 dilution series. Bound antibodies were detected using HRP-labelled rabbit anti-human IgG and IgM antibodies. Colorimetric development and absorbance measurement was performed as described above. The autoantibodies II-1 and I-9 were used as positive controls.[6,7,14,16] The OD value of the binding of antibody II-1 to rhADAMTS13 was used as a reference and set as 1 to calculate relative OD values.

Exposure of Cryptic Epitopes by Capturing ADAMTS13

To be able to explain selection of anti-ADAMTS13 autoantibodies against cryptic epitopes, the conformation of ADAMTS13 had to be changed during the selection of specific anti-ADAMTS13 B cell clones. Therefore, we finally investigated if cryptic epitopes become exposed in ADAMTS13 when captured by murine anti-ADAMTS13 antibody 3H9. Therefore, a 96-well microtiter plate was coated with the murine anti-ADAMTS13 antibody 1C4[22] or 3H9 (5 µg/mL).[21,24] After blocking, pure NHP was added in a 1 in 2 dilution series. Bound ADAMTS13 was detected with the biotinylated antibodies 3H9 or 1C4, respectively, and HRP-labelled streptavidin (1/10,000; Sigma Aldrich). Colorimetric development and absorbance measurement was performed as described above. Relative OD values were calculated by putting the OD value of 1C4 binding to 3H9 captured ADAMTS13 as 1.

Results

Isolation of Anti-ADAMTS13-Specific B Cell Clones from iTTP Patients

We aimed at cloning anti-ADAMTS13 autoantibodies from iTTP patients to further expand the knowledge on epitopes and inhibitory characteristics of anti-ADAMTS13 autoantibodies. This will help to further elucidate how anti-ADAMTS13 autoantibodies cause ADAMTS13 deficiency. To do so, anti-ADAMTS13 autoantibody expressing B cells were sorted from PBMCs of 13 iTTP patients (►**Table 1**) and the VH and VL sequences of the antibodies expressed by the selected B cells were determined. We previously have shown that this technology was successful to isolate both IgG and IgM expressing anti-RhD-specific B cells from peripheral blood of donors hyperimmunized with D^+ erythrocytes.[23,31] The PBMCs of the 13 iTTP patients were used in 1 or 2 sorts, resulting in a total of 17 sorted samples. After sorting, 8,521 B cells were retrieved (►**Table 1**) and were incubated for 9 days in the presence of EL4.B5 cells to induce proliferation of the B cells and production of the antibodies. Cell supernatant of all wells was tested for the presence of anti-ADAMTS13 autoantibodies using an ELISA where rhADAMTS13 was captured on the antibody 3H9. This assay revealed that 96 of the 8,521 wells contained B cells that produced detectable anti-ADAMTS13 autoantibodies (1–19 positive wells per patient; ►**Table 1**). The same supernatant was also tested in ELISA to determine the IgM or IgG isotype. Of the 96 B cell clones producing anti-ADAMTS13 autoantibodies, 27 were of the IgG isotype, 32 of the IgM isotype and 37 of both the IgG and IgM isotype. B cells producing both IgG and IgM antibodies originate from IgM

producing cells that class switched to IgG producing cells during the incubation with the EL4.B5 cells.[31] The high number of isolated IgM expressing anti-ADAMTS13-specific B cells from PBMCs of iTTP patients is in line with previous findings, where the frequency of circulating IgM expressing antigen-specific B cells in peripheral blood was also high.[31,38] The viability of the B cells was tested on 11 of the 17 sorted samples by screening for the presence of IgM or IgG in all sorted wells (and not only in the anti-ADAMTS13 autoantibody positive wells). These data show a great variability in viability of the B cells between the patient samples (►**Table 1**). In some sorted samples, more than 90% of the wells did not contain IgM or IgG antibodies implying that more than 90% of the cells died during incubation or that the cells could not secrete antibodies, while in other samples only 40% of the wells did not contain IgM or IgG antibodies. Hence, the delicate process of isolating, freezing, storing and thawing of the samples greatly influences the viability of the cells.

Antibody Sequence Determination of Isolated B Cell Clones

In a second step, we isolated RNA, performed 5′ RACE PCR and nested PCR to obtain the sequences of the antibody variable regions from the 96 anti-ADAMTS13 autoantibody producing B cell clones. The DNA sequence of the variable regions of both heavy and light chains from 35 antibodies of 6 patients was obtained (►**Table 2**). From the other 61 clones, no DNA sequences for (both) heavy and light chains could be retrieved, likely because not enough good quality RNA could be isolated from the B cells. Thirty of the 35 variable heavy chain antibody sequences were retrieved from IgM producing B cells, while 5 variable heavy chain DNA sequences belonged to an IgG producing B cell clone (►**Table 2**). For the variable fragments of the light chains, 25 DNA sequences encoded for a kappa light chain and 10 DNA sequences encoded a light chain of the lambda sub-type (►**Table 2**). All DNA sequences were analysed using the IMGT database (http://www.imgt.org) to identify their corresponding human germline sequence. Sixteen different immunoglobulin heavy chain variable (IGHV) germline sequences were identified in the antibody sequences from the IgM isotype (►**Table 2**). Remarkably, one-third (10 out of 30) of the IGHV sequences were derived from the same germline sequence, namely, IGHV1–69. This is in line with previous studies in which several different germline sequences were identified, but antibodies encoded by this IGHV1–69 germline sequence were isolated multiple times in the majority of the investigated iTTP patients.[4,5,7,9,16] In the IgM antibody sequences, replacement (R) or silent (S) mutations in the framework or complementarity determining regions of the antibody sequences were rather limited. Indeed, in 17 of the 30 antibody sequences (57%) no single somatic hypermutation (SHM) was found in both the VH and VL sequence (►**Table 2**), while 13 sequences did show SHM (►**Table 2**). All five antibody sequences encoding an IgG antibody (TTP73–1, ELH2–1, TR8C11, BM4H3, ELH5B9) were derived from different germlines (►**Table 2**). Also, here one antibody sequence (TR8C11) was derived from the IGHV1–69 germline. No R or S mutations were found for the antibody sequences TR8C11 and BM4H3 while the antibody sequences TTP73–1, ELH2–1 and ELH5B9 contain up to 6 SHM (►**Table 2**). For this study, we selected the 5 IgG antibody sequences (TTP73–1, ELH2–1, TR8C11, BM4H3, ELH5B9; ►**Table 3**), as anti-ADAMTS13 autoantibodies of the IgG isotype have been found in 80 to 97% of the iTTP patients while only 10% of the patients have detectable IgM anti-ADAMTS13 autoantibodies in their plasma.[39,40]

Cloning and Production of Specific Anti-ADAMTS13 IgG Autoantibodies

To characterize the 5 IgG antibodies, we first cloned and expressed the antibodies. The sequences encoding the VH and VL of the 5 selected anti-ADAMTS13 IgG autoantibodies (►**Table 3**) were first codon optimized and chemically synthesized. Next, the VH and kappa VL sequences were cloned in a plasmid containing the constant IgG_1 or kappa sequence, respectively. Finally, the complete antibody heavy (IgG_1) and light chain sequences (kappa or lambda) were cloned in a pcDNA3.1 (+) expression plasmid. Thereafter, HEK293 F suspension cells were transfected with the expression plasmids encoding TTP73–1, ELH2–1, TR8C11, BM4H3 and ELH5B9 antibodies.[36] Antibodies were purified and SDS-PAGE analysis showed that both heavy and light chains were efficiently expressed for all antibodies, except for antibody ELH5B9 where no antibody chains were detected indicating a secretion deficiency (►**Fig. 1A**). Three of the cloned antibodies TTP73–1, ELH2–1 and TR8C11 did retain their binding activity as they were able to bind to ADAMTS13 captured by 3H9 in ELISA (►**Fig. 1B**), while antibody BM4H3 did not (►**Fig. 1B**). The previously cloned anti-ADAMTS13 autoantibodies II-1 and I-9, were used as positive controls (►**Fig. 1B**).[6,14–16]

In conclusion, we generated three novel anti-ADAMTS13 autoantibodies from iTTP patients.

Epitope Mapping and Inhibitory Capacity of Novel Cloned Anti-ADAMTS13 Autoantibodies

We next determined the epitopes and the inhibitory potential of the anti-ADAMTS13 autoantibodies TTP73–1, ELH2–1 and TR8C11. Initial screening revealed that antibodies TTP73–1 and TR8C11 are directed against the N-terminal part (MDTCS), while ELH2–1 targets the C-terminal part (T2C2) of ADAMTS13 (data not shown). Using a series of ADAMTS13 truncation (M, MD, MDT, MDTCS, T5, T6, T7, T8 and T2C2; ►**Fig. 2A**) and deletion mutants (delT2, delT3, delT4, delT5, delT8 and delCUB1; ►**Fig. 2A**),[21] we further refined the epitope mapping. Antibodies TTP73–1 and TR8C11 only recognized MDTCS and not M, MD or MDT, demonstrating that both antibodies are directed against the cysteine–spacer domains (►**Figs. 2B** and **C**). Antibody ELH2–1 bound to the C-terminal truncation or deletion mutants T5, T6, T7, T8, delT4, delT5, delT8 and delCUB1, while binding to delT2 and delT3 was abrogated, showing that ELH2–1 recognized the T2–T3 domains of ADAMTS13 (►**Fig. 2D**). Western blot revealed that the autoantibodies TTP73–1, ELH2–1 and TR8C11 recognize denatured ADAMTS13 under non-reducing conditions. Although detection by TR8C11 was relatively weak, we could define a light band in contrast to our negative control (►**Fig. 2E**). As expected, the control autoantibodies II-1 and I-9 recognized ADAMTS13 in western blot under non-reducing conditions

Table 2 Overview of 35 anti-ADAMTS13 autoantibody sequences retrieved from the sorted B cells

B cell ID	Heavy chain				FR		CDR1 + CDR2		Length CDR3	Light chain			FR		CDR1 + CDR2		Length CDR3
	Isotype	V germline	J germline	D germline	R	S	R	S		Isotype	V germline	J germline	R	S	R	S	
HA-3	μ	IGHV1–18*01F	IGHJ2*01F	IGHD2–21*02F	4	0	1	0	16	κ	IGKV3–20*01F	IGKJ4*01F	1	1	0	1	9
TR6-B11	μ	IGHV1–2*02F	IGHJ6*02F	IGHD2–2*01F	0	0	0	0	28	κ	IGKV1–12*01F	IGKJ4*01F	0	0	0	0	9
TTP783-G3	μ	IGHV1–2*02F	IGHJ3*02F	IGHD2–15*01F	1	0	0	0	5	κ	IGKV4–1*01F	IGKJ1*01F	0	0	0	0	9
HA3-C5	μ	IGHV1–69*01F	IGHJ4*02F	IGHD5–12*01F	0	0	0	0	11	κ	IGKV3–20*01F	IGKJ4*01F	1	0	0	0	9
TR4-F9	μ	IGHV1–69*01F	IGHJ5*02F	IGHD6–19*01F	0	0	0	0	10	κ	IGKV4–1*01F	IGKJ1*01F	0	0	0	0	9
TR7-F5	μ	IGHV1–69*01F	IGHJ5*02F	IGHD7–27*01F	0	0	0	0	12	λ	IGLV3–25*03F	IGLJ2*01F	0	0	0	0	10
TTP73–5	μ	IGHV1–69*01F	IGHJ5*02F	IGHD3–22*01F	0	0	0	0	13	κ	IGKV2–28*01F	IGKV5*01F	0	0	0	0	9
BM2-D8	μ	IGHV1–69*02F	IGHJ3*02F	IGHD1–26*01F	0	0	0	0	14	κ	IGKV2–28*01F	IGKJ1*01F	0	0	0	0	9
BM5-B12	μ	IGHV1–69*02F	IGHJ4*02F	IGHD3–22*01F	0	0	0	0	12	λ	IGLV3–21*02F	IGLJ1*01F	0	0	0	0	11
BM5-G6	μ	IGHV1–69*02F	IGHJ4*02F	IGHD2–8*01F	0	0	0	0	9	λ	IGLV3–1*01F	IGLJ1*01F	0	0	0	0	9
HA3-B1	μ	IGHV1–69*02F	IGHJ4*02F	IGHD3–22*01F	0	0	0	0	17	κ	IGKV2–28*01F	IGKJ1*01F	0	0	0	0	9
TTP73–3	μ	IGHV1–69*02F	IGHJ6*02F	IGHD3–22*01F	0	1	0	0	11	κ	IGKV2–28*01F	IGKJ5*01F	0	1	0	0	9
TR4-G8	μ	IGHV1–69*06F	IGHJ4*02F	IGHD5–24*01	0	0	1	0	12	λ	IGLV3–25*03F	IGLJ3*02F	1	0	0	0	10
BM3-F5	μ	IGHV2–5*02F	IGHJ4*02F	IGHD2–15*01F	3	2	2	0	12	λ	IGLV1–51*01F	IGLJ3*01F	3	1	2	0	11
TTP73–8	μ	IGHV3–07*01F	IGHJ4*02F	IGHD6–13*01F	0	0	0	0	14	κ	IGKV4–1*01F	IGKJ3*01F	0	0	0	0	8
TTP73–2	μ	IGHV3–20*01F	IGHJ6*02F	IGHD2–2*02F	0	0	0	0	24	κ	IGKV2–28*01F	IGKJ4*01F	0	0	0	0	9
HA3-F9	μ	IGHV3–21*01F	IGHJ4*02F	IGHD3–22*01F	0	0	0	0	14	κ	IGKV4–1*01F	IGKJ1*01F	0	0	0	0	9
TTP73–18	μ	IGHV3–23*01	IGHJ4*03F	IGHD1–26*01F	0	0	0	0	11	λ	IGLV1–51*01F	IGLJ1*01F	5	0	0	0	11
BM2-C5	μ	IGHV3–30	IGHJ5*02F	IGHD3–9*01F	0	0	1	0	18	κ	IGKV1–13*02F	IGKJ4*01F	0	0	0	0	9
TR9-B12	μ	IGHV3–33*02F	IGHJ6*02F	IGHD6–19*01F	3	0	0	0	22	κ	IGKV1–16*02F	IGKJ2–02F	0	0	0	0	9
TR9-F7	μ	IGHV3–53*01F	IGHJ6*03F	IGHD6–13*01F	4	7	6	2	13	κ	IGKV6–21*02F	IGKJ4*01F	2	1	2	0	9
TR6-A7	μ	IGHV4–30–2*01F	IGHJ4*02F	IGHD3–3*01F	0	0	0	0	14	λ	IGLV2–23*01F	IGLJ1*01F	0	0	0	0	10
TR4-A8	μ	IGHV4–31*03F	IGHJ6*02F	IGHD3–16*01F	0	0	0	0	18	κ	IGKV1–39*01F	IGKJ1*01F	0	0	0	0	9
TR9-D7	μ	IGHV4–31*03F	IGHJ6*02F	IGHD3–10*01F	0	0	0	0	17	λ	IGLV3–25*03F	IGLJ3*02F	0	0	0	0	10
TTP73–6	μ	IGHV4–31*03F	IGHJ4*02F	IGHD1–26*01F	0	0	0	0	13	κ	IGKV1–8*01F	IGKJ4*01F	0	0	0	0	9
TR9-B7	μ	IGHV4–39*01F	IGHJ4*02F	IGHD3–22*01F	0	0	0	0	17	λ	IGLV1–51*01F	IGLJ3*02F	0	0	0	0	12
BM5-H1	μ	IGHV4–61*02F	IGHJ6*02F	IGHD6–13*01F	0	0	0	0	19	κ	IGKV3–20*01F	IGKJ5*01F	4	1	1	0	10
HA4-D6	μ	IGHV4–61*04F	IGHJ5*01F	IGHD3–22*01F	9	3	4	1	16	κ	IGKV1–39*01F	IGKJ2*01F	4	3	0	0	8
HA4-E7	μ	IGHV6–1*01F	IGHJ6*02F	IGHD3–22*01F	0	0	0	0	25	κ	IGKV1–39*01F	IGKJ4*01F	0	0	0	0	9
TR5-D8	μ	IGHV6–1*01F	IGHJ6*03F	IGHD6–19*01F	0	0	0	0	20	κ	IGKV3–15*01F	IGKJ4*01F	7	2	2	1	10
TTP73–1	γ	IGHV4–39*01F	IGHJ3*02F	IGHD3–22*01F	0	0	1	0	22	κ	IGKV1–39*01F	IGKJ2*01F	0	0	0	0	11
ELH2–1	γ	IGHV4–31*03F	IGHJ6*02F	IGHD2–15*01F	4	0	2	0	24	κ	IGKV3–11*01F	IGKJ3*01F	0	1	0	0	7
TR8C11	γ	IGHV1–69*01F	IGHJ4*02F	IGHD6–13*01F	0	0	0	0	11	λ	IGLV3–21*02F	IGLJ2*01F	0	0	0	0	11
BM4H3	γ	IGHV4–59*08F	IGHJ4*02F	IGHD3–22*01F	0	0	0	0	22	κ	IGKV4–1*01F	IGKJ-1*01F	0	0	0	0	10
ELH5B9	γ	IGHV1–18*01F	IGHJ4*02F	Not identified	0	0	0	0	22	κ	IGKV1–5*03F	IGKJ1*01F	2	0	1	1	9

Abbreviations: ADAMTS13, a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13; ID, identity; iTTP, immune-mediated thrombotic thrombocytopenic purpura.

Note: Isotype (γ or μ for heavy and λ or κ for light chain), V, D and J germline sequence, replacement (R) or silent (S) mutations in the framework (FR) regions or complementarity determining regions (CDR) 1 and 2, and the length of the CDR3 are given for both heavy and light chain sequences of the 35 anti-ADAMTS13 autoantibodies isolated from B cells of iTTP patients.

Table 3 Alignment of the 5 antibody variable sequences (TTP73–1, ELH2–1, TR8C11, BM4H3 and ELH5B9) of the IgG isotype with their corresponding germline sequence

Heavy Chain								
	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
IGHV1-18*01	QVQLVQSGA EVKKP GASVKVSCKAS	GYTF TSYG	ISWVRQAP GQGLEWMGW	ISAY NGNT	NYAQKLQ G RVTMTTDTST STAYMELRSLRS DDTAVYYC		
ELH5-B9	--------- ----- ----------	---- ----	-------- ---------	---- ----	------- - ---------- ---------- --------	ARERPASPYYDFWSGYYLLNDY	CGQGTLVTVSS
IGHV1-69*01	QVQLVQSGA EVKKP GSSVKVSCKAS	GGTF SSYA	ISWVRQAP GQGLEWMGG	IIPI FGTA	NYAQKFQ G RVTITADEST STAYMELSSLRS EDTAVYYC		
TR8-C11	--------- ----- ----------	---- ----	-------- ---------	---- ----	------- - ---------- ---------- --------	ARVYSSSWLDY	WGQGTLVTVSS
IGHV4-31*03	QVQLQESGP GLVKP SQTLSLTCTVS	GGSIS SGGYY	WSWIRQHP GKGLEWIGY	IYYS GST	YYNPSLK S RVTISVDTSK NQFSLKLSSVTA ADTAVYYC		
ELH2-1	--------- ----- --------A--	-D--- -----	-N------ -----S---	---- -N-	------- - -----L---- ---------- --------	ARSNSPTKYCSGGTCVYYYYGMDV	WGQGTTVTVSS
IGHV4-39*01	QLQLQESGP GLVKP SETLSLTCTVS	GGSIS SSSYY	WGWIRQPP GKGLEWIGS	IYYS GST	YYNPSLK S RVTISVDTSK NQFSLKLSSVTA ADTAVYYC		
TTP73-1	--------- ----- ----------	----- -----	-------- ---------	L--- ---	------- - ---------- ---------- --------	ARHSLIYYYDSSGYYPNDAFDI	WGQGTMVTVSS
IGHV4-59*08	QVQLQESGP GLVKP SETLSLTCTVS	GGSI SSYY	WSWIRQPP GKGLEWIGY	IYYS GST	NYNPSLK S RVTISVDTSK NQFSLKLSSVTA ADTAVYYC		
BM4-H3	--------- ----- ----------	---- ----	-------- ---------	---- ---	------- - ---------- ---------- --------	ARHKGYYYDSSGYYYG LGYFDY	WGQGTLVTVSS
Light chain							
	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
IGKV1-39*01F	DIQMTQSPSSLSASV GDRVTITCRAS	QSI SSY	LNWYQQKP GKAPKLLIY	AA S	SLQSGVP S RFSGSG SG TDFTLTISSLQP EDFATYYC		
TTP73-1	--------------- ----------	--- ---	-------- ---------	-- -	------- - ------ -- ---------- --------	QQSYSTRYPYT	FGQGTKLEI
IGKV1-5-03F	DIQMTQSPSTLSASV GDRVTITCRAS	QSI SSW	LAWYQQKP GKAPKLLIY	KA S	SLESGVP S RFSGSG SG TEFTLTISSLQP DDFATYYC		
ELH5-B9	--------------- ----------	--- -G-	-------- ---------	-* -	TI----- - ------ -- ---------- --------	QQYNSYSLT	FGQGTKXEIK
IGKV3-11*01F	EIVLTQSPATLSLSP GERATLSCRAS	QSV SSY	LAWYQQKP GQAPRLLIY	DA S	NRATGIP A RFSGSG SG TDFTLTISSLEP EDFAVYYC		
ELH2-1	--------------- ----------	--- ---	---*---- ---------	-- -	------- - ------ -- ---------- --------	QQRRGFT	FGPGTKVDI
IGKV4-1*01F	DIVMTQSPDSLAVSL GERATINCKSS	QSVLYSSNNKNY	LAWYQQKP GQPPKLLIY	WA S	TRESGVP D RFSGSG SG TDFTLTISSLQA EDVAVYYC		
BM4-H3	--------------- ----------	----------	-------- ---------	-- -	------- - ------ -- ---------- --------	QQYYSTPPWT	FGQGTKXEI
IGLV3-21*02F	SYVLTQPPS VSVAP GQTARITCGGN	NIG SKS	VHWYQQKP GQAPVLVVY	DD S	DRPSGIP E RFSGSN SG NTATLTISRVEA GDEADYYC		
TR8-C11	--------- ----- ----------	--- ---	-------- ---------	-- -	------- - ------ -- ---------- --------	QVWDSSSDHVV	FGGGTKLTV

Abbreviation: IgG, immunoglobulin G.
Note: Replacement mutations are indicated with letters, silent mutations are indicated with * and identical codon usage is represented with –.

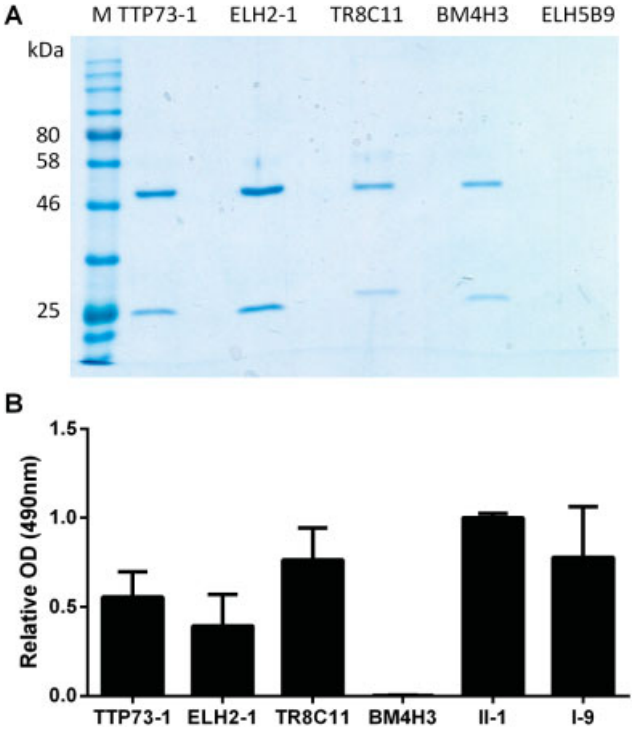

Fig. 1 The cloned antibodies TTP73–1, ELH2–1 and TR8C11 bind to ADAMTS13. (**A**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of the newly cloned antibodies TTP73–1, ELH2–1, TR8C11, BM4H3 and ELH5B9. (**B**) Binding of biotinylated TTP73–1, ELH2–1, TR8C11, BM4H3, II-1 and I-9 to ADAMTS13 captured from normal human plasma pool (NHP) by 3H9 (5 µg/mL) coated on a 96-well microtiter plate. Detection of the cloned anti-ADAMTS13 autoantibodies was done with horseradish peroxidase (HRP)-labelled streptavidin. The optical density (OD) value corresponding to the binding of antibody II-1 was set as 1 to calculate relative OD values.

(►**Fig. 2E**). However, TTP73–1, ELH2–1 and TR8C11 did not detect ADAMTS13 under reducing conditions (data not shown).

The effect of the autoantibodies TTP73–1, ELH2–1 and TR8C11 on ADAMTS13 activity was tested in the FRETS-VWF73 assay. Autoantibodies TTP73–1, ELH2–1 and TR8C11 did not inhibit ADAMTS13 activity (►**Fig. 2F**). As expected, also autoantibody I-9 could not inhibit ADAMTS13 activity at the concentration used (►**Fig. 2F**), while autoantibody II-1 had an inhibitory effect of approximately 70% (►**Fig. 2F**).[6]

To conclude, we expanded the panel of cloned anti-ADAMTS13 autoantibodies[4–10] by generating two novel anti-cysteine–spacer antibodies that are non-inhibitory and an anti-T2–T3 antibody that is also non-inhibitory in the fluorescence resonance energy transfer (FRETS) assay.

Anti-ADAMTS13 Autoantibodies Recognize Cryptic Epitopes in ADAMTS13

The spacer domain of ADAMTS13 is known to be partly shielded by the CUB domains through direct interaction of the spacer and the CUB domains.[19–21] Abolishment of this interaction exposes cryptic epitopes in the so-called 'open' conformation of ADAMTS13, which we recently showed to be specifically present during the acute phase of iTTP patients.[22] Hence, we next wanted to analyse whether our three newly cloned autoantibodies could potentially recognize cryptic epitopes of ADAMTS13. To investigate if autoantibodies TTP73–1, ELH2–1 and TR8C11 recognize cryptic epitopes, the capacity of the autoantibodies to capture ADAMTS13 or to bind to coated ADAMTS13 was compared in ELISA (►**Fig. 3A**). Antibodies that recognize cryptic epitopes will not be able to capture 'closed' ADAMTS13 from solution (►**Fig. 3A**, left), while those antibodies will bind to coated ADAMTS13 (►**Fig. 3A**, right), because coating of ADAMTS13 changes its conformation.[22,41] Interestingly, none of the newly cloned antibodies (TTP73–1, ELH2–1 and TR8C11) were able to capture ADAMTS13 (►**Fig. 3B**), while they did bind to coated ADAMTS13 (►**Fig. 3C**), indicating that they indeed recognize cryptic epitopes in ADAMTS13. In contrast, the control autoantibody II-1 did both capture ADAMTS13 and bind to coated ADAMTS13 (►**Figs. 3B** and **C**). Intriguingly, control autoantibody I-9 could also not capture ADAMTS13 (►**Fig. 3B**) but could bind to coated ADAMTS13 (►**Fig. 3C**), showing for the first time, that the known antibody I-9 also recognizes a cryptic epitope in the spacer domain.

In conclusion, we specifically checked for the first time if cloned anti-ADAMTS13 autoantibodies can recognize cryptic epitopes in ADAMTS13 and showed that iTTP patients can indeed have autoantibodies recognizing cryptic epitopes in the cysteine–spacer and T2–T3 domains.

Murine Anti-ADAMTS13 Antibody 3H9 Induces a Conformational Change in ADAMTS13

Initially, we did not expect to identify anti-ADAMTS13 autoantibodies recognizing cryptic epitopes as the anti-ADAMTS13 autoantibodies were selected on ADAMTS13 captured by our murine anti-human ADAMTS13 antibody 3H9. Capturing ADAMTS13 from solution was anticipated to exhibit ADAMTS13 in a folded conformation. However, since the autoantibodies TTP73–1, ELH2–1 and TR8C11 recognize cryptic epitopes in ADAMTS13, this implies that capturing ADAMTS13 by 3H9 induces a conformational change in ADAMTS13 thereby exposing cryptic epitopes. To prove this, we used our murine anti-ADAMTS13 antibody 1C4, which was previously found to recognize a cryptic epitope in the spacer domain of ADAMTS13.[22] Indeed, ADAMTS13 in solution could not be captured by antibody 1C4 (►**Fig. 3D**). However, when ADAMTS13 was captured by antibody 3H9, antibody 1C4 did bind to ADAMTS13 (►**Fig. 3D**), proving that capturing ADAMTS13 on 3H9 indeed induces a conformational change in which cryptic epitopes become exposed. Hence, our anti-ADAMTS13 autoantibodies were selected on open ADAMTS13. Our results also suggest that labelling of the ADAMTS13 used for B cell sorting must have altered the conformation of ADAMTS13.

To conclude, selecting human anti-human ADAMTS13 antibodies that recognize cryptic epitopes in ADAMTS13 can be explained through the capturing of ADAMTS13 by murine anti-ADAMTS13 antibody 3H9 that apparently induces an open conformation in ADAMTS13.

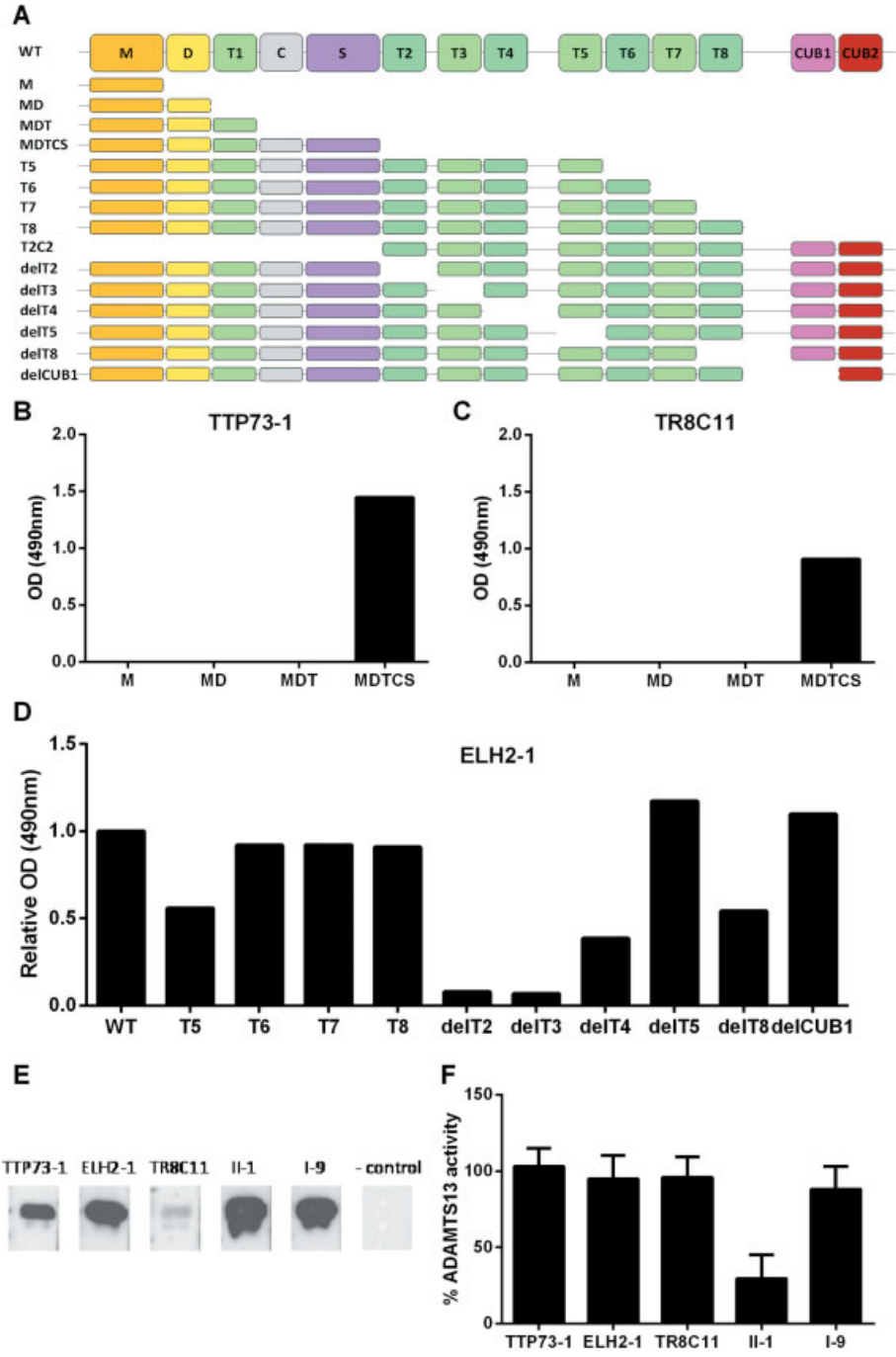

Fig. 2 The cloned anti-ADAMTS13 autoantibodies TTP73–1 and TR8C11 recognize the ADAMTS13 cysteine–spacer domains while ELH2–1 is directed against the T2–T3 domains and none of the autoantibodies inhibit ADAMTS13 activity. (**A**) Structure of wild-type (WT) ADAMTS13 and its truncation (M, MD, MDT, MDTCS, T5, T6, T7, T8 and T2C2) and deletion variants (delT2, delT3, delT4, delT5, delT8 and delCUB1) that were used for the epitope mapping of the cloned anti-ADAMTS13 autoantibodies TTP73–1, TR8C11 and ELH2–1. The different domains in ADAMTS13 are: a metalloprotease domain (M), a disintegrin-like domain (D), a thrombospondin type 1 repeat (T1), a cysteine-rich domain (C), a spacer domain (S), another 7 T domains and 2 CUB domains. In the ADAMTS13 variants one or more domains have been deleted. (**B–D**) Epitope mapping of anti-ADAMTS13 autoantibodies TTP73–1 (**B**), TR8C11 (**C**) and ELH2–1 (**D**). The ADAMTS13 variants were captured by antibody 3H9 (5 μg/mL) on 96-well microtiter plates and the cloned human anti-ADAMTS13 autoantibodies were added. Bound antibodies were detected using horseradish peroxidase (HRP)-labelled rabbit anti-human immunoglobulin G (IgG) and IgM antibodies. For antibody ELH2–1 (**D**), relative optical density (OD) values were calculated by putting the OD value of the binding of ELH2–1 to WT ADAMTS13 as 1. (**E**) Western blot was performed to test whether the anti-ADAMTS13 autoantibodies recognize denatured ADAMTS13. A 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was run with 50 nM of rhADAMTS13 under non-reducing conditions and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking, the cloned antibodies TTP73–1, ELH2–1 and TR8C11 were added (1.5 μg/mL) and bound antibodies were detected using HRP-labelled rabbit anti-human IgG and IgM antibodies. The autoantibodies II-1 and I-9 were used as a positive control. Detection with only secondary antibody (– 'control') was used as negative control. (**F**) The inhibitory capacity of the cloned anti-ADAMTS13 autoantibodies TTP73–1, ELH2–1 and TR8C11 was tested in the FRETS-VWF73 assay. Antibodies II-1 and I-9 were included as controls. The autoantibodies (10 μg/mL) were incubated with 0.03 μg/mL of plasma ADAMTS13. Activity of ADAMTS13 in normal human plasma pool (NHP) was set as 100%.

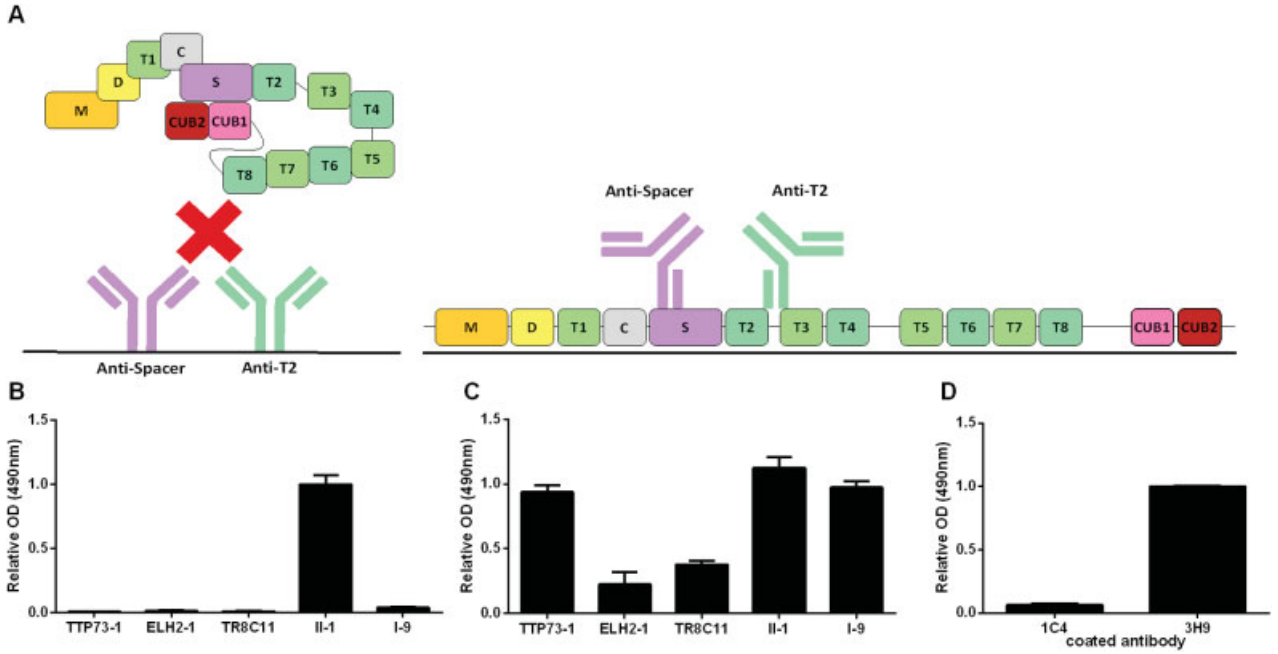

Fig. 3 Anti-ADAMTS13 autoantibodies recognize cryptic epitopes in ADAMTS13. (**A**) Antibodies that recognize cryptic epitopes in ADAMTS13 cannot capture 'closed' ADAMTS13 from solution (left). Coating of ADAMTS13 induces conformational changes where cryptic epitopes become exposed. Antibodies recognizing those cryptic epitopes will now be able to bind (right). (**B**) A 96-well microtiter plate was coated with autoantibodies (TTP73–1, ELH2–1 and TR8C11) and control antibodies II-1 and I-9 (5 µg/mL). Next, human plasma was added and bound ADAMTS13 was detected with biotinylated 3H9 and streptavidin-horseradish peroxidase (HRP). The optical density (OD) value corresponding to ADAMTS13 captured by II-1 was set as 1 and used to calculate the relative OD values. (**C**) A 96-well microtiter plate was coated with rhADAMTS13 (15 nM). Autoantibodies TTP73–1, ELH2–1 and TR8C11 or controls II-1 and I-9 were added (6.25 µg/mL) and bound antibodies were detected with HRP-labelled rabbit anti-human immunoglobulin G (IgG) and IgM antibodies. Relative OD values were calculated by putting the OD value represented by binding of II-1 to coated rhADAMTS13 at 1. (**D**) A 96-well microtiter plate was coated with antibody 1C4 or 3H9 (5 µg/mL). Plasma ADAMTS13 was added and bound ADAMTS13 was detected, using biotinylated 3H9 or 1C4, respectively, followed by addition of streptavidin-HRP. The OD value corresponding to the binding of 1C4 to ADAMTS13 captured by 3H9 was set at 1 and used to calculate relative OD values. The murine anti-ADAMTS13 antibody 1C4 recognizes a cryptic epitope in plasma ADAMTS13. This epitope becomes exposed when ADAMTS13 is captured by 3H9.

Discussion

We recently showed that ADAMTS13 in acute iTTP adopts an open conformation resulting in exposure of a cryptic epitope in the spacer domain.[22] In this study, we now cloned three anti-ADAMTS13 autoantibodies, TTP73–1, ELH2–1 and TR8C11, from PBMCs of iTTP patients through B cell sorting and showed that all three antibodies recognize cryptic epitopes, either in the cysteine–spacer domains (TTP73–1 and TR8C11) or in the T2–T3 domains (ELH2–1).

We isolated 96 B cell clones from PBMCs of 13 iTTP patients of which 27 produced anti-ADAMTS13 IgG antibodies and 69 IgM antibodies (32 produced anti-ADAMTS13 IgM antibodies and 37 produced IgM antibodies that class switched to IgG antibodies during the incubation period). Hence, 72% of the viable anti-ADAMTS13 B-cells were of the IgM isotype, although IgM's are only found in approximately 10% of iTTP patients.[39,40] Sequence determination of 30 IgM antibodies revealed that they were generated from 16 different IGHV germline sequences and that 57% of the complete antibody sequences contained no SHM (derived from naïve IgM clones), while 43% did show SHM (generated after antigen encounter). Our data are in line with those described in our previous study, where anti-RhD antibodies were isolated from donors hyper-immunized with D^+ erythrocytes using the same B cell sorting technology. Despite the almost complete absence of IgM anti-RhD antibodies in these donors, a high frequency (77%) of IgM expressing anti-RhD-specific B cells was found.[23,31] These IgM-producing clones were either naïve (no SHM) or were hypermutated by antigen encounter.[23] These results supported data generated in mice suggesting that a large pool of long-lasting IgM memory cells circulate in the peripheral blood and that these cells continuously replenish the IgG memory pool after repeated re-encounter of the antigen.[31,42] As the role of IgM presenting B cells and therefore also IgM antibodies could be larger than initially thought, also cloning of IgM antibodies could be useful in the future to better understand their role in iTTP and in (auto)immunity.

The remaining 27 anti-ADAMTS13 positive B cells produced IgG antibodies. The complete sequence of 5 of these IgG antibodies could be determined and revealed that they were generated from 5 different germlines. The antibody sequences of anti-cysteine–spacer autoantibodies TR8C11 and TTP73–1 were derived from the germline sequence VH1–69 and VH4–39, respectively. Although the VH1–69 germline sequence was shown to be dominant in anti-spacer autoantibodies,[6] our and other studies show that anti-spacer antibody germline sequences are not restricted to the VH1–69 sequences.[4,5,7,9,14]

The SHM rate in the ADAMTS13-specific antibodies TR8C11, TTP73–1 and ELH2–1 was low or absent suggesting that additional antigen encounter might increase affinity for ADAMTS13. Indeed, it has been shown that affinity maturation of antibodies does occur during the disease course.[43] Selecting two antibodies (BM4H3 and ELH5B9) that were in the end not specific for ADAMTS13 or could not be expressed is also in line with our previous study[23] and stresses the importance of cloning and expressing antibodies selected from B cells before labelling them as antigen specific.

We previously showed that ADAMTS13 has an altered (or open) conformation, where a cryptic epitope in the spacer domain becomes exposed when patients are suffering from acute iTTP.[22] In this study, we isolated two anti-ADAMTS13 autoantibodies that recognize cryptic epitopes in the cysteine–spacer domains and one antibody that recognizes a cryptic epitope in the T2–T3 domains. Interestingly, Underwood et al recently presented a study at the International Society on Thrombosis and Haemostasis in Berlin where patient samples were screened for anti-ADAMTS13 autoantibodies against open and closed ADAMTS13. They showed that more autoantibodies could bind to open ADAMTS13 compared with closed ADAMTS13.[44] These data are in line with our study and show that iTTP patients indeed have anti-ADAMTS13 autoantibodies recognizing cryptic epitopes. How the open ADAMTS13 conformation and the circulating autoantibodies against cryptic epitopes contribute to ADAMTS13 deficiency remains to be determined. It is tempting to speculate that both the open ADAMTS13 conformation as well as the antibodies against cryptic epitopes contribute to ADAMTS13 clearance, an important determinant in the pathophysiology of iTTP[18,45] by a multistep process. During acute iTTP, a—so far unknown—trigger opens ADAMTS13, cryptic epitopes become exposed and could induce autoantibody development recognizing these cryptic epitopes which will subsequently bind. Binding of these autoantibodies together with the (already) bound autoantibodies recognizing non-cryptic epitopes might lead to large immune complexes resulting in ADAMTS13 clearance. This might explain why some iTTP patients in remission still have anti-ADAMTS13 autoantibodies but no ADAMTS13 clearance. Hence, specifically targeting autoantibodies recognizing cryptic epitopes might be an effective immune therapy. Alternatively, targeting the factors that open ADAMTS13 might be another valuable strategy.

In this study, we also used the previously cloned anti-ADAMTS13 spacer domain autoantibodies I-9 and II-1 as control antibodies. We now demonstrated that also I-9 recognizes a cryptic epitope in the spacer domain as I-9 could not capture ADAMTS13 from solution but did bind to coated ADAMTS13 in ELISA. These data are in line with our previous findings that I-9 recognizes the same epitope as the murine monoclonal antibody 1C4, which we previously identified to recognize a cryptic epitope in the spacer domain of ADAMTS13.[14,22] Although antibody II-1 has an overlapping epitope with I-9 and 1C4, II-1 can capture 'closed' ADAMTS13 from solution in our ELISA. This is in line with the data of South et al[20] who could immunoprecipitate ADAMTS13 from solution by antibody II-1. However, inducing an open ADAMTS13 conformation increased the binding of II-1,[20] showing that epitope of II-1 is partially cryptic.

In conclusion, we were able to clone three novel specific anti-ADAMTS13 autoantibodies (TTP73–1, ELH2–1 and TR8C11) by sorting B cells from iTTP patients. In vitro characterization revealed that these anti-ADAMTS13 autoantibodies target cryptic epitopes in ADAMTS13. Whether these autoantibodies develop after exposure of the cryptic epitopes and if and how they contribute to antigen clearance, remain to be determined.

What is known about this topic?

- Immune-mediated thrombotic thrombocytopenic purpura (iTTP) patients are characterized by an absent ADAMTS13 activity, presence of polyclonal anti-ADAMTS13 autoantibodies and an open ADAMTS13 conformation with a cryptic epitope in the spacer domain exposed during an acute episode.
- Cloning of anti-ADAMTS13 autoantibodies helps to gain insight into how the anti-ADAMTS13 autoantibodies induce ADAMTS13 deficiency.

What does this paper add?

- Anti-ADAMTS13 autoantibodies can be isolated and cloned through B cell sorting.
- iTTP patients have anti-ADAMTS13 autoantibodies that recognize cryptic epitopes in the spacer and T2T3 domains of ADAMTS13.
- Capturing ADAMTS13 by antibodies can induce conformational changes in ADAMTS13.

Authors' Contributions

E.R., G.V., O.J.H.M.V., F.P. and K.V. designed the experiments. E.R., K.K., O.J.H.M.V., L.D., I.P., N.V. and A.V. performed the experiments. I.M., C.V., M.A.A., L.G., M.S., D.D. and F.P. provided clinical information and PBMCs from iTTP patients. A.S.S. developed the antibody 1C4 and J.V. provided the antibodies II-1 and I-9. E.R., G.V., K.K., O.J.H.M.V., I.M., H.D. and S.F.D.M. and K.V. interpreted the data. E.R. and K.V. wrote the manuscript, which was critically reviewed by G.V., K.K., O.J.H.M.V., I.M., A.S.S., J.V., M.A.A., L.G., M.S., D.D., H.D., S.F.D.M. and F.P. All authors approved the final version of the manuscript. K.V. provided funding.

Funding

This work was supported by the European Framework Program for Research and Innovation (Horizon 2020 Marie Sklodowska Curie Innovative training network PROFILE grant 675746) and KU Leuven grants OT/14/071 and PF/10/014 awarded to K.V. A.S.S. is supported by a PhD grant from the Agency Innovation And Entrepreneurship (VLAIO, www.iwt.be), Flanders, Belgium (141136). Academische Stichting Leuven is acknowledged for providing a travel grant to E.R. to present this study at the ISTH 2017, Berlin, Germany.

Conflict of Interest
F.P. is a member of the scientific advisory boards of Ablynx and Shire.

Acknowledgements
The SV40 Large T coding region DNA sample is a gift from Genmab B.V. and has been generated based on the information provided in the publication entitled 'A simple, robust and highly efficient transient expression system for producing antibodies'[36] in which the material was first described. The authors would like to thank Remco Vissers, Magdalena A. Berkowska and Janne Caron for their technical assistance. The authors also acknowledge Erik de Graaf and Simon Tol for their assistance during the B cell sorting. M, MD, MDT and MDTCS mutants were a kind gift of Dr. Kenji Soejima (The Chemo-Sero-Therapeutic Research Institute, Kikuchi, Kumamoto, Japan).

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