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The ADAMTS13¹²³⁹⁻¹²⁵³ peptide is a dominant HLA-DR1-restricted CD4⁺ T-cell epitope

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ABSTRACT

Acquired thrombotic thrombocytopenic purpura is a rare and severe disease characterized by auto-antibodies directed against "A Disintegrin And Metalloproteinase with Thrombospondin type 1 repeats, 13th member" (ADAMTS13), a plasma protein involved in hemostasis. The implication of CD4⁺ T cells in the pathogenesis of the disease is suggested by the IgG isotype of the antibodies. However, the nature of the CD4⁺ T-cell epitopes remains poorly characterized. Here, we determined the HLA-DR-restricted CD4⁺ T-cell epitopes of ADAMTS13. Candidate T-cell epitopes were predicted *in silico* and binding affinities was confirmed in competitive ELISA. ADAMTS13-reactive CD4⁺ T-cell hybridomas were generated following immunization of HLA-DR1 transgenic mice (Sure-L1 strain) and used to screen the candidate epitopes. We identified the ADAMTS13¹²³⁹⁻¹²⁵³ peptide as the single immunodominant HLA-DR1-restricted CD4⁺ T-cell epitope. The peptide is located in the CUB2 domain of ADAMTS13. It was processed by dendritic cells, stimulated CD4⁺ T cells from Sure-L1 mice and was recognized by CD4⁺ T cells from a HLA-DR1-positive patient with acute thrombotic thrombocytopenic purpura. Interestingly, the ADAMTS13¹²³⁹⁻¹²⁵³ peptide demonstrated promiscuity towards HLA-DR11 and HLA-DR15. Our work paves the way towards the characterization of the ADAMTS13-specific CD4⁺ T-cell response in patients with thrombotic thrombocytopenic purpura using ADAMTS13¹²³⁹⁻¹²⁵³-loaded HLA-DR tetramers.

Keywords: ADAMTS13, TTP, T-cell epitope, HLA-DR1, T-cell hybridoma

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a rare and severe autoimmune disease characterized by the occurrence of IgG autoantibodies against the metalloprotease "A Disintegrin And Metalloproteinase with Thrombospondin type 1 repeats, 13th member" (ADAMTS13).¹⁻³ ADAMTS13 cleaves multimers of von Willebrand factor (VWF), a glycoprotein involved in hemostasis. Inhibition of ADAMTS13 by IgG leads to an accumulation of hyper-adhesive VWF multimers causing microthrombi that occlude the lumen of the capillaries in the microcirculation, thus inducing red cell hemolysis and down-stream organ ischemia. TTP is thus characterized by a combination of microangiopathic hemolytic anemia, peripheral thrombocytopenia and organ failure of variable severity with typically neurological involvement.⁴

The physiopathological mechanisms underlying TTP and responsible for the synthesis of anti-ADAMTS13 antibodies, and particularly the mechanisms involved in the loss of tolerance of the immune system towards ADAMTS13, are poorly understood. Polyclonal anti-ADAMTS13 antibodies are directed against different domains of ADAMTS13.⁵ In most patients, anti-ADAMTS13 antibodies are of the IgG isotype with a predominance of the IgG₄ subclass.⁶ IgG from all patients recognize immunodominant B-cell epitopes located in the spacer domain of ADAMTS13.⁷ The B-cell epitopes have been proposed to be located between the 660-661 and 665 amino-acids.⁸ The fact that anti-ADAMTS13 antibodies are of the IgG isotype, of high affinity and have undergone affinity maturation, strongly suggests the requirement of a CD4⁺ T-cell help in the development of the disease.⁹ Besides, the HLA-DRB1*11 (DR11) haplotype was independently identified as a strong risk factor by three

research groups.^{10–12} However, while CD4⁺ T cells are thought to play a major role, the specificity and the properties of the CD4⁺ T lymphocytes involved in the pathogenesis of TTP have not been studied. Importantly, the HLA restriction hints at the existence of immuno-dominant peptides in ADAMTS13.

Naive CD4⁺ T-cell activation is initiated by the interaction of the T-cell receptor (TCR) with a peptide/MHC class II complex on professional antigen-presenting cells (APC). Extracellular antigens are endocytosed, degraded into peptides in the early endosome and loaded onto MHC class II $\alpha\beta$ heterodimer molecules. Sorvillo *et al.* identified, through mass spectrometry, ADAMTS13-derived peptides that are localized within the HLA-DR molecules expressed by dendritic cells derived from human monocytes (Mo-DCs) and incubated with ADAMTS13 *in vitro*.¹³ The identified peptides belonged mainly to the CUB1 and CUB2 domains of ADAMTS13. The core sequence FINVAPHAR from the CUB2 domain was the only sequence detected for 6 donors with the DR11 haplotype among the 17 donors included in the study. In addition, the same group recently demonstrated that a peptide containing the FINVAPHAR amino-acid sequence is able to stimulate CD4⁺ T cells from a HLA-DR11 TTP patient.¹⁴ These studies did however not investigate the recognition of other peptides by CD4⁺ T cells. To address this question, we focused on the HLA-DR1 haplotype for which a humanized transgenic HLA-DR1 mouse is available, and investigated the CD4⁺ T-cell epitopes for the HLA-DR1 allele. The identified epitopes were then studied in the context of the HLA-DR11 allele.

METHODS

Peptides and antigen

ADAMTS13-derived peptides were selected based on their binding capacity to HLA-DRB1*01:01 molecules. HLA class II-binding predictions of human ADAMTS13 (GenBank reference: AAL11095.1) were made on November 10, 2011, using the Immune Epitope Database analysis resource consensus tool, on the dedicated website www.iedb.org.^{15–17} Strong HLA-DRB1*01:01 binders were predicted *in silico* using overlapping 15-mer peptides that span the whole ADAMTS13 sequence. Altogether, 99 15-mer peptides were predicted to be strong binders to HLA-DRB1*01:01 with binding scores below 10 (i.e., with a probability for being good binders greater than 90%). Some of the predicted peptides shared common HLA-DRB1*01:01-binding core sequences (9-mer peptides). When considering only unique core sequences and after exclusion of two peptides located in the prodomain of ADAMTS13, the list came down to 15 9-mer core peptides (Table 1). The peptides were synthesized at greater than 80% purity (GL Biochem, Shanghai, China) and included the 9-mer core sequences with addition of the 3 residues from the N-terminal end and the 3 residues of the C-terminal end. Individual peptides were solubilized at 1 mg.mL⁻¹ in DMSO/water. Recombinant full-length human ADAMTS13 (rhADAMTS13) was a kind gift from Baxter (Vienna, Austria).¹⁸

HLA-peptide-binding assays

HLA-DR molecules were purified from homozygous EBV cell lines by affinity-chromatography using the monomorphic mAb L243. The binding to HLA-DR molecules was assessed by competitive ELISA, using an automated workstation, as previously reported.^{19, 20} Briefly, HLA heterodimers were incubated with a biotinylated indicator peptide and serial dilutions of competitor peptides. As reference, the unlabelled form of biotinylated reporter peptide was used as internal control. After

24 hr incubation at 37°C, samples were neutralized with 450 mM Tris HCl (pH 7.5) (Sigma, St Quentin-Fallavier, France), 0.3% BSA (Sigma), 1 mM n-dodecyl β -D-maltoside buffer (Sigma) and applied to 96-well MaxiSorp ELISA plates (Nunc A/S, Roskilde, Denmark) coated with 10 $\mu\text{g.mL}^{-1}$ L243. Bound biotinylated peptide was detected by streptavidin-alkaline phosphatase conjugate (GE Healthcare, Saclay, France) and after adding 4-methylumbelliferyl phosphate substrate (Sigma). Emitted fluorescence was measured at 450 nm upon excitation at 365 nm. The peptide concentration that prevented binding of 50% of the biotinylated peptide (IC_{50}) was evaluated. The sequence of the biotinylated reporter hemagglutinin (HA) peptide was $^{306}\text{PKYVKQNTLKLAT}^{318}$; the mean IC_{50} values of the $\text{HA}^{306-318}$ peptide for HLA DRB1*01:01 and DRB1*11:01 binding were 4.61 and 37.42 nM, respectively. For HLA DRB1*15:01, the sequence of the reporter biotinylated peptide $\text{A3}^{152-166}$ was $^{152}\text{EAEQLRAYLDGTGVE}^{166}$ with an IC_{50} value of 41.46 nM. Means and standard errors of the means were calculated from three independent experiments. Binding data are estimated as relative affinities defined as the ratio of the IC_{50} of each individual peptide to the IC_{50} of the reference peptide. Peptides with relative affinities of 20 or less were considered as high binders to the HLA-DRB1 molecules and were retained for the rest of the study.

Human samples

Heparinized blood from healthy donors (EFS, Ile-de-France, Créteil, France) or from TTP patients (French national reference centre for TMA, Prof. P. Coppo, St Antoine Hospital, Paris, France and Dr L. Galicier, St Louis Hospital, Paris, France) was obtained after written informed consent. The use of samples for research purposes has been approved by the ethical committee from Saint-Antoine Hospital

(Authorization n°04807/12/2005). Acquired TTP criteria were those previously reported,⁴ and included a severe deficiency (<5% of normal activity) in ADAMTS-13 activity along with a detectable inhibitor and/or detectable serum antibodies as assessed by ELISA (Technozym R ADAMTS13 INH, Technoclone, Vienna, Austria; threshold positivity > 15 U mL⁻¹).

Blood processing

All blood samples were shipped at room temperature within 4 hr after drawing. HLA-DR screening was performed by molecular typing using the Olerup SSP HLA*DR kit (Olerup SSP, Stockholm, Sweden) after DNA extraction from total blood (Qiagen, Vienna, Austria). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on lymphocyte separation medium (PAA Laboratories GmbH, Les Mureaux, France) and immediately frozen in pooled human male AB serum containing 10% DMSO (Sigma).

Immunisation of mice

HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II (IA β -/- β 2m-/-)-knockout mice (Sure-L1 mice) were 8-12 week-old.²¹ Mice were immunized subcutaneously with 150 μ g of rhADAMTS13 in complete Freund's adjuvant (Sigma), and fifteen days later with 150 μ g rhADAMTS13 in incomplete Freund's adjuvant (Sigma). Blood was drawn by retro-orbital bleeding 4 days after the second administration of rhADAMTS13 and cells from the spleen and draining lymph nodes were collected. Animals were handled in agreement with local ethical authorities (Comité Régional d'Éthique p3/2005/002).

Titration of anti-ADAMTS13 IgG

Antibodies against human ADAMTS13 were measured in mice serum using enzyme-linked immunosorbent assays (ELISA). Briefly, ELISA plates were coated with rhADAMTS13 ($1 \mu\text{g.mL}^{-1}$) for 1 hr at 37°C and subsequently blocked for an additional hour with PBS-1% BSA. Serum serially diluted in PBS-BSA (dilution factor: 1/3) was then incubated during 1 hr at room temperature. Bound IgG was revealed using a secondary horseradish peroxidase-coupled goat F(ab')_2 anti-mouse IgG antibody (Southern Biotech, Birmingham, AL, USA) and substrate. The mouse monoclonal anti-human ADAMTS13 IgG mAb 20A5 (HYCULT, Uden, NL) was used as positive control.

HLA-DRB1*01:01 positive antigen-presenting cells (APC)

Three different sources of APCs were used for T-cell presentation assays: 1) purified splenocytes from Sure-L1 mice inactivated with mitomycin C ($50 \mu\text{g.mL}^{-1}$, Sigma), 2) immature Mo-DCs from HLA-DRB1*01:01 healthy donors inactivated with mitomycin C ($50 \mu\text{g.mL}^{-1}$), or 3) artificial APCs derived from NIH-3T3 cell line expressing HLA-DRB1*01:01 (AAPC^{DR1}). To generate AAPC^{DR1}, NIH-3T3 cells were transduced with the common HLA-DR α chain and the specific HLA-DR β 1*01:01 chain, and with the co-stimulatory and adhesion human molecules B7.1, ICAM-1 and LFA-3.²² AAPC^{DR1} does not produce murine IL-2 and therefore does not require inactivation with mitomycin C. Its ability to present and stimulate efficiently HLA-DRB1*01:01-restricted CD4⁺ T-cells has been demonstrated.²² In the case of Mo-DCs, human monocytes were isolated from PBMCs using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were cultured in RPMI-1640 medium supplemented with 10% human AB Serum (Sigma), L-glutamine and

antibiotics (Life Technologies - Invitrogen, Carlsbad, CA, USA) in the presence of 500 IU human recombinant interleukin 4 (IL-4, ImmunoTools, Friesoythe, Germany) and 1000 IU human recombinant GM-CSF (ImmunoTools) per 10^6 cells. After 5 days of culture, the non-adherent immature Mo-DC-enriched fraction was harvested and the immature status was confirmed by the expression of the following surface phenotypic markers: CD40, CD80, CD86, CD83, CD1a and HLA-DR (Becton Dickinson, Le Pont de Claix, France), by flow cytometry as explained previously.²³

Proliferation response to ADAMTS13-derived peptide

Cells from the spleen and draining lymph nodes from immunized Sure-L1 mice were pooled. Cells (10^5 cells/well) were incubated alone, with each individual synthetic ADAMTS13-derived peptide ($10 \mu\text{g.mL}^{-1}$) or with a pool containing all the peptides ($2.5 \mu\text{g.mL}^{-1}$ of each peptide) in AIMV medium (Life Technologies, Invitrogen, Carlsbad, CA, USA) for 4 days. Cell proliferation was determined by incorporation of ^3H -thymidine ($1 \mu\text{Ci}$ per well, specific activity of 6.7 Ci.mmol^{-1}) added during the final 16 hr and measured as counts per minute (cpm) on a micro- β -counter (Perkin Elmer Applied Biosystems). Stimulation indexes (SI) were calculated as the ratio between the cpm in the presence of a stimulus, and the average cpm of the same cells incubated alone.

Generation of mouse ADAMTS13-specific CD4⁺ T-cell hybridomas

Cells from draining lymph nodes of immunized Sure-L1 mice were stimulated 72 hr with rhADAMTS13 ($1 \mu\text{g.mL}^{-1}$) and fused with the BWZ.36 fusion (TCR^{-/-}) partner cell line²⁴ (a kind gift from Prof N Shastri, University of California, Berkeley, CA, USA) using polyethylene glycol. ADAMTS13-specific CD4⁺ T-cell hybridomas were

obtained after culture in HAT and HT selective media (both from Sigma). Specificity for ADAMTS13 was evaluated using a T-cell stimulation assay.

T-cell hybridoma stimulation assay

Mouse HLA-DR1-restricted CD4⁺ T-cell hybridomas (10⁵ cells/well) were cultured in 96 round bottom plates with 10⁴ (ratio 1:10) DRB1*01:01 APC (splenocytes or immature Mo-DCs) in the presence of rhADAMTS13 (10 µg.mL⁻¹) for 24 hr at 37°C. Murine interleukin 2 (IL-2) secreted by activated T-cells was measured in the supernatant by an ELISA Kit (eBioscience, Paris, France) according to manufacturer protocol. Positive control used was concanavalin A (Sigma) at 1 µg.mL⁻¹. For each hybridoma cell line growing in HAT media, stimulation index (SI) was calculated. Hybridoma clones with an at least two-fold increase in IL-2 release (ratio between ADAMTS13 stimulated cultures and control medium) were considered to be ADAMTS13-specific and were subsequently sub-cloned by limiting dilution (0.5 cell/well). Among ADAMTS13-specific T-cell hybridomas, screening for peptide specificity was realized with AAPC^{DR1} co-cultured for 24 hours with peptides at 10 µg.mL⁻¹, and IL-2 secretion by activated T-cells hybridoma was measured in the supernatant with the same ELISA Kit.

Generation of ADAMTS13-specific human CD4⁺ T-cell lines

Using the accelerated co-cultured DC (acDC) assay,²⁵ PBMCs from a TTP patient were stimulated for 10 days with the pool of 16 ADAMTS13-derived peptides, (2.5 µg.mL⁻¹ for each peptide). Briefly, cells (5.10⁶ cells.mL⁻¹) were thawed and cultured in AIM-V medium culture supplemented with 1000 U.mL⁻¹ of GM-CSF, 500 U.mL⁻¹ of IL-4 (ImmunoTools). After 24 hr (day 1), the pool of ADAMTS13-

derived peptides was added along with TNF- α (1000 U.mL⁻¹), IL-1 β (10 ng.mL⁻¹), IL-7 (0.5 ng.mL⁻¹) (ImmunoTools) and prostaglandin E2 (1 μ M; Merck Calbiochem, Saint-Quentin-en-Yvelines, France). On day 10, non-adherent cells were collected, washed, and CD4⁺ T lymphocytes were isolated by negative magnetic purification with a CD4⁺ T-cell isolation kit (Miltenyi). Cells were then incubated with irradiated DRB1*01 Mo-DCs (45 Gy) from healthy donors (ratio 1:10), pulsed with the pool of ADAMTS13-derived peptides, in fresh AIM-V at 37°C for 6 hours. Subsequently, CD4⁺ T-cells reactive to ADAMTS13-derived peptides were isolated using the IFN- γ secretion assay-cell detection kit (Miltenyi) according to manufacturer protocol. Cell lines were obtained after culture of the IFN- γ -secreting cells in AIM-V medium supplemented with 10% human AB serum, IL-2 (20 U.mL⁻¹), IL-4 (5 ng.mL⁻¹), anti-CD3 mAb (OKT3, 30 ng.mL⁻¹) (ImmunoTools) and irradiated (45Gy) PBMC from 2 unrelated donors. Cells were fed every 7 days with fresh cytokines. The stimulated CD4⁺ T cells were tested after ~3 weeks for peptide specificity by IFN- γ ELISPOT after incubation with peptide-pulsed or non-pulsed APC.

ELISPOT assays

The human IFN- γ ELISPOT was performed according to the manufacturer's recommendations. Ninety-six-well polyvinylidene fluoride plates (Millipore-Merck) were pre-wetted with ethanol. The individual ADAMTS13-derived peptides (10 μ g.mL⁻¹), the peptide pool, or medium alone were plated in triplicate wells, into the plates that had been pre-coated overnight with the anti-IFN- γ capture antibody (U-CyTech, Utrecht, the Netherlands) and blocked with AIMV plus 10% human serum. Irradiated AAPC^{DR1} (45 Gy) were then seeded and incubated (3x10⁴/well) with the CD4⁺ T-cell line (3x10⁵/well) for 24 hr at 37°C in a CO₂ incubator. Following PBMC removal, IFN-

γ secretion was visualized with a biotin-conjugated anti-IFN-γ antibody (U-CyTech), alkaline phosphatase-conjugated ExtrAvidin, and Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma). Plates were then air dried and scanned on an ImmunoSpot Analyzer (CTL, Shaker Heights, Ohio). Spot-forming cells (SFCs) were automatically calculated by the ImmunoSpot Software using the SmartCount™ and Autogate™ functions ²⁶. ELISPOT readouts are expressed as SFCs/10⁶ CD4⁺ T-cells. Peptides that generated at least twice the SFCs over the control wells were defined as eliciting a positive response.

RESULTS

Few ADAMTS13-derived peptides are high binders to HLA-DRB1*01:01 molecules

We hypothesized that the T-cell epitopes of rhADAMTS13 correspond to ADAMTS13-derived peptides that are stably binding to the groove of class II MHC molecules. The candidate peptides should thus bind with a high affinity to HLA-DR molecules. Using the IEDB consensus method prediction tool for MHC class II binding, we first predicted the array of ADAMTS13-derived 15-mer peptides that bind to the HLA-DRB1*01:01 (DR1) allele. Altogether, fifteen 15-mer peptides with unique core sequences were predicted to be strong binders to HLA-DR1 with binding scores below 10 (Table 1). The corresponding synthetic 15 peptides were used in binding assays to determine *in vitro* affinities for purified HLA-DRB1*01:01 molecules by competition ELISA. The calculated IC₅₀ values ranged from 0 to 5000 nM (Table 1). Five peptides bound to DR1 with an IC₅₀ below 4 nM, an affinity greater than that of the influenza HA³⁰⁶⁻³¹⁸ control peptide (IC₅₀ = 4.61 nM). Three peptides revealed a

poor binding capacity with IC₅₀ 20-fold greater than that of the HA control peptide, despite a good IEDB-predicted binding score.

ADAMTS13-derived peptides activate ADAMTS13-specific mouse CD4⁺ T-cell hybridomas restricted to HLA-DRB1*01:01

To assess whether the ADAMTS13-derived HLA-DR1 binding peptides are T-cell epitopes and are presented to T-cells by antigen-presenting cells, we generated ADAMTS13-specific CD4⁺ T-cell hybridomas restricted to the HLA-DR1 allele. Induction of an anti-rhADAMTS13 immune response in a humanized transgenic HLA-DR1 mouse was confirmed by ELISA (Figure 1A). Cells from the draining lymph nodes were fused to a TCR^{-/-} partner (BWZ36) and 95 CD4⁺ T-cell hybridomas were selected. Among them, 26 hybridomas produced IL-2 following incubation with splenocytes from Sure-L1 mice in the presence of rhADAMTS13. The 26 hybridomas were subsequently sub-cloned.

We next evaluated the activation of the T-cell hybridomas by the 15-mer peptides. The ADAMTS13¹²³⁹⁻¹²⁵³ peptide was the only peptide to induce a significant secretion of IL-2 in all the hybridoma cell lines tested (tested for 6 of the 26 rhADAMTS13-reactive T-cell hybridomas; representative result shown for one T cell-hybridoma in Figure 1B). To delineate more precisely the T-cell epitope recognized by the T-cell hybridomas, overlapping 15-mer peptides that span aa 1235 to 1256 were synthesized and were assessed for their capacity to activate one of the T-cell hybridomas (Clone 2G10δ). Significant T-cell activation was observed for peptides 1237-1251, 1238-1252, 1239-1253, 1240-1254 and 1241-1255 (Figure 1C). Conversely, peptides 1235-1249, 1236-1250 and 1242-1256 failed to activate the T-cell hybridoma. Similar results were obtained with another T-cell hybridoma (clone

1F5γ, data not shown). These results demonstrate that the minimal core peptide sequence recognized by the ADAMTS13-specific DRB1*01:01-restricted T-cell hybridomas is ¹²⁴1MLLLWGRLTWR¹²⁵1. Anchor residues for binding of peptide 1239-1353 to the HLA groove are highlighted in Table 1: L1, W4, R6 and W9.

The ADAMTS13¹²³⁹⁻¹²⁵³ peptide is recognized by CD4⁺ T-cells from humanized Sure-L1 HLA-DRB1*01:01 mice

Next, we asked whether other peptides than the one identified using the hybridoma technology may be recognized by polyclonal CD4⁺ T-cells from Sure-L1 mice immunized with rhADAMTS13. Splenocytes and cells from the draining lymph nodes from three immunized Sure-L1 mice were stimulated with the individual ADAMTS13-derived peptides previously identified to bind to HLA-DRB1*01:01 with a high affinity. A significant cell proliferation was observed in the case of ADAMTS13¹²³⁹⁻¹²⁵³ peptide (Figure 2A), confirming that this peptide is a dominant CD4⁺ T-cell epitope, at least in mice.

The ADAMTS13 T-cell epitope identified in humanized Sure-L1 HLA-DRB1*01:01 mice is presented by human antigen-presenting cells

We then investigated whether the ADAMTS13¹²³⁹⁻¹²⁵³ peptide is generated by professional human antigen-presenting cells and presented to CD4⁺ T-cells in a HLA-DRB1*01:01 context. To this end, immature HLA-DRB1*01:01 Mo-DCs were incubated with rhADAMTS13 and with T-cell hybridomas of known specificity for the 1239-1253 peptide. T cells incubated with Mo-DCs in the presence of the ADAMTS13¹²³⁹⁻¹²⁵³ peptide presented with a stimulation index of 22.2±1.9 (Figure 2B). In contrast, incubation of the cells with the ADAMTS13¹¹¹⁻¹²⁵ control peptide did

not result in T-cell activation (stimulation index: 1.1 ± 0.0). Interestingly, incubation of T cells and Mo-DCs with rhADAMTS13 induced T-cell activation with a stimulation index of 3.3 ± 0.6 , indicating that rhADAMTS13 is efficiently processed by human antigen-presenting cells, and that the ADAMTS13¹²³⁹⁻¹²⁵³ peptide is readily generated.

The ADAMTS13¹²³⁹⁻¹²⁵³ peptide is a dominant T-cell epitope in a TTP patient with the HLA-DRB1*01:01 allele

We then assessed whether the ADAMTS13¹²³⁹⁻¹²⁵³ peptide is recognized by CD4⁺ T-cells from patients with acquired TTP. CD4⁺ T-lymphocytes were isolated from the blood of a 43 years-old HLA-DRB1*01:01 male, 14 days after the onset of acute acquired TTP. In the patient, the ADAMTS13 activity was less than 5% of normal value due to the presence of inhibitory antibodies directed to ADAMTS13. A CD4⁺ T-cell line specific for ADAMTS13-derived peptides was amplified from the patient's PBMCs as explained. After 32 days of culture, the CD4⁺ T-cell line produced IFN- γ , as assessed by ELISPOT, following incubation with Mo-DCs of HLA-DR1 healthy donors, pulsed with the pool of ADAMTS13-derived peptides with high binding affinity for HLA-DR1, but failed to produce IFN- γ when incubated with Mo-DCs alone (data not shown). We then incubated the T-cell lines with AAPC^{DR1} and with each individual peptide. Together with the ADAMTS13¹³⁹²⁻¹⁴⁰⁶ peptide, the ADAMTS13¹²³⁹⁻¹²⁵³ was the only peptide able to activate human IFN- γ -producing CD4⁺ T-cells (Figure 2C). Similar results were obtained when the T-cells and ADAMTS13¹²³⁹⁻¹²⁵³ peptide were incubated in the presence of HLA-DR1 Mo-DCs (data not shown). Further evaluation of the ADAMTS13¹²³⁹⁻¹²⁵³ peptide by IEDB predicted good binding scores to HLA-DR11, 15, 13, 8, 3 and 4, which was confirmed in competitive ELISA at least in the

case of HLA-DR11 and DR15 (Table 2). T-cell lines were also generated from one TTP female patient in acute phase (aged 19, Figure 3A), one TTP female patient in remission phase (aged 34, Figure 3B) and one 25-year old male healthy donor (Figure 3C), all with the HLA-DRB1*11:01 allele. Reactivity towards the ADAMTS13¹²³⁹⁻¹²⁵³ peptide was detected in the case of the three individuals. Together, our data suggest that the ADAMTS13¹²³⁹⁻¹²⁵³ peptide is a dominant and promiscuous T-cell epitope in TTP patients.

DISCUSSION

In the present study, we delineated the ADAMTS13-derived peptides that are potent T-cell epitopes in the context of the HLA-DR1 haplotype. An array of ADAMTS13-derived peptides that are processed and presented by Mo-DCs had been previously reported in the case of healthy donors with different HLA-DR haplotypes.¹³ As many as 11 core peptides had thus been retrieved from the HLA-DR-binding groove; the peptides spanned 6 different domains of ADAMTS13. In order to identify HLA-DR-restricted T-cell epitopes of ADAMTS13, we took advantage of the humanized Sure-L1 mouse strain. Sure-L1 mice are invalidated for murine MHC class I and II molecules and transgenic for HLA-DRB1*01:01. HLA transgenic mice have been proven an appropriate human counterpart for the characterization of T-cell epitopes derived from pathogens²¹ as well as therapeutic proteins.²⁷ In agreement with this, our results demonstrate that the ADAMTS13¹²³⁹⁻¹²⁵³ peptide identified as an immunodominant T-cell epitope using *in silico* prediction, *in vitro* binding assays and T-cell hybridoma generation, is processed by human Mo-DCs *in vitro* and activates CD4⁺ T cells both *in vivo* in Sure-L1 mice, and *ex vivo* in the case of cells from patients with TTP.

The *in silico* selection of the ADAMTS13-derived peptides was based on the hypothesis that the T-cell epitope of rhADAMTS13 binds with a high affinity to HLA-DR molecules. The prediction efficiency of IEDB for HLA class II-binding peptides has been validated for various antigens, including human plasma protein, and correlates adequately with HLA binding, as measured by competitive ELISA.^{15–17} For instance, T-cell epitopes of the human pro-coagulant factor VIII were identified using the hybridoma technology in an HLA-DR15 transgenic mouse model upon screening a peptide library spanning the entire sequence of the protein.²⁷ Our unpublished data demonstrate a strong correlation between the predicted IEDB binding score for HLA-DR15 and the CD4⁺ T-cell epitope found in this work through hybridoma technology (area under the ROC curve=0.90). *In silico* prediction may nevertheless be over-predictive and positively select peptides that do not activate T cells.²⁸

In the case of ADAMTS13, HLA-DR11 has been identified as a major risk factor for TTP. Sorvillo *et al.* identified 11 hypothetical T-cell epitopes following purification from HLA-DR molecules expressed by ADAMTS13-loaded Mo-DCs of healthy donors with several HLA haplotype.¹³ The ADAMTS13-derived core sequence ¹³²⁸FINVAPHAR¹³³⁶ was shown to be preferentially presented by HLA-DR11 molecule,¹³ and stimulated CD4⁺ T cells from at least one TTP patient.¹⁴ In our hands, the ADAMTS13^{1325–1339} peptide, that contains the same core sequence, was predicted as a high binder HLA-DR1 peptide. It however failed to bind HLA-DRB1*01:01 and to activate human CD4⁺ T cells from a DRB1*01:01 patient. Besides, the ADAMTS13^{1325–1339} peptide did not activate CD4⁺ T cells from 2 TTP patients with the HLA-DRB11 allele. Whether this results from differences in peptide length and in the nature of the residues adjacent to the core peptide, remains to be determined.^{29,30}

Our rationale for focusing our study on the HLA-DRB1*01:01 allele is justified by the availability of an appropriate transgenic HLA-DRB1*01:01 mouse model, and by the fact that the HLA-DRB1*01:01 allele is found in 18% of the patients with acquired TTP¹⁰ and in 15 to 30% of the Caucasian population.³¹ Interestingly, 11 of the 15 peptides predicted to bind HLA-DR1 by IEDB were also predicted to have a high affinity for the HLA-DRB1*11:01 allele. In particular, the unique immunodominant HLA-DRB1*01:01-associated T-cell epitope characterized in our work, ADAMTS13¹²³⁹⁻¹²⁵³, demonstrated an excellent binding score to HLA-DRB1*11:01 (score of 1.2) and HLA-DRB1*15:01, strong affinity for HLA-DRB1*11:01 and HLA-DRB1*15:01 in competitive ELISA (Table 2), and was a target epitope of CD4⁺ T cells from 2 TTP patients and 1 healthy donor with the HLA-DRB11 allele. Of note, the ADAMTS13¹²⁴⁰⁻¹²⁵² peptide, that shares the identical core peptide with the ADAMTS13¹²³⁹⁻¹²⁵³ peptide, was identified by the study of Sorvillo *et al.* in the case of Mo-DCs from a patient with the HLA-DR4/DR13 haplotype.¹³ Together with our IEDB prediction, the data suggest that the ADAMTS13¹²³⁹⁻¹²⁵³ peptide exhibits promiscuity for at least three HLA-DR alleles: DR1, DR11 and DR15. Additional work including more HLA-DR alleles is required to determine whether pathogenic recognition of ADAMTS13 by CD4⁺ T cells is principally directed to promiscuous immunodominant epitopes such as the ADAMTS13¹²³⁹⁻¹²⁵³ peptide, or whether private T-cell epitopes dominate in the case of HLA-DR alleles that are predominantly associated with acquired TTP.

We cannot exclude the existence of additional immuno-dominant T-cell epitopes potentially overlooked in our experimental approach. Besides, additional work is required to link our findings to the Th2 polarization of CD4⁺ T cell and production of anti-ADAMTS13 autoantibodies. Yet, identification of the ADAMTS13¹²³⁹⁻¹²⁵³ peptide

as a promiscuous immuno-dominant T-cell epitope may have clinical implications at term. It shall pave the way towards the characterization of the ADAMTS13-specific CD4⁺ T-cell response in TTP patients using ADAMTS13¹²³⁹⁻¹²⁵³-loaded HLA-DR tetramers. Such a tool would provide new insights on the mechanisms implicated in the breakdown of tolerance to ADAMTS13 and should be of clinical relevance to follow in a quantitative manner populations of ADAMTS13-specific T cells in the course of TTP treatment and anticipate relapse. In particular, confirmation of our results would lead to identification of prognostic factors (persistence of specific Th17 T cells, generation of regulatory T cells) that would help in the decision process for the pre-emptive use of Rituximab to prevent relapse.³²

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AUTHOR CONTRIBUTION

LG, PC, SK and SLD designed the research and wrote the paper. LG, SD, IP, MI, YL, BG, BM, YK, MS, AP, JBL, MH, OT, PL performed the research. PC and LGa provided patients' samples. AV performed the ADAMTS13 investigation for medical

diagnosis in human samples and reviewed the manuscript. LG, BM, PC and SLD analysed the data.

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TABLES

Table 1. Affinity of ADAMTS13-derived peptides for HLA-DRB1*01:01 molecules

	ADAMTS13 peptide	Domain	Sequence [#]	IC ₅₀ (nM) [†]	Predicted binding score [‡]
1	589-603	Spacer	RPL F THLAVRIGGRY	0.46	7.01
2	267-281	Metalloprotease	RRQ LLSLLSAG RARC	0.91	1.36
3	371-385	Desintegrin	CRS LVELTPIAA VHG	1.59	4.22
4	1239-1253	CUB1	GDM LLLWGRLT WRKM	1.61	7.08
5	1392-1406	CUB2	EGF LKAQASLR GQYW	3.74	1.62
6	1355-1369	CUB2	ASY ILIRDTHSL RRTT	5.65	6.65
7	774-788	TSR 3	GSL LKTLP PARC	7.11	5.65
8	570-584	Spacer	YVT FLTVTPNLT SVY	13.10	0.79
9	914-928	TSR 5	ELR FLCMD SALRVPV	12.6	3.24
10	1224-1238	CUB1	VVT LRVLESSLN CSA	13.68	6.21
11	1281-1295	CUB1	GVL LRYGSQLA PETF	28.34	6.90
12	464-478	Cys-Rich	GAS FYHWGA AVPHSQ	31.07	8.42
13	1325-1339	CUB2	CRL F INVAPHARIAI	147.97	1.99
14	140-154	Metalloprotease	APN ITANLTSS LLSV	279.67	9.74
15	308-322	Desintegrin	NEQ CRVAFGPKA VAC	5000	8.11
16*	111-125	Metalloprotease	GAEL LLRDP SLGAQFR	>10000	20.93

[†]ADAMTS13-derived peptides are sorted based on the measured IC₅₀ values compared to the HA³⁰⁶⁻³¹⁸ control peptide (IC₅₀=4.61 nM). IC₅₀ values are shown in nM. A lower IC₅₀ value indicates stronger binding. IC₅₀>10000 indicates no detectable binding in the assay.

[‡]Binding scores were generated by IEDB and reflect the expected binding affinities, wherein lower scores indicate stronger predicted affinities.

[#]The 9-residue sequences (core peptides) predicted to fit into the HLA-DRB1*01:01 binding groove are underlined for each peptide. Anchor residues are highlighted in bold.

*This ADAMTS13-derived peptide is used as a negative control, without detectable binding to HLA-DRB1*01:01.

Table 2. Affinity of ADAMTS13¹²³⁹⁻¹²⁵³ peptide for HLA-DRB1 molecules.

HLA-DR molecule [#]	Predicted binding score [†]	Relative affinity [‡]
DRB1*11:01	1.2	16.04
DRB1*15:01	1.40	3.48
DRB1*13:01	1.72	ND
DRB1*08:01	4.47	ND
DRB1*01:01	7.08	0.27
DRB1*03:01	7.92	ND
DRB1*04:01	12.10	ND
DRB1*12:01	25.89	ND
DRB1*07:01	31.19	ND
DRB1*09:01	33.67	ND

[#]HLA-DRB1 molecules are classified based on their predicted binding score.

[†]Binding scores were generated by IEDB using the consensus method prediction tool.

[‡]Binding data are estimated as relative affinities defined as the ratio of the IC₅₀ of the ADAMTS13¹²³⁹⁻¹²⁵³ peptide to the IC₅₀ of each reference peptide. Relative affinities of 20 or less were considered high binders to the HLA-DR molecule. The sequences and mean IC₅₀ values of the reference peptides were the following: HA³⁰⁶⁻³¹⁸ (PKYVKQNTLKLAT) for DR1 (4.61 nM) and DR11 (37.42 nM); A3¹⁵²⁻¹⁶⁶ (EAEQLRAYLDGTGVE) for DR15 (41.46 nM).

ND: not done.

FIGURE LEGENDS

Figure 1

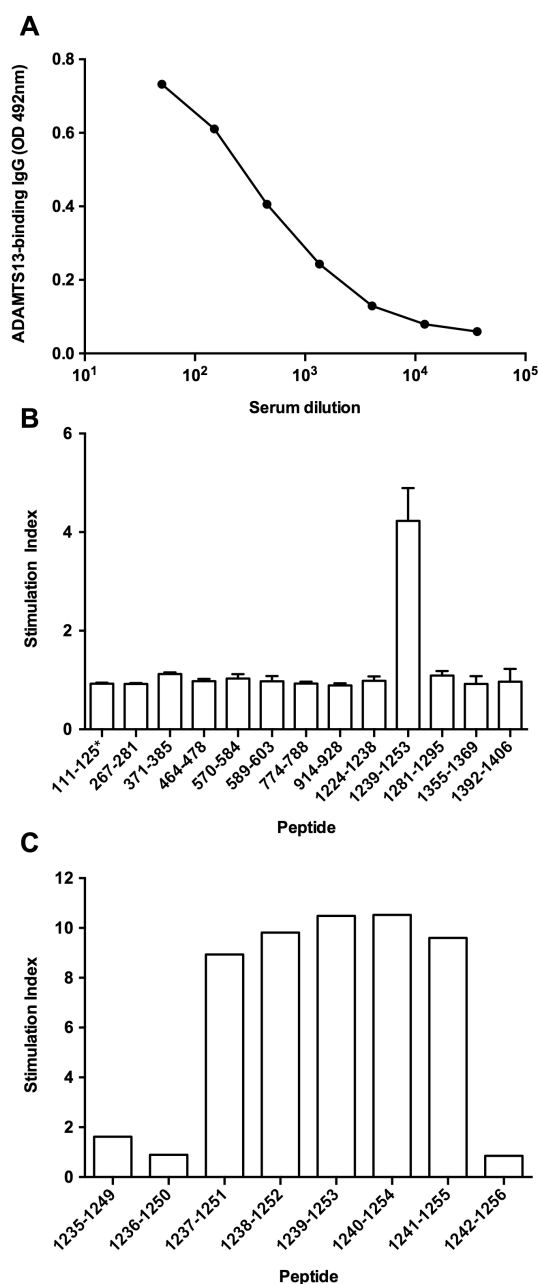


Figure 1. Identification of HLA-DR1-restricted T-cell epitopes using ADAMTS13-specific CD4⁺ T-cell hybridomas. **(A)** Anti-ADAMTS13 IgG titers in Sure-L1 mice immunized with rhADAMTS13. The serum from an HLA-DR1-transgenic Sure-L1 mouse immunized with rhADAMTS13 was incubated in serial dilutions on ADAMTS13-coated ELISA wells. ADAMTS13-bound IgG were detected using horseradish peroxidase-coupled polyclonal goat anti-mouse IgG and substrate. Results are expressed in arbitrary units as optical density measured at 492 nm. **(B)** Activation of ADAMTS13-specific CD4⁺ T-cell hybridomas. A representative ADAMTS13-specific CD4⁺ T-cell hybridoma (clone 2G10δ) was incubated with AAPC^{DR1} (10:1 ratio) and ADAMTS13-derived peptides as listed in Table 1. Stimulation indexes represent the ratio of IL-2 secreted by the T-cells measured by ELISA upon incubation with ADAMTS13 peptides over IL-2 secreted in the absence of peptide. Means±SD are from two independent experiments. **(C)** Delineation of the core peptide for ADAMTS13-specific CD4⁺ T-cell hybridomas. A representative ADAMTS13-specific CD4⁺ T-cell hybridoma (clone 2G10δ) was incubated with AAPC^{DR1} and overlapping 15-mer peptides spanning the 1235-1256 peptide sequence. Stimulation indexes were assessed as explained above.

Figure 2

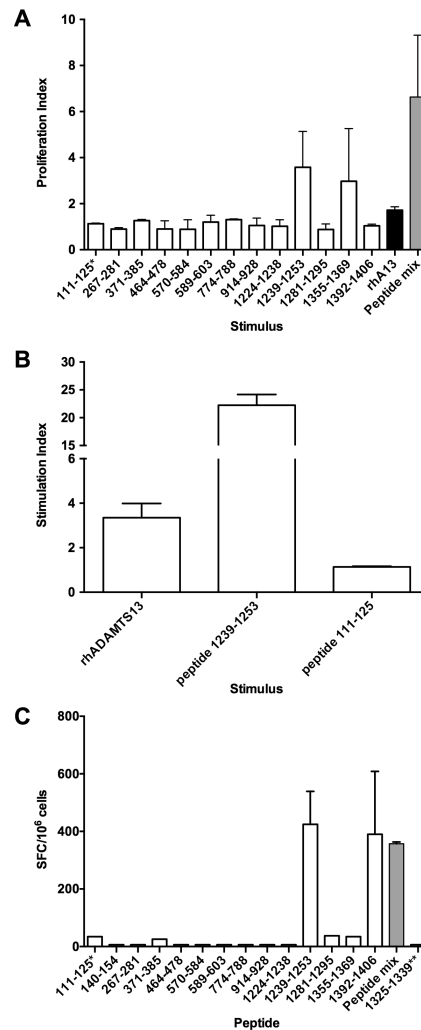


Figure 2. The ADAMTS13¹²³⁹⁻¹²⁵³ peptide is an immunodominant T-cell epitope both for Sure-L1 mice as well as for a patient with TTP. (A) Stimulation by ADAMTS13-derived peptides of spleen and lymph node cells from Sure-L1 mice immunized with rhADAMTS13. Cells (3×10^5 /well) were incubated with each individual peptide ($10 \mu\text{g} \cdot \text{mL}^{-1}$), with the pool of peptides (Peptide mix) or with rhADAMTS13. Proliferation was assessed by incorporation of tritiated thymidine. Proliferation indexes are defined as the ratio of cpm of stimulated cells versus cpm of control cells, and are expressed as means \pm SD for two Sure-L1 mice. **(B)** The ADAMTS13¹²³⁹⁻¹²⁵³ peptide is processed and presented to T-cells by human APCs. A representative ADAMTS13-specific CD4⁺ T-cell hybridoma (clone 1F5y) was incubated with immature dendritic cells from a healthy HLA-DR1⁺ blood donor and with the ADAMTS13¹²³⁹⁻¹²⁵³ peptide, with rhADAMTS13 or with the ADAMTS13¹¹¹⁻¹²⁵ peptide. Stimulation indexes represent the ratio of secretion of IL-2 by T-cells stimulated in the presence of antigen versus the secretion of IL-2 by T-cells incubated with Mo-DCs alone. Means \pm SD are from two independent experiments. Similar results were obtained with other ADAMTS13-CD4⁺ T-cell hybridoma clones (not shown). The peptide incubated alone or with DR15-positive artificial antigen presenting cells failed to activate the T cells (not shown). **(C)** ADAMTS13 epitope specificity of CD4⁺ T-cell lines from a patient with acquired TTP. CD4⁺ T-cell lines were generated after stimulation with pooled ADAMTS13-derived peptides as defined in Table 1. T-cells (3×10^5 /well) were then incubated with AAPC^{DR1} (3×10^4 cells/well) alone or pulsed with individual peptide ($10 \mu\text{g} \cdot \text{mL}^{-1}$), or with the peptide pool (Peptide mix). The number of cells producing IFN- γ was then assessed by ELISPOT and is expressed as mean number of spot forming cells (SFC) per million cells calculated in the case of peptide-stimulated T-cells minus the SFC obtained in the case of un-stimulated cells. Means \pm SD are from two independent experiments. *The 111-125 ADAMTS13-derived peptide that did not bind to HLA-DRB1*01:01 was used as a negative control.

Figure 3

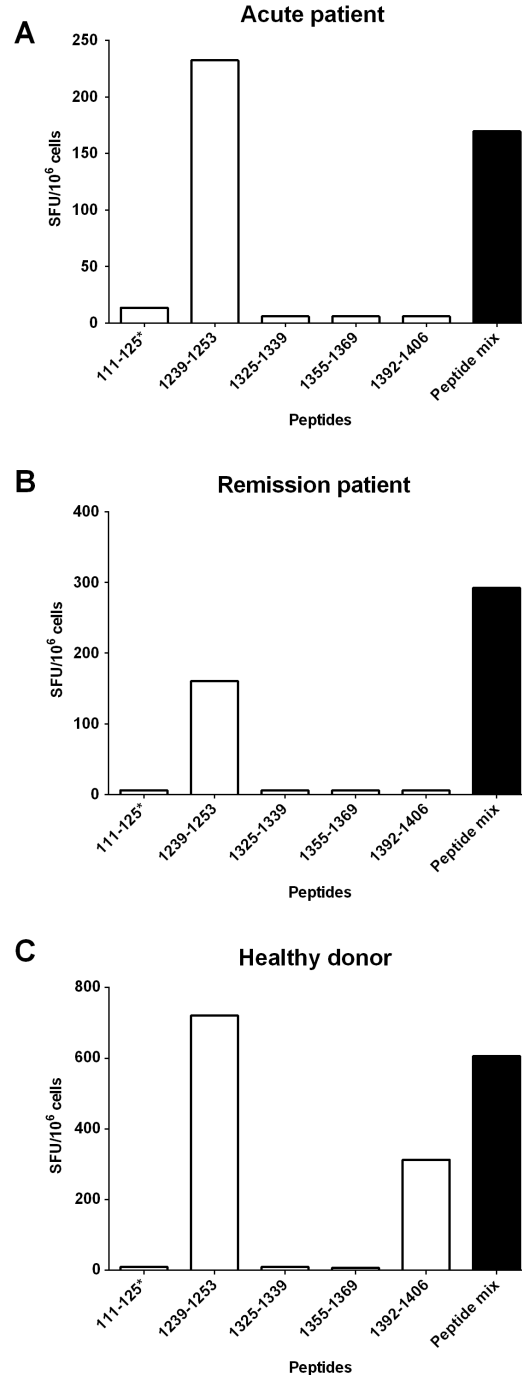


Figure 3. The ADAMTS13¹²³⁹⁻¹²⁵³ peptide is an immunodominant T-cell epitope for HLA-DRB1*11 individuals. CD4⁺ T-cell lines from HLA-DRB1*11 TTP patients at (A) acute phase or (B) in remission phase of the disease, respectively, or from (C) a HLA-DRB1*11 healthy donor, were stimulated with the ADAMTS13-derived peptides identified as immuno-dominant for HLA-DRB1*01. T cells (3.10⁵ cells/well) were incubated with AAPC^{DR11} (3.10⁴ cells/well) alone or pulsed with individual peptide (10 µg.mL⁻¹), or with the peptide pool (Peptide mix). The number of cells producing IFN-γ was then assessed by ELISPOT and is expressed as mean number of spot forming cells (SFC) per million cells calculated in the case of peptide-stimulated T-cells minus the SFC obtained in the case of unstimulated cells. *The 111-125 ADAMTS13-derived peptide is used as a negative control, without detectable binding to HLA-DRB1*11:01 (data not shown).