Monoclonal antibodies targeting the disintegrin-like domain of ADAMDEC1 modulates the proteolytic activity and enables quantification of ADAMDEC1 protein in human plasma

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ABSTRACT

Decysin-1 (ADAMDEC1) is an orphan ADAM-like metalloprotease with unknown biological function and a short domain structure. ADAMDEC1 mRNA has previously been demonstrated primarily in macrophages and mature dendritic cells. Here we generated

monoclonal antibodies (mAbs) against the mature ADAMDEC1 protein, as well as mAbs specific for the ADAMDEC1 pro-form, enabling further investigations of the metalloprotease. The generated mAbs bind ADAMDEC1 with varying affinity and represent at least six different epitope bins. Binding of mAbs to one epitope bin in the C-terminal disintegrin-like domain efficiently reduces the proteolytic activity of ADAMDEC1. A unique mAb, also recognizing the disintegrin-like domain, stimulates the caseinolytic activity of ADAMDEC1 while having no significant effect on the proteolysis of carboxymethylated transferrin. Using two different mAbs binding the disintegrin-like domain, we developed a robust, quantitative sandwich ELISA and demonstrate secretion of mature ADAMDEC1 protein by primary human macrophages. Surprisingly, we also found ADAMDEC1 present in human plasma with an approximate concentration of 0.5 nM. The presence of ADAMDEC1 both in human plasma and in macrophage cell culture supernatant were biochemically validated using immunoprecipitation and Western blot analysis demonstrating that ADAMDEC1 is secreted in a mature form.

KEYWORDS: ADAMDEC1, Metalloprotease, disintegrin, plasma, quantification, ELISA.

INTRODUCTION

ADAMDEC1 is an unusual ADAM-like metzincin metalloprotease with unknown function *in vivo*. Macrophages and mature dendritic cells express ADAMDEC1 mRNA,[1, 2] and differential regulation of mRNA expression has been demonstrated in a number of cancer types and diseases of the immune system.[3-13] ADAMDEC1 is therefore predicted to serve a role in modulating the immune response. This is further substantiated by a recent study demonstrating that *Adamdec1*$^{-/-}$ mice exhibited enhanced susceptibility toward bacterial- and chemically induced colitis.[14] A new study show ADAMDEC1 being present in human

platelets where it is released upon platelet activation.[15] *In vitro*, ADAMDEC1 has been shown to react with human α_2-acroglobulin (α_2M) in a fashion consistent with proteolysis of the α_2M "bait region".[16] In addition, the ADAMDEC1 proteolytic activity has also been demonstrated through cleavage of bovine casein and human carboxymethylated transferrin.[16, 17] ADAMDEC1 is expressed as a zymogen with a large, partially inhibitory N-terminal prodomain.[1, 16] However, in contrast to the canonical ADAM metalloproteases, which contain several ancillary domains C-terminal to the metalloprotease domain, including a transmembrane domain, the mature ADAMDEC1 protein consist only of a metzincin-type metalloprotease domain and a short disintegrin-like domain.[1, 18] Thus, ADAMDEC1 is secreted rather than membrane-bound like other ADAMs. The metalloprotease domain of ADAMDEC1 contains a rare ADAM-like zinc-binding active site, where the catalytic zinc ion is coordinated by two histidine and an aspartic acid residue, rather than three histidine residues.[1] This rare active site architecture has been shown to be sufficient for catalyzing substrate hydrolysis by ADAMDEC1.[16]

To generate molecular tools for studying ADAMDEC1 in greater detail, we produced monoclonal antibodies against the pro- and mature domain(s) of ADAMDEC1. We obtained monoclonal antibodies with varying affinities for ADAMDEC1 and which had significantly different impacts on ADAMDEC1 proteolytic activity against its reported macromolecular substrates. The antibodies enabled detection of ADAMDEC1 protein both in human plasma and macrophage conditioned medium using immunoprecipitation and Western blot analysis. Finally, we developed a quantitative sandwich ELISA and quantified the ADAMDEC1 concentration in human plasma to 0.5-0.6 nM.

RESULTS

Generation of anti-proADAMDEC1 monoclonal antibodies- Full-length proADAM-DEC1 consists of an N-terminal prodomain (PRO) followed by the mature protein comprising a metalloprotease (MP) and a disintegrin-like (DIS) domain (Fig. 1A). We previously showed that the prodomain of ADAMDEC1 is subjected to post-translational limited proteolysis at two independent proprotein convertase (PC) recognition sites at Arg56 and Arg203 during expression in HEK293 cells; Cleavage at the latter site causes the removal of the entire prodomain. The prodomain is also subject to auto-proteolysis in at least one location between Pro161-Leu162.[16] Removal of both PC recognition sites (the R56A/R200K/R203A variant) generates full-length proADAMDEC1 (57 kDa, fragment a). Removing only the PC2 site (the R200K/R203A variant) results in the secretion of multiple fragments of ADAMDEC1, where the autoproteolyzed form beginning at Leu162 (39 kDa, fragment c) is the predominant fragment. Removing the PC2 site in a catalytically inactive ADAMDEC1 variant (R200K/R203A/E353A) generates a near full-length protein starting at Glu57 (54 kDa, fragment b). The different proforms of ADAMDEC1 described in Table 1 and Fig. 1A allow for identification of antibody epitopes.[16]

The prodomain of ADAMDEC1 was produced in *E. coli*, purified and utilized as antigen for generation of monoclonal antibodies (mAbs). Of 82 ELISA-positive hybridoma culture super-natants, 67 contained antibody which recognized forms of proADAMDEC1 in a Western blot analysis using a mixture of the previously described proforms produced in a mammalian expression system.[16] The remaining antibodies were not characterized further. Based on the recognition pattern obtained with a mixture of proADAMDEC1 variants, the antibodies were divided into three different bins and, based on expression levels, one clone from each bin was chosen for mAb production. The three different proADAMDEC1 forms

(R56A/R200K/R203A, R200K/R203A/E353A and R200K/R203A) were used for mapping the epitopes of the anti-proADAMDEC1 mAbs using Western blot analysis (Table 1 and Fig. 1B). mAb43 only recognized the full-length protein, mAb60 recognized two bands in hR200K/R203A and mAb21 recognized all proADAMDEC1 forms (Fig. 1B). The recognition patterns of the three antibodies were consistent with mAb43 binding between residues 31-56, mAb60 between residues 57-162 and mAb21 between residues 163-200 (Fig. 1A).

Transfecting HEK293 cells with a full-length, wild-type ADAMDEC1 expression construct resulted in the release of a 30-kDa fragment recognized by mAb60 and mAb21 in Western blot analysis, suggesting that the prodomain was secreted into the media along with the mature protein (Fig 1B). As previously shown the prodomain is glycosylated at two sites, which likely causes the observed increase in the apparent MW compared to the *E. coli*-expressed antigen.[16] mAb43 only poorly recognized the secreted prodomain fragment indicating that the fragment may be trimmed in the N-terminus. A commercial anti-ADAMDEC1 mAb (Ab57224, Abcam, UK), which recognises an epitope in the C-terminal part of ADAMDEC1 between residue 361-471, was included as a control for equal sample load.

Generation of anti-mature ADAMDEC1 mAbs- To raise antibodies against the mature ADAMDEC1 protein, *E. coli*-expressed MBP-ADAMDEC1 fusion protein was used for immunization. Hybridoma clone supernatants containing antibody were tested for binding to immobilized antigen using ELISA. Clones recognizing the isolated MBP fusion protein were discarded. Seven supernatants contained antibodies binding mammalian expressed ADAMDEC1 in Western blot analysis were selected for mAb production. The epitope-containing domain was determined by Western blot analysis where binding to mature, wild-

type ADAMDEC1 (WT) and to a truncated variant containing only the MP domain was compared (Fig. 1C). Based on this analysis, the seven mAbs were divided into two bins where mAb110, mAb134, mAb137, mAb186 and Ab57224 (Abcam) recognized both ADAMDEC1 variants and thus bound to the MP-domain between residue 361 and 410 (denoted anti-ADAMDEC1-MP mAbs). The second bin consisted of mAb111, mAb129 and mAb177 which bound to full-length ADAMDEC1, but not the isolated MP-domain, indicating that their epitopes were positioned in the C-terminal DIS domain (denoted anti-ADAMDEC1-DIS mAbs). No signal was observed with the supernatant from mock transfected HEK293 cells for any of the mAbs (Fig. 1C).

To further divide the anti-ADAMDEC1 mAbs into epitope bins, a cross competition binding assay was employed using surface plasmon resonance (SPR). Mature ADAMDEC1 with an N-terminal HPC4-tag (HPC4-ADAMDEC1[16]) was captured on immobilized anti-HPC4-tag mAb and bound by one mAb before competitive binding of all mAbs was measured. Using this method, it was found that mAb129 and mAb177 competed with each other for binding to the DIS-domain, but not with mAb111 (supplementary figure S1). Capturing HPC4-ADAMDEC1 on the anti-HPC4 mAb abrogated binding of the anti-ADAMDEC1-MP mAbs (mAb110, mAb134, mAb137 and mAb186) and prevented further binning of these four mAbs using this approach (data not shown).

Binding kinetics of mAbs recognizing mature ADAMDEC1- To investigate the binding kinetics of anti-ADAMDEC1 mAbs, a surface plasmon resonance (SPR) analysis was performed. Briefly, the FAb fragments were generated by limited proteolysis of the anti-ADAMDEC1 mAbs, and binding to immobilized ADAMDEC1 protein was measured (Table 2 and supplementary figure S2). The fraction of monomeric FAb fragments was in general 90-93% (supplementary table S3). Interestingly, all the FAb fragments exhibited comparable

and relatively slow dissociation rates. However, the association rates varied greatly, spanning at least two orders of magnitude. Notably, the three FAbs recognizing the DIS-domain demonstrated the highest binding affinities (FAb111, FAb129 and FAb177). The estimated binding affinities of the anti-ADAMDEC1 mAbs were not affected by either the N-terminal prodomain or by an N-terminal HPC4-tag (data not shown).

mAb sensitivity to ADAMDEC1 disulfide bond reduction- The importance of an intact disulfide bond structure for antibody recognition was investigated by Western blot analysis using reduced and non-reduced ADAMDEC1 protein (Fig. 2). Three anti-ADAMDEC1-MP mAbs (mAb110, mAb134 and mAb137) preferentially bind the reduced form of ADAMDEC1. However, the two remaining MP-binding mAbs, mAb186 and the commercial Ab57224, were not affected by disulfide reduction, indicating at least two separate bins of anti-ADAMDEC1-MP mAbs. In contrast, the three anti-ADAMDEC1-DIS mAbs (mAb111, mAb129 and mAb177) preferentially bound to the non-reduced form of ADAMDEC1 (Fig. 2) indicating that binding of these antibodies rely on intact disulfide bonds in the disintegrin-like domain.

Anti-ADAMDEC1 mAb effect on ADAMDEC1 proteolytic activity- ADAMDEC1 cleaves α_2-macroglobulin (α_2M), azo-labelled casein and carboxymethylated transferrin (Cm-Tf).[16, 17] The effects of anti-ADAMDEC1 mAbs under saturating conditions on the proteolytic activity of ADAMDEC1 against the different substrates were analyzed (Fig. 3).

Cleavage of α_2M by ADAMDEC1 results in thioester-mediated trapping of the protease, which is thought to require the protease to access the internal cavity of α_2M capable of accommodating a protease up to 80-90 kDa.[16, 19] The three anti-ADAMDEC1-DIS mAbs (mAb111, mAb129 and mAb177) efficiently inhibited α_2M-mediated trapping of ADAMDEC1. Interestingly, the DIS-domain of ADAMDEC1 is not necessary for the α_2M-mediated

covalent trapping of ADAMDEC1, as the isolated MP-domain was as efficiently trapped by α_2M as ADAMDEC1 WT (Fig. 3A). Thus, the inhibitory effect of the anti-ADAMDEC1-DIS mAbs is most likely due to steric hindrance preventing ADAMDEC1 for entering the α_2M prey chamber. In contrast, the four anti-ADAMDEC1-MP mAbs did not block α_2M trapping. However, the presence of these mAbs appeared to affect the α_2M-ADAMDEC1 complex band pattern (Fig. 3A).

ADAMDEC1-mediated cleavage of azo-labelled casein results in the release of TCA-soluble, azo-labelled peptides which can be quantified.[16] The caseinolytic activity was almost completely blocked by the two anti-ADAMDEC1-DIS mAbs with overlapping epitopes (mAb129 and mAb177). Surprisingly, the third anti-ADAMDEC1-DIS mAb (mAb111) appeared to exert a stimulatory effect on the ADAMDEC1 caseinolytic activity (Fig. 3B). The stimulatory effect of mAb111 could not be explained by background caseinolytic activity in the mAb preparation (supplementary figure S4). These results demonstrate that binding to different regions of the DIS-domain of ADAMDEC1 can elicit opposite functional effects. The anti-ADAMDEC1-MP mAbs did not have a statistically significant effect on ADAMDEC1 activity (supplementary figure S5).

Further, ADAMDEC1 cleaves Cm-Tf between Cys(alkylated)194-Leu195 generating two main product bands, α and β (Fig. 3C).[17] Anti-ADAMDEC1-DIS mAb129 and mAb177 both inhibit this proteolytic activity, whereas mAb111 had only limited effect (Fig. 3C-D).

Again, the DIS-domain of ADAMDEC1 is not necessary for the proteolysis of Cm-Tf (Fig. 3C), suggesting the inhibitory effect of mAb129 and mAb177 may be due to steric hindrance of substrate binding. As for the other substrates, the four anti-ADAMDEC1-MP mAbs displayed only minor effects on the proteolytic activity of ADAMDEC1 against Cm-Tf (supplementary figure S6).

Macrophage secretion of mature ADAMDEC1 protein- Previously, expression of ADAMDEC1 mRNA by immune cells has been reported by methods such as RT-PCR and *in situ* hybridization. ADAMDEC1 has been demonstrated to be constitutively expressed in macrophages and up-regulated by LPS and 1α,25-dihydroxy vitamin D_3.[2] ADAMDEC1 mRNA is absent in immature dendritic cells and expression is induced by spontaneous, CD40- or LPS-dependent maturation.[1, 2] To investigate the expression of ADAMDEC1 protein, supernatants from primary human M0-, M1- and M2-macrophages as well as immature human dendritic cells were analyzed by Western blot analysis (Fig. 4). For this purpose, we tested all identified anti-ADAMDEC1 mAbs and found mAb111 to be of superior sensitivity. ADAMDEC1 was found in the supernatant of unpolarised M0 macrophage cells. Polarization of the macrophages by IFN-γ (M1) and IL-4 (M2) did not significantly change the secretion of ADAMDEC1 protein into the cell medium. ADAMDEC1 protein was not observed in the supernatant of immature dendritic cells (DC), in line with previous studies of transcriptional regulation (Fig. 4). The secreted ADAMDEC1 protein from macrophages displayed an apparent molecular weight comparable to the 32 kDa mature recombinant ADAMDEC1, indicating similar proprotein processing and post translational modifications.

Establishment of a quantitative ADAMDEC1 sandwich ELISA- The identification of several anti-ADAMDEC1 mAbs with diverse epitopes enabled establishment of an ADAMDEC1-specific quantitative sandwich ELISA. To find pairs of anti-ADAMDEC1 mAbs suitable for a quantitative sandwich ELISA, all possible combinations of mAbs were tested by cross-matching. Three combinations of mAbs (coating/detecting: mAb111/mAb177, mAb111/mAb129 and mAb177/mAb111) were functional in the sandwich ELISA setup. Only anti-ADAMDEC1-DIS mAbs were represented in the functional pairs, in line with these

mAbs displaying the highest affinities in the SPR analysis. Further, all three mAb pairs represent combinations of two mAbs from distinct epitope bins. The most sensitive dose response curve was obtained by coating with 2 µg/mL of mAb111 and detecting with 1.2 µg/mL biotin labelled mAb177. A minimal required dilution of plasma was identified as 1:10, based on acceptable accuracy (relative error (%RE) <15%) and precision (coefficient of variation (%CV) <15%) - data not shown. Lower limit of quantification (LLOQ) was <0.013 nM in buffer and 0.024 nM in 10% plasma corresponding to 0.24 nM in undiluted plasma. Cross-reactivity towards related plasma proteins was examined using 0.65 nM of either ADAMTS-5 (without the C-terminal thrombospondin domain), Coagulation Factor VIIa, or the MP-domain of ADAMDEC1. No reactivity was seen towards any of these proteins (data not shown). The dynamic range of the assay was found to be from 0.01 nM to 1.55 nM (Fig. 5).

Detection of mature ADAMDEC1 protein in human plasma- To investigate the presence of ADAMDEC1 in human plasma, the developed sandwich ELISA was applied to plasma and serum samples from seven healthy donors, prepared within two hours after collection. The plasma concentration of ADAMDEC1 was determined to be 0.5-0.6 nM in serum, heparin plasma and citrate plasma, but significantly lower in EDTA plasma (Fig. 6A). The latter finding led to further investigation of the effect of EDTA on quantification of ADAMDEC1 in the assay. Measuring ADAMDEC1 in buffer, heparin plasma and serum in the presence of EDTA showed a max reduction from baseline of 75% ADAMDEC1 in plasma at 0.75 mM EDTA, but only a 25% reduction in buffer (Fig. 6B). Freeze/thaw effect (3 cycles) was tested in plasma and serum from 7 donors and showed no effect on the ADAMDEC1 quantification. Assay robustness was tested using repeated analysis over several months of pooled citrate human plasma from Siemens: 0.51 nM (%CV = 11.5), HemosIL: 0.60 nM (%CV = 7.1) and

Cryocheck: 0.50 nM (%CV = 9.4). Here, the ADAMDEC1 plasma concentration was determined consistently with low %CV. The integrity of the ADAMDEC1 present in human plasma was verified through immunoprecipitation from pooled plasma using mAb111-conjugated Dynabeads and subsequent Western blot analysis utilizing the anti-ADAMDEC1 mAb mAb177 as primary antibody (Fig. 6C). Immunoprecipitation of ADAMDEC1, both from plasma and recombinant expressed in conditioned medium, resulted in two specific bands of 33 kDa and ~60 kDa, respectively, consistent with a mono- and dimer of mature ADAMDEC1. Further, the ~60 kDa band was not observed in Western blot analysis of immunoprecipitate using anti-proADAMDEC1 mAb21 as secondary mAb (supplementary figure S7) suggesting that it does not contain the pro domain. The blood plasma monomer migrates with identical molecular weight as recombinant ADAMDEC1, indicating similar post translational processing. An ADAMDEC1 dimer was not observed in the non-immunoprecipitated recombinant protein preparation under these conditions. Instead a minor band of ~57 kDa consistent with unprocessed proADAMDEC1 was observed (Fig. 6C).

DISCUSSION

To enable further characterization of the ADAMDEC1 protein, we generated molecular tools in the form of monoclonal antibodies (mAb) binding the mature or pro-form of ADAMDEC1 and characterized the effect of selected mAbs on ADAMDEC1 functionality *in vitro*.

We generated and characterized 10 mAbs with epitopes covering the entire full-length ADAMDEC1 protein. Three mAbs recognize unique epitopes in the 172 amino acid prodomain (anti-proADAMDEC1 mAbs), four mAbs bind the 204 residue metalloprotease domain (anti-ADAMDEC1-MP mAbs) and three mAbs recognize the 60 residue disintegrin-like domain (anti-ADAMDEC-DIS mAbs). Although, the mAbs were raised against

ADAMDEC1 domains expressed in *E. coli*, they recognize recombinant, mammalian expressed ADAMDEC1 and are well suited for detection of endogenous ADAMDEC1 using ELISA or Western blot analysis.

Using the anti-proADAMDEC1 mAbs, we noted that a prodomain containing fragment appeared to be secreted with the mature protein during recombinant expression in HEK293 cells. Whether this is also the case *in vivo* might be questioned, due to the extent of overexpression obtained with HEK293 cells. However, for some ADAMs the prodomain is thought to serve as a chaperone assisting folding of the protease in the secretory pathway.[20, 21] Alternatively, the fragment may arise from limited proteolysis of an ADAMDEC1 proform secreted from the HEK293 cells.

Three of the four anti-ADAMDEC1-MP mAbs preferentially bind to the reduced form of ADAMDEC1 in Western blot analysis, whereas the fourth (mAb186) binds equally well to the reduced and non-reduced forms of ADAMDEC1. None of the MP-binding mAbs perturb trapping of ADAMDEC1 by α_2M which is surprising, since the covalent trapping of a protease by α_2M is believed to require proteolysis of the "bait-region" located inside the α_2M cavity,[19, 22] and binding of an antibody to a protease is likely to block its entrance to the "prey chamber". This observation may be explained by a combination of the long incubation period used in the assay and the relative low binding affinity for these mAbs to ADAMDEC1. The theoretical occupancy is estimated to be 55-80% which likely enables unbound ADAMDEC1 to react with α_2M. However, the anti-ADAMDEC1-MP mAbs do not influence the proteolytic activity of ADAMDEC1 against azocasein and Cm-Tf either, despite a high theoretical occupancy in the respective assays. Thus, the anti-ADAMDEC1-MP mAbs likely bind an epitope away from the catalytic site cleft.

In contrast to the MP-binding mAbs, the three anti-ADAMDEC-DIS mAbs are all capable of inhibiting thioester-mediated trapping of the protease by human α_2M. They divided into two sub-bins, where mAb129 and mAb177 compete with each other for binding to ADAMDEC1, and mAb111 is in a separate epitope bin. ADAMDEC1 does not require the disintegrin-like domain to be proteolytically active against Cm-Tf. However, mAb129 and mAb177 are both able to significantly reduce proteolysis of Cm-Tf suggesting that binding of mAb129 and mAb177 result in steric hindrance of substrate binding. Furthermore, mAb129 and mAb177 inhibited the cleavage of azocasein by ADAMDEC1 which may indicate that the disintegrin-like domain is located in the vicinity of the active site similar to ADAMTS-1 and not opposite the active site as in ADAM22.[23, 24] Interestingly, mAb111 has little effect on Cm-Tf cleavage, consistent with it representing a different functional bin than mAb129 and mAb177, and indicating that mAb111 can bind the disintegrin-like domain without negatively interfering with the active site. Furthermore, mAb111 appears to stimulate the cleavage of casein. The peculiar stimulatory properties of mAb111 may arise from a potential extension of ADAMDEC1 half-life in solution *in vitro*, perhaps by reducing protein aggregation or break-down. This effect would probably be most pronounced in the azocasein assay since this cleavage is rather slow and requires incubation for 72 hr before proteolysis can be measured. Alternatively, we suggest that mAb111 binds to ADAMDEC1 and promotes a conformation that increases activity towards casein.

ADAMDEC1 mRNA has been shown to be weakly expressed in monocytes, while high constitutive expression has been observed in macrophages. ADAMDEC1 mRNA expression in macrophages was previously shown to be up-regulated by stimulation with LPS or 1α,25-dihydroxyvitamin D_3. ADAMDEC1 is not detectable in immature dendritic cells, but the expression is induced by spontaneous or CD40- and LPS-mediated maturation.[1, 2] We were

able to extend on reported ADAMDEC1 mRNA expression findings by demonstrating secretion of mature ADAMDEC1 protein by unpolarised M0 macrophages. The apparent molecular weight of the endogenous ADAMDEC1 is near identical to the recombinant ADAMDEC1 expressed in HEK293 cells, indicating similar processing and glycosylation. The expression of ADAMDEC1 was unchanged in macrophages polarized using IFN-γ (M1) or IL-4 (M2). We do not observe secreted ADAMDEC1 protein in immature dendritic cells consistent with the findings of Mueller *et al.*[1]

Previously, a glycoproteomics study identified a glycosylated peptide in human plasma corresponding to the N-linked glycan in mature ADAMDEC1.[25] Using the identified anti-ADAMDEC1-DIS mAbs, we developed a robust, quantitative sandwich ELISA capable of measuring the ADAMDEC1 concentration in human plasma samples. The concentration determination was found to be dependent on the presence of EDTA in the plasma sample, where 1 mM EDTA is sufficient to reduce the quantification of ADAMDEC1 dramatically. We quantify ADAMDEC1 to be present in approximately 0.5 nM in pooled plasma/serum samples. As a biochemical validation, mature ADAMDEC1 protein was shown to be present in plasma by a pull-down experiment using mAb111. Compared to recombinant ADAM-DEC1 protein, plasma-derived ADAMDEC1 is of identical electrophoretic mobility suggesting similar or identical processing and glycosylation. The potential unprocessed ADAMDEC1 observed during recombinant expression is not seen in plasma pull-down suggesting that this species is an artefact of overexpression.

In conclusion, we generated monoclonal antibodies to human ADAMDEC1 representing different epitope bins. Antibodies binding the disintegrin-like domain have significant influence on the observed proteolytic activity of ADAMDEC1 and enabled visualization of

ADAMDEC1 protein secretion from primary macrophage cells. Moreover, mature ADAM-DEC1 is found to be present in human plasma.

MATERIALS AND METHODS

Materials- SDS-PAGE and Western blot materials (Novex/iBlot System) were from Invitrogen (CA, US). All Biacore reagents were from GE Healthcare (Uppsala, Sweden). Azocasein was from Sigma Aldrich (MO, US). Anti-ADAMDEC1 Ab57224 mAb was from Abcam (Cambridge, UK). Plasma-derived human α_2-Macroglobulin (α_2M) was a kind gift from Prof. Lars Sottrup-Jensen, Department of Molecular Biology and Genetics, Aarhus University, Denmark and Henrik Østergaard, Department of Haemophilia Biochemistry, Novo Nordisk A/S. Supernatant form monocyte, macrophage and dendritic cells were provided by Heidi Schiøler Schultz, department of Immunobiology, Novo Nordisk A/S.

Generation of antigens for immunization- ADAMDEC1 prodomain (residues 31-203) and mature protein (residues 204-470) were individually expressed in *E. coli* SHuffle (New England Biolabs, MA, US) as fusion-proteins with N-terminal maltose binding protein (MBP). The target proteins were purified by amylose affinity chromatography. In case of the ADAMDEC1 prodomain, MBP was removed using recombinant TEV protease, and the prodomain was purified by anion exchange chromatography. For mature ADAMDEC1, the MBP fusion protein (MBP-ADAMDEC1) was purified using size exclusion chromatography.

Mice and immunization- Two cohorts of 8-week-old RBF or NMRCF1 mice were immunized three times with two week intervals and boosted three days before isolation of the spleen. One cohort was immunized with recombinant MBP-ADAMDEC1 while the other was immunized with the prodomain. The first immunization was performed subcutaneously and consisted of 20 µg protein in 100 µl PBS (Invitrogen, MA, US) mixed with 100 µL Complete

Freund's adjuvant while the latter two was performed intraperitoneally with Incomplete Freund's adjuvant. The booster injections contained 20 µg protein in a total volume of 200 µL PBS and were injected intraperitoneally. Animal care and experiments were performed in accordance with institutional and national guidelines, and all mice were found to be free of specified pathogens in accordance with the FELASA recommendations for health monitoring of experimental units. The experiments have been approved by the Danish Animal Experiments Inspectorate (2012-15-2934-00352).

Generation of hybridomas by electrofusion- Spleen cells from the immunized mice and X63.Ag8.653 myeloma cells (ATCC #CRL-1580, for NMRCF1 derived splenocytes) or FOX-NY (ATCC #CRL-1732, for RBF derived splenocytes) were diluted in DMEM culture media to a concentration of 1.0E07 cells/mL. The cells were mixed 1:1 and washed three times followed by resuspension in Cyto Pulse Cytofusion Media (Cyto Pulse Sciences, Inc. MD, US) to a final concentration of 1.0E7 cells/mL. 9 mL were transferred to the fusion chamber and the electrofusion performed using a BTX ECM 2001 Electro cell manipulator (Harvard Apparatus, MA, US). After the fusion cells were left for 5 min in the fusion chamber followed by gentle transfer to 40 mL RPMI 1640 Glutamax^{TM} (Invitrogen, MA, US) media supplemented with 100 U/mL penicillin (Invitrogen, MA, US), 100 µg/mL streptomycin (Invitrogen, MA, US), 1 mM Na-pyruvate (Invitrogen, MA, US), 50 µM 2-mercaptoethanol (Invitrogen, MA, US), 2 % FCS (Gibco, MA, US), 0.2 % HFCS (Roche, Switzerland), 100 µM hypoxanthine (Sigma-Aldrich, MO, US), 0.4 µM aminopterin (Sigma-Aldrich, MO, US), and 16 µM thymidine (Sigma-Aldrich, MO, US) and incubation at 37 ˚C, 5 % CO_2 for 1 hr. Subsequently, the cells were diluted to 150,000 cells/mL and dispensed into 96-well plates (Greiner, Germany). During cultivation, the RBF-FOX hybridomas were kept under constant selection pressure using media supplemented with thymedine, adenine and

aminoptherin. The hybridomas were screen using ELISA against immobilized antigen and Western blot analysis 10 days after the fusion.

Subcloning, expression and purification of antibodies- The antibody-producing cells were subcloned by limiting dilution, seeding 1 and 5 cells/well in individual 96 well plates. The cell cultures were incubated at 37 ˚C and 5 % CO_2 for one week and visually inspected for single clones during the incubation period. The supernatants of the monoclonal cultures were tested in ELISA against the antigen, and one ELISA-positive clone from each culture was selected for mAb production. The conditioned medium was harvested by centrifugation at 4500xG and 4 ˚C for 8 min, filtered through at 0.22 µm filter and purified using a 1 mL mAb-select column (GE Healthcare, UK) according to manufacturer's protocol. The mAbs were dialyzed at 4 ˚C against 100 mM Hepes (pH 7.4) and 100 mM NaCl.

Antibody characterization- For testing antibody epitopes and binding affinity, human ADAMDEC1 variants were expressed in HEK293-6E using the FreeStyle 293 expression system as described previously.[16] For domain specific epitope mapping, the different ADAMDEC1 variants were blotted onto nitrocellulose membranes before blocking with 3 % (w/v) BSA. The antibodies to be tested were mixed with blocking buffer, added to the individual membranes and incubated for 16 h at 4 ˚C. For detection, 1 µg/mL HRP-conjugated anti-mouse IgG pAb (Dako, Denmark) was used. FAb fragments of anti-ADAMDEC1 antibodies were generated using the Mouse IgG1 FAb and $F(ab')_2$ Preparation Kit (Thermo Fisher Scientific, MA, US), and the prepared FAb fragments were analyzed for undigested mAb, F(Ab)2 fragment and free light chain using SEC-HPLC equipped with a MiniDawn Treos (Wyatt, CA, US) multi-angle light scattering detector. Binding affinities toward mature ADAMDEC1-WT immobilized on a CM5 chip were obtained using the Biacore T200 system. Binding experiments were performed in HBS-P^+ running buffer supple-

mented with 1 mg/mL BSA at 30 µL/min flow rate. The association and dissociation curves are fitted to a 1:1 binding model. Further, antibodies recognizing the disintegrin-like domain of ADAMDEC1 were separated into bins using a cross-competition assay analyzed by surface plasmon resonance. First ADAMDEC1 with an N-terminal HPC4 tag (HPC4-ADAMDEC1) was captured using immobilized anti-HPC4-tag mAb and the anti-ADAMDEC1 antibodies were analyzed for competitive binding by first capturing the individual mAbs before measuring binding to all mAbs one by one.

ADAMDEC1 proteolytic assays- The relative concentrations of ADAMDEC1 variants were evaluated by densitometric analysis of Coomassie Brilliant Blue stained SDS-PAGE using the Phoretix 1D software (Totallab, UK). In all functional assays, the antibodies were added to ADAMDEC1 samples in 2.2-5 fold molar excess and incubated for 1 h at 37 ˚C before the substrate was added. Proteolysis of human plasma-derived α_2M was carried out by incubating 25 nM ADAMDEC1 variant proteins with 1.25 µM α_2M in reaction buffer (50 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM $CaCl_2$, 5 µM $ZnCl_2$) for approx. 20 h at 37 ˚C. Cross linking of ADAMDEC1 to α_2M was visualized by reducing Western blot analysis using 1.31 µg/mL anti-HPC4-tag antibody and 1 µg/mL HRP-labeled goat anti-human IgG pAb (Perkin Elmer, MA, US). Carboxymethylated transferrin (Cm-Tf) was produced as previously described and used as a protease substrate by incubating with a final concentration of 500 nM ADAMDEC1 variants. The samples were analyzed by reducing SDS-PAGE, and densitometry analysis was carried out using 1D Phoretix (Totallab). Proteolytic activity against azocasein was measured by incubating 7.8 µM ADAMDEC1 and 17 µM antibody before adding 94 µM substrate. Reaction mixtures were incubated at 37 ˚C for 3 days. The proteolysis was terminated by the addition of 6 % (w/v) TCA followed by a 30-min incubation on ice and removal of undigested protein by centrifugation at 10,000xG and 4 ˚C

for 10 min. 0.26 mM NaOH was added to the supernatants and the absorbance was measured at 440 nm using a SpectraMax 190 (Molecular Devices, CA, US). Absorption data were subtracted buffer contribution and compared with the data of ADAMDEC1 wild-type (WT) in absence of any mAb using a one-way ANOVA analysis with Bonferroni's adjustment.

Establishment of sandwich ELISA- Nunc Immuno MaxiSorp 96 well plates (Thermo Scientific, MA, US) were coated with 1 µg/mL anti-ADAMDEC1 mAb in PBS buffer (pH 7.4) overnight at 4 ^{o}C and blocked with PBS blocking buffer (PBS buffer (pH 7.4) supplemented with 0.05 % Tween-20 (v/v) and 2 % (w/v) BSA) followed by incubation with 50 µL sample. For establishment and all standard curves the sample constituted recombinant ADAMDEC1 in PBS blocking buffer. Subsequently, each well was incubated first with 1 µg/mL biotinylated anti-ADAMDEC1 mAb and afterwards for 30 minutes with streptavidin-HRP (BD Pharmingen, USA) diluted 1:5000 in PBS blocking buffer. Binding was detected by addition of 100 µL TMB substrate (Kem-En-Tech Diagnostics A/S, Denmark) for 20 minutes before quenching the enzymatic reaction with 2M H_3PO_4. The absorbance was measured at 450 nm with 620 nm as reference on an Infinite M200 PRO NanoQuant (Tecan Trading AG, Switzerland) or a Spectramax plus 384 plate readers (Molecular Devices, CA, US). All incubations were performed at room temperature, shaking at 350 rpm and for 1 hour unless otherwise stated. Between each step, the wells in the plates were washed 4 times in PBS buffer supplemented with 0.05 % tween-20 at pH 7.4. Antibodies found suitable for coating where immobilized at different concentrations (0.5-5 µg/mL) and cross-matched against biotinylated detection mAb (0.2-2.4 µg/mL) to search for cooperating antibody pairs. The final ADAMDEC1 sandwich ELISA was established using 2 µg/mL mAb111 for coating and 1.2 µg/mL HRP-labelled mAb177 for detection.

The concentration of recombinant ADAMDEC1 used for calibration was evaluated by densitometric analysis of Coomassie Brilliant Blue stained SDS-PAGE using the Phoretix 1D software (Totallab, UK) with protein standard curves made from fully characterized preparations of both Maltose Binding Protein and soluble Tissue Factor. ADAMDEC1 concentration was estimated using gel densitometry analysis as previously described.[17] Spike recovery analysis was performed in both buffer and human pooled citrate plasma, and the lower limit of quantification (LLOQ) was identified where the total error (sum of nominal %RE and %CV) was less than 30 %.

Blood sampling and handling- Three different sources of standard pooled human citrate plasma were used: SMN 10446238 (Siemens AG, Germany), HemosIL calibration plasma 0020003700 (Instrumentation Laboratory, USA) and CRYOcheck pooled normal plasma CCN10 (Precision BioLogic Incorporated, USA). In addition, complete sets of serum and plasma stabilized with EDTA, citrate and heparin, respectively, from 7 different donors where analyzed using the established sandwich ELISA. The stability of native ADAMDEC1 in plasma where analyzed by subjecting the plasma and serum samples to three freeze/thaw cycles with thawing at either 37 ^{o}C for 2 minutes or on ice for 40 minutes and freezing at -80 ^{o}C. The plasma and serum was prepared from blood obtained from healthy Danish volunteers according to the agreement H-D-2007-0055 approved by the Danish Scientific Ethical Committee Region Hovedstaden.

Statistical analysis- ADAMDEC1 concentration in samples were calculated from a standard curve of log(concentration) vs. absorbance, fitted with a four parameter logistic equation in GraphPad Prism 6. The lower limit of quantification (LLOQ) was defined as the lowest used rADAMDEC1 concentration in which the recovery was 100 % +/-20 % and the

coefficient of variance (%CV) below 20 % along with the total error (defined by the sum of the accuracy and the %CV) below 30 %.

Immunoprecipitation of ADAMDEC1 from plasma- Dynabeads M-280 Tosylactivated (Life technologies, MA, USA) were coated with mAb111 for 18 hours at 37 ^{o}C according to the manufacture protocol. HemosIL calibration plasma 0020003700 were diluted 1:2 in Hepes buffer (20 mM Hepes, 150 mM NaCl at pH 7.4) with 5 mM EDTA, 0.4 mM hirudin and 1x cOmplete EDTA-free protease inhibitor cocktail (Roche Diagnostics, USA). 10 µL beads were washed three times in Hepes buffer before incubated with either 4 mL Hepes buffer in both absence and presence of 32 ng rADAMDEC1 or 1:1 diluted plasma. The samples were incubated overnight at 4 ^{o}C while rotating. The beads were retained by a magnet and the supernatants removed before the beads were washed three times with Hepes buffer supplemented by 0.05 % Tween-20. The protein was eluted by adding 10 µL 2x LDS sample buffer (Life technology, MA, USA) and heated for 10 minutes at 70 ^{o}C before the supernatants were subjected to Western blot analysis using mAb177 as primary antibody.

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Author contributions: JL, ME, MTO and HHP designed the study. JL and MGR generated the antibodies, JL performed activity experiments with guidance from LT and HN. AMEB and ML established the ELISA assay and measured plasma samples. All authors contributed to writing the manuscript.

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FIGURE LEGENDS

Figure 1. Mapping of anti-proADAMDEC1 and anti-ADAMDEC1 mAb epitope region. A: The ADAMDEC1 protein fragments (a-e) utilized for epitope mapping (see Table 1) [16]. ADAMDEC1 is modified by N-linked glycosylations at Asn61, Asn184 and Asn237 (black lollipops) [16]. B: Non-reducing Western blot analyses of the three anti-proADAMDEC1 mAbs (mAb21, mAb43 and mAb60) using four different ADAMDEC1 variants: Recombinant ADAMDEC1 R56A/R200K/R203A (RRR), R200K/R203A/E353A (RRE), R200K/R203A (RR) and wild-type (WT). Negative control: Supernatant from mock transfected HEK293 cells. Positive control: The *E. coli* expressed prodomain (the immunization antigen). C: Non-reducing Western blot analysis of anti-ADAMDEC1 mAbs against recombinant ADAMDEC1 wild-type (WT) and metalloprotease domain (MP) in HEK293 supernatant. As a negative control, the supernatant from mock transfected HEK293 cells was included.

Figure 2. Dependency of ADAMDEC1 disulfide bonds on mAb recognition. Western blot analysis comparing the binding of each anti-ADAMDEC1 mAb to non-reduced (NR) and reduced (R) recombinant ADAMDEC1 wild-type, respectively. The epitope-containing domain (MP or DIS) is indicated below the individual mAb names. A commercial anti-ADAMDEC1 mAb (Ab57224, Abcam, Cambridge, UK) was included.

Figure 3. Effect of anti-ADAMDEC1 mAbs on ADAMDEC1 proteolytic activity. A: Covalent trapping of HPC4-ADAMDEC1 WT by plasma-derived human α_2M in the absence or presence of the anti-ADAMDEC1 mAbs recognizing either the metalloprotease (MP) or the disintegrin-like (DIS) domain (stated in the figure). Trapping is visualised by reducing Western blot analysis using an anti-HPC4 mAb. B: Proteolytic activity of ADAMDEC1 WT

against azo-labelled casein in absence and presence of anti-ADAMDEC1-DIS mAbs. The individual measurements are shown in open circles, the mean as a horizontal line and standard deviation as vertical lines (n = 3). Roman numerals indicate statistically unique ($P < 0.005$) mean values as determined from one-way ANOVA analysis with Bonferroni's adjustment. C: Left part: Proteolysis of Cm-Tf in absence and presence of anti-ADAMDEC1-DIS mAbs. Right part: Proteolysis of Cm-Tf by inactive (E353A) and mature WT-like (C392S) forms of human ADAMDEC1 as well as the isolated MP-domain (C392S/A411stop). Cleavage is analyzed by reducing SDS-PAGE. D: Gel densitometric analysis of the Cm-Tf cleavage (β band) in triplicate. The generation of product band β is quantified and roman numerals indicate statistically unique values as determined from one-way ANOVA analysis with Bonferroni's adjustment.

Figure 4. Expression of ADAMDEC1 by human macrophages and immature dendritic cells. Expression of mature, human ADAMDEC1 was detected by non-reducing Western blot using biotinylated anti-ADAMDEC1-DIS mAb111 and avidin-HRP. The expression levels in macrophage (M0-2) and dendritic cell (DC) supernatants were compared to approx. 5 ng recombinant ADAMDEC1 wild-type (WT) expressed in HEK293 cells.

Figure 5. Dynamic range of ADAMDEC1 sandwich ELISA in buffer. Mean standard curve of mAb111/mAb177-HRP ELISA with the ADAMDEC1 concentration ranging from 0.01 nM to 1.55 nM. Error bars represent standard deviation (n = 6).

Figure 6. Identification and quantification of ADAMDEC1 in plasma. A: Quantification of ADAMDEC1 in plasma stabilised by EDTA, heparin or citrate along with serum from 7

donors. Lines connecting markers represent the individual donors. Concentration below LLOQ (grey area) is depicted as 0.5 times LLOQ (here 0.12 nM) in the graph. B: The effect of EDTA on the quantification (mean recovery) of ADAMDEC1 spiked into buffer, serum and plasma stabilized with heparin (n = 3-4). C: Immunoprecipitation of ADAMDEC1 protein from plasma and conditioned expression medium evaluated using Western blot analysis. ADAMDEC1 was precipitated using mAb111-conjugated Dynabeads and compared to approx. 32 ng recombinant ADAMDEC1 WT. mAb111-conjugated Dynabeads incubated with buffer was added as a control and exhibits elution of the capture mAb.

TABLE LEGENDS:

Table 1. ADAMDEC1 constructs used for evaluating domain-specific recognition of the anti-ADAMDEC1 and anti-proADAMDEC1 mAbs. Five ADAMDEC1 protein variants with different length was created by perturbing PC-mediated prodomain processing at PC1 (Arg56), PC2 (Arg203), and/or auto-proteolysis at the Pro161-Leu162 scissile bond as well as truncating the protein upstream of the disintegrin-like domain. SP: Signal peptide (residue 1-30). PRO: Prodomain (residue 31-203), MP: Metalloprotease domain (residue 204-410), DIS: Disintegrin-like domain (residue 411-470). Full-length numbering of human ADAMDEC1 is used.

Table 2: Binding kinetics of anti-ADAMDEC1 FAb fragments to immobilized ADAMDEC1 measured by SPR. The constants are calculated by fitting sensorgrams to a 1:1 binding model (n = 3) and the FAb fragments are sorted based on the measured binding affinity.

S1: Binding competition of Disintegrin-like domain binding anti-ADAMDEC1 mAbs

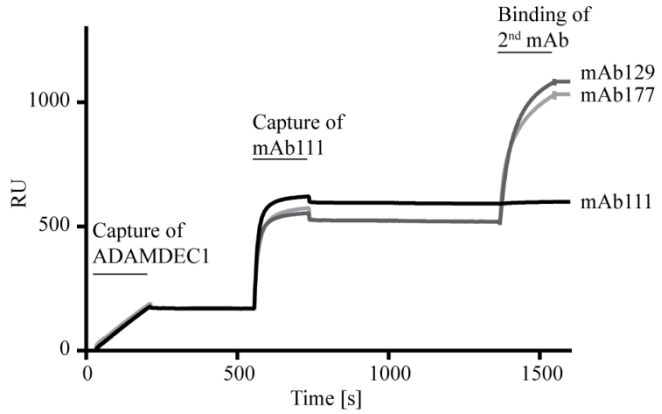

Figure S1: Example of competition binding experiment using Biacore T200. First ADAMDEC1 with an N-terminal HPC4 tag was captured using immobilized anti-HPC4-tag mAb. Cross-competitive binding was measured by saturating the captured ADAMDEC1 with one mAb (in this case mAb111) and subsequently measuring binding of the remaining anti-ADAMDEC1 mAbs.

S2: Surface plasmon resonance sensorgrams of anti-ADAMDEC1 FAb binding to immobilized ADAMDEC1

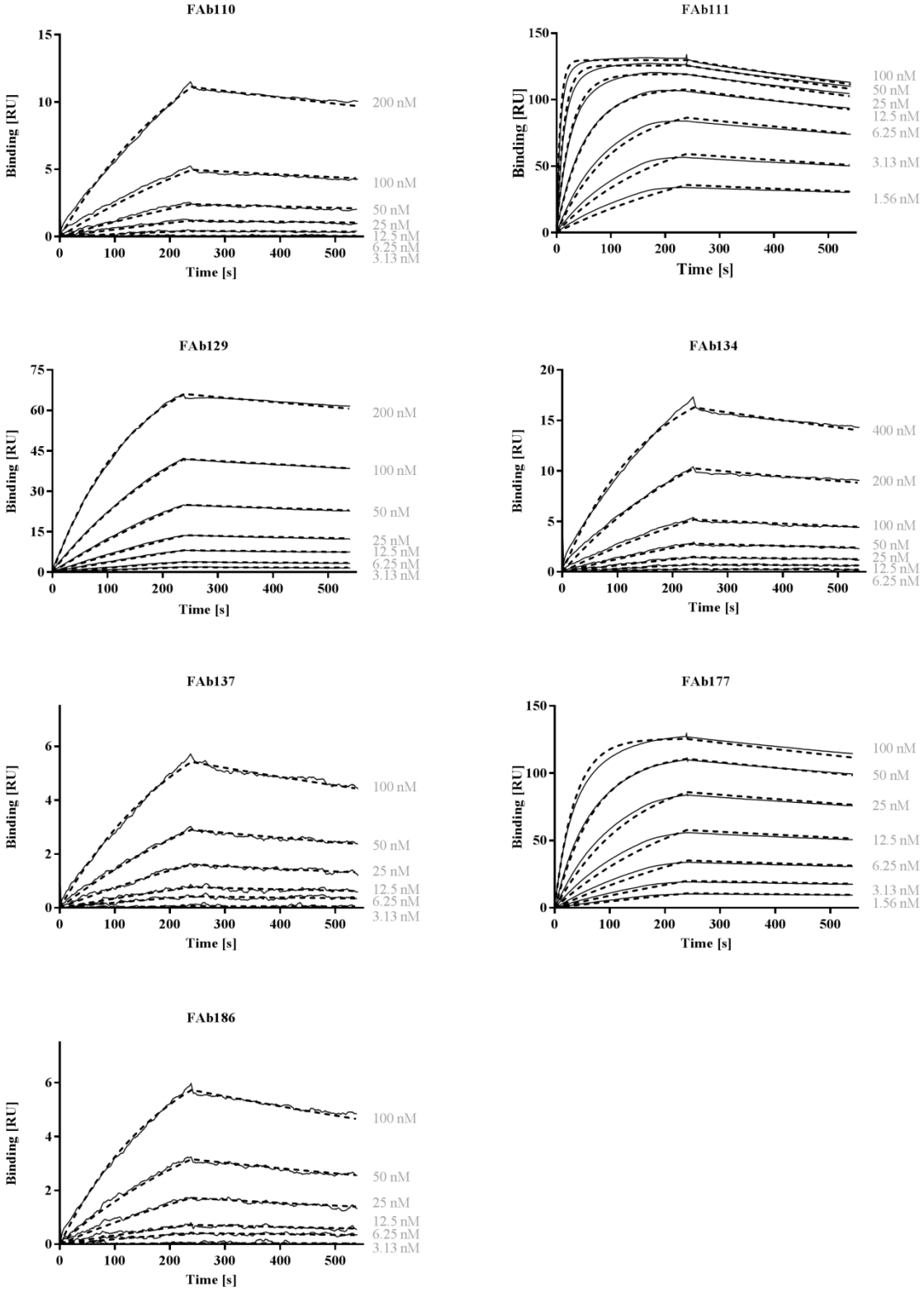

Figure S2: Binding of anti-ADAMDEC1 FAb-fragments to immobilized ADAMDEC1. Kinetic binding data (thin, black line) was fittet to a 1:1 binding model (dotted, black line). Fab concentrations are stated in grey font.

S3: Analysis of anti-ADAMDEC1 FAb fragment preparation using SEC-HPLC with multi-angle light scattering size-determination

Sample	Undigested mAb (%)	$F(Ab)_2$ fragment (%)	Fab fragment (%)	Free light chain (%)
FAb111	0.8	5.2	93.0	1.0
FAb137	0.4	7.3	91.5	0.9
FAb177	0.4	5.8	93.0	0.8
FAb186	0.3	5.0	93.3	1.3
FAb134	6.9	8.8	83.4	0.9
FAb110	1.0	7.3	90.8	0.9
FAb129	1.1	5.5	91.3	2.1

Table S3: Content of undigested mAb, $F(Ab)_2$ fragment and free light chain in the FAb fragment preparations as analysed by SEC-HPLC. The contented is stated in % of total protein content.

S4: The effect of mAb preparetion on Azocasein proteolysis in the presence and absence of ADAMDEC1

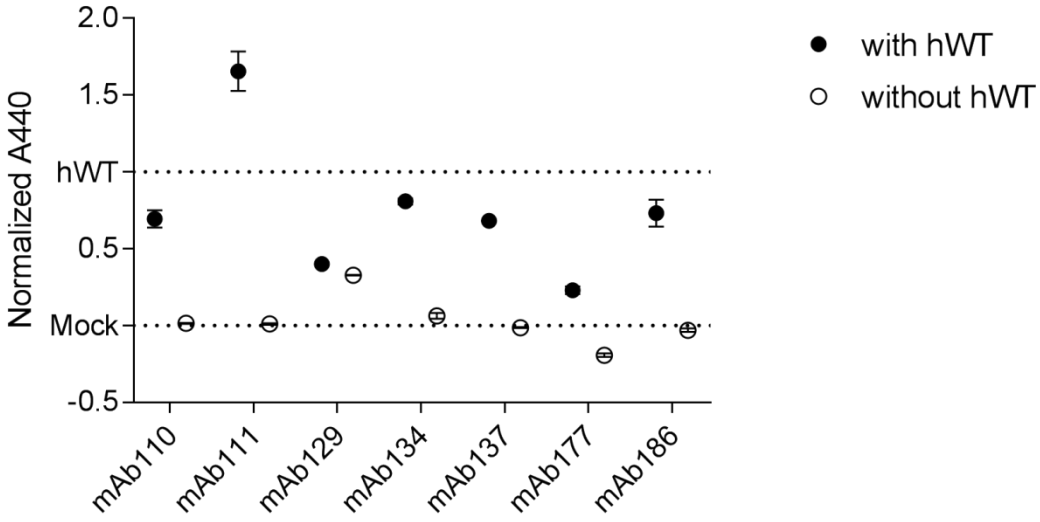

Figure S4: Effect of anti-ADAMDEC1 mAbs on Azocasein proteolysis in the absence and presence of ADAMDEC1 (hWT).

S5: Effect of all anti-ADAMDEC1 mAbs on ADAMDEC1 caseinolytic activity

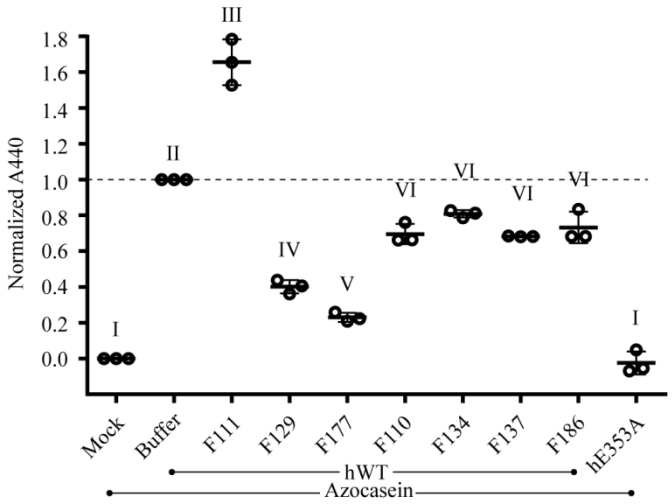

Figure S5: The effect of all identified anti-ADAMDEC1 mAbs on the caseinolytic activity of ADAMDE1 WT (normalized to 1, indicated by horizontal dotted line). The roman numerals describe different groups where the mean casienolytic activity is statistically significantly different ($P < 0.005$) between the different groups.

S6: Effect of all anti-ADAMDEC1 mAbs on ADAMDEC1 limited proteolysis of carboxymethylated transferrin

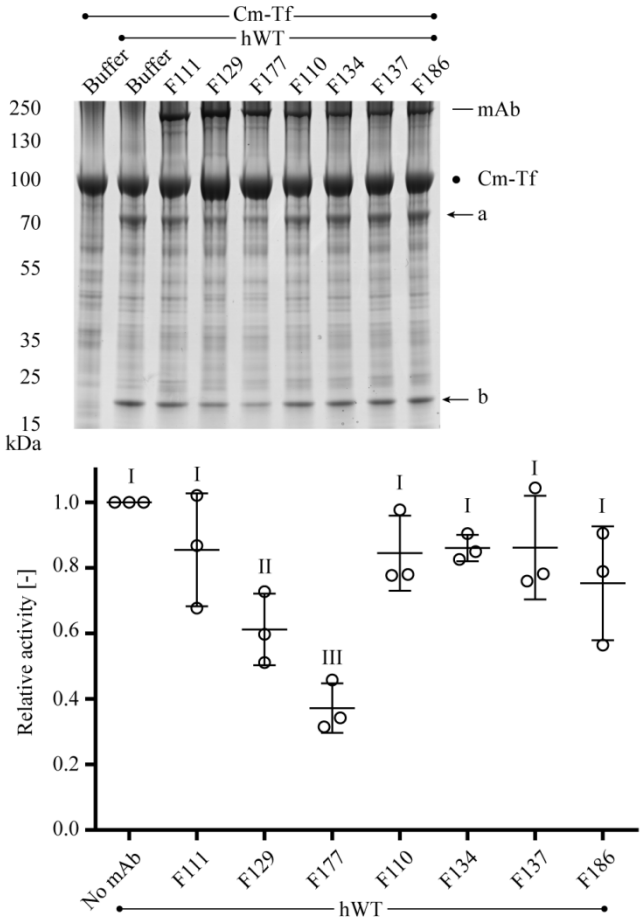

Figure S6: The effect of all identified anti-ADAMDEC1 mAbs on the limited proteolysis of carboxymethylated transferrin by ADAMDE1 WT (normalized to 1). The roman numerals describe different groups where the mean casienolytic activity is statistically significantly different ($P < 0.005$) between the different groups.

S7: Immunoprecipitation of ADAMDEC1 from plasma and conditioned expression medium using anti-ADAMDEC1-DIS and anti-proADAMDEC1 mAbs

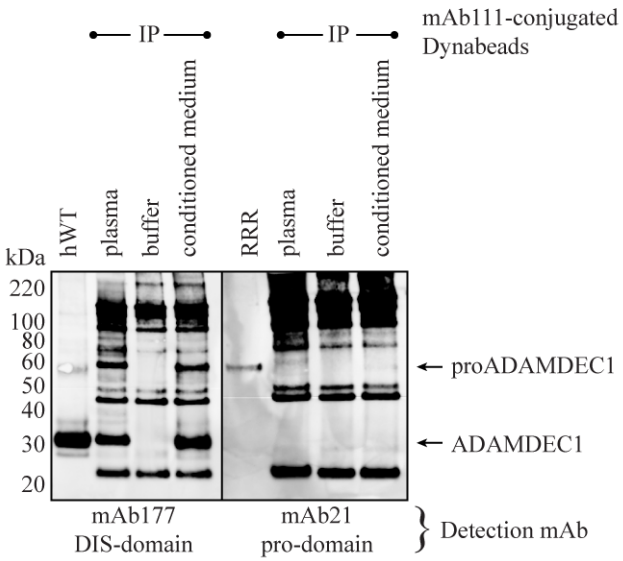

Figure S7: Immunoprecipitation of ADAMDEC1 protein from plasma and conditioned expression medium evaluated using Western blot analysis. ADAMDEC1 was precipitated using mAb111-conjugated Dynabeads and evaluated by Western blot analyses using both anti-ADAMDEC1-DIS mAb177 and anti-proADAMDEC1 mAb21. Immunoprecipitated protein was compared to recombinant ADAMDEC1 (hWT) and proADAMDEC1 (RRR, R56A/R200K/R203A). mAb111-conjugated Dynabeads incubated with buffer was added as a control and exhibits elution of the capture mAb.