JBC Papers in Press. Published on March 27, 2007 as Manuscript M608414200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M608414200

THE LOW AFFINITY IgE RECEPTOR (CD23) IS CLEAVED BY THE METALLOPROTEINASE ADAM10

George A. Lemieux[*], Fernando Blumenkron[§], Nolan Yeung[†], Pei Zhou[‡], Jason Williams[**], Amrie C. Grammer[§], Robert Petrovich[**], Peter E. Lipsky[§], Marcia L. Moss[¶‖] and Zena Werb[*‖]

[*]Department of Anatomy and Biomedical Sciences Program, University of California, San Francisco, CA 94143-0452

[†]Cognosci, Research Triangle Park, NC 27710

[‡]Duke University Medical Center, Durham, NC 27710

[§]B Cell Biology Group in the Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892.

****National Institute for Environmental Health and Sciences, Research Triangle Park, NC, 27710**

[¶] BioZyme Inc., Apex, NC 27523

Running Title: ADAM10 is a CD23 sheddase

[‖] To whom correspondence should be addressed: (M. L. M) Biozyme Inc. Apex, NC 27523. E-mail: mmoss@biozyme-inc.com. Phone: (919)362-1339. FAX: (919)362-1339, (Z. W.) Department of Anatomy, University of California, San Francisco, CA 94143-0452. Phone: (415) 476-4622. FAX: (415)476-4565. E-mail: zena.werb@ucsf.edu.

The low affinity IgE receptor, FcεRII (CD23) is both a positive and negative regulator of IgE synthesis. The proteinase activity that converts the membrane bound form of CD23 into a soluble species (sCD23) is an important regulator of the function of CD23 and may be an important therapeutic target for the control of allergy and inflammation. We have characterized the catalytic activity of ADAM (a disintegrin and metalloproteinase) 10 towards human CD23. We found that ADAM10 efficiently catalyzes the cleavage of peptides derived from two distinct cleavage sites in the CD23 backbone. TIMPs (tissue inhibitors of metalloproteinases) and a specific prodomain-based inhibitor of ADAM10 perturb the release of endogenously produced CD23 from human leukemia cell lines as well as primary cultures of human B cells. Expression of a mutant metalloproteinase-deficient construct of ADAM10 partially inhibited the production of sCD23. Similarly, siRNA (small inhibitory RNA) knockdown of ADAM10 partially inhibited CD23 release and resulted in the accumulation of the membrane-bound form of CD23 on the cells. ADAM10 contributes to CD23 shedding and thus could be considered a potential therapeutic target for the treatment of allergic disease.

The low affinity IgE receptor, FcεRII (CD23) is a 46 kDa type II membrane protein that is expressed on B-cells and cells of the myeloid lineage (1). CD23 has multiple functions. It is both a positive and negative regulator of IgE synthesis (2). It facilitates IgE-dependent antigen presentation through its binding of IgE-antigen complexes (3,4). Moreover, the release of proinflammmatory cytokines from macrophages is stimulated by CD23 binding to CD18/11b and 11c (complement receptor 3 and 4) (5-7).

In humans, two isoforms of CD23 that differ by only seven amino acids in the short N-terminal cytoplasmic domain are observed (8). CD23a is expressed only on B-cells. Stimulation of B-cells and cells of the myeloid lineage with interleukin-4 (IL-4) induces the expression of CD23b. The C-terminal extra cellular domain consists of a globular fold that has homology to the C-type lectin family (9). This globular domain has been shown to contain two distinct binding sites, one for IgE and a second that recognizes CD21 (complement receptor 2) (10,11). At the cell surface CD23 self assembles to form homotrimers that have a higher affinity for IgE than the CD23 monomer (12,13). The self association is driven by a leucine zipper-like domain (14) that connects the N-terminal cytoplasmic and transmembrane domains to the C-terminal globular domain.

Homotrimeric CD23 molecules exhibit a

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15 nm α-helical coiled coil stalk that extends the globular C-terminal domains from the plasma membrane (15). Cleavage in the stalk region by a membrane-associated endoproteolytic activity generates soluble fragments of CD23 (sCD23) that possess apparent molecular weights of 37, 33 and 29 kDa (16). All three of these sCD23 fragments exist as homotrimers (15). Smaller fragments of CD23 (25 and 16 kDa) are known. However, these are thought to be formed from cleavage of the larger soluble fragments by a cysteine proteinase (16). *In vitro* sCD23 promotes the differentiation of germinal center B-cells into plasma cells (17), stimulates IgE synthesis in B cells (18) and induces the secretion of proinflammatory cytokines in monocytes (6,19,20). *In vivo*, inhibition of proteolytic shedding of CD23 using small molecule metalloproteinase inhibitors has been shown to suppress IgE synthesis in *Scid* mice that have transplanted human peripheral blood lymphocytes (PBL) (21,22).

The identities of the proteinase activities responsible for generating the 33 and 37 kDa forms of CD23 remain elusive. Release of the 33 kDa fragment from the human B-cell line RPMI 8866 has been shown to be mediated by a membrane-associated metalloproteinase activity (23). It has been demonstrated that members of the disintegrin/metalloproteinase family (ADAM8, 15 and 28) are capable of cleaving CD23 (24). However, the peptide sequence used to test proteolytic activity was not derived from the known 33 and 37 kDa cleavage sites of CD23 (16). In this paper, we provide evidence that ADAM10 is catalytically competent in cleaving human CD23 at the known physiologic sites and sheds CD23 from human cell lines and primary cultures of human B cells.

EXPERIMENTAL PROCEDURES

Reagents - Commercial reagents were obtained from the following sources: Recombinant human IL-4 and recombinant human catalytic/disintegrin domains of ADAMs 8, 10 and 17 were from R&D systems (Minneapolis, MN). Tissue inhibitors of metallopreoteinases (TIMPs) were from R&D systems**.** TAPI-2 was obtained from Calbiochem (La Jolla, CA). Complete proteinase inhibitor cocktail was from Roche. All other chemicals, unless specified were obtained from Sigma/Aldrich (St. Louis, MO). DNP peptides (Table I) were either purchased from the University of North Carolina Chapel Hill Peptide Chemistry Department, or purchased from SynPep (Dublin, CA). Recombinant prodomain constructs of ADAM10 (A10 23-213 and A10 23-181) were expressed in *E.coli* and purified by Ni-Histidine affinity chromatography followed by gel filtration (M.L.M. manuscript in preparation).

Antibodies - Rabbit polyclonal antisera directed against ADAM8, 10 and 17 were from Chemicon (Temecula, CA). Rabbit polyclonal anti-hemagglutinin (HA) and anti-FLAG tags were from Sigma. Monoclonal anti-FLAG (M2) was from Stratagene (La Jolla, CA). Monoclonal anti-HA (HA.11) was from Covance (Berkeley, CA). Rabbit polyclonal ant-CD23 was from Anaspec (San Jose, CA). Human CD23 ELISA kits were from BD Biosciences (San Jose, CA) and Invitrogen (Carlsbad, CA).

PCR and Cloning - A clone of full length mouse ADAM8 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and used as a PCR template. The primers 5'-ATGCTTGGCCTCTGGCTGCTC-3' (forward) and 5'-CCTCTTCTGGATGGGGACCTTC-3' (reverse) were used to generate a PCR product that was cloned into a gateway recombinational cloning entry vector (pCR8/GW/TOPO, Invitrogen). Sequence encoding amino acids 228-397 was removed by digestion of the full length mADAM8 clone in pCR8 with *Acl*I and *Pml*I yielding a 1 kB fragment that was cloned into a 3.8 kB fragment of ADAM8-pCR8 produced by digestion with *Acl*I and *Hinc*II. The resulting metalloproteinase domain deleted (amino acids 228-397) ADAM8 (ADAM8ΔMP) construct was directionally recombined into a modified pQCXIP (Clontech, Mountain View, CA) that contained a Gateway acceptor cassette (Invitrogen) followed by a 3' FLAG epitope tag. Full length mouse ADAM10 and mADAM10ΔMP (amino acids 56-457 deleted) were amplified from plasmid templates using the primers 5'-ATGGTGTTGCCGACAGTGTTA-3' (forward) and 5'-GCGTCGCATGTGTCCCATT-3' (reverse). Mouse ADAM17 and ADAM17ΔMP (amino acids 189-378 deleted) were amplified using the primers 5'-ATGAGGCGGCGTCTCCTCATC-3' (forward)

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and 5'-AGCACTCTGTCTCTTTGCTGTCAACTCG-3'. All PCR products were cloned into pCR8/GW/TOPO, the sequences verified and directionally recombined into the gateway-modified pQCXIP vector. The catalytically inactive point mutant of ADAM10 (Glu385Ala) was generated by site directed mutagenesis of ADAM10 in pCR8/GW/TOPO using the mutagenic primers 5'-TCTCATATTACGTTTGCTCATGCAGTTGGA CATAACTTTGGATCT-3' (forward) and 5'-AGATCCAAAGTTATGTCCAACTGCATGAG CAAACGTAATATGAGA-3' (reverse) and the polymerase *Pfu*-turbo (Stratagene). Full length human CD23b was amplified from IL-4 stimulated U937 cell cDNA using the primers 5'TCAGACTCGAGCAGGAACTTGGAACAA-GCAGAATTTAGC-3' (forward, contains a 5' *Xho*I site) and 5'-TAGCAGGATCCCTATG-CGTAGTCTGGCACATCATATGGGTAAGAG TGGAGAGGGGCAGAG-3' (reverse, encodes a C-terminal hemagglutinin (HA) epitope tag followed by a *BamH*I site). The PCR product was digested with *XhoI/BamH*I and cloned into pIRES2eGFP (Clontech). The human ADAM10 siRNA construct is based on a previously described sequence (29). The construct targets the sequence 5'-GACAUUUCAACCUACGAAU-3'. Complementary oligonucleotides encoding the above sequence followed by a hairpin sequence (5'-TTCAAGAGA-3') then the reverse complement of the target sequence were annealed and cloned into the retroviral siRNA expression vector pSIREN-retro-Q (Clontech) The negative control construct pSIREN-retro-Qneg is commercially available from Clontech.

Determination of enzyme concentration - The substrate Dabcyl-LAQA(HomoPhe)RSC(Fluorescein)-NH2, 5-10 μM, was added from a 5 mM DMSO stock into buffer, 20 mM Tris, pH 7.5, and 0.0075% Brij 35. Enzyme (10 μl of a 1/30 diluted stock solution; 200 μg/ml), was added to a 96-well black-coated Costar plate. TIMP1, from a 7.6 μM stock solution was diluted to 1 μM in the above buffer. It was then serially diluted in 3-fold increments from 1 μM-3 nM. The diluted TIMP1, 10μl, was added to the enzyme and allowed to preincubate for 10-15 minutes, after which substrate in buffer, 80 μl, was added to initiate the reaction. Reaction progress was monitored continuously in a Cytofluor plate reader by following a change in fluorescence using an excitation of 485 nm and an emission of 530nm. Data were fit to the Morrison equation to calculate the enzyme concentration (45).

k_{cat}/K_M determination for DNP-peptide substrates - The specificity constant k_{cat}/K_M ($s^{-1}M^{-1}$)is calculated as:

Formula: $k_{cat}/K_M = M/(3600*Ae*Ce)$

M (counts/hr) is the slope of the net absorbance *vs* time curve in the early linear range,

Ae (counts) is the net increase in absorbance reading at reaction end, Ce (M) is the concentration of enzyme in reaction and 3600 is a conversion factor (seconds/hr)

Cleavage of DNP-peptide substrates - Recombinant ADAM proteinase (ADAM 8, 10 or 17), 5 μM, was diluted into 50 μl of buffer containing 20-100μM DNP substrate. The final concentration of enzyme varied from 50nM to 500nM. The reaction progressed at 37°C for 40-120 min. The reactions were quenched with an equal volume of 1% HFBA and spun to remove precipitated protein. Reaction products were run on a C18 Vyadac column using solvents containing 0.1% HFBA with an acetonitrile/water gradient. Peaks were detected with a wavelength of 350 nM. Cleavage sites were determined either by LC/MS (Synpep and Duke University) or by co-chromatography with standard products using two HPLC systems.

Cell Culture - U937 (UCSF cell culture core) and Ramos (provided by A. DeFranco, UCSF) cells were cultured in RPMI-1640 supplemented with glutamax (Invitrogen) and 10% fetal bovine serum (FBS) and penicillin/streptomycin. HEK293T (ATCC) and GP2-293T (Clontech) were cultured in DME-H21 (UCSF-cell culture core) supplemented with 2 mM glutamine and 10% FBS. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult normal volunteers by centrifugation of leukapheresis fractions over diatrizoate/Ficoll gradients (Sigma) and lysing red blood cells with ACK buffer (Quality Biological, Gaithersburg, MD). B cells were purified by negative selection using the Rosette-sep technique following the manufacturer's instructions (StemCell Technologies, Vancouver, Canada) and cultured (1 X 10^5cells/well, 0.5 X 10^6 cells/ml) in 96-well

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microtiter plates (Costar, Corning Life Sciences, Acton, MA) in RPMI medium (Invitrogen) supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml) and 5% FBS, 200 μl final volume, and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Retroviral transduction - Stable cell lines expressing dominant negative ADAM constructs and siRNA's were produced by retroviral transduction using vesicular stomatitis virus type G (VSV-G) enveloped viruses. The viruses were produced using a pantropic packaging system (Clontech). Retroviral expression vectors (pQCXIP or pSIREN-retro-Q) were cotransfected (Fugene-6, Roche, Palo Alto, CA) with a vector encoding the VSV-G envelope protein into GP2-293T packaging cells; 48 hr later, U937 cells were suspended in the conditioned medium from the packaging cells that was supplemented with 4 μg/ml polybrene; 24 hr after transduction, stable cells were selected with 2 μg/ml puromycin for 5 days.

CD23 Release from HEK293T cells - HEK293T cells (250,000/well) were seeded into a 24-well plate and allowed to attach overnight. Using Transfectin (Bio-Rad, Hercules, CA) cells were co-transfected with plasmids encoding C-terminal HA tagged CD23b (CD23-HA) and either full length ADAM10, ADAM10 (Glu385Ala), ADAM8, ADAM17 or empty vector (pQCXIP). The medium was replaced with culture media containing reduced FBS (1%) 24 hr after transfection. The reduced-serum supernatant was harvested 24 hr later. The conditioned medium was cleared by centrifugation (10,000 x g for 10 min) and supplemented with proteinase inhibitor cocktail (Complete). The supernatants were then concentrated 10-fold by centrifugal ultrafiltration (Microcon YM-3, Millipore, Billerica, MA). Cells were washed with cold PBS then lysed in RIPA buffer: 140 mM NaCl, 50 mM Tris-HCl pH 7.5, 10% glycerol, 2 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF and Complete proteinase inhibitor cocktail. The cell lysate was clarified by centrifugation (16,500 x g for 10 min). RIPA lysates and concentrated conditioned media were separated by SDS-PAGE (12% acrylamide gels) transferred onto PVDF membrane for western blotting using a monoclonal anti-HA antibody (HA.11, Covance), followed by secondary detection using a goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate and chemiluminescence detection using the ECL substrate (Amersham Biosciences, Piscataway, NJ).

CD23 release from U937 and Ramos cells - Cells (100,000 per well) were seeded in round-bottomed 96-well plates (Sarstedt, Newton, NC) in 0.2 ml of media supplemented with IL-4 and either inhibitors (A10 23-213 150-600 nM, GM6001 10-50 μM, TAPI-2 10-50 μM, TIMPs 250 nM) or vehicle controls (A10 23-213 buffer: 25 mM NaH_2PO_4 pH 7.0, 125 mM KCl, 75 mM NaCl 20% glycerol, PBS, or dimethyl sulfoxide). Cells were cultured for 24 hr. The conditioned medium was harvested by centrifugation (2000xg, 15 min). The conditioned medium was assayed by ELISA for soluble CD23 according to the manufacturers' instructions (BD Biosciences, Invitrogen).

Flow cytometric analysis of primary B-cell culutures treated with wtA10 23-213 - Purified B-cells were cultured for 12 hr in the presence or absence of IL-4 (100 ng/ml), wtADAM10 23-213 (1-5 μM) or matched diluent control (25 mM Tris-HCl pH 8, 100 mM NaCl, 10% glycerol, 0.1% β-mercaptoethanol). Cells were stained for expression of surface antigens following washing with 1% BSA/PBS. Cells were incubated for 30 minutes at 4°C in the dark with fluorochrome-conjugated mAbs: APC-CD19 (CALTAG, Burlingame, CA), CD23-PE, or an isotype matched control IgG1-PE (BD Biosciences, San Jose, CA) before washing to remove unbound mAb. Cells were resuspended in PBS and immediately analyzed with the use of the FACS Calibur (BD Biosciences, San Jose, CA). The software program FlowJo (Ashland, OR) was used to analyze data generated by flow cytometry.

Co-immunoprecipitation - HEK293T cells were seeded into 10 cm plates and allowed to adhere overnight. The cells were then co-transfected (Transfectin, Biorad) with expression vectors encoding ADAM10ΔMP-FLAG and CD23-HA or vector controls (pQCXIP or pIRES2eGFP). Forty-eight hr post transfection the cells were washed twice with PBS (all procedures at 4 °C), then lysed in co-ip buffer (50 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM CHAPS, 10% glycerol, 2 mM EDTA, 1 mM PMSF, Complete proteinase inhibitor cocktail). Lysates were clarified by centrifugation (16,500 x g, 20 min). Total protein concentration of the supernatants was adjusted to 1

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mg/ml with lysis buffer. Polyclonal sera (5 μg, Sigma) against either the HA or FLAG epitopes was added to 1 ml of lysate, incubated four 2 hr, then precipitated using protein-A-agarose (Invitrogen). The agarose beads were washed (3 x 1 ml) with co-ip buffer then treated with loading buffer, separated by SDS-PAGE and blotted onto PVDF. Membranes were probed with mouse monoclonal antibodies against the FLAG epitope (M2, Stratagene) or against the HA epitope (HA.11, Covance).

Stimulated CD23 release - U937 cells that were cultured for 24 hr with IL-4 (20 ng/ml) were washed and resuspended in PBS (5 x 10^6/ml). Either A10 23-213, A10 23-181, TAPI-2 or vehicle control (A10 23-213 buffer) was then added. Following addition of stimulus (ionomycin 1 μM, lysophosphatidic acid 10 μM, sphingosine-1-phosphate, phorbol myristate acetate 100 nM, adenosine triphosphate 0.25 mM, or vehicle controls: DMSO, PBS) cells were incubated at 37 °C for 45 min. The supernatants harvested following centrifugation (10 min at 1,000 x g) were then assayed for CD23 content by ELISA.

Time course for CD23 shedding - U937 cells (both vector control and ADAM10 siRNA transduced cells) were cultured with IL-4 overnight. Cells were washed twice with PBS and resuspended at a density of 500,000/ml in fresh growth medium. Cells were incubated at 37 °C for 1, 3, 5 and 7 hour time periods. After each time point clarified supernatants were obtained by centrifugation (10 min at 1,000 x g) and stored at -80°C until CD23 levels could be determined by ELISA.

RESULTS

In human serum, there are five observed molecular weights of sCD23: 37, 33, 29, 25 and 16 kDa. The cleavage sites that give rise to the 37 and 33 kDa forms of the receptor are known and were used to design N-terminal 2, 4 dinitrophenyl-labeled (DNP) peptide substrates. Cleavage of the peptides by recombinant human ADAM10 (rhADAM10) was monitored by HPLC separation of the cleavage reactions followed by colorimetric detection of the DNP-chromophore. The peptides used and resulting specificity constants (k_{cat}/K_M) that were determined for each cleavage reaction are reported in Table 1. The specificity constant for a DNP-labeled peptide spanning the TNF-α cleavage site was determined to be 1200 $M^{-1}s^{-1}$ and is comparable to the literature value of 2000 $M^{-1}s^{-1}$ reported for the N-terminal-acetylated peptide (25). The substrate DNP-HGDQMAQKSQST-NH2 is based on the cleavage site that gives rise to the 37 kDa form of sCD23. This substrate was cleaved by rhADAM10 with a specificity constant of 250 $M^{-1}s^{-1}$. The specificity constant of 250 $M^{-1}s^{-1}$ indicated that efficiency of the cleavage reaction was comparable in efficiency to that of DNP-EVHHGKLVTFFAE (350 $M^{-1}s^{-1}$) a peptide derived from the α-secretase cleavage site of amyloid precursor protein (APP) a known ADAM10 substrate (25). The peptide corresponding to the 33 kDa cleavage-site sequence of CD23 DNP-RAEQQRLKSQDL was cleaved in two places by rhADAM10 having specificity constants of 190 and 90 $M^{-1}s^{-1}$. The proteolysis by rhADAM10 appeared to be selective as other DNP-labeled peptides derived from the known cleavage sites of TNFRI (Table 1) and EGF, HB-EGF, IL-6R, TNFRII and L-selectin (data not shown) were not processed by this enzyme.

We also determined the cleavage site specificity that rhADAM10 exhibited toward the 37 and 33 kDa CD23 peptides and compared it to that of recombinant human ADAM8 (rhADAM8) and recombinant human ADAM17 (Table 2). The 37 kDa peptide was cleaved by both rhADAM10 and rhADAM8 at the physiological Ala-Gln site. The 33 kDa peptide was cleaved by rhADAM10 at both the physiological Arg-Leu site and a non-physiological Gln-Gln site. In contrast, rhADAM8 cleaved the 33 kDa peptide only at non-physiological Gln-Gln and Glu-Gln sites. Recombinant human ADAM17, the closest family member of ADAM10 did not possess activity towards either the 37 kDa or 33 kDa peptides but was able to cleave a peptide derived from the cleavage site of TNF-α (data not shown).

To examine ADAM10's ability to cleave CD23 in a cell based assay, we transiently co-transfected ADAM10 and CD23 into HEK293T cells. Either wild-type ADAM10 (A10), ADAM10 containing a point mutation (Glu385Ala) that renders the proteinase inactive (A10EA) or vector alone were co-transfected with C-terminal hemagglutinin (HA)-tagged CD23 (CD23-HA). After 48 hr, the conditioned medium was

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harvested and the cells were washed and solubilized in RIPA buffer. Samples from each transfection were separated by SDS-PAGE, transferred to PVDF membranes and analyzed by western blotting for soluble and membrane bound CD23-HA. The upper and lower panels in Fig. 1*A* show the conditioned medium and cell lysates respectively probed with an anti-HA antibody for the presence of CD23-HA. Transfection of A10 enhanced the accumulation of CD23-HA in the supernatant. Both the A10EA and the vector control tranfectants exhibited a background level of CD23 shedding that was partially inhibited using the broad spectrum metalloproteinase inhibitor TAPI-2. Quantification of the band intensity in each lane from multiple experiments indicated that transfection with A10 increased shedding by about fifty percent over the vector control transfection (Fig. 1*B*). In contrast, transfection with the catalytically inactive A10EA did not increase sCD23 production relative to the vector control. Cotransfection of HEK293T cells with ADAM8 (A8) and CD23-HA resulted in a marked increase in soluble CD23 in the medium (Fig. 1*C*), as reported previously (24). In contrast, cotransfection of ADAM17 (A17) marginally affected shedding of CD23-HA from these cells.

The expression of endogenous CD23 is positively regulated by IL-4 in many types of leukocytes. However, the effect of IL-4 on ADAM10 expression is unknown. We cultured U937 promonocytic leukemia cells in the presence of IL-4 for 24 hr and analyzed the expression of ADAM10 in cell lysates by western blotting (Fig. 2, *top panel*). IL-4 slightly increased the amount of ADAM10 present in the lysates. However, it was a much stronger positive regulator of CD23 (Fig. 2, *middle panel*). Increases in sCD23 upon exposure to IL-4 are more likely due to the elevated expression of CD23 than to changes in the levels of ADAM10.

The effect of inhibitors of metalloproteinases on CD23 shedding was assessed in U937 promonocytic leukemia cells and Ramos cells, a human B-cell line and primary cultures of human peripheral blood B cells. The cell lines were cultured for 24 hr in the presence of IL-4 and each inhibitor. The amount of sCD23 in the conditioned medium was then determined by ELISA. In both cell lines the broad spectrum metalloproteinase inhibitor GM6001 appeared to inhibit 50-60% of CD23 shedding (Fig. 3*A*, 3*B*). A second broad spectrum inhibitor TAPI-2 was slightly more potent than GM6001 (70% inhibition) in U937 cells (Fig. 3*A*). Recombinant tissue inhibitors of metalloproteinases (TIMP) 1, 2 and 3 all exhibited modest inhibitory activity toward CD23 shedding in U937 cells, with TIMPs 1 and 3 being more potent than TIMP-2. In Ramos cells TIMP-1 inhibited CD23 release (Fig. 3*B*) to a similar extent as in U937 cells.

The recombinant pro-domain of ADAM10 (amino acids 23-213, wtA10 23-213) is a potent, selective inhibitor (K_i = 48 nM) of ADAM10 *in vitro* and in cell culture (M.L.M. manuscript in preparation). Mutation of the cysteine switch region (Cys173Ser) improves the stability of the reagent and maintains the potency (K_i = 36 nM) and selectivity towards ADAM10. At concentrations of 600 and 300 nM the Cys173Ser mutant (A10 23-213) inhibited shedding of CD23 from U937 cells by about 30% and Ramos cells by about 20% (Fig. 3*A*, *B*). At lower concentrations the inhibitory activity diminished in this cellular assay of CD23 shedding. A truncated prodomain (A10 23-181), based on the full length C to S mutant of ADAM10, is a poor inhibitor of the metalloproteinase *in vitro* (Ki = >5μM) and weakly inhibited sCD23 production (Fig. 3*A*, *B*).

Primary cultures of peripheral blood B cells isolated from normal healthy adults were treated either with wtA10 23-213 or vehicle control and cultured with and without IL-4 for 12 hr at 37 °C. The cultures were then stained with fluorochrome-labeled anti-CD19 and anti-CD23 and analyzed by flow cytometry. In the absence of IL-4, treatment of the cultures with wtA10 23-213 induced a shift in the percentage of CD23/CD19 double-positive cells from 38% in the diluent control sample to 56% in the wtA10 23-213 treated sample (Fig. 3*C*). Conversely, the percentage of CD19 positive, CD23 negative cells decreased from 53% in the diluent control sample to 36% in the wtA10 23-213 treated sample. Treatment with the proteolytically inactive A10 23-181 did not affect the percentage of positive cell (data not shown). The density (determined by mean fluorescence intensity) of CD23 on the double positive cells was found to be insensitive to the presence of wtA10 23-213. IL-4 treated cells responded differently to wtA10 23-213 in that treatment with wtA10 23-213 did not affect the

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percentage of CD23/CD19 double positive cells in the sample, but it increased the density of CD23 present on the CD23/CD19 double positive cells (Fig. 3*D*). IL-4 stimulated, wtA10 23-213 treated CD19+ cells exhibited 2.5 fold greater mean density of cell surface CD23 than the vehicle control treated cells. Prolonged treatment of IL-4 stimulated cells (36 hr) with wtADAM10 23-213 increased the percentage of cells that were CD23 positive from 83% in the diluent control to 98% in the inhibitor treated sample (data not shown)

The ADAMs family of proteinases is also involved in the shedding of cell surface proteins that is stimulated by various small molecule agonists. Cells cultured overnight with IL-4 were washed and treated with various agonists for 45 min at 37 °C. After centrifugation, the sCD23 in the cleared supernatants was determined by ELISA (Fig. 4*A*). Both phorbol 12-myristate 13-acetate (PMA) and lysophosphatidic acid (LPA) strongly induced shedding. Other reported agonists of shedding such as adenosine triphosphate (ATP) and ionomycin only weakly induced shedding of CD23 from U937 cells. Sphingosine-1-phosphate (S-1-P) did not stimulate shedding. Both A10 23-213 and TAPI-2 were used to evaluate the contribution of ADAM10 and metalloproteinases in general to the stimulated shedding of CD23 (Fig. 4*B*). The weakly induced shedding by ATP and ionomycin was inhibited slightly more by A10 23-213 than the unstimulated control whereas inhibition by TAPI-2 was relatively constant. In the samples strongly stimulated by LPA and PMA, A10 23-213 was a poorer inhibitor of CD23 release than in the unstimulated control. While TAPI-2 inhibited PMA induced shedding in a manner comparable to the unstimulated control, it was a somewhat weaker inhibitor of LPA-induced shedding.

Mutant versions of ADAM proteins that lack metalloproteinase domains have been shown to function as dominant-negative alleles that suppress shedding of proteinase substrates (26-28). We examined the effect of metalloproteinase-deleted alleles of ADAM8 (A8ΔMP), ADAM10 (A10ΔMP) and ADAM17 (A17ΔMP) on the shedding of CD23 from U937 cells. The mutant alleles were cloned into a retroviral expression vector (pQCXIP) and transduced into U937 cells by retroviral delivery. Puromycin-resistant cells were then stimulated with IL-4 overnight, and the conditioned medium was assayed for the presence of sCD23 (Fig. 5*A*). Both A8ΔMP and A10ΔMP exhibited significantly reduced (20-25%) production of sCD23 compared to the vector control cells. The A17ΔMP transduced cells produced similar amounts of sCD23 to the vector control cells. These results are consistent with both ADAM8 and 10 being CD23 sheddases and ADAM17 having no activity towards this protein.

We also found that A10ΔMP physically associated with CD23 (Fig. 5*B*). A C-terminal FLAG-tagged ADAM10 construct (A10ΔMP-FLAG) was co-transfected with CD23-HA into HEK293T cells. The transfected cells were cultured for 48 hr then washed with PBS and lysed in co-ip buffer. The extracts were then cleared by centrifugation and the supernatants were immuno-precipitated using polyclonal sera against the respective epitope tags. The precipitated complexes were separated by SDS-PAGE and transferred to PVDF membranes. When CD23-HA and A10ΔMP-FLAG were co-transfected, immunoprecipitation of CD23-HA resulted in the co-precipitation of A10ΔMP (Fig. 5*B*, top left panel). In the absence of transfected CD23-HA, A10ΔMP-FLAG was not detectable following precipitation. In both lysates A10ΔMP-FLAG was expressed, although A10ΔMP-FLAG expression levels were considerably higher in the absence of CD23-HA (Fig. 5*B* lower left panel). Immunoprecipitation of A10ΔMP-FLAG resulted in the co-precipitation of CD23-HA in cotransfected cells (Fig. 5*B* upper right panel). In the absence of transfected A10ΔMP-FLAG, a residual amount of CD23-HA still precipitated. The expression level of CD23-HA was similar in both lysates.

Endogenous ADAM10 in U937 cells was depleted using RNA interference. A construct encoding a hairpin siRNA specific for human ADAM10 (29) was cloned into a retroviral RNAi vector and transduced into U937 cells along with a control siRNA. The stably transduced cells exhibited ADAM10 protein levels depleted approximately 50 % compared to the control cells (Fig. 6A). Overnight stimulation of these cells with IL-4 followed by assay of the conditioned media using a CD23 ELISA revealed that the amount of CD23 produced was reduced by 35% in the conditioned medium from the ADAM10

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siRNA-treated cells versus the control cells (Fig. 6*B*). Both the ADAM10 siRNA cells and the control cells were cultured with and without IL-4 and/or TAPI-2 for 24 hr. The concentrated conditioned medium and whole cell lysates were separated by SDS-PAGE and transferred to PVDF membrane for western blotting with CD23 antiserum (Fig. 6*C*, top two panels) or actin antiserum (loading control, Fig. 4*C*, bottom panel). In the absence of IL-4, very little CD23 was present in both the conditioned medium (top panel) and cell lysates (middle panel). In the presence of IL-4, CD23 was induced in the both the conditioned medium and in the cell lysate. In the conditioned medium the CD23 appeared to migrate with an apparent molecular weight of 37 kDa. In the cell lysate CD23 appeared to migrate at approximately 45 kDa. The accumulation of the 37 kDa form of CD23 in the conditioned medium was inhibited using the broad spectrum metalloproteinase inhibitor TAPI-2. Conversely, treatment of IL-4 stimulated cells with TAPI-2 led to an increase in the amount of higher molecular weight form CD23 associated with the cells. Compared to the control cells, the ADAM10 depleted cells produced less of the 37 kDa soluble form of CD23 than the control cells. However, the ADAM10 depleted cells possessed more of the cell-associated 45 kDa form of CD23 than the wild-type cells. Quantification of the blots revealed that the ratio of sCD23 to mCD23 in the ADAM10 depleted cells was approximately 0.4 while that of the control cells was 1.4.

We also assessed the rate of release of soluble CD23 from ADAM10 depleted versus wild-type cells. Cells were stimulated for 24 hr then washed with medium and re-suspended in fresh medium and incubated at 37^{o}C s for 1-7 hr. The conditioned medium at each time point was then used to assay soluble CD23 production by ELISA. A plot of CD23 concentration in the conditioned medium versus time shows that the rate of sCD23 production is lower in the ADAM10 depleted cells versus the control cells (Fig. 6*D*) The control cells produced CD23 at a rate of 0.19±0.04 ng•ml^{-1}•hr^{-1}. In contrast, the ADAM10 depleted cells released soluble CD23 at a rate of 0.11±0.02 ng•ml^{-1}•hr^{-1} and the TAPI-2 treated control cells released sCD23 at a rate of 0.02±0.01 ng•ml^{-1}•hr^{-1}.

DISCUSSION

Proteolytic shedding of membrane proteins is an evolutionarily conserved post-translational modification that regulates the physiological activity of numerous proteins (30). The ADAMs family of disintegrin/metalloproteinases has been shown to catalyze many of these cell surface endoproteolytic events. The cellular and physiological processes that are directly regulated by ADAMs include growth factor activation, chemokine mobilization, cell fate specification and cell adhesion (31-33). Release of the 33 kDa sCD23 fragment from the human B-cell line RPMI-8866 has been shown to be mediated by a membrane-associated metalloproteinase activity (23). ADAM 8 has been shown to be capable of cleaving CD23 when co-transfected in HEK293 cells(24). However, the peptide sequence used to test activity of ADAM8 on CD23 was not derived from the known 33 and 37 kDa cleavage sites of CD23 (16). In spite of sCD23's potential role in allergic and inflammatory diseases, the identity of the proteinase activities responsible for generating sCD23 remains elusive.

We investigated shedding of CD23 in human cells because the functional significance of mouse sCD23 is unclear. Serum IgE levels are elevated in CD23 knockout mice indicating that CD23 is a negative regulator of IgE *in vivo* (34). However, transgenic mice that over-express mouse sCD23 exhibit no apparent IgE phenotype (35). Several biochemical differences between human and mouse CD23 may explain this disparity. Mouse CD23 is 55% identical to human CD23 at the amino acid level. The location and identity of the cleavage sites of mouse sCD23 are not conserved with human CD23 (36). Human sCD23 stimulates IgE synthesis *in vitro* through binding CD21 (18). Recent structural data predict that mouse sCD23 lacks a CD21 binding site (10). In addition, mouse sCD23 binds to IgE with an affinity that is more than 10-fold weaker than human CD23-IgE interaction (36). Because of these disparities, mice may not adequate models for the function of sCD23 in IgE homeostasis.

Identification of the cellular proteinase that cleaves a particular substrate is facilitated when the physiological cleavage site of that substrate is known. The peptide cleavage data indicate that ADAM10's catalytic domain

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possesses the catalytic capability to cleave CD23 directly at sites that are known to be physiologically relevant. There is evidence that cleavage of protein substrates by ADAMs likely involves ADAM-substrate interactions outside of the catalytic domain (37). We have demonstrated that a metalloproteinase-deficient mutant of ADAM10 co-immunoprecipitates with CD23 suggesting that ADAM10 and CD23 are present within the same complex in the cell. Thus, there is strong support for a direct action of ADAM10 on CD23.

Cell based assays support ADAM10's cellular role in shedding CD23. While overexpression of ADAM10 enhanced shedding of CD23, inhibition of ADAM10 function through either the use of specific inhibitors, dominant negative ADAM10 constructs or depleting ADAM10 by siRNA led to a reduction in CD23 shedding. However, depletion of cellular ADAM10 was not complete in cells transduced with the ADAM10 siRNA. Because targeted interference with ADAM10 function did not reduce CD23 shedding as much as the use of broad spectrum metalloproteinase inhibitor TAPI-2, inhibition of its activity may have been incomplete. Shedding of integral membrane proteins may take place within intracellular compartments as well as at the cell surface (38). While ADAM10 23-213 is a potent, specific ADAM10 inhibitor *in vitro,* its polypeptide composition may render it cell impermeable and thus may not efficiently inhibit ADAM10 in a cell-based assay. The residual ADAM10 activity may account for at least part of the remaining CD23 sheddase activity in these cells.

A second reason for the failure of targeted inhibition of ADAM10 to inhibit CD23 shedding completely in the cell-based assays may be the presence of other proteinases that are functionally redundant with ADAM10. This phenomenon has been observed for other ADAMs substrates (39). For example, utilizing both cell-based assays and peptide cleavage assays, it has been demonstrated that the amyloid precursor protein APP can be cleaved by ADAM8**,** ADAM9, ADAM10 and ADAM17 (40). Mouse embryonic fibroblasts from *Adam10* $^{-/-}$ mice process APP normally (41). However, the phenotypes resulting from the overexpression of ADAM10 as well as dominant negative ADAM10 in a mouse model of Alzheimer's disease are consistent with ADAM10 being a physiological APP sheddase (42). Stimulated shedding of CD23 from U937 cells by different small molecule agonists was differentially inhibited by the A10 23-213, indicating that different stimuli activate different CD23 processing activities. This cellular behavior has been observed with other ADAMs substrates such as CD44 (43). Both the Ramos and U937 cells express RNA transcripts for almost every proteolytically active member of the ADAMs family including several previously characterized CD23 sheddases. The fact that CD23 is processed physiologically at several sites and several ADAMs are expressed in the cell types that express CD23 make it probable that CD23 shedding is a process controlled by several enzyme activities.

In summary, we have shown that ADAM10 is a physiological CD23 sheddase. The targeted inhibition of ADAM10 *in vivo* may induce the accumulation of mCD23 and reduce levels of sCD23. Since mCD23 inhibits IgE synthesis and sCD23 stimulates IgE synthesis, alteration of the proteolytic balance between mCD23 and sCD23 through targeted inhibition of ADAM10 may prove to be an important therapeutic intervention point for controlling allergic disease.

Note added in proof:

While this manuscript was under review, an article was published (46) that found ADAM10 to be the major CD23 sheddase activity in mice.

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FOOTNOTES

This work was supported by funds from the National Institutes of Health (AI053194, HL75602 to ZW), the Sandler Family Sustaining Foundation and an Institutional National Research Service Award (HL07731).

Abbreviations: ADAM, a disintegrin and metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; DNP, 2, 4-dinitrophenyl; siRNA, small inhibitory RNA; TAPI-2, N-(R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-*t*-butyl-alanyl-L-alanine, 2-aminoethyl amide.

FIGURE LEGENDS

Fig. 1. Co-transfection of ADAM10 with CD23 enhances sCD23 production. *A,* HEK293T cells were transiently transfected with CD23-HA and A10, A10EA, or the empty expression vector as a control. Conditioned medium *top panel* and cell lysates *middle panel* were subjected to western blotting using an anti-HA monoclonal antibody. Lysate blots were also probed with and anti-FLAG antibody *bottom panel* to detect the tagged ADAM10 constructs. *B,* Densitometric analysis was performed on blots from three different experiments. The A10 and A10EA lanes were normalized to the vector control lanes to allow comparisons across multiple experiments. *C,* HEK293T cells were transiently transfected with CD23-HA and either A8, A17 or empty expression vector as a control. Conditioned medium *top panel* and cell lysates *middle panel* were subjected to western blotting using an anti-HA monoclonal antibody. The expression of the metalloproteinases were analyzed using ADAM8 and ADAM17 antisera.

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Fig 2. Influence of IL-4 on CD23 and ADAM10 expression. U937 cells were cultured with and without IL-4 (20 ng.ml). After 24 hr the cells were lysed and separated by SDS-PAGE. The western blot was probed with ADAM10 antisera *top panel*, CD23 antisera *middle panel,* and actin antisera *bottom panel*.

Fig. 3. CD23 release is inhibited in both human leukemia cell lines and primary human B-cell cultures by metalloproteinase inhibitors. *A*, U937 cells were cultured with IL-4 (20 ng/ml) and in the presence of broad spectrum metalloproteinase inhibitors TAPI-2 and GM6001 (20 μM), recombinant TIMPs 1-3 (250 nM), a potent, specific ADAM10 inhibitor (A10 23-213, 150, 300 and 600 nM) and a weak ADAM10 inhibitor (ADAM10 23-181, 600 nM). After 24 hr the conditioned medium was harvested and soluble CD23 was assayed by ELISA. *B*, Ramos cells treated treated as described in panel *A*. In panels A and B data are the mean +/- the SEM of three independent experiments *C*, Primary peripheral blood B-cells were cultured with wtA10 23-213 (5 μM, black line) or vehicle control (gray line) for 12 hr and stained with APC-anti-CD19, anti-CD23-PE or isotype matched control antibody (dotted line). Data represent CD23 expression by CD19+ B cells. *D*, Primary peripheral blood B-cells were cultured with IL-4 (50 ng/ml) and either wtA10 23-213 (1 μM, black line) or vehicle control (gray line) for 12 hr. Cells were stained with APC-anti-CD19, anti-CD23-PE or isotype matched control antibody (dotted line). Data represent CD23 expression by CD19+ B cells.

Fig. 4. Stimulated shedding of CD23 in U937 cells is inhibited by metalloproteinase inhibitors *A*, sCD23 was quantified in supernatants by ELISA from IL-4 stimulated U937 cells that were treated for 45 min at 37 °C with either no stimulus, ATP (0.2 mM), ionomycin (1 μM), sphingosine-1-phosphate (S-1-P, 1 μM), lysophosphatidic acid (LPA, 10 μM), phorbol ester (PMA, 100 nM). *B*, Inhibition of stimulated CD23 shedding assayed as in A by A10 23-213 (500 nM) and TAPI-2 (20 μM). Data are the mean ± standard deviation in both *A* and *B*.

Fig. 5. A dominant-negative construct of ADAM10 interacts with CD23 and partially inhibits shedding of CD23. *A* U937 cells expressing mutant ADAMs lacking the proteinase domains (A8ΔMP: amino acids 228-397 deleted, A10ΔMP: amino acids 56-457 deleted, A17ΔMP: amino acids 189-378 deleted) or empty expression vector were cultured with IL-4 (20 ng/ml) for 24 hr. The amount of sCD23 in the conditioned medium was assessed by ELISA. Two-tailed unpaired *t*-test: * $P = 0.0031$, ** $P < 0.0001$. *B,* HEK293T cells were co-transfected with CD23-HA and FLAG-tagged A10ΔMP or empty vector. Two days later, cell lysates were immunoprecipitated with anti-HA and western blots were probed with anti-FLAG (*left panel*) or immunoprecipitated with anti-FLAG and blots were probed with anti-HA (*right panel*).

Fig. 6. Depletion of endogenous ADAM10 by RNAi reduces the amount of 37 kDa sCD23 that is produced by U937 cells, and increases the cell associated 46 kDa CD23. *A*, Western blot of whole cell lysates from A10 siRNA cells and control cells using anti-ADAM10 antibodies (*top panel*) the pro and mature forms of the enzyme are indicated with arrowheads. The blot was stripped and reprobed with anti-ADAM17 antibodies (*lower panel*). *B*, Relative amounts of sCD23 produced by control and A10 siRNA cells determined by ELISA *C*, Western blots of conditioned medium (*top panel*) and whole cell lysate (*middle panel*) from A10 siRNA and control cells probed with anti-CD23 antibodies. Cells were cultured in parallel with and without IL-4 (20 ng/ml) as well as TAPI-2 (50 μM). The western blot of the whole cell lysate was stripped and reprobed with anti-actin antibodies to control for loading (*bottom panel*). *D*, The amount of sCD23 released from A10 siRNA cells (*triangles*), control cells (*squares*), and control cells treated with TAPI-2 (50 μM, *circles*) as a function of time. Cells were cultured with IL-4 overnight, then washed and incubated at 37 °C for 1, 3, 5 and 7 hr. sCD23 in the supernatant was determined by ELISA.

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TABLES

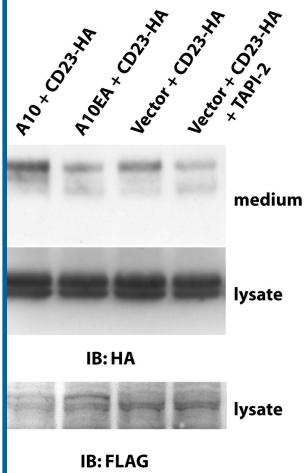
Table 1. Recombinant human ADAM10 processing of peptide substrates.
* indicates non physiological cleavage. Units for k_{cat} / K_M are in $M^{-1}s^{-1}$

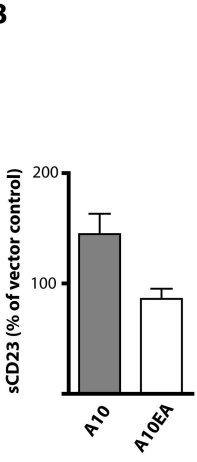
Protein	Peptide	k_{cat} / K_M
APP	DNP-EVHHQKLVFFAE	350 +/-150
CD23 33 kDa	DNP-RAEQQRLKSQDL	190 +/- 20 90 +/-10*
CD23 37 kDa	DNP-HGDQMAQKSQST	250 +/- 100
TNFRI	DNP-LPQLENVKGTED	<5
TNF-alpha	DNP-SPLAQAVRSSSR	1200 +/- 100

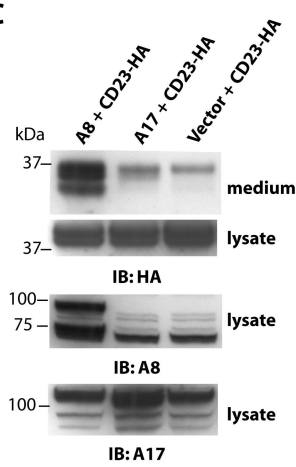
Table 2. Recombinant ADAM processing of peptides corresponding to the 33 and 37 kDa cleavage sites in CD23. * Indicates cleavage site.

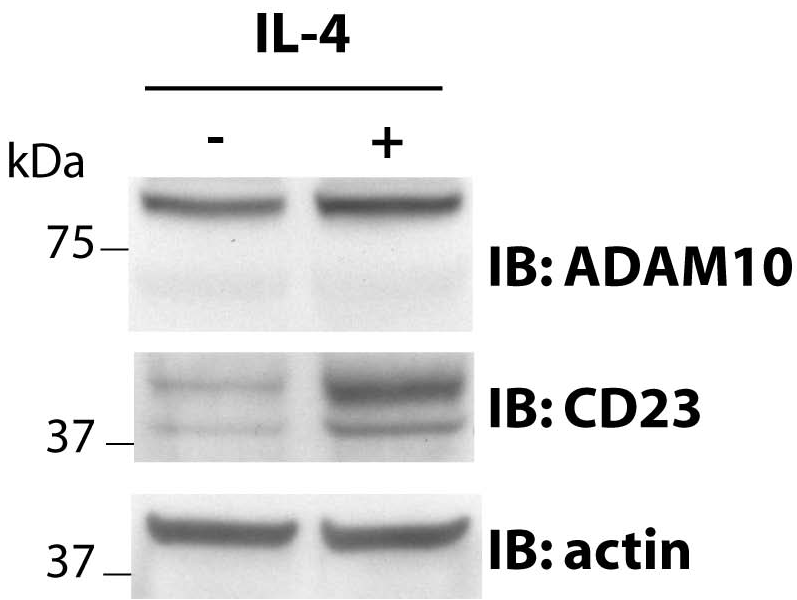
ADAM	*33 kDa*	Phys?	*37 kDa*	Phys?
8	AEQ*QRL	No	QMA*QKS	Yes
	AE*QQRL	No		
10	QQR*LKS	Yes	QMA*QKS	Yes
	EQ*QRLK	No		
17	Not cleaved	-	Not cleaved	-

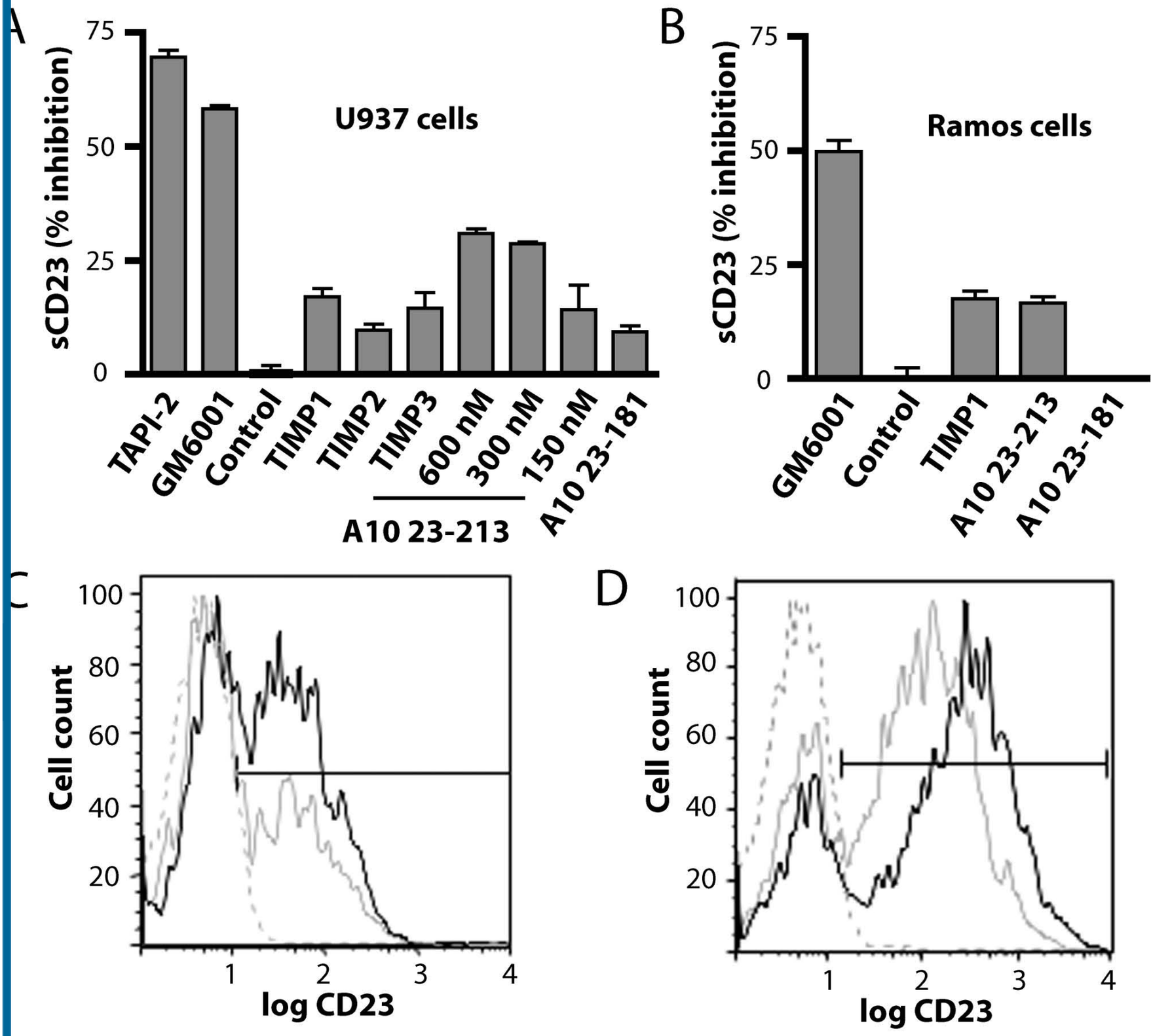
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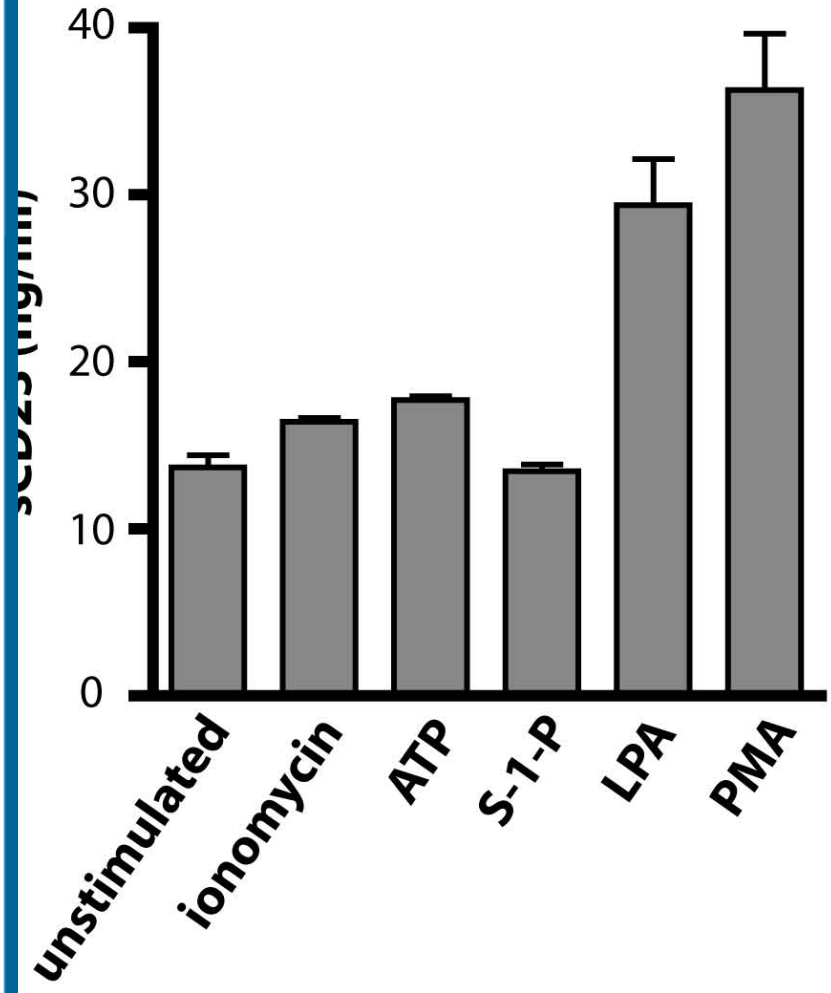

A10 + CD23-HA
A10EA + CD23-HA
Vector + CD23-HA
Vector + CD23-HA + TAPI-2
medium
lysate
IB: HA
lysate
IB: FLAG

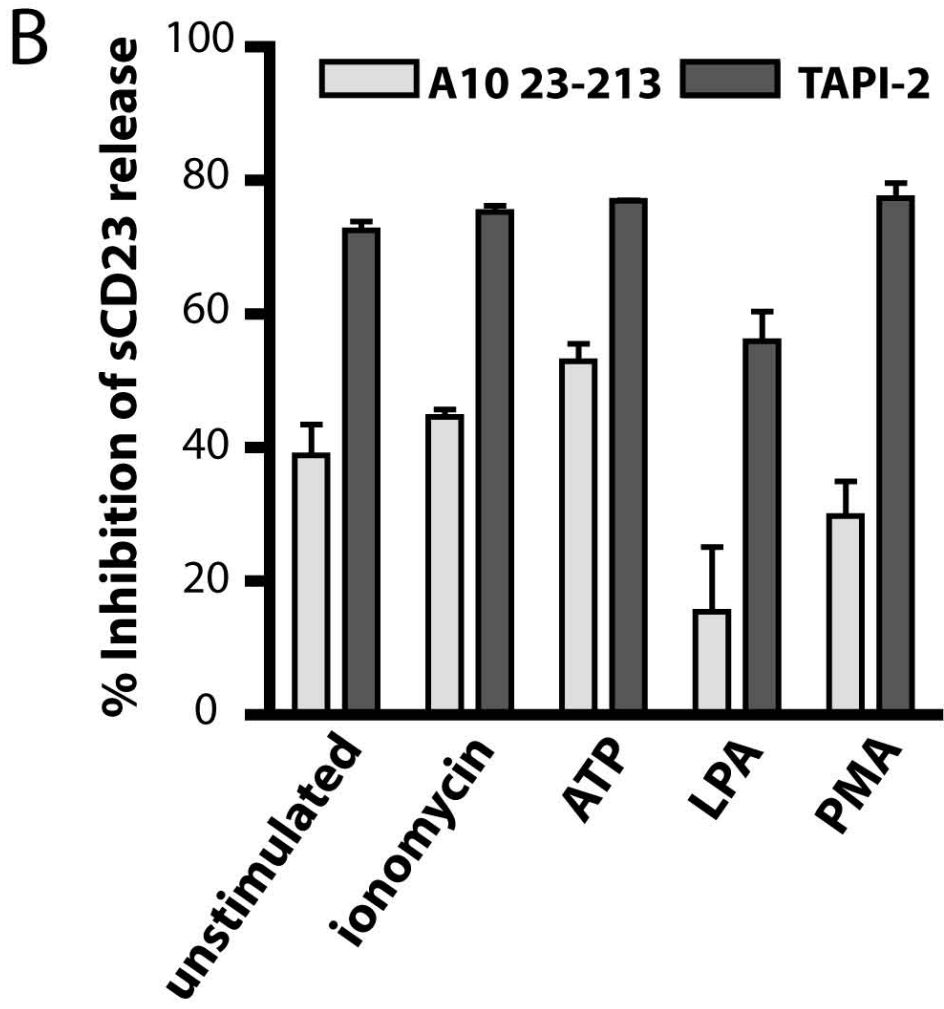

B
200
100
sCD23 (% of vector control)
A10
A10EA

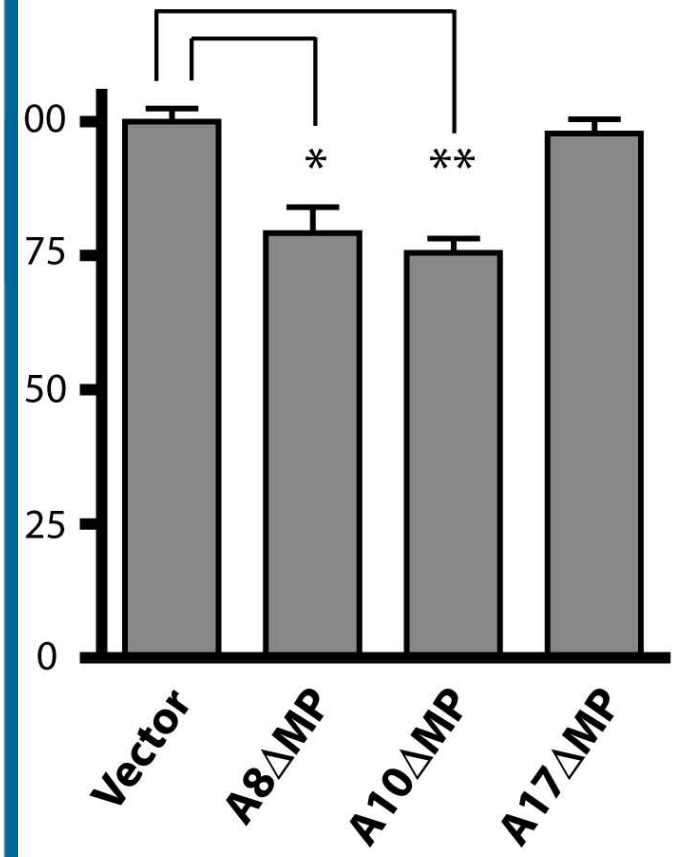

C
kDa
A8 + CD23-HA
A17 + CD23-HA
Vector + CD23-HA
37–
37–
medium
lysate
IB: HA
100–
75 –
lysate
IB: A8
100 –
lysate
IB: A17

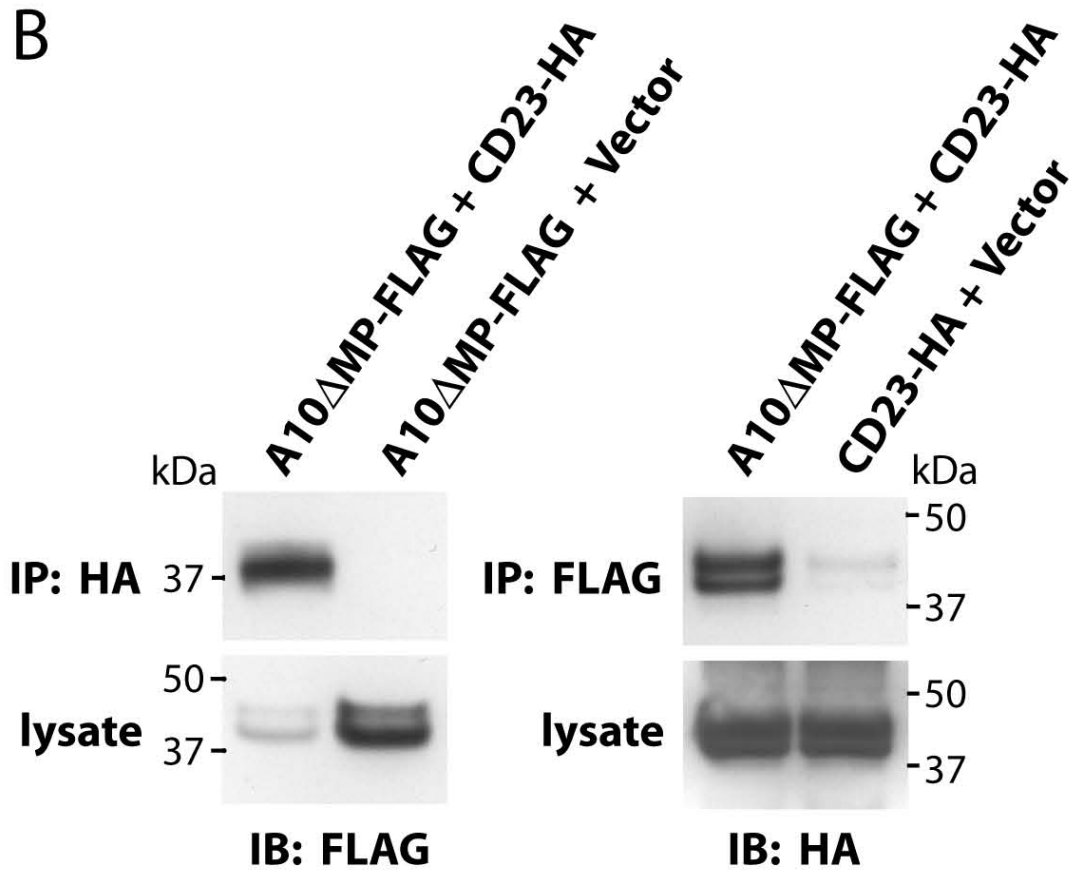

IL-4
-
+
kDa
75
IB: ADAM10
37
IB: CD23
37
IB: actin

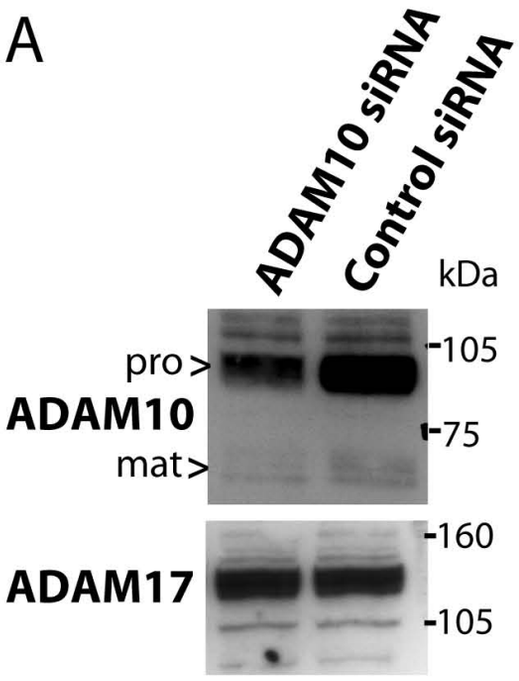

A
sCD23 (% inhibition)
75
50
25
0
U937 cells
TAPI-2
GM6001
Control
TIMP1
TIMP2
TIMP3
600 nM
300 nM
150 nM
A10 23-181
A10 23-213
B
sCD23 (% inhibition)
75
50
25
0
Ramos cells
GM6001
Control
TIMP1
A10 23-213
A10 23-181
C
Cell count
100
80
60
40
20
1
2
3
4
log CD23
D
Cell count
100
80
60
40
20
1
2
3
4
log CD23

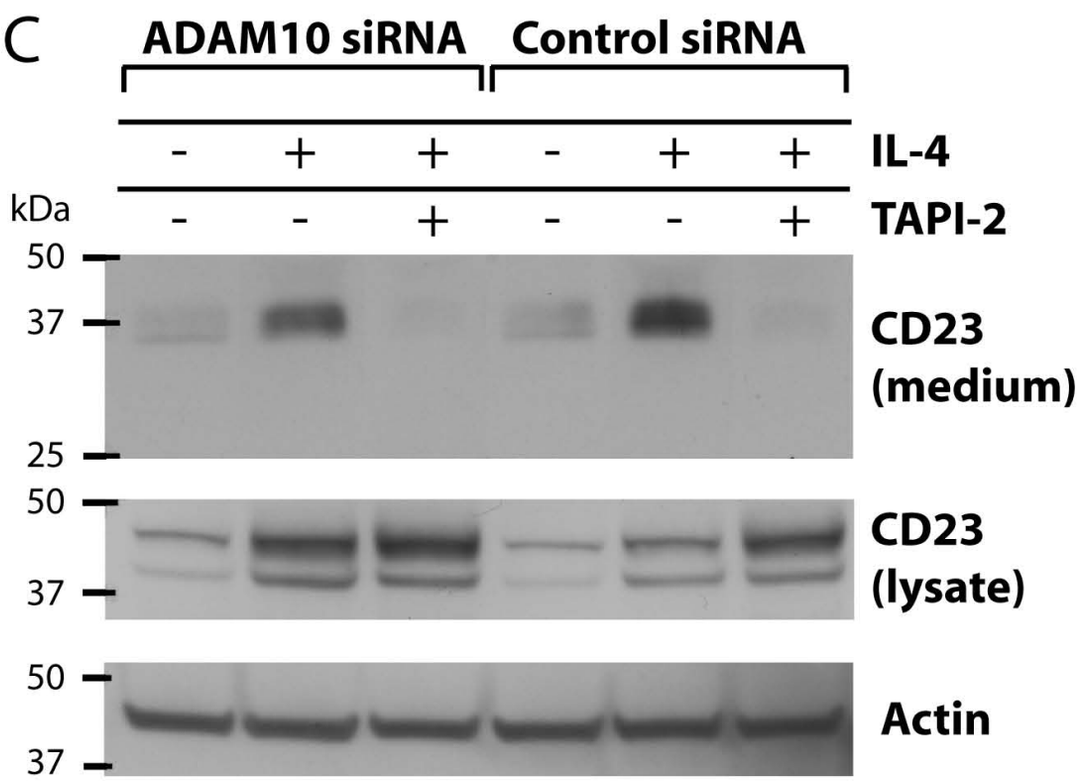

40
30
20
10
0
unstimulated
ionomycin
ATP
S-1-P
LPA
PMA

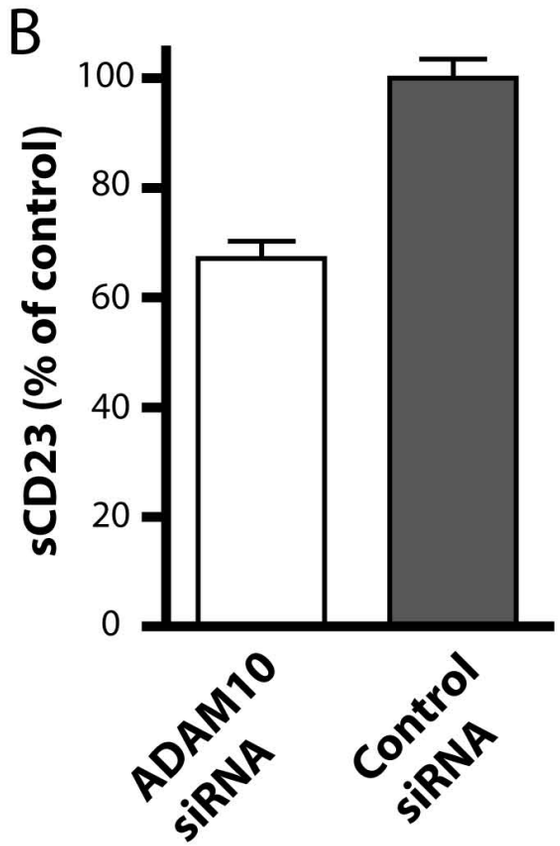

B
100
80
60
40
20
0
% Inhibition of sCD23 release
A10 23-213
TAPI-2
unstimulated
ionomycin
ATP
LPA
PMA

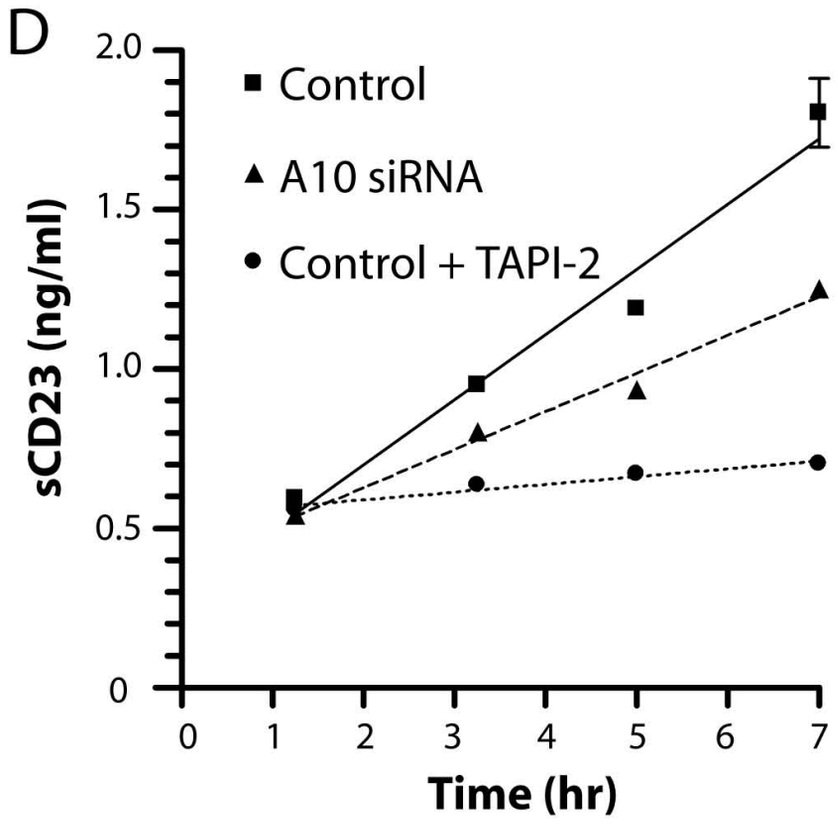

00
75
50
25
0
*
**
Vector
A8ΔMP
A10ΔMP
A17ΔMP


B
A10ΔMP-FLAG + CD23-HA
A10ΔMP-FLAG + Vector
A10ΔMP-FLAG + CD23-HA
CD23-HA + Vector
kDa
IP: HA
37
50
lysate
37
IB: FLAG
kDa
IP: FLAG
50
37
lysate
50
37
IB: HA


A
ADAM10 siRNA
Control siRNA
kDa
pro
ADAM10
mat
105
75
ADAM17
160
105


C
ADAM10 siRNA
Control siRNA
IL-4
TAPI-2
kDa
50
37
25
CD23 (medium)
CD23 (lysate)
Actin


B
sCD23 (% of control)
100
80
60
40
20
0
ADAM10 siRNA
Control siRNA


D
sCD23 (ng/ml)
2.0
1.5
1.0
0.5
0
Control
A10 siRNA
Control + TAPI-2
0
1
2
3
4
5
6
7
Time (hr)