Online material

Macrophage ADAM17 deficiency augments CD36-dependent apoptotic cell uptake and the linked anti-inflammatory phenotype

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Running title: ADAM17 limits CD36-mediated apoptotic cell uptake

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DETAILED METHODS

Hematopoietic chimeric mice

Adam17$^{\Delta Ex5/\Delta Ex5}$ (*Adam17* -/-) or wildtype hematopoietic chimeras were generated as previously described using C57BL/6 ES cells.[1, 2] All animals used for studies were second generation hematopoietic chimeras (C57BL/6, Jackson Laboratory stock #000664) repopulated with bone marrow from first generation fetal liver chimeras. To prepare mixed hematopoietic chimeras, bone marrow cells for transplantation were a 50:50 mixture of Ly5.1-expressing C57BL/6J bone marrow (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ, Jackson Laboratory stock #002014) and *Adam17*-/- cells (Ly5.2) to repopulate C57BL/6J recipients (C57BL/6, Jackson Laboratory stock #000664). All mouse experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

Preparation of bone-marrow-derived macrophages

Bone marrow was harvested from wildtype and *Adam17*-/- hematopoietic chimeras, plated and incubated with 1,000,000 U/L human macrophage colony-stimulating factor (gift from Chiron) for 7 days. Bone-marrow-derived macrophages were incubated under different conditions for evaluation CD36 levels in conditioned media and cell lysates or used for liposome binding studies, following replating to establish comparable cell densities.

Flow cytometry

Staining of freshly isolated cells for flow cytometric analyses used the following antibodies: anti-CD36 (clone CRF D-2712, gift from Roy Silverstein),[3] guinea pig polyclonal anti-SR-A (gift from Debra Rateri),[4] anti-SR-BI (Novus, NB400-113), anti-Mer tyrosine kinase (R&D Systems, AF591), PE-anti-LOX-1 (R&D Systems, FAB1564P), and PerCP-Cy5.5-anti-CD45.2 (BD Pharmingen, 552950). For analysis of intracellular staining, cells were fixed on ice with 2% paraformaldehyde for 45 minutes, permeablized with ice-cold methanol for 8 hours or more at -80°C and stained with antibodies for arginase I (BD Transduction Laboratories, 610708) and inducible nitric oxide synthetase (iNOS; Abcam, ab15323).[5] Nonspecific binding was blocked with anti-CD16/32 (BD Pharmingen, 553142). Unconjugated antibodies were fluorescently labeled using Zenon Antibody Labeling Kits (Invitrogen), or detected with FITC-anti-IgY (AnaSpec, 29709-FITC), FITC-anti-IgG (Molecular Probes, A21441), or FITC-anti-IgA (eBioscience, 11-4204-81). Stained cells were analyzed on a FACScan (BD Pharmingen), and 10,000-50,000 events were collected for each analysis. Flow data were analyzed using FlowJo 7.5 software (TreeStar).

Fab preparation

Protein-L affinity purified IgA from hybridoma culture media (anti-CD36, Clone CRF-D 2717, gift from Dr. Roy Silverstein), or non-immune IgA (Sigma Aldrich, M-1421), was partially reduced to facilitate papain cleavage.[6] Immobilized papain (Pierce, 20341) was used to generate Fab fragments. The amount of Fab (80 µg per cavity) used for *in vivo* blocking studies was based on *in vitro* titrations of Fab sufficient to block phosphatidylserine liposome binding to thioglycollate-elicited macrophages. Endotoxin

levels in Fab preparations were determined by bioassay using THP1-XBlue reporter cells (Invivogen). Levels were below 10 pg endotoxin/80 µg Fab; a dose known not to affect the cellular influx into the peritoneal cavity.

In vivo efferocytosis

Thymuses harvested from 4-6 week old C57BL/6 mice were dissociated by mechanical disruption and filtered using a 70 µm cell strainer (BD Falcon, 352350) to yield a single-cell suspension. Thymocytes were labeled using the red fluorescent TAMRA-SE dye (Molecular Probes, C-1171).[7] Briefly, 100 µg of TAMRA-SE was used to label 60 x 10^6 thymocytes suspended in DMEM at 37° C for 15 minutes. Excess label was quenched with fetal bovine serum, and the cells were washed. Apoptosis was then induced by treatment with 1 µmol/L dexamethasone in RPMI 1640 medium with 10% fetal bovine serum and 3.4 µl/L β-mercaptoethanol for 5 hrs at 37° C, yielding a population of thymocytes that were 60-80% Annexin V positive.[8] Opsonized control cells did not receive dexamethasone treatment, and were generated by incubation with 5 µg anti-CD45 antibody (Pharmingen, 01111D) per 10x10^7 cells for 15 minutes at 4° C.[9] To evaluate apoptotic cell uptake, 1x10^7 fluorescently labeled apoptotic thymocytes were injected into the peritoneum of ADAM17 null or wildtype chimeric mice 4 days after peritoneal injection of thioglycollate, in some experiments with 80 µg receptor-blocking Fab or isotype control Fab. Peritoneal cells were harvested 30 minutes after thymocyte injection with 5 ml PBS containing 5 mmol/L EDTA, and the percent of F4/80-stained macrophages positive for TAMRA-labeled thymocytes was assessed by flow cytometry. In trial experiments, we quantified uptake by both flow cytometry and by fluorescent microscopy (Online Figure I; only yellow apoptotic cells were counted, both TAMRA+ and F4/80+), and the data showed good agreement between the two methods (data not shown).

Liposome binding and uptake

Phospholipids were purchased from Avanti Polar Lipids. Phosphatidylserine (PS) rich liposomes, composed of equal parts PS to phosphatidylcholine, were prepared with a 1% mole fraction of the fluorescent dye, 1-dioctadecyl-3,3-tetramethylindocarbocianin perchlorate (DiI, Sigma, 42364) by extrusion through a 0.1 µm polycarbonate membrane.[10] Thioglycollate-elicited cells from wildtype or ADAM17 null hematopoietic chimeras, or 5-7 day bone-marrow-derived macrophages were plated on tissue culture dishes and macrophages (> 95%) adherent after 2 hours were used for binding and uptake studies. Macrophages were incubated with 160 µmol/L fluorescently-labeled PS liposomes and 5 mg/L of either receptor blocking (anti-CD36, CRF D-2712; anti-SR-A, R&D Systems AF1797) or isotype control antibodies. The cells were incubated for one hour at 37° C, and fluorescent liposome binding/uptake was assessed by flow-cytometry. Acetylated-low-density lipoprotein (acLDL) was prepared by treatment of 10 mg of LDL (1.063 > *d* > 1.019 g/ml) in 50% saturated ice-cold sodium acetate with 15 mg acetic anhydride.[11] For some *in vitro* binding analyses, adherent macrophages were treated with or without acLDL for 15 minutes prior to the addition of 40 µmol/L fluorescently-labeled PS liposomes and a 4-hour incubation at 4° C. Fluorescent liposome binding was evaluated by flow-cytometry.

Soluble CD36 characterization

Thioglycollate-elicited macrophages were plated 2 hours in tissue culture dishes and adherent cells were treated with 1,000,000 U/L human macrophage colony-stimulating factor (gift from Chiron) in Opti-MEM (Invitrogen) for 24 hours at 37° C. Conditioned media was removed and centrifuged at 300 x g for 10 minutes to remove cell debris. The media was then centrifuged at 28,300 x g for 140 minutes at 4°C to deplete microparticle content.[12] The resulting media was concentrated 20-fold by ultrafiltration (Orbital Biosciences AP2000910), and immuno-precipitated with anti-CD36 antibody (CRF D-2712) covalently coupled to agarose (Pierce 26198). Immunoprecipitated protein was eluted by boiling in non-reducing SDS- sample buffer, and precipitated overnight at 4° C in 15% TCA (Sigma T0699). The precipitate was washed with acetone, dried, re-suspended, and digested with PNGase F (New England Biolabs, P0704S). SDS-PAGE was carried out using 10% acrylamide gels, and Western blotting was performed following semi-dry transfer onto PVDF membranes using biotinylated anti-CD36 (R&D BAF2519), streptavidin conjugated horseradish peroxidase (Jackson ImmunoResearch 016-030-084), and SuperSignal West Femto developing reagent (Thermo).

Identification of the putative CD36 cleavage site

Gel bands corresponding to CD36 were detected by Coomassie staining, and were verified by CD36 immunoblot analysis of adjacent lanes in the same gel. Bands corresponding to CD36 were excised and subjected to standard in-gel digestion with trypsin. Digested peptides extracted from the gel pieces were dried down and reconstituted for liquid chromatography- mass spectrometry (LCMS) analysis in 5% acetonitrile/0.1% trifluoroacetic acid. The peptides were then injected onto a C18 trap column (XBridge C18 100A, 5 µm, 0.1 x 30 mm, Waters), desalted for 15 minutes with water/0.1% formic acid (4 µL/minute), eluted onto an analytical column (XBridge C18 100A, 3.5 µm, 0.1 x 100 mm, Michrom Bioresources, Inc.) heated to 45º C and separated at a flow rate of 0.5 µL/minute over 90 minutes, using a linear gradient of 5% to 35% acetonitrile/0.1% formic acid in 0.1% formic acid on a NanoAquity HPLC (Waters, Milford, MA). Positive ion mass spectra were acquired with electrospray ionization in a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap XL, Thermo Fisher, San Jose, CA) or Q Exactive mass spectrometery (Thermo Fisher, Dan Jose, CA) with data-dependent acquisition of MS/MS scans (linear ion trap) on the 8 most abundant ions (20 for Q Exactive) in the survey scan (orbitrap, resolution 30,000). An exclusion window of 45 seconds was used after 2 repeated acquisitions of the same precursor ion.

For protein identification, MS/MS spectra were matched against the mouse Uniprot/SwissProt database using the SEQUEST (v 2.7) search engine with fixed Cys carbamidomethylation and variable Met oxidation modifications and no enzyme specificity (semi-specific restriction was applied on the results of the database search). The mass tolerance for precursor ions was 50 ppm (MS1 data); SEQUEST default tolerance was accepted for product ions. SEQUEST results were further validated with PeptideProphet and ProteinProphet, using an adjusted probability of ≥ 0.90 for peptides and ≥ 0.95 for proteins. Each charge state of a peptide was considered a unique identification. Identity of the semi-specific trypsin proteolytic fragment was further

confirmed by Mascot database search (v 2,1, mouse SwissProt database, Matrix Science,UK) on the MS/MS spectrum of the m/z 1045.5 (semi-tryptic specificity, mass tolerance 50 ppm precursor, 0.4 Da fragments, modifications - fixed Cys+57.021, variable Met+15.99).

CD36 ELISA

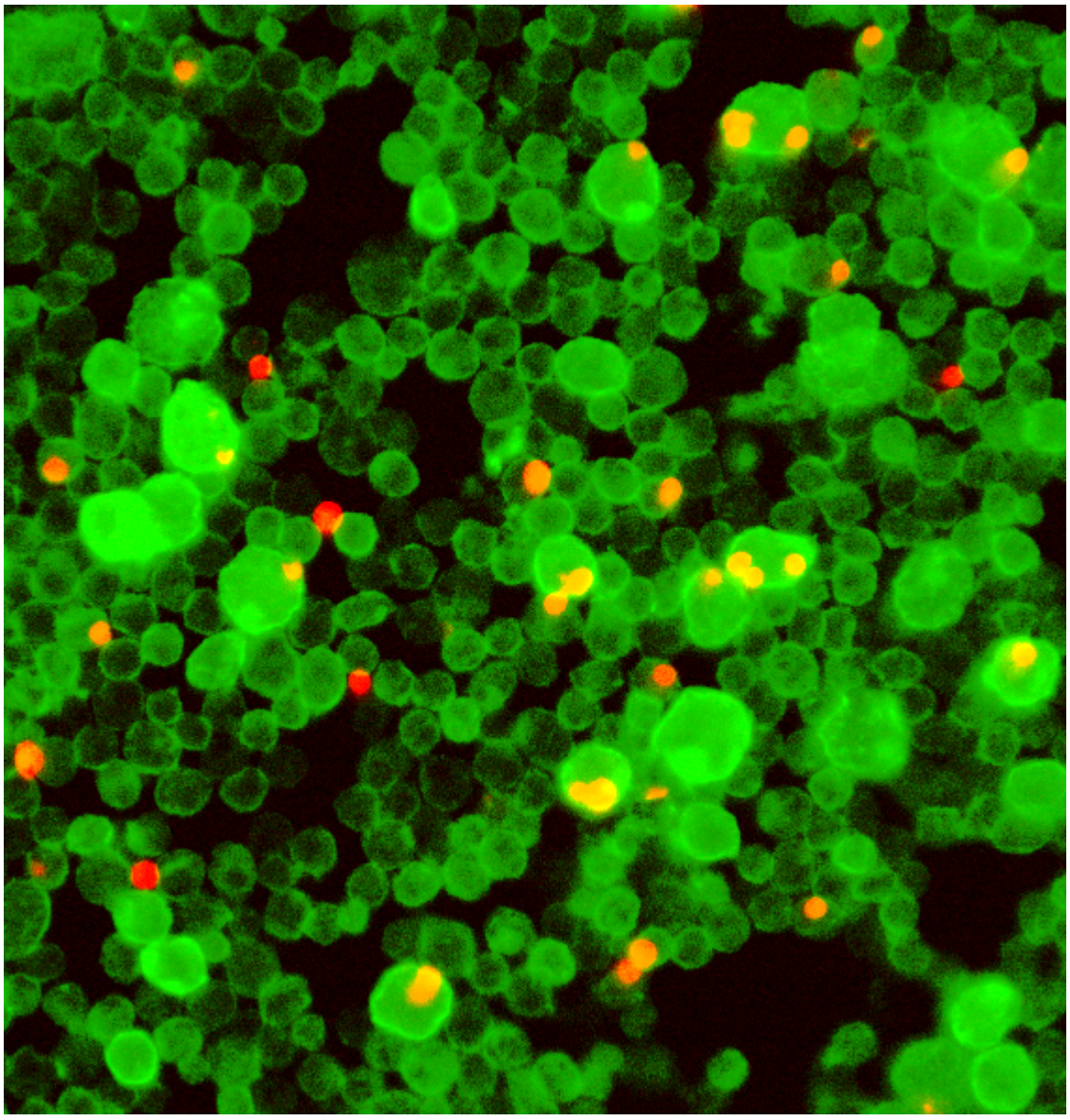
Both antibodies used for the ELISA recognize the extracellular domain of CD36. 96-well plates (Nunc-Immuno 62409-003) were coated overnight at room temperature with 50 µl/well of 0.5 µg/L anti-CD36 capture antibody (CRF D-2712) in PBS. Wells were blocked with 100ul of 1% BSA in 0.05% Tween-20 PBS for 2 hrs at room temperature. Samples were diluted in 20 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 0.1% BSA, 0.05% Tween-20, and added to the plate in a volume of 50 µl and incubated for 2 hours. A reference standard was prepared by dilution of pooled 96-hour thioglycollate-elicited peritoneal macrophage lysate. CD36 was detected using 400 µg/L biotinylated anti-CD36 (R&D BAF2519) for 2 hours, followed by 1.5 mg/L streptavidin-HRP (Jackson ImmunoResearch, 016-030-084) for 30 minutes. Plates were developed by the addition of 50 µl tetramethylbenzidine substrate reagent (R&D, DY999). After 15 minutes, the reaction was stopped by the addition of 2N sulfuric acid, and absorbance at 450 nm was measured using a SpectraMax 2Me spectrophotometer.

Primer pairs

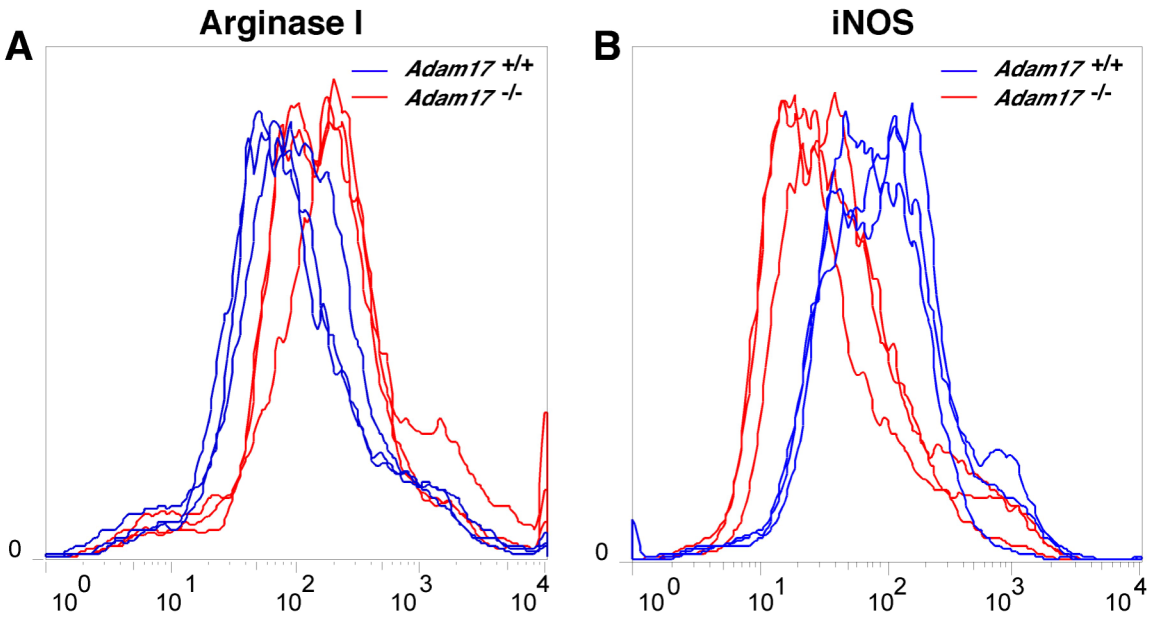
All primer pairs to detect different ADAM proteases were designed to generate ~ 400bp fragments:

ADAM2:	forward 5'-ACC GAT TAT GTT GGC GCT AC-3',
	reverse 5'-TTT CTG CAC AGC CCT TCT TT-3';
ADAM8:	forward 5'-TGT CCT GGA GGG AAC AGA AC-3',
	reverse 5'-AAC CGG TTG ACA TCT GGA AC-3';
ADAM9:	forward 5'-CAT GAA TTG GGG CAT AAC CT-3',
	reverse 5'-CTC ACT GGT CTT CCC TCT GC-3';
ADAM10:	forward 5'-AGC AAC ATC TGG GGA CAA AC-3',
	reverse 5'-TAA AGT TGG GCT TGG GAT CA-3';
ADAM12:	forward 5'-AGA GAA AGG AGG CTG CAT CA-3',
	reverse 5'-ACA CAT TGG CTG GAC AGT GA-3';
ADAM15:	forward 5'-ACA AGC ATC TTA GGC GTT GC-3',
	reverse 5'-TTT GAC AAC AGG GTC CAT CA-3';
ADAM17:	forward 5'-TTG AGC GAT TTT GGG ATT TC-3',
	reverse 5'-GGT CCT TCT CAA ATC CGT CA-3';
ADAM19:	forward 5'-AAT GCC TCC AAC TGC ACT CT-3',
	reverse 5'-ATC TTC CCA CAC TTG GCA TC-3';
ADAM33:	forward 5'-CCA CAC GAC TCC ACA CAA CT-3',
	reverse 5'-CTT CCA AGA AGC CGT TTC C-3'.

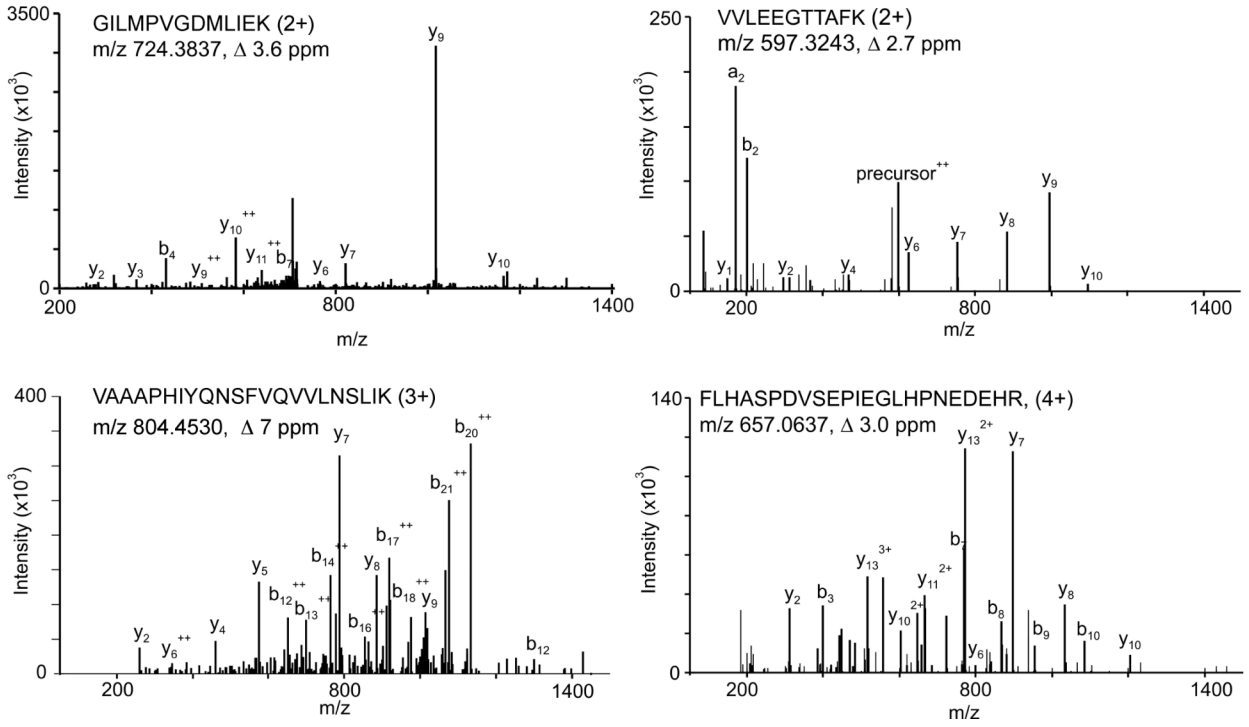
ONLINE FIGURES



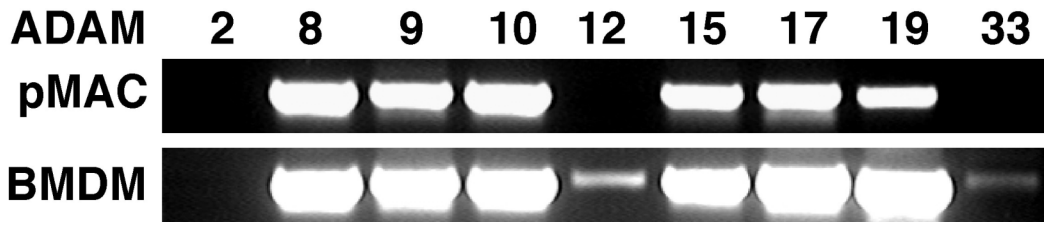
Online Figure I: Immunofluorescent microscopy is consistent with uptake of apoptotic cells. Representative microphage of F4/80-stained macrophages (green) obtained from a wildtype ADAM17 hematopoietic chimera injected intraperitoneally with TAMRA-labeled (red) apoptotic thymocytes as described in Figure 1A. Quantification of thymocyte uptake by fluorescence microscopy was in agreement with flow cytometry data (data not shown).



Online Figure II: Representative histograms of intracellular Arginase I and iNOS show the shift to a less inflammatory phenotype in macrophages lacking ADAM17. Histograms of intracellular levels of **A.** Arginase I and **B.** iNOS for wildtype macrophages (blue) and *Adam17*-null macrophages (red).



Online Figure III: Spectra from three unique N-terminal peptides with non-tryptic N-terminal sequences and one C-terminal peptide, which were identified with high confidence in soluble CD36. The spectra shown were identified with high confidence in at least 2 of 3 separate samples: **A**. N1, PeptideProphet probability 0.98, delta mass 3.59 ppm; **B.** N2, PeptideProphet probablility 0.975, delta mass 2.7 ppm; **C.** N3, PeptideProphet probability 0.95, delta mass 6.96 ppm; and **D**. C1, PeptideProphet probability 1.0, delta mass 3.0 ppm.



Online Figure IV: Multiple ADAMs containing protease domains are expressed by mouse peritoneal and bone marrow-derived macrophages. Total RNA was isolated from peritoneal (pMAC) and bone marrow-derived (BMDM) macrophages, reverse transcribed and the resulting cDNA was amplified with primer pairs specific to the indicated ADAMs. Products were separated on a 1.2% agarose gel and stained with ethidium bromide.

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