Macrophage ADAM17 Deficiency Augments CD36-Dependent Apoptotic Cell Uptake and the Linked Anti-Inflammatory Phenotype

Will S. Driscoll, Tomas Vaisar, Jingjing Tang, Carole L. Wilson, Elaine W. Raines

Rationale: Apoptotic cell phagocytosis (efferocytosis) is mediated by specific receptors and is essential for resolution of inflammation. In chronic inflammation, apoptotic cell clearance is dysfunctional and soluble levels of several apoptotic cell receptors are elevated. Reports have identified proteolytic cleavage as a mechanism capable of releasing soluble apoptotic cell receptors, but the functional implications of their proteolysis are unclear.

Objective: To test the hypothesis that ADAM17-mediated cleavage of apoptotic cell receptors limits efferocytosis in vivo.

Methods and Results: In vivo comparison of macrophage efferocytosis in wild-type and Adam17-null hematopoietic chimeras demonstrates that ADAM17 deficiency leads to a 60% increase in efferocytosis and an enhanced anti-inflammatory phenotype in a model of peritonitis. In vitro uptake of phosphatidylserine liposomes identifies the dual-pass apoptotic cell receptor CD36 as a major contributor to enhanced efferocytosis, and CD36 surface levels are elevated on macrophages from Adam17-null mice. Further, temporal elevation of CD36 expression with inflammation may also contribute to its impact. Soluble CD36 from macrophage-conditioned media comprises 2 species based on Western blotting, and mass spectrometry identifies 3 N-terminal peptides that represent probable cleavage sites. Levels of soluble CD36 are decreased in Adam17-null conditioned media, providing evidence for involvement of ADAM17 in CD36 cleavage. Importantly, enhanced efferocytosis in vivo by macrophages lacking ADAM17 is CD36 dependent and accelerates macrophage clearance from the peritoneum, thus promoting resolution of inflammation and highlighting the impact of increased apoptotic cell uptake.

Conclusions: Our studies demonstrate the importance of ADAM17-mediated proteolysis for in vivo efferocytosis regulation and suggest a possible mechanistic link between chronic inflammation and defective efferocytosis.

Key Words: apoptotic cells ■ inflammation ■ macrophage ■ metalloproteinase ■ proteolysis

Efficient phagocytosis of apoptotic cells (efferocytosis) is an essential component of tissue homeostasis, wound healing, and the resolution of inflammation. Professional phagocytes, such as macrophages, use a variety of transmembrane receptors to rapidly recognize and ingest apoptotic cells.1 After engulfing an apoptotic cell, macrophages actively dampen inflammation by releasing anti-inflammatory cytokines, such as transforming growth factor-β and interleukin-10, as well as proresolving lipid mediators and eicosanoids that promote macrophage efflux and the resolution of inflammation.2,3 However, if apoptotic cells are not rapidly cleared, secondary necrosis ensues, resulting in leakage of toxic intracellular antigens, tissue damage, and amplified inflammation. Defective efferocytosis is frequently observed in the context of chronic inflammation, with pathological sequelae ranging from nonresolving foot ulcers in diabetes mellitus to necrotic core expansion in atherosclerosis.4,5 However, the underlying mechanisms responsible for deficient apoptotic cell uptake are poorly understood.

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Biological fluids from patients with chronic inflammatory diseases also show elevated levels of soluble apoptotic cell receptors, including CD36, Mer tyrosine kinase (MerTK), and lectin-type oxidized low-density lipoprotein (LDL)
LOX-1 can be proteolytically cleaved by the transmembrane type II transmembrane proteins, respectively. MerTK and ADAM17 membrane proximal cleavage of its stalk length and distance from the membrane seem to be important. ADAM17 activity have hampered progress. Unlike soluble enzymes that understand, and lack of reagents to monitor its levels and proteolysis substrates releases almost the entire extracellular domain, and has been shown for LOX-1 and MerTK. Thus, proteolysis of CD36 in vivo is sufficient to abolish enhanced efferocytosis and its concomitant anti-inflammatory consequences. The current investigation tests the hypothesis that efferocytosis and its anti-inflammatory consequences are limited by proteolytic cleavage of apoptotic cell receptors from the macrophage surface. We show that ADAM17 deletion enhances macrophage-mediated efferocytosis in vivo, resulting in an augmented anti-inflammatory response. In addition, we report that macrophage CD36 surface levels are elevated in the absence of ADAM17, and that there are 3 probable N-terminal cleavage sites in CD36. The absence of ADAM17 leads to a decrease in soluble CD36 levels. Blockade of CD36 in vivo is sufficient to abolish enhanced efferocytosis by Adam17-null macrophages, a process which also accelerates resolution of inflammation. Together these studies establish that ADAM17-mediated proteolysis of CD36 is an important post-translational mechanism controlling apoptotic cell phagocytosis, inflammation, and its resolution.

Methods
An expanded Methods section describing all procedures is available in the Online Data Supplement.

Hematopoietic Chimeric Mice
Adam17−/−embryonic (Adam17−/−) or wild-type (WT) hematopoietic chimeras were generated as previously described using C57BL/6 embryonic stem cells. All mouse experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

Sterile Peritonitis Model
Thioglycollate peritonitis was induced by injection of 1 mL of 4% sterile thioglycollate (BD Diagnostic, 2321398). Peritoneal cells (thioglycollate-elicited cells) were collected after 4 days by injection and removal of 5 mL phosphate buffered saline (PBS) containing 5 mmol/L EDTA.

Flow Cytometry
Staining of freshly isolated cells for flow cytometric analyses (FACScan, BD Pharmingen; 10–50,000 events) used antibodies listed in the Online Data Supplement. Flow data were analyzed using FlowJo 7.5 software (TreeStar).

Fab Preparation
Protein-L purified IgAs from hybridoma media (anti-CD36, Clone CRF-D 2717, Roy Silverstein), or nonimmune IgA (Sigma Aldrich, M-1421), were partially reduced to facilitate papain cleavage and incubated with immobilized papain (Pierce, 20341) to generate Fab fragments.

In Vivo Efferocytosis
Thymuses (4- to 6-week C57BL/6 mice) were harvested, mechanically dissociated and filtered to yield a single-cell suspension. Thymocytes were labeled with mixed isomers of 5-((and)-6)-carboxytetramethylrhodamine, succinimidyl ester (TAMRA-SE) (Molecular Probes, C-1171), and in vivo analysis of apoptotic cell uptake was performed.

Soluble CD36 Characterization
Thioglycollate-elicited macrophages were plated 2 hours and adherent cells were cultured with 10,000 U/L human macrophage colony-stimulating factor (gift from Chiron), or other stimulants, in Opti-MEM (Invitrogen) for 4, 6, or 24 hours at 37°C. Conditioned media was centrifuged at 300,000 g for 140 minutes at 4°C to minimize microparticle content, and levels of CD36 were determined by ELISA using antibodies recognizing the extracellular domain of CD36. The resulting media were directly run on SDS-PAGE for Western analysis, or immunoprecipitated and run on SDS-PAGE for mass spectrometry (MS).

Identification of Potential Cleavage Sites in Soluble CD36
Gel bands corresponding to CD36 were detected by Coomassie staining and were verified by CD36 immunoblot analysis of adjacent lanes. CD36 fractions were excised, subjected to standard in-gel digestion with trypsin, and digested peptides were analyzed by liquid chromatography–MS analysis.

Statistical Analysis
For statistical analysis, the Student t test was performed using the InStat software, version 3.0b. All error bars represent SE of the mean.

Results
In Vivo Efferocytosis Is Enhanced in Macrophages Lacking ADAM17
To test the hypothesis that efferocytosis and its downstream anti-inflammatory response are limited by proteolytic cleavage of macrophage apoptotic cell receptors, we evaluated the effect of leukocyte-specific deletion of the transmembrane protease ADAM17 on apoptotic cell phagocytosis in vivo.
Adam17-null and WT hematopoietic chimeras were injected intraperitoneally with the sterile irritant thioglycollate to promote inflammatory monocyte influx. At 96 hours after thioglycollate, levels of cytokines released early in the response (2–4 hours) have abated and the influx of neutrophils has cleared (48 hours), leaving F4/80+ macrophages as the major leukocyte (>85%) in the peritoneal cavity.21 Fluorescently labeled control or apoptotic thymocytes were injected into the peritoneum to evaluate efferocytosis (Figure 1A). No difference in thymocyte uptake between Adam17-null and WT macrophages is observed after injection of either healthy leukocyte (>85%) in the peritoneal cavity.21 Fluorescently labeled apoptotic thymocytes is shown (intraperitoneal [IP]). After in vivo uptake of apoptotic cells, Adam17-null macrophages still display enhanced efferocytosis (Figure 1B). These results indicate that the increase in apoptotic cell phagocytosis by Adam17-null macrophages is cell intrinsic and directly impacts apoptotic cell receptor function.

Efferocytosis has been shown to actively inhibit the macrophage inflammatory response.3 To evaluate whether elevated apoptotic cell uptake alters the inflammatory phenotype of Adam17-null macrophages, intracellular protein levels of arginase I and inducible nitric oxide synthase were compared by flow cytometry after injection of apoptotic thymocytes (representative histograms in Online Figure 2), healthy thymocytes, or PBS. After in vivo uptake of apoptotic cells, Adam17-null macrophages show a 34% elevation in arginase I levels relative to WT (Figure 1C). In addition, subsequent inflammatory stimulation ex vivo leads to a 45% reduction in inducible nitric oxide synthase induction in the null macrophages (Figure 1D). No differences in arginase I or inducible nitric oxide synthase levels were observed in Adam17-null or WT macrophages injected with control thymocytes (Figure 1C and 1D). Although we cannot eliminate the possibility that prior uptake of apoptotic neutrophils during the thioglycolate response may alter macrophage phenotype and efferocytosis, Adam17-null macrophages did not demonstrate altered arginase signaling detectable at 96 hours. Overall, ADAM17 deletion significantly augments the macrophage efferocytosis-induced anti-inflammatory phenotype.

Increased Phosphatidylserine Liposome Binding/ Uptake by Adam17-Null Macrophages Is CD36 Dependent and Is Associated With Elevated CD36 Surface Levels

Macrophages express a variety of apoptotic cell receptors that may be modulated by ADAM17. Because macrophage efferocytosis is dependent on apoptotic cell receptor recognition of PS exposed by cells undergoing apoptosis,2 PS liposomes were used as a surrogate for apoptotic cells for an in vitro screen of potential receptors responsible for the enhanced efferocytosis by Adam17-null macrophages. Adam17-null peritoneal macrophages bind more PS liposomes in vitro (Figure 2A and 2C), but not phosphatidylcholine liposomes (data not shown), suggesting that ADAM17 deletion results in elevated levels of receptors that recognize PS exposed.

Figure 1. Increased in vivo efferocytosis of apoptotic thymocytes by Adam17-null macrophages is cell autonomous and shifts them to a less inflammatory phenotype. A. The scheme for in vivo peritoneal macrophage uptake of TAMRA-labeled apoptotic thymocytes is shown (intraperitoneal [IP]). Healthy thymocytes and opsonized thymocytes were evaluated as controls. B. Wild-type (WT), Adam17-null, or mixed hematopoietic chimera mice that have 50% WT and 50% Adam17-null leukocytes were injected with the indicated thymocytes. The percentage of TAMRA-positive macrophages was assessed by flow cytometry. C. After in vivo exposure to phosphate buffered saline (PBS), healthy or apoptotic thymocytes, Adam17-null, or WT macrophages were analyzed immediately after harvest for intracellular levels of arginase I. Overall, Adam17 deletion significantly augments the macrophage efferocytosis-induced anti-inflammatory phenotype.
on apoptotic cells. Similar results were obtained with Adam17-null versus WT bone-marrow–derived macrophages (Figure 2B). Although many apoptotic cell receptors can bind PS, a unique subset of receptors shares an affinity for acetylated LDL as a ligand. When acetylated LDL is added with PS liposomes, the ADAM17-dependent difference in PS liposome binding is abolished (Figure 2A). This finding suggests that the receptors responsible for elevated liposome binding by Adam17-null macrophages have the ability to directly bind both acetylated LDL and PS liposomes—a trait shared by CD36 and scavenger receptor (SR)-B1.27 However, SR-B1 surface levels were not detectable above isotype control staining of WT or Adam17-null macrophages (data not shown), thus focusing our attention on CD36.

To first directly test the role of CD36 in the enhanced in vitro liposome binding and uptake by Adam17-null macrophages, the extent of inhibition by anti-CD36 or isotype control antibody was evaluated (Figure 2C). CD36 blockade abolishes the differential liposome uptake by Adam17-null peritoneal macrophages and significantly reduces uptake by both Adam17-null and WT macrophages. In contrast, blocking antibody to SR-A, a prominent scavenger receptor, has no significant effect. Together these data identify CD36 as the primary apoptotic cell receptor leading to enhanced PS liposome binding and uptake in vitro by Adam17-null macrophages.

To more broadly screen for possible changes in apoptotic cell receptor surface levels, freshly isolated thioglycollate-elicited peritoneal macrophages from WT and Adam17-null chimeras were analyzed by flow cytometry. ADAM17 deletion results in a 30% increase in macrophage surface CD36 but no difference in other apoptotic cell receptors (Figure 3A), although MerTK, LOX-1, and SR-phosphatidylserine and oxidized LDL are reported substrates of ADAM17.12,13,28 CD36 surface levels are elevated to a similar extent on Adam17-null macrophages from mixed hematopoietic chimeras (data not shown), indicating a cell autonomous trait. Interestingly, CD36 surface levels are low in resident peritoneal macrophages but increase after thioglycollate injection, reaching a maximum at 96 hours (Figure 3B–3D). These results suggest that CD36 may be important during the resolution phase of inflammation.2,29 Also, no difference in CD36 mRNA levels is observed by quantitative polymerase chain reaction analysis of Adam17-null and WT macrophages (data not shown), suggesting that ADAM17-dependent mechanisms regulate CD36 surface levels post-translationally. The combined data showing an acetylated LDL–sensitive increase in PS liposome binding and uptake by Adam17-null macrophages, as well as elevated CD36 surface levels, implicate CD36 as a major target of ADAM17.

**ADAM17 Deletion Reduces Levels of Soluble CD36**

Elevated macrophage CD36 surface levels would be expected to coincide with decreased release of soluble CD36. To evaluate whether levels of soluble CD36 are altered by ADAM17 deficiency, 24-hour conditioned media were collected from adherent Adam17-null and WT peritoneal macrophages. To reduce potential microvesicle content, the media were centrifuged at high speed, which depletes microvesicles by ≈75%.26 Quantification of media CD36 levels by ELISA using antibodies to the CD36 extracellular domain shows that ADAM17 deletion reduces the ratio of soluble/cellular CD36 by 25.8% (Figure 4A). Soluble forms of CD36 are not a result of alternative splicing because this has been shown to occur in the 5′ noncoding region in all identified variants.30 Release of soluble CD36 from WT peritoneal macrophages was also investigated after 4-hour stimulation with PBS, lipopolysaccharide, and interleukin-4 (Figure 4B). Although shedding of tumor necrosis factor receptor II, another substrate of ADAM17, was increased >5-fold after lipopolysaccharide stimulation (Figure 4B), neither lipopolysaccharide nor interleukin-4 increased levels of soluble CD36 relative to PBS, suggesting its release is primarily constitutive.

Because CD36 is a highly glycosylated protein, Western analysis was performed after PNGase F treatment of media samples to assess molecular species (Figure 4C and 4D).
in 6-hour conditioned media (Figure 4D), consistent with it not being a degradation product of the longer 24-hour incubation. Together, these observations provide the first evidence for a role of ADAM17 in the proteolytic release of soluble CD36.

**MS Identifies 3 Probable N-Terminal Cleavage Sites in Soluble CD36**
CD36 is predicted to be a dual-pass transmembrane protein with considerable extracellular topology (Figure 5A). To further characterize the species in soluble CD36 detected by Western analysis, conditioned media from WT macrophages were immunoprecipitated with anti-CD36 and 3 fractions were cut (Figure 5B) from the Coomassie-stained gel to include the α52-kDa region (fraction A), the zone between the 2 regions (fraction B), and the α47-kDa region (fraction C). In-gel tryptic digest followed by MS was performed on each of the excised gel fractions. MS unequivocally identified CD36 in all fractions with high sequence coverage (A, 31%; B, 34%; and C, 32%), and all peptides included segments of the extracellular domain (Figure 5C). The prediction would be that peptides from both the N- and C-terminal regions of the extracellular domain with nontryptic cleavage sites should be detected for soluble CD36.

Mass spectrometric analysis identified 3 N-terminal semitryptic peptides (Figure 5A and 5C; Online Figure III). Peptide N1 results from a nontryptic proteolytic cleavage between Gly23 and Gly24. This unique cleavage site is estimated to be 6 amino acids within the extracellular side of the putative N-terminal transmembrane domain (http://uniprot.org), which is not a typical cleavage site for ADAM17. Cleavage 12 amino acids from the transmembrane domain between Glu41 and Val42, a more typical pattern for ADAM17, gives rise to peptide N2. Peptide N3 consists of amino acids 139 to 163 and contains the ADAM17 preferred amino acids alanine at the P1 position and valine at the P1’ position of the cleavage site as observed for several ADAM17 substrates, including tumor necrosis factor-α. Although the N3 peptide is a greater distance from the transmembrane domain than other reported ADAM17 cleavage sites, it is adjacent to a hydrophobic region between amino acids 184 and 204 (Figure 5A and 5C) that may interact with the plasma membrane.

Only 1 semitryptic peptide (C1) was identified in the C-terminal portion of CD36 (Figure 5A and 5C; Online Figure IIIID). Because its cleavage site is 95 amino acids from the C-terminal transmembrane domain, ADAM17 cleavage at this site is unlikely. However, because ADAM17 may share cleavage sites with trypsin (http://merops.sanger.ac.uk), it is possible that some tryp tic peptides actually result from ADAM17 cleavage, such as tryptic peptides with a C-terminal cleavage site at K426 and K430 (Figure 4C). The use of alternative enzymes for proteomics analysis would help address this issue. Nevertheless, our data provide strong evidence for proteolytic cleavage of CD36.

**Increased In Vivo Apoptotic Cell Phagocytosis by Adam17-Null Macrophages Is CD36 Dependent and Promotes Accelerated Resolution of Inflammation**
We next sought to directly test the contribution of CD36 to enhanced efferocytosis by Adam17-null macrophages in vivo.
To investigate in vivo relevance of enhanced efferocytosis by Adam17-null macrophages, we asked whether resolution of the inflammatory response was enhanced after injection of apoptotic thymocytes. Administration of apoptotic thymocytes led to a 33% reduction in the number of WT macrophages within 30 minutes (Figure 6B), indicating that apoptotic cell uptake enhances macrophage clearance and inflammation resolution. Injection of healthy thymocytes or PBS had no effect on macrophage numbers, suggesting no cell-intrinsic acceleration of macrophage exiting at 96 hours. In contrast to WT macrophages, Adam17-null macrophage numbers were reduced 56% in response to efferocytosis (Figure 6B), demonstrating accelerated exiting and highlighting the in vivo impact of increased apoptotic cell uptake in the absence of ADAM17.

Discussion

This study demonstrates for the first time that in vivo efferocytosis and its associated anti-inflammatory effects are enhanced by ADAM17 deletion, suggesting that ADAM17 normally functions to limit apoptotic cell phagocytosis. Unexpectedly, the major target of ADAM17 seems to be the dual-pass SR CD36; its surface levels are elevated in the absence of ADAM17 and the ratio of soluble to cell-associated CD36 is decreased by 25.8%. Further, in the sterile peritonitis model, we detect no Adam17-dependent alterations in surface levels of any other major apoptotic cell receptors, including LOX-1 and MerTK, which were previously implicated as substrates of ADAM17.12,13 Using MS, 3 novel N-terminal peptides and 1 C-terminal peptide were detected in soluble CD36 that represent probable cleavage sites. In addition, we establish that enhanced in vivo efferocytosis in the absence of ADAM17 is CD36 dependent and leads to accelerated clearance of macrophages. These data provide evidence for ADAM17 involvement in the shedding of a dual-pass transmembrane protein and demonstrate the importance of proteolysis in controlling apoptotic cell uptake and resolution of inflammation associated with apoptotic cell uptake.

Recent reports have shown that ADAM17 can cleave MerTK and LOX-1 in vitro, and thus they were particularly likely targets.12,13 However, we demonstrate no change in surface levels of either of these receptors in vivo, whereas CD36 levels are elevated in the absence of ADAM17. A likely explanation for the lack of effect on either MerTK or LOX-1 is that in vitro analyses have shown that their shedding requires stimulation with lipopolysaccharide or tumor necrosis factor-α, respectively.12,13 Thus, ADAM17 cleavage of MerTK and LOX-1 may play a more significant role in responses to pathogens and on classically activated macrophages.

The ADAM17-dependent correlation between macrophage CD36 surface levels and in vivo enhanced efferocytosis further highlights the importance of CD36 as an apoptotic cell receptor. Previous experiments in CD36-deficient mice established that apoptotic cell burden in wound tissue is 2- to 3-fold greater than in WT mice, and in vitro efferocytosis was reduced by ≈40% in CD36-deficient macrophages.33,34 In our studies, elevated surface levels of CD36 in the absence

Figure 4. Adam17 deletion results in a 25% decrease in the ratio of soluble to cell-associated CD36. CD36 content and biochemical characteristics of microparticle-depleted conditioned medium and cell lysates from Adam17-null and wild-type (WT) peritoneal macrophages after the indicated incubation times were analyzed. A, CD36 levels in lysate and media were quantified by ELISA and expressed as the ratio of soluble to cellular CD36, and numbers within the bars indicate the number of mice of different genotypes whose macrophages were evaluated. These data are representative of 2 experiments. B, Soluble levels of CD36 and tumor necrosis factor (TNF) receptor II were evaluated by ELISA after stimulation of WT peritoneal macrophages for 4 hours. Data are expressed relative to levels in phosphate buffered saline (PBS). Values are from 2 different experiments using macrophages from different mice determined in duplicate. C and D, Western blot analysis of conditioned media from Adam17-null or WT peritoneal macrophages after deglycosylation with peptide-N-glycosidase F. Each lane represents media collected from macrophages of different mice after the indicated time, and the collections for C are from a different experiment than shown in A. IL-4 indicates interleukin-4; and LPS, lipopolysaccharide.
of ADAM17 led to increased apoptotic cell uptake by inflammatory macrophages in vivo, and we demonstrate that the majority of ADAM17-dependent enhanced efferocytosis is abolished by CD36 blockade. Low levels of expression of CD36 on resident tissue and peritoneal macrophages and highest CD36 expression on inflammatory macrophages during the resolution phase suggest that its role in efferocytosis may be more important for inflammatory responses than for normal homeostasis. Further, we show that acceleration of macrophage clearance from the peritoneum is dependent on apoptotic cell uptake. Together these data suggest a temporal and context-dependency for ADAM17 modulation of the resolution of inflammation.

To biochemically characterize soluble CD36, we analyzed media collected from cultured WT peritoneal macrophages, and our MS analysis uncovered 3 probable N-terminal cleavage sites. ADAM17 expression on inflammatory macrophages during the resolution phase suggest that its role in efferocytosis may be more important for inflammatory responses than for normal homeostasis. Further, we show that acceleration of macrophage clearance from the peritoneum is dependent on apoptotic cell uptake. Together these data suggest a temporal and context-dependency for ADAM17 modulation of the resolution of inflammation.

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To biochemically characterize soluble CD36, we analyzed media collected from cultured WT peritoneal macrophages, and our MS analysis uncovered 3 probable N-terminal cleavage sites. ADAM17 normally cleaves the extracellular stalk of its substrates proximal to the transmembrane domain (typically within 15 amino acids) with some preferences for particular residues flanking the cleavage site.15 The non-tryptic N terminus of peptide N1 is estimated to be 6 amino acids into the putative transmembrane domain, and, therefore, is less likely to be a direct target of ADAM17. Because we cannot distinguish between ADAM17 involvement in primary and secondary cleavage events, it is possible that initial ADAM17-dependent cleavage of CD36 at N2, N3, or another site leads to activation of regulated intramembrane proteolysis, such as that mediated by γ-secretase,17 or that ADAM17 modulates cleavage by another protease and does not directly target CD36. With a cleavage site 12 amino acids from the transmembrane domain, peptide N2 is more typical of ADAM17 cleavage. In contrast, peptide N3 is 113 amino acids from the transmembrane domain, but 45 amino acids from a hydrophobic region of CD36 that may interact with the plasma membrane,9 and thus may localize the N3 cleavage site in a juxtamembrane position that favors interaction with ADAM17 (Figure 5A). It also contains alanine and valine preferred by ADAM17 in the P1 and P1′ position, respectively.18 Functionally, both N2 and N3 cleavage would likely disrupt the domain of CD36 (amino acids 155–183) implicated in macrophage phagocytosis of apoptotic cells.15 We identified only 1 C-terminal non-tryptic peptide (C1), and because it is 95 amino acids from the C-terminal transmembrane domain, it likely results from cleavage by an enzyme other than ADAM17. Because ADAM17 deletion only inhibits release of soluble CD36 by 25%, other proteases may
be involved in liberation of soluble CD36, such as ADAM10 or multiple other ADAM proteases expressed by peritoneal and bone marrow–derived macrophages (Online Figure IV). Detailed structural data for CD36 and a better understanding of its processing would facilitate interpretation of our data. Although we do not yet understand how soluble CD36 is released from the cell, our data collectively implicate ADAM17 in the shedding of CD36 and establish a role for ADAM17 in the regulation of surface levels of the ditopic transmembrane receptor CD36.

CD36 levels in serum are increased in several chronic inflammatory diseases and have been found to positively correlate with mortality, type 2 diabetes mellitus, and atherosclerotic disease severity. However, the biochemical nature of soluble CD36 has not been evaluated in these contexts. A recent study suggested that the soluble CD36 found in the plasma of healthy donors is full-length CD36 and is a component of microparticles. Although this study analyzed isolated fractions by Western blot analysis and failed to detect CD36 in the microparticle-depleted platelet-free plasma, technical issues with the analysis of plasma may have limited the sensitivity of detection. Given the already robust correlations of soluble levels of CD36 with disease severity and early detection, we plan to investigate the relative distribution of soluble CD36 in microparticles and cleaved soluble forms in different disease states, which could significantly enhance their usefulness as biomarkers. Our analysis of WT macrophage conditioned media in vitro showed a relative distribution of 0.48±0.038% in media depleted of microparticles and 0.042±0.001% in the microparticle pellet after ultracentrifugation, both relative to cell lysate CD36 (n=3 per group). In the absence of ADAM17, CD36 in media was decreased 25.8% (P=0.011; n=3) and microparticle pellet content was increased by 14.3% (P=0.023; n=3). Our data suggest that under these in vitro conditions, shed CD36 in media is a more significant contributor than microparticle-derived CD36. More detailed biochemical analysis is needed to determine the extent to which soluble CD36 in chronic inflammatory diseases may result from ADAM17-mediated shedding.

Our studies have focused on enhanced uptake of apoptotic cells in the absence of macrophage ADAM17 and identified CD36 as the primary apoptotic cell receptor targeted by ADAM17. The increased ADAM17-mediated CD36 shedding uncovered in our study may provide a mechanistic link between the nonresolving nature of certain diseases and defective apoptotic cell phagocytosis. However, in addition to apoptotic cell uptake, CD36 has several other functions, such as uptake of pathogens and modified LDLs important in inflammatory responses, including atherosclerosis, mediation of long-chain fatty acid uptake and transport into cells involved in metabolic disorders, and binding thrombospondin and related proteins to inhibit angiogenesis in wound healing and various pathologies. Essential to these other functions is CD36 assembly of signaling complexes, most likely mediated by the C-terminal cytoplasmic domain. Proteolysis would disable this downstream pathway, and thus it is likely that this mechanism has even broader implications for inflammation and disease pathogenesis than has been uncovered in our investigations. Therefore, it will be important for future studies to define the role of proteolysis in the multiple additional functions of CD36 in normal homeostasis and pathology.

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Disclosures

None.

References


What Is Known?
- Uptake of dying cells decreases inflammation and promotes its resolution.
- In chronic inflammation, uptake of dying cells is impaired and the levels of the soluble forms of their receptors are elevated.
- The proteolysis of cell surface receptors for dying cells can regulate the levels of these receptors on the surface of macrophages.

What New Information Does This Article Contribute?
- Deficiency of the transmembrane protease ADAM17 in macrophages increases in vivo uptake of dying cells.
- ADAM17 mediates the cleavage of the dual-pass apoptotic receptor CD36 and in Adam17-deficient mice enhanced uptake of dying cells is CD36 dependent.
- ADAM17 limits the uptake of dying cells, and thereby the resolution of inflammation.

Dysfunctional uptake of dying cells during chronic inflammation impairs resolution of inflammation, but the underlying mechanisms are poorly understood. Here, we show that in vivo proteolysis by ADAM17 controls the levels of the scavenger receptor CD36 on macrophage surface and, therefore, limits the uptake of dying cells by this receptor. As a post-translational mechanism, ADAM17 proteolysis can rapidly regulate the inflammatory response, but our studies suggest that ADAM17-mediated effects are dependent on both temporal and contextual modulation of CD36 expression. These findings suggest a potential mechanistic link among proteolysis, chronic inflammation, and defective uptake of dying cells.
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**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^{ΔEx5/ΔEx6} (Adam17^{-/-}) or wildtype hematopoietic chimeras were generated as previously described using C57BL/6 ES cells. 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<sup>a</sup> Pepc<sup>b</sup>/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), guinea pig polyclonal anti-SR-A (gift from Debra Rateri), 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, permeabilized 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). 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 FACSscan (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. 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). Briefly, 100 µg of TAMRA-SE was used to label 60 x 10⁶ 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. Opsonized control cells did not receive dexamethasone treatment, and were generated by incubation with 5 µg anti-CD45 antibody (Pharmingen, 01111D) per 10x10⁷ cells for 15 minutes at 4° C. To evaluate apoptotic cell uptake, 1x10⁷ 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-tetramethylinodocarbocianin perchlorate (Dil, Sigma, 42364) by extrusion through a 0.1 µm polycarbonate membrane. 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. 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. 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 45° 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 spectrometry (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 100µl 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 CCT CCC TCT GC-3';
- **ADAM10**: forward 5'-AGG 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 TCTCAA ATC CGT CA-3';
- **ADAM19**: forward 5'-ATTG GCC TCC AAC TGG 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 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 probability 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.
ONLINE REFERENCES


