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

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Abstract

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

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Rationale: Phagocytosis of apoptotic cells (efferocytosis) is mediated by apoptotic cell 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 is 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 uptake of apoptotic cells in wildtype and *Adam17*-null hematopoietic chimeras demonstrates that ADAM17 deficiency leads to a 60% increase in efferocytosis and an enhanced anti-inflammatory phenotype. *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. Soluble CD36 from macrophage-conditioned media is comprised of two ~47 and ~52 kDa species based on Western blotting, and mass spectrometry identifies two non-tryptic, N-terminal peptides in the ~47 kDa species and one non-tryptic, C-terminal peptide in the ~52 kDa fraction, which represent probable cleavage sites. Levels of soluble CD36 are decreased in *Adam17*-null conditioned media, providing the first evidence for involvement of ADAM17 in CD36 cleavage. Importantly, blockade of CD36 *in vivo* abrogates enhanced efferocytosis in macrophages lacking ADAM17.

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.

Chapter 1: Apoptotic cell receptors in chronic inflammatory diseases: Possible regulation by proteolysis

Introduction

The efficient removal of non-essential and damaged cells is a vital aspect of tissue maintenance, wound healing, and the resolution of inflammation.[2] Professional phagocytes, such as macrophages, employ a variety of transmembrane receptors and adaptor molecules to rapidly recognize and ingest apoptotic cells.[3] The evolutionary importance of dead and dying cell removal is evidenced by the number and redundancy of receptors that facilitate apoptotic cell phagocytosis (also termed efferocytosis). Notwithstanding this redundancy, deficient efferocytosis is observed in the context of several chronic inflammatory diseases.

If apoptotic cells are not swiftly cleared, their membranes become increasingly permeable as they undergo secondary necrosis.[4] Leakage of intracellular contents from post-apoptotic cells can have substantial inflammatory consequences. The release of intracellular toxins and proteases damage surrounding cells and amplifies inflammation. Leakage of 'self' antigens from non-phagocytosed dead cells into this inflamed environment can also promote the generation of autoantibodies, resulting in autoimmunity.[5]

Defective apoptotic cell phagocytosis is thought to exacerbate inflammation in atherosclerosis, diabetes, systemic lupus erythematosus, and rheumatoid arthritis.[2] Interestingly, biological fluids from patients with these chronic inflammatory diseases contain increased amounts of soluble apoptotic cell receptors, with levels that reflect disease severity. In vitro studies reveal that a number of transmembrane apoptotic cell receptors are proteolytic cleaved, or shed from the cell surface. Although the biological ramifications of apoptotic cell receptor cleavage and generation of a soluble receptor are unclear, receptor cleavage could inhibit efferocytosis in the context of inflammatory diseases.

This chapter will focus on perspectives gained from recent reports evaluating efferocytosis in chronic inflammatory diseases, levels of soluble apoptotic cell receptors in these diseases, and proteolytic cleavage of apoptotic cell receptors. We will also explore the potential biological ramifications of apoptotic

cell receptor cleavage, and evaluate data from the literature that provides insights as to the possible biological roles of shed apoptotic cell receptors.

Efferocytosis as an important mechanism to maintain homeostasis

As professional phagocytes, macrophages contribute to tissue homeostasis by swiftly and efficiently removing apoptotic cells in a non-inflammatory manner. Macrophages are recruited to the vicinity of apoptotic cells in response to chemoattractant gradients released as a result of caspase activation during programed cell death. Nucleotide triphosphate release can be sensed by the $P2Y_2$ receptor on vascular endothelial cells, inducing expression of vascular cell adhesion molecule (VCAM)-1.[6] VCAM-1 binds to the integrin VLA-4 on leukocytes,[7] promoting monocyte extravasation to the vicinity of apoptotic cells. Tissue macrophages can also detect nucleotide gradients using the $P2Y_2$ receptor, and chemotax towards the apoptotic cell source.[8] Other chemoattractant molecules released by apoptotic cells include the chemokine CX3CL1,[9] as well as the lipids lysophosphatidylcholine (LPC)[10] and sphingosine-1-phosphate (S1P).[11] Macrophages sense CX3CL1 via the CX3CR1 receptor,[9] while LPC and S1P are detected by the G-protein-coupled receptors G2A[12] and S1PR1-5, respectively.[13] The redundancy of chemoattractant signals ensures that macrophages are rapidly recruited to the site of apoptosis to facilitate the phagocytic removal of dying cells.

During apoptosis, the surface of the cell undergoes a transformation. Healthy cells express surface molecules that signal to inhibit phagocytosis, such as CD47[14] and CD31.[15] For example, CD47 binds to the immune inhibitory receptor SIRP-*α* on macrophages, which inhibits myosin-driven formation of the phagocytic cup.[16] Loss or redistribution of cell surface CD47 during apoptosis prevents SIRP-α mediated inhibition of phagocytosis.[17] Similarly, the interaction between CD31 (PECAM-1) on healthy cells and macrophages prevents phagocytosis.[15] Remodeling of cell surface expression of CD47 and CD31 during apoptosis removes inhibitory phagocytosis signals, and permits macrophage-mediated efferocytosis.

Apoptosis not only removes inhibitory phagocytic signals, but also results in the exposure of molecules that promote the recognition of apoptotic cells for phagocytosis (Figure 1). The anionic phospholipid phosphatidylserine (PS) is exposed on the outer-leaflet of an apoptotic cell's membrane early in the apoptotic cascade, and is the best characterized signal for apoptotic cell recognition by macrophages.[18] Several macrophage receptors can bind directly to PS, including lectin-type oxidized LDL receptor (LOX)-1,[19] scavenger receptor for oxidized LDL and PS (SR-PSOX),[20] and CD36.[21] Other macrophage receptors require a bridging molecule for PS-dependent recognition of apoptotic cells. Growth arrest-specific growth factor-6 (GAS-6) and protein S are soluble proteins that can form a bridge between PS and the mer tyrosine kinase (MerTK) receptor on macrophages.[22] Milk fat epidermal growth factor (MFG-E8) is another PS bridging molecule, and facilitates apoptotic cell recognition by binding to macrophage integrins.[23] An additional class of molecules is thought to facilitate macrophage recognition of apoptotic cells by functioning as 'tethering' molecules. Among this family, both CD14[24] and CD93[25] have both been shown to be important for macrophage recognition of apoptotic cells experimentally, however, little is known regarding the nature of their interaction with apoptotic cells.

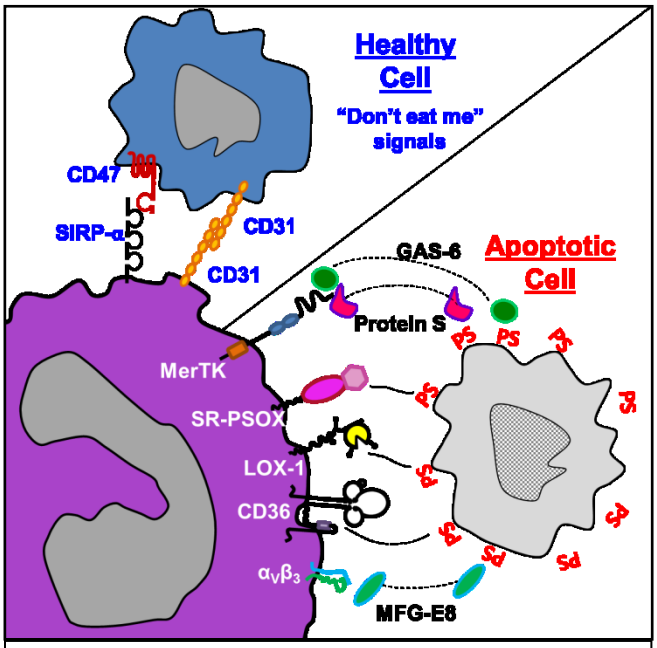

Figure 1: Apoptotic cell receptors, ligands, and bridging molecules
Macrophage phagocytosis is inhibited by 'don't eat me' signals expressed on viable cell, such as CD31 and CD47. During apoptosis, CD31 and CD47 are lost, and phosphatidylserine (PS) is exposed on the surface of the cell. PS is directly recognized for phagocytosis by some apoptotic cell receptors, whereas other receptors require bridging molecules to interact with apoptotic cells.

PS-dependent phagocytosis of apoptotic cells by macrophages is anti-inflammatory.[26] Efferocytosis not only sequesters apoptotic cells, preventing the release of toxic/immunogenic intracellular contents from the dying cell, but macrophages also actively dampen inflammation by releasing anti-inflammatory cytokines such as interleukin (IL)-10[27] and transforming growth factor-β (TGF-β),[28] as well as pro-resolving lipid mediators and eicosanoids[29, 30] following phagocytosis of apoptotic

cells. Continued macrophage recruitment and activation is further limited by anti-inflammatory cytokine release that suppresses Toll-like receptor (TLR)-dependent production and release of IL-1β, IL-6, IL-8 and TNF-α.[31-33] Lipoxins, resolvins, and protectins are pro-resolving lipid mediators released in response to efferocytosis that augment continued apoptotic cell uptake, while promoting macrophage efflux and the resolution of inflammation.[29, 34] The mechanisms of apoptosis and efferocytosis have evolved to provide a rapid, non-inflammatory system for the removal of damaged and non-essential cells.

Defective efferocytosis in chronic inflammatory diseases

Although non-phagocytosed apoptotic cells are exceedingly rare in healthy tissue, increased apoptotic cell burden is observed in tissue from patients with several chronic inflammatory diseases, including atherosclerosis,[35-38] diabetes, [31, 32] and systemic lupus erythematosus (SLE).[39-41] If apoptotic cells are not rapidly cleared, the dying cell's plasma membrane becomes increasingly permeable as secondary necrosis ensues. Leakage of intracellular antigens and toxins from the post-apoptotic cell results in damage to the surrounding tissue, and amplified inflammation. The continued recruitment of inflammatory cells in the presence of intracellular antigens and DNA-fragments is thought to give rise to autoantibody production, and promote the development of autoimmune diseases like SLE and rheumatoid arthritis.[2] Impaired efferocytosis creates an environment where pro-inflammatory cytokine release is not suppressed by the efferocytosis-dependent production of TGF-β[28] and IL-10[27], and the resolution of inflammation is not promoted by eicosanoid release.[29] Defective efferocytosis may contribute to the non-resolving nature of chronic inflammatory diseases.

Atherosclerosis is a chronic inflammatory disease that developing over decades, and is responsible for more deaths per year in the industrialized world than any other disease. Apoptotic bodies are rare in early lesions; however dead cell debris is hallmark of an advanced lesion's necrotic core.[35-38] Defective efferocytosis by lesional macrophages is thought to contribute to the development and expansion of the necrotic core. Of all atherosclerosis related deaths, 75% result from plaque rupture,[42, 43] and post-mortem studies reveal that culprit plaques contain larger necrotic cores than non-ruptured plaques.[44-46] Necrotic core expansion resulting from impaired apoptotic cell phagocytosis may contribute to increased plaque vulnerability and elevated mortality risk.

Several other chronic inflammatory diseases also exhibit defects in apoptotic cell phagocytosis. Compromised efferocytosis is thought to impede wound healing in diabetics who frequently develop non-resolving ulcers that can lead to limb amputation.[47, 48] Experimental models of type I and II diabetes reveal that macrophages from diabetic mice have a reduced ability to phagocytose apoptotic cells *in vitro*,[49, 50] as well as *in vivo*.[48, 51, 52] There is also ample experimental evidence linking autoimmune disorders to defective efferocytosis; increased levels of apoptotic cells are found in patients with active SLE,[39, 40] and macrophages from these patients display impaired efferocytosis.[40, 41]

Through genetic analysis of patients, as well as animal studies evaluating targeted deletion of apoptotic cell receptors, it has become clear that impaired efferocytosis contributes to chronic inflammatory disease pathology. Although the underlying mechanisms responsible defective efferocytosis in most patients remains elusive, experimental interference with a number of different apoptotic cell receptors in mouse models of disease has revealed the close link between impaired efferocytosis and the pathology of atherosclerosis, diabetes and SLE. Defective efferocytosis resulting from the genetic deletion of the apoptotic cell-bridging molecule MFG-E8 results in accelerated atherosclerosis,[53] diabetes,[54] and a lupus-like phenotype in mice.[23, 55] Similarly, mutation of the apoptotic cell receptor MerTK results in a lupus-like phenotype,[56] and mutant mice on an atherogenic background develop plaques with larger necrotic cores and elevated levels of non-phagocytosed apoptotic cells,[57, 58] Although the molecular mechanisms that compromise efferocytosis in atherosclerosis, diabetes and SLE are not yet fully understood, mounting evidence (as outlined above) suggests that certain apoptotic cell receptors can become inactivated in the context of chronic inflammation.

Levels of soluble apoptotic cell receptors are elevated in several chronic inflammatory diseases

Loss of apoptotic cell receptors from the surface of phagocytic cells is a potential mechanism that could alter the efficiency of efferocytosis in chronic inflammatory diseases. Interestingly, elevated levels of soluble apoptotic cell receptors are found in biological fluids from patients with a variety of chronic inflammatory diseases that display impaired efferocytosis (Table 1). Deficient efferocytosis in these diseases could be directly related to increased levels of circulating soluble receptors: loss of apoptotic cell receptors from the surface of phagocytes could result in impaired apoptotic cell recognition, and reduce

the overall rate of apoptotic cell phagocytosis. Consequently, less efferocytosis could result in increased tissue damage by post-apoptotic cells, and the sustained inflammatory environment characteristic of chronic inflammatory diseases.

Table 1: Increased soluble apoptotic cell receptors in chronic inflammatory diseases

Receptor	Disease	Fluid	Correlation with disease severity	Reference
CD36	atherosclerosis	serum, plasma	yes	59-61
	diabetes	plasma	yes	62-64
	scleroderma	serum	yes	65
CD93	atherosclerosis	plasma	yes	66
	rheumatoid arthritis	synovial	not tested	67
	lupus	serum	not tested	68
	scleroderma	serum	yes	68
LOX-1	atherosclerosis	serum, plasma	yes	69-71
	diabetes	serum	yes	72
	rheumatoid arthritis	synovial, plasma	yes	73
MerTK	rheumatoid arthritis	plasma	not tested	74
	lupus	plasma	not tested	74
SR-PSOX	atherosclerosis	serum, plasma	yes	75-81
	diabetes	serum	not tested	82
	rheumatoid arthritis	synovial, urine, serum	yes	83-85
	lupus	urine, serum	yes	82, 84
	scleroderma	serum	yes	82

Primarily, two major mechanisms can lead to the release of soluble transmembrane receptors; receptors are either proteolytically shed from the cell surface, or released from the cell as membrane-bound components of microvesicles. The potential that soluble apoptotic cell receptors result from proteolytic cleavage will be discussed in detail in following sections, and is a focus of this dissertation. Alternatively, inflammation could cause increased microparticle release, yielding elevated levels of soluble apoptotic cell receptors. Several studies have established a positive correlation between

microparticle levels and inflammation: increased microparticle concentrations are detected in the plasma and synovial fluid from RA patients,[86] as well as in the plasma from patients with SLE,[87] systemic sclerosis (SSc; scleroderma),[88] diabetes,[89] and atherosclerosis.[90] While the majority of microparticles in blood originate from platelets, phagocytes are also able to release microparticles.[91] Activation of macrophages with a variety of TLR ligands greatly enhances microparticle release *in vitro*.[92, 93] Apoptotic cells are another potential source of microparticle in disease since PS exposure during apoptosis can lead to microparticle release.[94]

Although the studies referred to in table 1 did not differentiate between microvesicle-derived and proteolytically cleaved receptors in the context of disease, several of these receptors are detected in healthy plasma as cleaved proteins (Table 2). For example, ELISA analysis reveals that soluble CD93 in healthy serum does not react with antibodies raised against the cytoplasmic domain of CD93, suggesting that the receptor has been cleaved from the cell surface.[95] Another approach to differentiate cleaved receptors from full-length microvesicle-bound receptors is to evaluate the electrophoretic mobility of the soluble receptor. Western blot analysis of soluble receptors reveals an increased electrophoretic mobility for plasma soluble MerTK,[96, 97] as well as for LOX-1,[98] and SR-PSOX[99] in conditioned cell culture media as compared to their full-length counterparts. Although evidence for the proteolytic shedding of several apoptotic cell receptors exists, biochemical characterization of these soluble receptors has not been performed in the context of disease. The relative contribution of microparticle release and proteolytic shedding to the increased soluble receptor levels found in disease remains poorly defined.

Proteolysis as a regulatory mechanism of efferocytosis

Apoptotic cell receptors could be shed from the cell surface by either soluble or transmembrane proteases. Matrix metalloproteinases (MMPs) are the best described members of the heterogeneous family of extracellular proteases. Known for their ability to cleave extracellular matrix, various soluble MMPs are released into the tissue by infiltrating macrophages, as well as many other cell types.[100-102] Soluble MMP-mediated cleavage of proteins often occurs at specific amino acid consensus sequences,[103] whereas transmembrane proteases are less dependent on sequence motifs.

Table 2: Apoptotic cell receptors with evidence of proteolytic cleavage

Soluble receptor	Evidence of cleavage *in vitro*	Evidence of cleavage *in vivo*	Suspected protease
CD36	**ND**	**no**[104, a, b]	**ND**
CD93	**yes**[95]	**yes**[95, b]	**MMPs**[103]
LOX-1	**yes**[98]	**ND**	**ADAM10**[105], **ADAM17**[106]
MerTK	**yes**[96, 97]	**yes**[96, 97, c]	**ADAM17**[97]
SR-PSOX	**yes**[99]	**ND**	**ADAM10**[107], **ADAM17**[108]

All evaluations showed a mobility shift for soluble receptor as compared to full-length by Western blot, unless otherwise noted. In [a], cleavage was determined by the absence of the cytoplasmic domain by ELISA. Analysis of healthy donor plasma is indicated by [b]. Mouse serum is indicated by [c].

ADAM10 and closely related ADAM17 are the best characterized transmembrane proteases, and have been identified as key proteases in most shedding events characterized to date.[109] ADAM-mediated ectodomain cleavage occurs near the plasma membrane, and does not require a lengthy consensus sequence.[97, 110] Both ADAM17 and ADAM10 are ubiquitously expressed, and are essential for development. Knock-out of either ADAM17 or ADAM10 is embryonically lethal.[111] While both proteases preferentially cleave specific substrates, genetic deletion studies have revealed that in the absence of ADAM17, ADAM10 can compensate by cleaving substrates that are usually shed by ADAM17 in murine cells.[111]

Proteins can be shed by ADAM17 in a both a constitutive and inducible manner.[111] A variety of factors can influence ADAM17-mediated cleavage. *In vitro*, macrophage activation with a variety of stimulants can induce the cleavage of specific substrates.[97] Both G-protein coupled receptor[112] and protein kinase C[113] activation have been shown to induce the shedding of ADAM17 substrates. Other ADAM17-mediated cleavage events are dependent on substrate co-localization on the plasma membrane; ADAM17 can associate with lipid rafts under certain conditions, which can promote cleavage of other raft-associated proteins.[114]

ADAM17-mediated cleavage releases the extracellular domain of a host of cell-surface proteins involved in inflammation, making it a likely protease for the cleavage of apoptotic cell receptors. Of note, ADAM17 has been implicated in the shedding of MerTK,[97] LOX-1,[106] and SR-PSOX.[108] However, the consequence of ADAM17-mediated shedding on apoptotic cell phagocytosis has not been evaluated.

Role of soluble apoptotic cell receptors

As outlined below, *in vitro* and *in vivo* evidence supports the concept that soluble receptors retain their ability to bind to apoptotic cells. For example, recombinant soluble LOX-1 binds directly to apoptotic cells,[19] and inhibits LOX-1 mediated phagocytosis *in vitro,*[19, 115] and *in vivo.*[73] Similarly, behaving as an antagonist, soluble forms of MerTK,[96, 116] and CD36[117] inhibit apoptotic cell phagocytosis *in vitro.* Although the efferocytosis-blocking activity of physiologically/pathologically relevant levels of soluble receptors has not been evaluated, the potential exists that elevated levels in disease could further dampen efferocytosis by blocking cell-associated receptor binding to apoptotic cells.

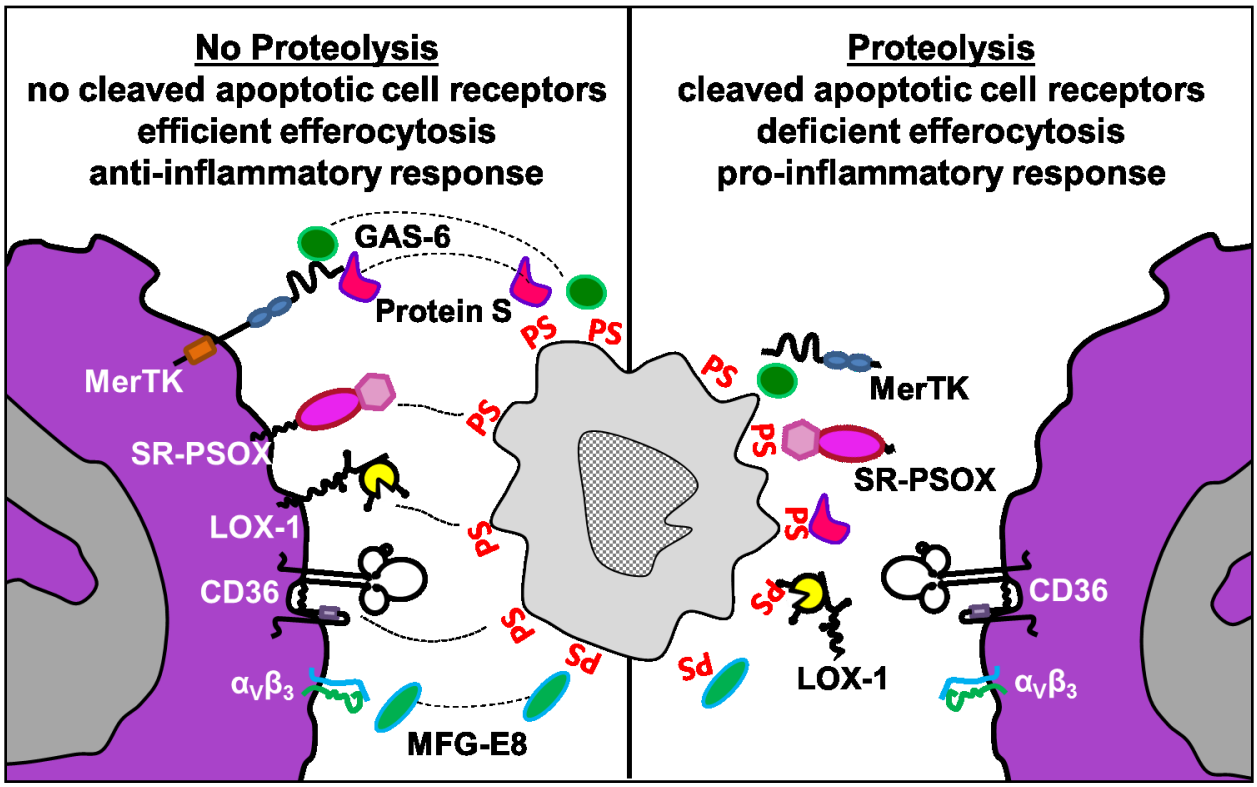

Figure 2: Apoptotic cell receptor shedding may limit phagocytosis. Proteolysis of apoptotic cell receptors from the phagocyte could impair apoptotic cell recognition. Additionally, soluble apoptotic cell receptors could function as antagonists to block apoptotic cell recognition by phagocyte-associated receptors.

It should be stressed that soluble receptors detected in assays of biological fluids do not directly reflect the amount of receptor released from the cell, but rather report the amount of 'non-bound' receptor. Since some soluble receptors have been shown to retain biological activity, it is quite possible that they can bind to cell-associated ligands following their release, and be drawn out of the soluble phase. Although

individual 'non-bound' soluble receptors are present in the low nanomolar range in disease fluids,[59, 74, 80, 95] the combined effect of elevated levels of several different soluble receptors may have significant pathological consequences on efferocytosis *in vivo*.

The apparent correlation between increased levels of soluble apoptotic cell receptors with diseases that exhibit impaired efferocytosis suggests that receptor loss from the cell surface could impair efferocytosis. If receptors are lost from the cell-surface by shedding, one might predict that the cell of origin may become a less effective phagocyte. Furthermore, soluble receptors may act as antagonists to inhibit apoptotic cell phagocytosis. In addition to being biomarkers of disease, soluble apoptotic cell receptors may also be intimately linked to the pathogenesis of the disease. However, we are unaware of any reports that have tested the functional contribution of proteolysis to the regulation of apoptotic cell uptake *in vivo.*

Hypothesis:

The data summarized above provides evidence for impaired efferocytosis in certain chronic inflammatory diseases, and demonstrates that levels of soluble apoptotic cell receptors are elevated in these diseases. We also summarize data demonstrating proteolytic cleavage of apoptotic cell receptors. We present the hypothesis that apoptotic cell receptor cleavage may provide a mechanistic link between elevated levels of soluble apoptotic cell receptors, impaired efferocytosis, and the chronic inflammatory nature of certain diseases.

This dissertation will specifically test the hypothesis that *in vivo* efferocytosis and its concomitant anti-inflammatory consequences are limited by proteolytic cleavage of apoptotic cell receptors from the macrophage surface.

Chapter 2: Macrophage ADAM17 deficiency augments CD36-dependent apoptotic cell uptake and the linked anti-inflammatory phenotype

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ABSTRACT

Rationale: Phagocytosis of apoptotic cells (efferocytosis) is mediated by apoptotic cell 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 is 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 uptake of apoptotic cells in wildtype and *Adam17*-null hematopoietic chimeras demonstrates that ADAM17 deficiency leads to a 60% increase in efferocytosis and an enhanced anti-inflammatory phenotype. *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. Soluble CD36 from macrophage-conditioned media is comprised of two ~47 and ~52 kDa species based on Western blotting, and mass spectrometry identifies two non-tryptic, N-terminal peptides in the ~47 kDa species and one non-tryptic, C-terminal peptide in the ~52 kDa fraction, which represent probable cleavage sites. Levels of soluble CD36 are decreased in *Adam17*-null conditioned media, providing the

first evidence for involvement of ADAM17 in CD36 cleavage. Importantly, blockade of CD36 *in vivo* abrogates enhanced efferocytosis in macrophages lacking ADAM17.

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.

INTRODUCTION

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, employ a variety of transmembrane receptors to rapidly recognize and ingest apoptotic cells.[3] 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 pro-resolving lipid mediators and eicosanoids which promote macrophage efflux and the resolution of inflammation.[26, 29, 30] 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 non-resolving foot ulcers in diabetes to necrotic core expansion in atherosclerosis.[38, 47, 48, 118] However, the underlying mechanisms responsible for deficient apoptotic cell uptake are poorly understood.

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 LDL receptor (LOX)-1.[60, 64, 65, 71, 73, 74] CD36 is a two-pass transmembrane receptor,[1] while MerTK[119] and LOX-1[120] are type I and type II transmembrane proteins, respectively. MerTK and LOX-1 can be proteolytically cleaved by the transmembrane protease ADAM17.[97, 106] Although ADAM17 was first described for its role in the shedding of cell-associated tumor necrosis factor-α, it has since been shown to cleave a variety of cell surface proteins involved in inflammation.[121, 122] Unlike soluble enzymes that cleave substrates at specific consensus sequences, ADAM17 cleaves its substrates at a membrane-proximal site in which stalk length and distance from the membrane appear to be important.[110] ADAM17 membrane proximal

cleavage of its substrates releases almost the entire extracellular domain, and the soluble ectodomain can often act as a antagonist, which has been shown for LOX-1 and MerTK.[19, 96] Thus proteolysis of apoptotic cell receptors has the potential to rapidly decrease receptor surface levels and release a soluble antagonist, both of which could exacerbate deficient efferocytosis. However, the functional contribution of proteolysis to the regulation of apoptotic cell uptake *in vivo* has not been tested.

The current investigation tests the hypothesis that efferocytosis and its concomitant anti-inflammatory consequences are limited by proteolytic cleavage of apoptotic cell receptors from the macrophage surface. We show for the first time that ADAM17 deletion enhances macrophage-mediated efferocytosis *in vivo*, resulting in an augmented anti-inflammatory response. Additionally, we report that macrophage CD36 surface levels are elevated in the absence of ADAM17, whereas MerTK and LOX-1 are not. In addition, we identify two probable N-terminal cleavage sites and one C-terminal site in soluble CD36 from macrophage-conditioned media, and the absence of ADAM17 leads to a decrease of soluble CD36 levels. Blockade of CD36 *in vivo* is sufficient to abolish enhanced efferocytosis by *Adam17*-null macrophages. Together these studies establish that ADAM17-mediated proteolysis of CD36 is an important post-translational mechanism controlling apoptotic cell phagocytosis.

METHODS

Hematopoietic chimeric mice

Adam17$^{\Delta Ex5/\Delta Ex5}$ (*Adam17* -/-) or wildtype hematopoietic chimeras were generated as previously described using C57BL/6 ES cells.[123] 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.

Sterile peritonitis model

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

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),[124] guinea pig polyclonal anti-SR-A (gift from Debra Rateri),[125] 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).[126] 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.[127] 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).[128] 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 ml/L b-mercaptoethanol for 5 hrs at 37° C, yielding a population of thymocytes that were 60-80% Annexin V positive.[18] 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.[26] 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.

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.[129] Thioglycollate-elicited cells from wildtype or ADAM17 null hematopoietic chimeras 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.[130] 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.[131] 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) with data-dependent acquisition of MS/MS scans (linear ion trap) on the 8 most abundant ions 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 (LTQ-Orbitrap 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, v.XX, 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

96-well plates (Nunc-Immuno 62409-003) were coated overnight at room temperature with 50 µl/well of 0.5 mg/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 ml 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 mg/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 ml 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.

Statistical analysis

For statistical analysis, the Student's *t*-test was performed using the InStat software, version 3.0b. All error bars represent standard error of the mean, unless otherwise noted.

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 wildtype hematopoietic chimeras were injected intraperitoneally with the sterile irritant thioglycollate to promote inflammatory monocyte influx. Four days after thioglycollate administration, a time point when greater than 85% of the elicited cells are F4/80$^+$ macrophages, 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 wildtype macrophages is observed following injection of either healthy control or opsonized thymocytes (Figure 1B). In contrast, administration of apoptotic thymocytes demonstrates a 1.6-fold increase in *Adam17*-null macrophage binding and uptake of apoptotic cells relative to wildtype (Figure 1B). This significant increase in macrophage-mediated efferocytosis suggests that ADAM17 may directly or indirectly change levels of one or more apoptotic cell receptors.

Mixed hematopoietic chimeras containing a 50% mixture of *Adam17*-null and wildtype bone marrow were evaluated to determine whether efferocytosis in the absence of ADAM17 is indirectly increased as a result of alteration of the extracellular inflammatory environment, for example by soluble mediators. Although both *Adam17*-null and wildtype cells in the mixed chimeras are exposed to an identical extracellular milieu, *Adam17*-null macrophages still display enhanced efferocytosis (Figure 1B, right

panel). These results indicate that the increase in apoptotic cell phagocytosis by *Adam17*-null macrophages is a cell-intrinsic feature and it directly impacts apoptotic cell receptor function.

Efferocytosis has been shown to actively inhibit the macrophage inflammatory response.[26] 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 (iNOS) were compared by flow cytometric analysis following injection of apoptotic thymocytes or a PBS sham injection. As shown in Figure 1C and 1D, the anti-inflammatory response is augmented in *Adam17*-null macrophages relative to wildtype as demonstrated by a 34% elevation of intracellular arginase I levels following *in vivo* uptake of apoptotic cells, and a 45% reduction in iNOS induction following subsequent inflammatory stimulus *ex vivo*. No differences in arginase I or iNOS levels were observed in *Adam17*-null or WT macrophages injected with control thymocytes (data not shown). Thus, ADAM17 deletion significantly augments the macrophage efferocytosis-induced anti-inflammatory phenotype.

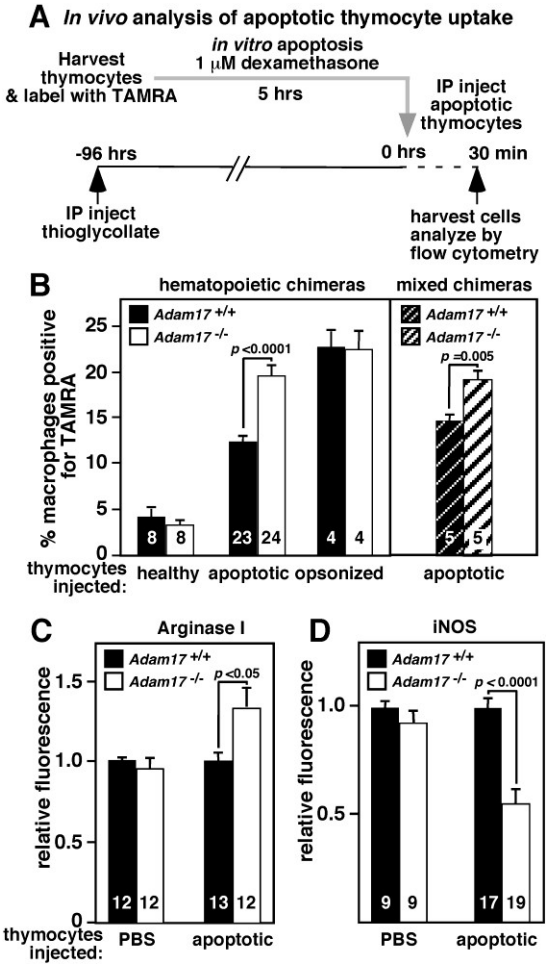


Figure 1: Increased *in vivo* efferocytosis of apoptotic thymocytes by *Adam17*-null macrophages is cell autonomous, and shifts them to a less inflammatory phenotype. **A**. The scheme for *in vivo* peritoneal macrophage uptake of TAMRA-labeled apoptotic thymocytes (*in vitro* treatment with dexamethasone) is shown. Healthy thymocytes and opsonized thymocytes were evaluated as controls. **B**. Wildtype, *Adam17*-null, or mixed hematopoietic chimera mice that have ~50% wildtype and ~50% *Adam17*-null leukocytes were injected with the indicated thymocytes. The percent of TAMRA-positive macrophages (with bound or phagocytosed thymocytes) was assessed by flow cytometry. **C**. Following *in vivo* exposure to PBS or apoptotic thymocytes, *Adam17*-null or wildtype macrophages were analyzed immediately following harvest for intracellular levels of arginase I, or **D**. cultured *ex-vivo* for 20 hours with lipopolysaccharide and IFNγ and analyzed for intracellular iNOS by flow cytometry. Numbers in the bars indicate the number of mice evaluated, and p values for significant differences are shown.

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. Since macrophage apoptotic cell uptake is dependent upon apoptotic cell receptor recognition of phosphatidylserine (PS) exposed by cells undergoing apoptosis,[26] PS liposomes were employed as a surrogate for apoptotic cells for an *in vitro* screen of potential receptors responsible for the enhanced efferocytosis by *Adam17*-null macrophages. As shown in Figures 2A and 2C, *Adam17*-null macrophages bind more PS liposomes *in vitro*, but not phosphatidylcholine liposomes (data not shown), suggesting that ADAM17 deletion results in elevated levels of receptors that recognize PS exposed on apoptotic cells. Although many apoptotic cell receptors can bind PS, a unique subset of receptors share an affinity for acetylated LDL (acLDL) as a ligand. When acLDL 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 and uptake by *Adam17*-null macrophages have the ability to directly bind both acLDL and PS liposomes – a trait shared by CD36 and SR-BI.[21]

To more broadly screen for possible changes in apoptotic cell receptor surface levels, freshly isolated thioglycollate-elicited peritoneal macrophages from wildtype and *Adam17*-null chimeras were analyzed by flow cytometry (Figure 2B). ADAM17 deletion results in a 30% increase in macrophage surface CD36 but no difference in other apoptotic cell receptors (Figure 2B), even though literature reports indicate MerTK, LOX-1 and SR-PSOX can be targets of ADAM17.[97, 106, 108] 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. Also, no difference in CD36 mRNA levels is observed by qPCR analysis of *Adam17*-null and wildtype macrophages (data not shown), suggesting that ADAM17-dependent mechanisms regulate CD36 surface levels post-translationally. SR-BI surface levels are not shown because they were not significantly elevated above isotype background staining on *Adam17*-null or wildtype macrophages. The combined data showing an acLDL-sensitive increase in PS liposome binding by *Adam17*-null macrophages and elevated CD36 surface levels implicate CD36.

To 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 wildtype 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 whose elevated surface levels lead to enhanced PS liposome binding and uptake *in vitro* by *Adam17*-null macrophages.

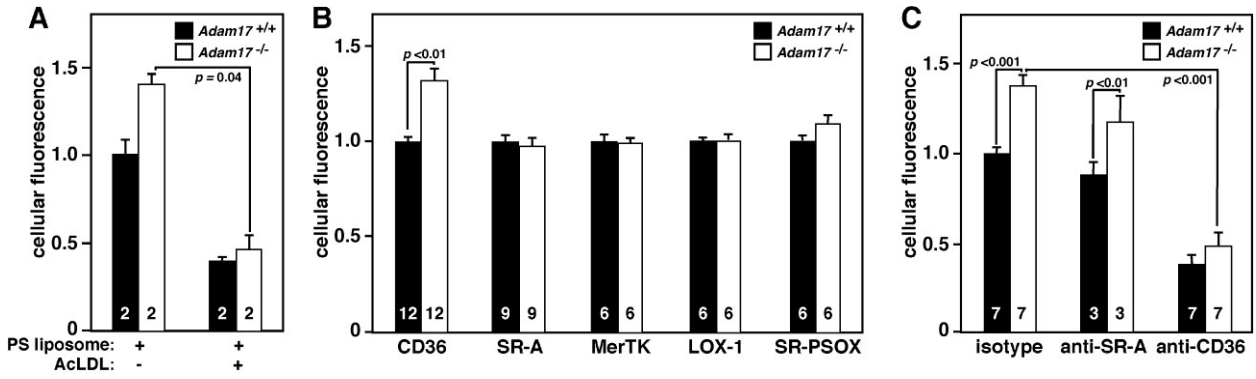
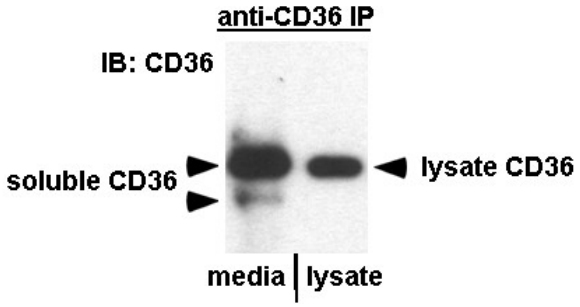


Figure 2: *Adam17*-null macrophages show increased CD36-dependent phosphatidylserine liposome binding/uptake, and increased CD36 surface levels. Peritoneal macrophages from wildtype or *Adam17*-null hematopoietic chimera mice were harvested 4 days after peritoneal thioglycollate injection, and either **A.** plated and treated with or without acetylated-LDL at 4°C for 15 minutes followed by incubation with 40 μmol/L fluorescently labeled phosphatidylserine (PS) liposomes for 4 hours at 4°C, or **B.** stained with antibodies to the indicated apoptotic cell receptors immediately after collection to assess receptor surface levels, or **C**. plated and incubated with 160 μmol/L fluorescently labeled PS liposomes and 5 mg/L of either receptor-blocking or isotype control antibody for one hour at 37°C. Fluorescence was assessed by flow cytometry, numbers within the bars indicate the number of mice evaluated, and *p* values for significant differences are shown.

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 wildtype peritoneal macrophages. To reduce potential microvesicle content, the media were centrifuged at high speed, conditions in which microvesicles are depleted by ~75%.[131] Quantification of media CD36 levels by ELISA shows that *Adam17* deletion reduces the ratio of soluble/cellular CD36 by 25.8% (Figure 3A). Since CD36 is a highly glycosylated protein, Western analysis was performed following PNGase F treatment of media samples (Figure 3B) to assess molecular species. Conditioned media from wildtype macrophages show primarily

two regions of staining with apparent molecular weights of ~52 and ~47 kDa (Supplemental Figure 1). The ~47 kDa region appears as a doublet, although detection of two distinct species is variable. However, CD36 levels in both the ~52 and ~47 kDa regions are reduced in conditioned media from *Adam17*-null macrophages relative to wildtype. Together, these observations provide the first evidence for a role of ADAM17 in the proteolytic release of soluble CD36.



Supplimental figure 1: A fraction of soluble CD36 has an increased electrophoretic mobility as compared to lysate. CD36 content in microparticle-depleted 24-hour-conditioned medium and cell lysate from wildtype peritoneal macrophages was analyzed by Western blot following immunoprecipitation and deglycosylation with PNGase F.

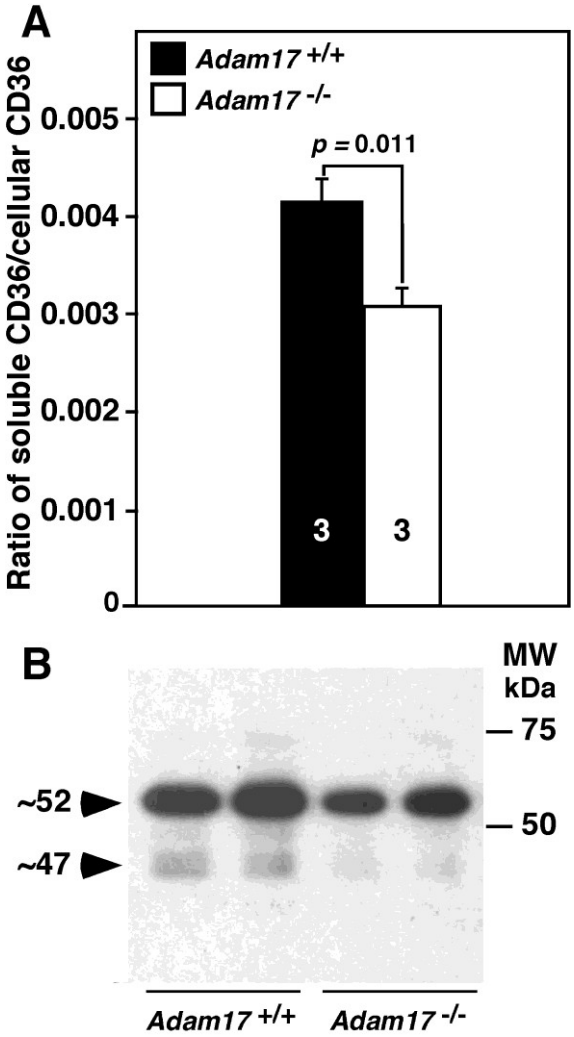


Figure 3: *Adam17* deletion results in a 25% decrease in the ratio of soluble to cell- associated CD36. CD36 content and biochemical characteristics of microparticle-depleted 24-hour-conditioned medium and cell lysates from *Adam17*-null and wildtype peritoneal macrophages were analyzed. **A.** CD36 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.** Western blot analysis of conditioned media from *Adam17*-null and wildtype peritoneal macrophages following deglycosylation with PNGase F. Each lane represents media collected from macrophages of different mice, and the collections are from a different experiment than shown in A.

Mass spectrometry identifies two probable N-terminal cleavage sites in soluble CD36

To determine the identity of the two regions in soluble CD36 detected by Western analysis, conditioned media from wildtype macrophages were immunoprecipitated with anti-CD36 (Figure 4A). Protein was detected by Coomassie staining and Western analysis from adjacent lanes, and three fractions were cut from the Coomassie-stained gel to include the ~52kDa region (fraction A), the zone between the two regions (fraction B) and the ~47kDa region (fraction C). In-gel tryptic digest followed by mass spectrometry was performed on each of the excised gel fractions (Figure 4A). Mass spectrometry 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 4C). The ability to distinguish between shed (proteolytically processed) and full-length transmembrane proteins is dependent upon detection of peptides from the cytosolic domain and/or peptide(s) with a non-tryptic terminus. Since CD36 is thought to be a dual pass transmembrane protein,[1] the prediction would be that peptides from both the N- and C-terminal regions of the extracellular domain with non-tryptic cleavage sites should be detected for soluble CD36. However, the short cytoplasmic domains (7-10 amino acids) and limited number of tryptic cleavage sites in both N-terminal and C-terminal stubs (transmembrane and cytosolic domains) of CD36 lowers the probability of identification of its cytosolic domains. Notably, peptides originating from the cytosolic and transmembrane domains were not identified previously in an analysis of isolated macrophage membranes.[132] Therefore, identification of peptides specific to soluble CD36 is primarily dependent upon detection of non-tryptic peptides.

Mass spectrometric analysis identified two N-terminal non-tryptic peptides N1 and N2 (Figure 4B-D) unique to the ~47-kDa region (fraction C). Peptide N1 results from a non-tryptic proteolytic cleavage between Gly23-Gly24 (Figure 4B). This unique, non-tryptic cleavage site is estimated to be six 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.[110] Peptide N2 (Figure 4B-D) consists of amino acids 139-163 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-a.[110] Although the N2 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

4C) that may interact with the plasma membrane.[1] Three other peptides with non-tryptic N-termini around the N2 peptide were also detected, and the longest peptide was found in both the ~47 kDa and ~52 kDa regions (Figure 4D).

Only one non-tryptic peptide (C1) was identified in the C-terminal portion of CD36, and it was detected only in the ~52 kDa region (Figure 4C, 4E). Because its cleavage site is 57-61 amino acids from the C-terminal transmembrane domain, ADAM17 cleavage at this site is unlikely. Detection of at least two distinct non-tryptic C-terminal peptides in the ~47 kDa region would be expected due to the presence of both N1 and N2 in the ~47 kDa region. A possibility is that fully tryptic peptides with a C-terminal cleavage site at K426 and/or K430 (Figure 4C) could result from ADAM17 rather than trypsin cleavage since K is found in the P1 position of several ADAM17 substrates (http://merops.sanger.ac.uk). The failure to detect additional C-terminal non-tryptic peptides could also be due to limitations of the LCMS approach to identify very short peptides determined by distribution of Lys and Arg residues. Although CD36 structure and the topology of its two proposed transmembrane domains are poorly understood,[1] our data provide strong evidence for proteolytic cleavage of CD36.

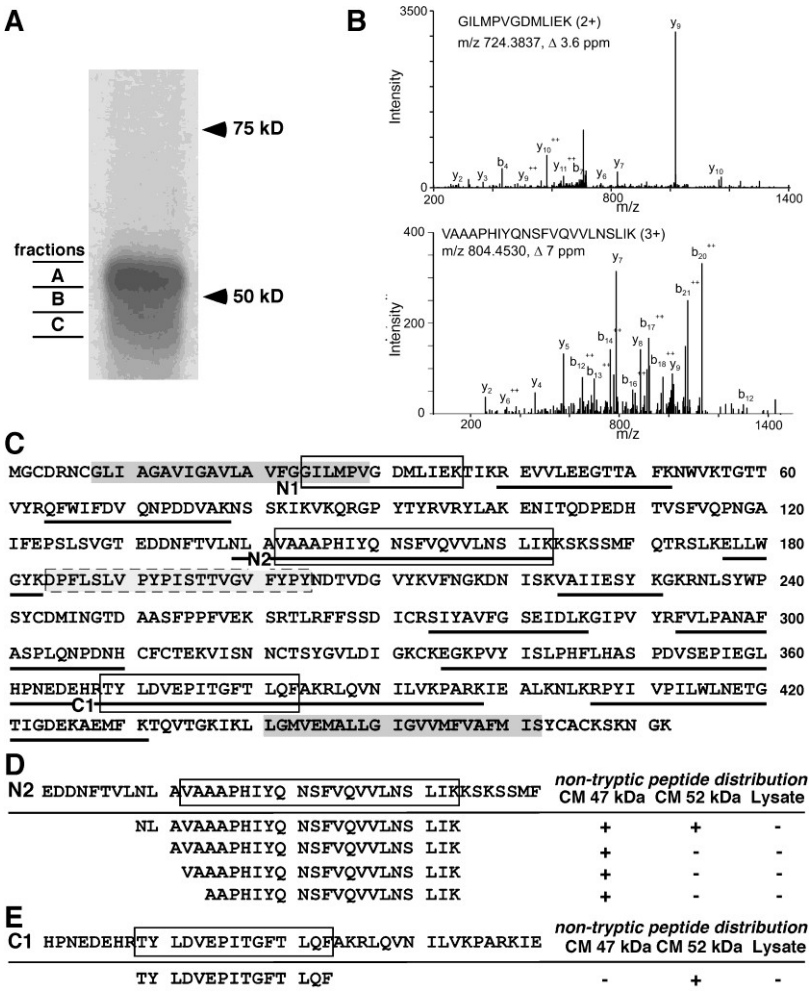


Figure 4: Identification of novel proteolytic cleavage sites in soluble CD36. **A**. Soluble CD36 immunoprecipitated from microparticle-depleted 24-hour-conditioned media were separated on SDS-PAGE following PNGase F treatment. **A**, Western showing three gel fractions (A, B, C) from the regions corresponding to the two major CD36 staining regions seen in Figure 3 and the area between them that were excised as indicated, in-gel digested and subjected to mass spectrometric analysis. **B.** Spectra from two unique peptides with non-tryptic N-terminal sequences were identified with high confidence (N1, PeptideProphet probability 0.98, delta mass 3.59 ppm; N2, Peptide Prophet probability 0.95, delta mass 6.96 ppm), both only in the ~47 kDa region. **C**. CD36 protein sequence is shown and peptides identified in the 3 gel slices are underlined, two putative transmembrane domains are in dark gray boxes, and a hydrophobic region that may interact with the plasma membrane is the gray box with dashed outline. The unique peptides whose spectra are shown in B (N1 and N2) and a C-terminal non-tryptic peptide (C1) are indicated by open black boxes. **D.** Four peptides with non-tryptic N-termini around the N2 peptide were detected and their distribution in different fractions is indicated. **E.** One peptide with a non-tryptic C-terminal cleavage (C1) between Phe383-Ala384 was detected only in the ~52 kDa regions with PeptideProphet probability of 0.95.

Increased *in vivo* apoptotic cell phagocytosis by *Adam17*-null macrophages is CD36-dependent

Since our data implicate CD36 as a primary target of ADAM17 based on *in vitro* evaluation of PS liposome uptake and biochemical analysis of conditioned media, we directly tested the contribution of CD36 to enhanced efferocytosis by *Adam17*-null macrophages *in vivo*. CD36-blocking or isotype control Fab were injected at the same time as the fluorescently-labeled apoptotic thymocytes into the peritoneum of chimeras. Fab was used to avoid CD36 dimerization and activation that occurs with whole IgA.[124] CD36 blockade significantly decreases efferocytosis by *Adam17*-null, but not wildtype, macrophages (Figure 5). The absence of an effect on apoptotic cell uptake by wildtype macrophages *in vivo* as compared with *in vitro* liposome uptake (Figure 2C) may be due to a disparate response to whole IgA used in the *in vitro* screen or differences in the effective dosage required *in vivo.* However, these *in vivo* data establish that CD36-mediated apoptotic cell phagocytosis is selectively enhanced in the absence of ADAM17.

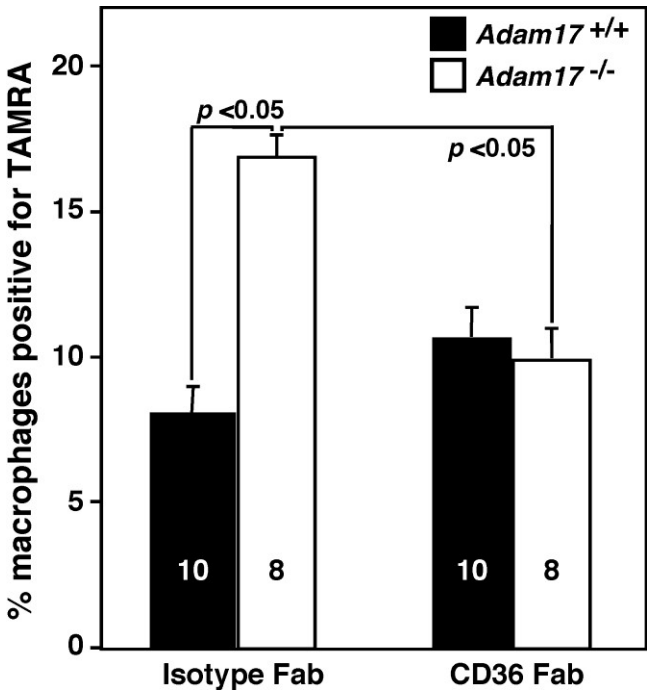


Figure 5: Enhanced efferocytosis by *Adam17*-null macrophages is abolished by CD36 blocking Fab. Fluorescently labeled apoptotic thymocytes, along with 80 μg of either isotype, or CD36-blocking Fab, were injected into the peritoneal cavity of *Adam17*-null and wildtype chimeric mice as shown in Figure 1A. The percent of TAMRA-positive macrophages was assessed by flow cytometry. The number of mice analyzed (within bars) and *p* values of significant differences are shown.

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 primary target of ADAM17 appears to be the dual-pass scavenger receptor 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, 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.[97, 106] Two novel N-terminal peptides with non-tryptic N-termini were identified in the lowest molecular weight fraction (~47 kDa) of soluble CD36, and one C-terminal non-tryptic peptide were detected in both fractions and represent probable cleavage sites. In addition, we establish that enhanced *in vivo* efferocytosis in the absence of ADAM17 is CD36 dependent. These data provide the first evidence for ADAM17 involvement in the shedding of a dual-pass transmembrane protein, and demonstrate the importance of proteolysis in controlling apoptotic cell uptake.

Recent reports have shown that ADAM17 can cleave MerTK and LOX-1 *in vitro*, and thus they were particularly likely targets.[97, 106] However, we demonstrate no change in surface levels of either of these receptors *in vivo*, while CD36 levels are elevated in the absence of ADAM17. A probable 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.[97, 106] Thus, ADAM17 cleavage of MerTK and LOX-1 may play a more significant role in responses to pathogens and/or in the presence of 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 in deficient mice, and *in vitro* efferocytosis was reduced by ~40% in CD36-deficient macrophages.[133, 134] In our studies, elevated surface levels of CD36 in the absence of ADAM17 lead to increased apoptotic cell uptake *in vivo,* and we demonstrate that the majority of ADAM17-dependent enhanced efferocytosis is abolished by CD36 blockade.

To biochemically characterize soluble CD36, we analyzed media collected from cultured wildtype and *Adam17*-null peritoneal macrophages. By Western blotting, soluble CD36 contains primarily two regions of CD36 staining with molecular weights of ~47 and ~52 kDa, with a doublet variably detected at ~47 kDa. Our MS analysis of immunoprecipitated soluble CD36 uncovered two probable N-terminal cleavage sites, only within the ~47 kDa region of soluble CD36. One cleavage site is close to the extracellular region of the predicted N-terminal transmembrane domain of CD36 (peptide N1) and the other (peptide N2) is adjacent to a hydrophobic region of CD36 (Figure 6). ADAM17 normally cleaves the extracellular stalk of its substrates proximal to the transmembrane domain with some preferences for particular residues flanking the cleavage site.[110] The non-tryptic N-terminus of peptide N1 is estimated to be six amino acids into the putative transmembrane domain, and therefore is less likely to be a direct target of ADAM17. Since we cannot distinguish between ADAM17 involvement in primary and secondary cleavage events, it is possible that initial ADAM17-dependent cleavage of CD36 at N2 or another site leads to activation of regulated intramembrane proteolysis, such as that mediated by g-secretases,[135] or that ADAM17 modulates cleavage by another protease and does not directly target CD36. Although peptide N2 contains alanine and valine preferred by ADAM17 in the P1 and P1' position,[110] respectively, it is 109 amino acids from the putative transmembrane domain, also atypical for ADAM17. However, the probable N2 peptide cleavage site is 45 amino acids from a hydrophobic region of CD36 that may interact with the plasma membrane,[1] and thus may localize the N2 cleavage site in a juxtamembrane position that favors interaction with ADAM17 (Figure 6). Functionally, the N2 cleavage would likely disrupt the domain of CD36 (amino acids 155-183) implicated in macrophage phagocytosis of apoptotic cells.[136] We identified only one C-terminal non-tryptic peptide (C1), whose identification was restricted to the ~52 kDa region. Since the cleavage site in C1 is 57 amino acids from the C-terminal transmembrane domain, C1 is a probable cleavage product of another enzyme. Our inability to conclusively identify other specific C-terminal cleavage sites and the apparent heterogeneity within the two major regions makes it difficult to predict the relationship of the soluble forms of CD36 to particular cleavage events involved in the release of soluble CD36. However, our data suggest that the two non-tryptic peptides detected in the ~47 kDa region must have been derived from molecular species with distinct C-termini to both migrate with a similar apparent molecular weight. In addition, the ~52 kDa region presumably contains close to the entire

extracellular domain, and at least in some molecular species have C1 cleavage, but its N-terminal cleavage site is unclear. Since ADAM17 deletion only inhibits release of soluble CD36 by 25%, it is likely that another enzyme(s) is involved in liberation of soluble CD36, perhaps utilizing the cleavage site in the C1 peptide. 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 for the first time a role for ADAM17 in the regulation of surface levels of the ditopic transmembrane receptor CD36.

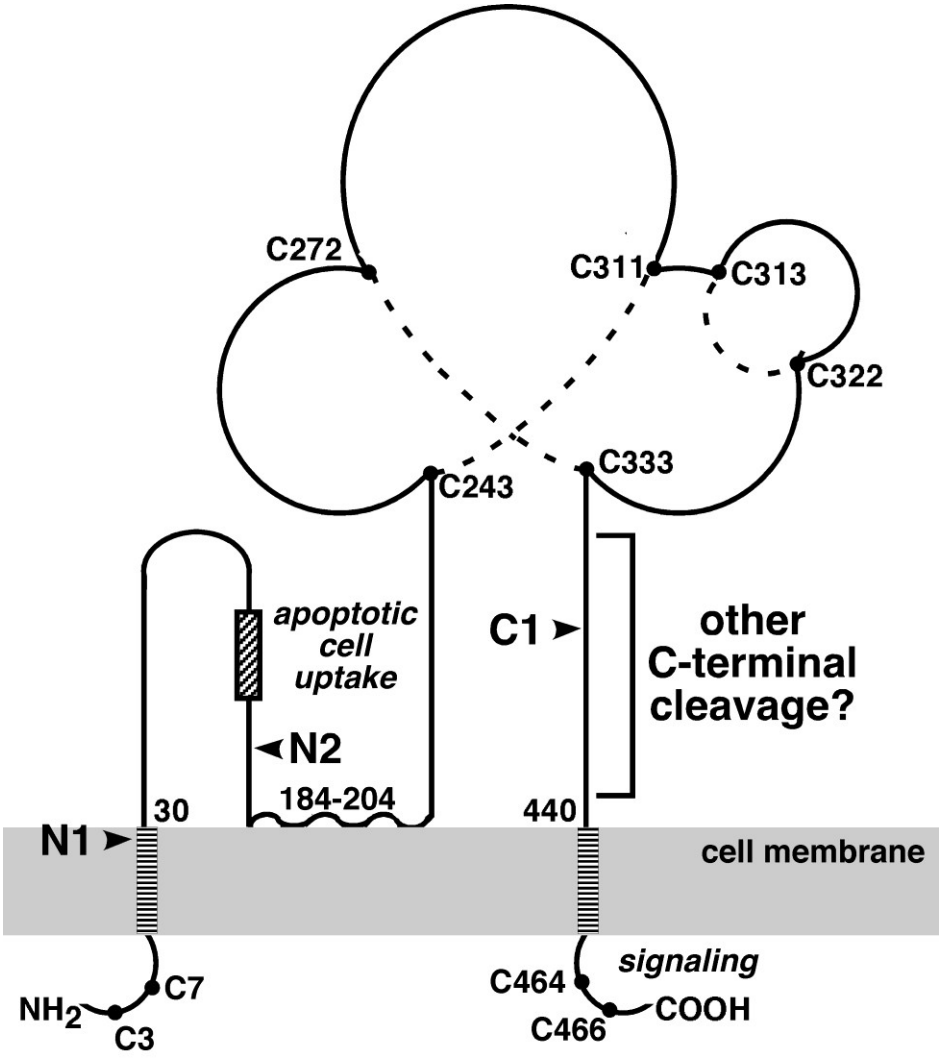


Figure 6: Topology and domains of CD36 and possible cleavage sites. Putative N1 and N2 cleavage sites in the N-terminal region and a putative C1 site in the C-terminal region of CD36 identified in this study are indicated with arrows. A hydrophobic region between amino acids 184-204 may interact with the plasma membrane,[1] which would create additional topology that may localize the N2 cleavage site in a juxtamembrane position more typical of ADAM17 cleavage sites. An additional C-terminal cleavage site would be expected to explain the mobility of the ~47 kDa fraction which contains peptides with the N1 and N2 cleavage sites, and a likely region within the C-terminal extracellular domain is indicated based on the disulfide-bonded structure of the CD36 extracellular domain and the apparent size of the soluble forms of CD36. All of the cysteine residues in CD36 are shown as a solid circle, and dotted lines indicate disulfide bonds determined from MS analysis.[137] CD36 domain data particularly implicate the C-terminal cytoplasmic domain of CD36 in assembly of dynamic signaling complexes that mediate its multiple functions,[138, 139] and disruption of this function would be expected with cleavage at C1. This diagram was adapted from Silverstein and Febbraio.[1]

CD36 levels in serum are increased in several chronic inflammatory diseases, and have been found to positively correlate with systemic sclerosis, type 2 diabetes, and atherosclerotic disease severity.[59-65] 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.[104] Although this study analyzed isolated fractions by Western blot analysis and failed to detect CD36 in the microparticle-depleted platelet-free plasma,[104] the monoclonal antibody used for immunoblotting may not detect soluble CD36 and the high protein content of plasma may have limited the sensitivity of detection. Our analysis of wildtype 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 following ultracentrifugation, both relative to cell lysate CD36 (n=3/group). In the absence of ADAM17, CD36 in media was decreased 25.8% (p =0.011, n=3) and microparticle pellet was increased by 14.3% (p =0.023, n=3). Our data suggest that under these *in vitro* conditions, soluble 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 non-resolving nature of certain diseases and defective apoptotic cell phagocytosis. However, in addition to apoptotic cell uptake, CD36 has a number of other functions, such as uptake of pathogens and modified low-density lipoproteins 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.[1] Essential to these other functions is CD36 assembly of signaling complexes, most likely mediated by the C-terminal cytoplasmic domain.[138, 139] Proteolysis would disable this downstream pathway, and thus it will be important to define the role of proteolysis in the multiple additional functions of CD36 in normal homeostasis and pathology.

Chapter 3: Major Findings and Unanswered Questions

Major Findings

This study demonstrates for the first time that *in vivo* efferocytosis and its associated anti-inflammatory consequences are enhanced by ADAM17 deletion, suggesting that ADAM17 can normally function to limit apoptotic cell phagocytosis. This finding reveals proteolysis to be an important mechanism regulating efferocytosis and impacting the inflammatory response.

Previous investigations have established that the anti-inflammatory effects of efferocytosis are dependent upon phosphatidylserine (PS) recognition.[26, 140, 141] Consistent with our observations of an enhanced efferocytosis-dependent anti-inflammatory phenotype in *Adam17*-null macrophages, this study also reveals that *Adam17*-null macrophages bind more PS liposomes *in vitro*. We demonstrate that the enhanced PS liposome binding and uptake by *Adam17*-null macrophages is abolished by the addition of acetylated-LDL, and, through the use of receptor-blocking antibodies, identify CD36 as the primary receptor mediating the differential PS liposome uptake by *Adam17*-null macrophages.

Evaluation of apoptotic cell receptor surface levels by flow cytometry reveals that ADAM17 deletion results in ~30% increase in surface CD36 levels. Though previous studies report ADAM17-mediated shedding of the apoptotic cell receptors MerTK[97] and LOX-1,[106] the current study finds no differences in their surface levels. Cleavage of ADAM17 substrates can occur in either an inducible or constitutive manner.[111] In the absence of specific stimulation, many ADAM17 substrates remain uncleaved.[112-114] ADAM17-mediated shedding of MerTK[97] and LOX-1[106] was only observed after macrophage activation with LPS or TNF-α respectively, indicating that these receptors are not constitutively shed by ADAM17. In contrast, elevated CD36 surface levels on *Adam17*-null thioglycollate-elicited macrophages suggests that CD36 is shed in a constitutive manner, independent of macrophage activation.[142]

Given that no other ditopic protein has been described as a target of ADAM17. The finding of elevated CD36 surface levels in the absence of ADAM17 was unexpected. CD36 is a dual-pass transmembrane protein with two short cytoplasmic tails, and a large extracellular domain (Figure 1). CD36 is a multi-functional receptor with a diverse range of ligands, including: apoptotic cells, oxidized lipoproteins, bacteria, viruses, microparticles, glycated proteins, thrombospondin-1, amyloid-forming peptides, and long-chain fatty acids.[1] In response to these varied ligands, CD36 is thought to initiate intracellular signaling events through protein complexes formed on its C-terminal cytoplasmic domain.[139] Any CD36 cleavage would likely disrupt ligand binding, as well as intracellular signaling.

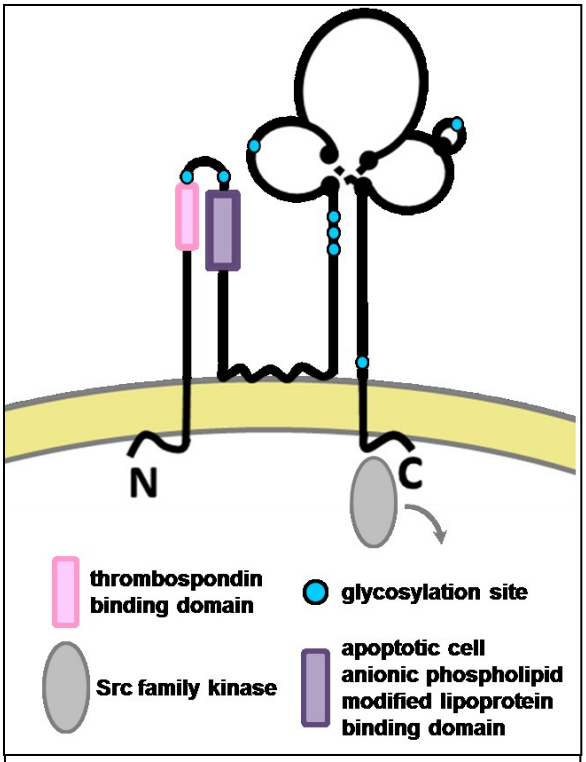


Figure 1: CD36 is a heavily glycosylated dual-pass transmembrane protein with multiple ligands and binding domains. Signalling occurs through complexes formed on the C-terminal domain. Figure adapted from Silverstein et al 2009.[1]

Our studies reveal that soluble CD36 is released by primary macrophages *in vitro*, and that ~90% of soluble CD36 is not associated with microvesicles. Although less than 1% of cell-associated CD36 is released over 24 hours of culture, Western blot analysis of microparticle-depleted conditioned media demonstrates that a fraction of soluble CD36 has an increased electrophoretic mobility, with an apparent molecular weight consistent with CD36 ectodomain cleavage near the transmembrane interface.

Analysis of microvesicle-depleted conditioned media from *Adam17*-null macrophages confirms that ADAM17 is involved in the release of soluble CD36; ADAM17 deletion decreases the ratio of soluble to cell-associated CD36 by 26%. This is the first example of a dual-pass transmembrane protein identified as a potential target of ADAM17. Although this finding indicates that ADAM17 is involved in CD36 shedding, it also illustrates that ADAM17 is not essential for CD36 release. Since ADAM17 deletion does not completely prevent CD36 release, it is likely that additional proteases can cleave CD36 as well.

Evaluation of soluble CD36 by mass spectrometry provides the first direct evidence for proteolytic shedding of CD36. We confirm that the bands detected by Western blot analysis of microparticle-depleted media are indeed CD36, and identify several putative cleavage sites for soluble CD36. Our analysis of soluble CD36 raises questions as to the proteases involved, and cleavage events required for CD36 shedding, and will be discussed in subsequent sections.

This study further demonstrates that enhanced *in vivo* efferocytosis in the absence of ADAM17 is CD36-dependent. CD36 blocking Fab abolishes ADAM17-dependent differences in efferocytosis *in vivo*, while isotype Fab has no effect. In accord with previous reports, the current investigation highlights the importance of CD36 as an apoptotic cell receptor. Studies performed by Greenberg et al. demonstrate the key role that CD36 plays in efferocytosis, reporting that apoptotic cell burden in wound tissue from mice lacking CD36 is 2 to 3-fold greater than that found in wildtype wound tissue.[133] The macrophage-specific influence of CD36 on *in vitro* efferocytosis was evaluated by Lucas et al., who report ~40% reduction in apoptotic cell phagocytosis by CD36 deficient macrophages.[134] Analysis of macrophages lacking ADAM17 suggests that normal ADAM17 expression decreases CD36 surface levels on macrophages, and significantly limits CD36-mediated efferocytosis *in vivo*. Taken together, these data suggest that ADAM17 limits CD36 surface levels, as well as CD36-mediated apoptotic cell phagocytosis and its associated anti-inflammatory phenotype.

Overall Significance

The findings of this study indicate that proteolytic shedding of CD36 by ADAM17 restricts efferocytosis, as well as its accompanying anti-inflammatory phenotype, demonstrating the importance of proteolysis in the regulation of efferocytosis *in vivo*.

Unanswered Questions

What is the relative role of proteolysis vs. microparticle release of soluble CD36 in chronic inflammatory disease?

Elevated levels of soluble CD36 have been identified in the plasma of patients with atherosclerosis,[59-61] diabetes mellitus,[60, 62-64] and scleroderma.[65] However, the biochemical structure of soluble CD36 has

not been evaluated in these diseases. Increased levels of soluble CD36 could result from proteolytic cleavage, or alternatively from elevated microvesicle release in inflammation.[88-90] A recent study by Alkhatatbeh et al. reports that CD36 found in the plasma of healthy donors is a full-length membrane-bound component of microparticles, rather than a product of proteolytic shedding.[104] A limitation of the study by Alkhatatbeh et al. is that it relied on electrophoretic mobility shifts as detected by Western blot to assess cleavage. In the current study, very subtle electrophoretic mobility shifts were detected in soluble CD36 by Western blot, whereas mass spectrometric analysis revealed evidence for potentially multiple proteolytic cleavage sites. Alkhatatbeh et al. also used a monoclonal antibody to detect CD36 by Western blot; it is possible that the epitope recognized by the antibody used is lost as a result of proteolysis.

An alternate method to clarify proteolytic vs. microparticle release of soluble CD36 in chronic inflammatory disease plasma is to use the sensitive and quantitative approach of targeted mass spectrometry. This would allow the simultaneous characterization and quantification of the N- and C-terminal domains of various soluble CD36 isoforms, including non-cleaved CD36. To perform this analysis, all cleavage sites must first be identified to allow targeted MS analysis of plasma CD36. As detailed below, more extensive characterization of soluble CD36 release in vitro is needed before findings can be applied to human samples.

Are the principal forms of cleaved, soluble CD36?

To further characterize soluble CD36 released *in vitro* by murine and human cells, alternate enzymes should be used for in-gel digestion and subsequent mass spectrometric analysis. If endogenous cleavage occurs *at* a tryptic digest site, the peptide fragment cannot be differentiated from one generated by trypsin cleavage *in vitro*. Conversely, if endogenous cleavage occurs *close to* a tryptic digest site, the resulting peptide fragment might be too small for positive identification by mass spectrometry. The use of alternate enzymes would allow us to evaluate whether or not CD36 is endogenously cleaved at or close to a tryptic digest sites, and enable us detect novel sites overlooked using the current approach.

A technical limitation to the current approach of in-gel digest followed by mass spectrometric analysis is that peptide detection is limited by the ability of individual peptides to ionize efficiently, and be detected by the mass spectrometer. Regardless of size, individual peptides with different amino acid compositions

can vary in their ionization efficiency by several orders of magnitude.[143] Particularly problematic hydrophobic protein sequences could be virtually undetectable using the current approach.[143]

As an alternative approach to identify N-terminal sequence of soluble CD36 proteins, gel-purified CD36 could be sequenced by Edman degradation coupled with mass spectrometry. This approach could be used to interrogate soluble CD36 for N-terminal proteolytic cleavage and sequence novel cleavage sites. A limitation of this approach is that it requires a fairly homogenous sample to obtain conclusive sequence data.

Soluble CD36 in conditioned murine macrophage media is heterogeneous by Western blot. It is possible that some of this heterogeneity could be limited by adjusting conditions used for sample collection and analysis. Future samples should include protease inhibitors during all steps of sample preparation, and should be reduced and alkylated to exclude potential disulfide bond formation as a source of sample heterogeneity. The extracellular domain of CD36 can also become phosphorylated,[144] which could impact electrophoretic mobility. Sample treatment with acid phosphatase prior to analysis would exclude this potential.[144] However, several lines of data indicate that multiple proteases are involved in CD36 shedding, and suggest that heterogeneity of soluble CD36 should be expected.

What is the role of ADAM17 in CD36 shedding?

Two potential ADAM17 cleavage sites for soluble CD36 are identified following in-gel tryptic digest and mass spectrometric analysis, but additional analysis using alternate enzymes for in-gel digestion are necessary to confirm our findings. Peptide N2 (Figure 2) consists of amino acids 139-163 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-□.[110] Although the N2 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 2) that is thought to interact with the plasma membrane.[1]

The second potential ADAM17 cleavage site, C2 (Figure 2), is located 10 amino acids from the C-terminal transmembrane domain. Although this site is also a trypsin cleavage site, no down-stream C-terminal peptides were detected from soluble CD36. Several other ADAM17 substrates contain cleavage sites with lysine in the P1 position, including L-selectin,[145] and amyloid precursor protein.[146] Additional cleavage site characterization with alternate enzymes used for in-gel digestion must be carried out before we can determine if C2 is an endogenous cleavage site.

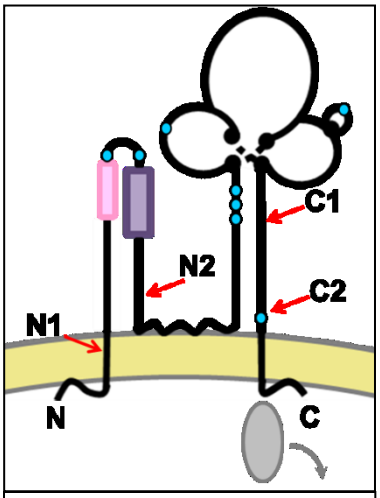


Figure 2: Putative cleavage sites on soluble CD36 suggested by mass spectrometry. Sites N2, and C2 are potential ADAM17 cleavage sites. Cleavage at N2, C1, or C2 would separate the ligand binding domain from the signaling domain on the C-terminus. Figure adapted from

To evaluate whether or not ADAM17 specifically mediates CD36 cleavage at positions N2 and C2, targeted mass spectrometric analysis of the N2 and C2 digest products can be performed on conditioned media from *Adam17*-null macrophages. A limitation to this approach is that in the absence of ADAM17, ADAM10 has been shown to cleave substrates preferred by ADAM17. Additionally, conditioned media from human macrophages should be evaluated to determine if a similar pattern of soluble CD36 cleavage sites are found.

What other proteases are involved in CD36 shedding?

C1 (Figure 2) is a non-tryptic peptide with a cleavage site located 57-61 amino acids from the C-terminal transmembrane domain. ADAM17 cleavage at this site is unlikely due to its distance from the plasma membrane, suggesting that a soluble extracellular protease cleaves CD36 at C1.

An additional cleavage site (N1) is detected six amino acids from the cell surface, within the putative N-terminal transmembrane domain of CD36 (Figure 2). Protein hydrolysis within the transmembrane domain is often mediated by the γ-secretase protease complex, as is the case with notch and amyloid precursor protein.[147] Although less likely, MMP-9 may be a candidate as well: β2 integrin subunit is released from the cell by proteolytic cleavage by MMP-9 at a site residing 3 amino acids within its putative transmembrane domain.[148]

To evaluate the role of other proteases in CD36 release, cells with targeted deletion of specific proteases could be used to evaluate CD36 cleavage at the C1 and N1 positions. Small molecule protease inhibitors could be used to broadly evaluate the role of different classes of proteases in CD36 cleavage; however, these inhibitors often lack specificity, and can be cytotoxic to cells.

What is the impact of CD36 shedding on CD36 signaling and its multiple other functions?

CD36 ectodomain shedding is a likely mechanism employed by the cell to limit ligand uptake and subsequent intracellular responses (Table 1). A single cleavage event is likely to result in a receptor that is inert in regards to ligand uptake and signaling. The C-terminal cytoplasmic domain is thought to participate in receptor mediated signaling[139] through interactions with Src family non-receptor protein tyrosine kinases and serine/threonine kinases of the MAPK family.[1] Given that multiple binding sites have been identified on the heavily glycosylated extracellular domain of CD36, it is conceivable that a single cleavage event could separate the ligand binding domain from the C-terminal domain involved in signaling, which could affect CD36 ligand-mediated signaling (Figure2).

CD36 is a multi-functional receptor expressed on a variety of cell types with a diverse range of ligands in addition to apoptotic cells. The cellular consequences of CD36 ligation are not only dependent upon the ligand, but upon the cell-type as well (Table 1), demonstrating that CD36 is involved in diverse biological processes. Since ADAM17 is ubiquitously expressed,[121] our findings raise the potential that CD36 shedding from a range of cell-types could impact the binding of a variety of CD36 ligands relevant to a number of different disease pathologies (Table 1).

Table 1: CD36 ligands and associated disease pathology

Cell type	Ligand	Consequence of binding	Relevant pathology	Anticipated outcome of cleavage
microvascular endothelial cells	thrombospondin (TSP)-1[149]	inhibits the influence of growth-factors by initiating apoptosis of activated microvascular endothelial cells.[150]	pannus formation in RA; tumor vascularization	promote angiogenesis
microglia	amyloid (A)-β[151]	internalizes inflammatory Aβ for degradation accompanied by pro-inflammatory cytokine production[152]	Alzheimer's disease	reduce inflammation; could also limit beneficial Aβ clearance
macrophage	oxidized LDL (oxLDL)	removal of inflammatory oxLDL;[153-156] formation of macrophage foam cells; inflammatory cytokine release.[33, 34]	atherosclerosis	limit foam cell formation and inflammation
macrophage	bacteria, fungus, and viral particles	opsonin-independent pathogen internalization	infection	increased susceptibility to infections[157]
cardiac and skeletal muscle cells, adipocytes and hepatocytes	fatty acids	fatty acid uptake for metabolism or storage	insulin resistance, obesity, and steatosis	reduced lipid uptake in muscle and liver
platelets	microvesicles;[158] oxLDL[159]	renders platelets more sensitive to activation[1]	hyperlipidemia, inflammation, and thrombosis	reduced thrombosis

What is the role of soluble CD36?

Though elevated levels of soluble CD36 can serve as a useful biomarker of disease (Table 1, Chapter 1), the biological ramifications of increased soluble CD36 are not clear. Although previous studies using recombinant soluble CD36 were not carried out using doses that match pathological levels of soluble CD36 (~25ng/ml),[59] *in vitro* and *in vivo* evidence supports the concept that soluble receptors retain their ability to bind to apoptotic cells. Behaving as an antagonist, recombinant soluble CD36 can inhibit apoptotic cell phagocytosis *in vitro,*[117] suggesting that cleaved CD36 might also dampen efferocytosis by blocking the cell-associated CD36 from binding to apoptotic cells.

Although recombinant forms of soluble CD36 have been previously used *in vitro* to assess CD36 binding properties,[117, 160, 161] the protein sequences used were not engineered to contain only the amino acids of

the shed portion of soluble CD36. To experimentally evaluate the potential biological roles of shed CD36 on the binding of various CD36 ligands to cell-associated receptors, soluble forms of recombinant CD36 corresponding to the shed portion of CD36 ectodomain can be generated and used to assay antagonist activity at physiologically relevant concentrations.

What is the effect of targeted inhibition of CD36 shedding?

Once the cleavage sites for CD36 have been more fully characterized, multiple approaches could be used to specifically prevent CD36 cleavage, as outlined below. Targeted inhibition of CD36 cleavage would allow us to analyze the specific impact of CD36 shedding in models of chronic or acute inflammation, and clarify the relationship between CD36 shedding and defective efferocytosis observed in the context of inflammatory disease.

A protease-resistant CD36 mutant mouse could be generated by altering small segments of protein sequence in the regions corresponding to sites of proteolysis. This approach has been used for several ADAM17 substrates, including MerTK,[97] to evaluate the biological consequences of their shedding *in vivo*. This approach will be useful for evaluating the role of CD36 shedding in the pathology of a number of diseases (discussed below).

Alternatively, the therapeutic consequence of specifically inhibiting CD36 cleavage could be evaluated by administering small molecule protease-resistant drugs that that could cloak sites of ADAM17-mediated proteolysis on CD36 by binding to them with high affinity. This class of drugs, known as peptoids, could be administered to specifically block ADAM17 cleavage sites on CD36. Peptoids are biologically stable drugs that can be generated at a fraction of the cost of antibodies.[162] Large peptoid libraries created by combinatorial chemistry can be screened for specific affinity to the cleavage site of CD36, and be used to evaluate impact of CD36 cleavage in established disease.

Elevated ADAM17-mediated CD36 shedding may provide a mechanistic link between the non-resolving nature of certain chronic inflammatory diseases and defective apoptotic cell phagocytosis. Our studies demonstrate that CD36 cleavage results in decreased phagocytosis of apoptotic cells, as well as a decreased anti-inflammatory response by macrophages. If increased shedding of macrophage CD36 occurs in the context of chronic inflammation, efferocytosis and its anti-inflammatory consequences may

be impaired as well. The use of uncleavable CD36 mutants or CD36 cloaking peptoids in murine models of disease will allow us to evaluate the role that CD36 shedding plays in the deficient efferocytosis and elevated levels of soluble CD36 frequently observed in chronic inflammatory disease pathology.

Soluble CD36 levels in serum are found to positively correlate type 2 diabetes, and atherosclerotic disease severity.[59-65] Increased apoptotic cell burden is a common trait of these diseases, which is indicative of impaired efferocytosis. The use of uncleavable CD36 mutants or CD36 cloaking peptoids in murine models of diabetes and atherosclerosis will clarify the role that CD36 shedding plays in the deficient efferocytosis observed in these diseases.

Summary

Our studies suggest that CD36 shedding may provide a mechanistic link between deficient apoptotic cell phagocytosis, elevated levels of soluble CD36, and potentially the non-resolving nature of certain chronic inflammatory diseases. The proposed studies will clarify the relationship between CD36 shedding and defective efferocytosis observed in the context of inflammatory disease.

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