Myocardial infarction (MI) is a significant contributor to the high morbidity and mortality rates associated with cardiovascular disease. After MI, the left ventricle (LV) undergoes a wound healing response comprised of robust infiltration of inflammatory cells that regulate extracellular matrix (ECM) turnover to remove necrotic debris and form an infarct scar. In the mouse model of MI, the inflammatory response begins to subside by day 7, leading the way for an increase in ECM production and scar formation. Matrix metalloproteinase (MMP)-9 is a key enzyme that regulates post-myocardial infarction left ventricular remodeling.

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that proteolytically process ECM proteins, cytokines, chemokines, growth factors, and adhesion molecules. MMP cleavage can activate or inactivate a protein; for example, cleavage of interleukin (IL)-8 increases its activity, whereas cleavage of Cxcl4 decreases its activity. MMP-9 is particularly important in the post-MI setting because protein concentrations increase 3-fold in the infarct region during the first week post-MI. MMP-9 deletion attenuates LV dilation post-MI, indicating a causal role for MMP-9. Although many in vitro MMP-9 substrates have been identified, including collagens (IV, V, VII, X, and XIV), gelatin, fibronectin, elastin, IL-8, Cxcl4, and IL-1β, the mechanisms whereby MMP-9 modulates post-MI LV remodeling has not been completely elucidated.

CD36 is a Matrix Metalloproteinase-9 Substrate That Stimulates Neutrophil Apoptosis and Removal During Cardiac Remodeling

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Background—After myocardial infarction, the left ventricle undergoes a wound healing response that includes the robust infiltration of neutrophils and macrophages to facilitate removal of dead myocytes as well as turnover of the extracellular matrix. Matrix metalloproteinase (MMP)-9 is a key enzyme that regulates post-myocardial infarction left ventricular remodeling.

Methods and Results—Infarct regions from wild-type and MMP-9 null mice (n=8 per group) analyzed by glycoproteomics showed that of 541 N-glycosylated proteins quantified, 45 proteins were at least 2-fold upregulated or downregulated with MMP-9 deletion (all P<0.05). Cartilage intermediate layer protein and platelet glycoprotein 4 (CD36) were identified as having the highest fold increase in MMP-9 null mice. By immunoblotting, CD36 but not cartilage intermediate layer protein decreased steadily during the time course post-myocardial infarction, which identified CD36 as a candidate MMP-9 substrate. MMP-9 was confirmed in vitro and in vivo to proteolytically degrade CD36. In vitro stimulation of day 7 post-myocardial infarction macrophages with MMP-9 or a CD36-blocking peptide reduced phagocytic capacity. Dual immunofluorescence revealed concomitant accumulation of apoptotic neutrophils in the MMP-9 null group compared with wild-type group. In vitro stimulation of isolated neutrophils with MMP-9 decreased neutrophil apoptosis, indicated by reduced caspase-9 expression.

Conclusions—Our data reveal a new cell-signaling role for MMP-9 through CD36 degradation to regulate macrophage phagocytosis and neutrophil apoptosis. (Circ Cardiovasc Genet. 2016;9:14-25. DOI: 10.1161/CIRCGENETICS.115.001249.)

Key Words: extracellular matrix • immunoblotting • infarction • inflammation • myocardial • proteomics

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Methods

Detailed methods are available in the Methods section in the Data Supplement.

Mice
C57BL/6j wild-type (WT) and MMP-9 null male and female mice of 4 to 7 months of age were used in this study (Table I in the Data Supplement). The MMP-9 null mice were generated by Zena Werb laboratory and backcrossed onto the C57BL/6j strain by Lynn Matrisian laboratory.11,12 Mice were kept in a light-controlled environment with a 12:12 hour light–dark cycle and given free access to standard mice chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the University of Mississippi Medical Center in accordance with the Guide for the Care and Use of Laboratory Animals. The mice underwent permanent coronary artery ligation surgery, to produce MI, as described previously.11,13 Three sets of mice (n≥5/group) were used (Table I in the Data Supplement): a first set was used for glycoproteomics; a second set was used for immunoblotting, immunohistochemistry, and immunofluorescence; and a third set was used for in vitro stimulation of macrophages and blood neutrophils. Figure I in the Data Supplement illustrates the workflow of the study. For all procedures, samples were randomized and analyzed in a blind manner.

Tissue Samples and Protein Extraction
The LV infarct (LVI) region was collected at 0, 1, 3, 5, and 7 days post-MI as described previously.1,13 Because the insoluble fraction is enriched for ECM, we used that fraction for the glycoproteomic analysis. Immunoblotting also used the insoluble fraction for confirmation of glycoproteomic findings.

Mass Spectrometry
The samples were digested with trypsin at a ratio of 1:50 (wt/wt, enzyme:protein) at 37°C overnight with gentle shaking. Peptide concentration was determined by bicinchoninic acid (BCA) assay. N-linked glycopeptides were isolated from the trypsic peptides using the solid-phase extraction of glycopeptides method as previously reported.14 The efficiency of glycopeptide capture was evaluated previously by Zhou et al.16 According to 2 standard proteins with isotope labeling, 85% of the glycopeptides were coupled to the hydrazide resin. Peptides were analyzed by liquid chromatography–mass spectrometry (MS/MS) using a Q Exactive. MS/MS spectra were searched with SEQUEST using Proteome Discoverer (version 1.4) against the mouse RefSeq database (released in November 2014) containing 57788 sequences. The False Discovery Rate was set at 0.01 to eliminate low-probability protein identifications. To eliminate false-positive identifications of N-glycopeptides, we determined the extent of spontaneous deamidation on Asn residues satisfying the N–X–S/T sequence by profiling the unconjugated fraction of hydrazide beads without PNGase F treatment.17,18 The rate of spontaneous deamidation in the consensus motif was 1.5% in our sample set. Glycosylation was confirmed for several proteins, including fibronectin, γ sarcoglycan, and peristin (Figure II in the Data Supplement). Peptides were quantified by label-free relative quantification based on integrated peptide peak intensities using the SIEVE software, version 2.1. Unsupervised clustering was performed using normalized peak areas of the identified glycopeptides.

Proteomic Data Repository
The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (http://www.ebi.ac.uk/pride/) with the data set identifier PXD001393.29

Bioinformatics Analysis
For gene ontology analysis, proteins were classified to gene ontology categories according to their cellular component and main biological processes using DAVID Bioinformatics tools (v6.7) with default settings.20,21 For network interaction analysis, the list of proteins was submitted to several protein–protein interaction databases, including Database of Interacting Proteins, Molecular INTeraction database, and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v9.1.22,23 STRING identified the most proteins from our list; therefore, it was used in this study to identify protein–protein interactions using database, literature, and experimental search parameters.24 The confidence score was set as high (0.7). The interaction network was visualized by Cytoscape.25

Immunoblot Analysis
Immunoblotting was used to validate proteomic findings. The insoluble fraction from the infarct area of WT or MMP-9 null mice (n=8 per group; 4 male and 4 female) were analyzed. Samples were pooled for time course analysis and analyzed individually for the day 7 post-MI analysis. The blots were examined using the IQ-TL image analysis software on luminescent image analyzer. The signal intensity of each sample was normalized to the densitometry value for the total protein of its corresponding lane.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)
RNA extraction was performed on the LVI tissue as previously described.26–28 Cartilage intermediate layer protein (CILP), CD36, Ltbp2, Col6a6, Col5a1, Lam5, and Fbn1 gene expression was assessed.

In Vitro Cleavage Assay
Mouse CD36-recombinant protein was incubated with active MMP-2–, MMP-9–, or MMP-12–recombinant protein at a ratio of 2:1. Two negative controls (MMP alone or recombinant protein alone) were included. Samples were run on SDS-PAGE, followed by silver staining.

Immunofluorescence Analysis or Immunohistochemistry
Five random images were captured at 40x magnification for analysis. Quantification was measured by Image-Pro Plus version 6.2. Representative images are shown at 40x or 60x magnification.

Phagocytosis Assay
Macrophages were isolated from day 7 post-MI LV of male mice, as previously described and stimulated with 1 ng/μL of active MMP-9, a CD36-blocking peptide (CD36i), or a combination of both (1:1 ratio).25,26 Phagocytosis was measured using the Vybrant Phagocytosis Kit (Life Technologies), with quantification measured on the SpectraMax M3 Plate Reader (excitation: 480 nm and emission: 520 nm). Representative images are shown at 40x magnification.

Blood Neutrophil Isolation and Stimulation
Neutrophils were isolated from the blood of male mice as previously described.25,26 Cells were stimulated with 0.5 ng/μL of active MMP-9 or CD36i and harvested for RNA isolation and RT-PCR examination.

Statistical Analysis
All analyses were performed blinded to groups, and data are presented as mean±SEM. For 2 group comparisons, the nonparametric Wilcoxon rank sum test was used. For multiple group comparisons, the nonparametric Kruskal–Wallis test with Dunns post-test was used. A 2-way ANOVA was used for CD36 time course analysis. A value of r>0.30 was considered a strong correlation for the linear regression of Cd36 and end-diastolic dimension. P<0.05 was considered statistically significant. Statistical analysis was performed using GraphPad InStat3 and Prism 5.

Results
Glycoproteomic Analysis Identified 45 MMP-9–Dependent Extracellular Protein Changes Post-MI
A total of 1161 unique N-linked glycopeptides were quantified by liquid chromatography–MS with a 1% false discovery rate.
rate and these sites represented 541 unique glycoproteins (Table II in the Data Supplement). Unsupervised clustering was used to analyze the identified glycopeptides. On the basis of the peptide abundance, 7 of 8 WT samples and 7 of 8 MMP-9 null samples were clustered together, indicating good grouping based on genotype (Figure 1A). There was poor clustering of males and females, indicating a lack of sex-related differences.

A total of 122 proteins showed significant changes in MMP-9 null mice compared with WT mice ($P<0.05$; Table III in the Data Supplement). Combining a minimum 2-fold change (fold change $\geq 2$ or $\leq 0.5$) and significance ($P<0.05$), there were 45 proteins (Table 1). Notably, 6 of the 45 proteins (fibronectin, collagen $\alpha$-1, carboxypeptidase N subunit 2, $\beta$-1 integrin, fibulin-2, and thrombospondin-1 [Thbs1]) had $>1$ glycopeptide identified with similar fold change, which increased the confidence for these proteins. Detailed information and quantification values can be found in Tables II and IV in the Data Supplement. For the 20 single glycopeptides identified only once, the annotated MS/MS spectra are provided in Figure III in the Data Supplement. RT-PCR analysis was performed for 7 of the top 12 proteins (Figure IV in the Data Supplement). Of note, none of the genes measured were significantly different between WT and MMP-9 null, indicating the glycoproteomic changes were not because of changes in transcription. This comparison between gene and protein levels highlights the importance of examining protein level changes in addition to gene level changes.

![Figure 1. Glycoproteomic analysis identified matrix metalloproteinase (MMP)-9–dependent extracellular protein changes post myocardial infarction. A, Unsupervised clustering of the identified glycopeptides was performed using normalized peak areas. Wild-type (WT) samples and MMP-9 null clustered separately, with the exception of 1 WT and 1 null sample. Sex differences were not observed. B, The changed proteins were analyzed by Search Tool for the Retrieval of Interacting Genes (STRING/Proteins v9.1). Protein–protein interaction software with high confidence (score $>0.7$) and visualized by Cytoscape. The majority of the changed proteins interacted with each other. Stronger associations are represented by thicker lines. Red nodes are the upregulated proteins, blue nodes are the downregulated proteins, and gray nodes present proteins associated with the changed proteins. MMP-9 is highlighted in green. CD36 and cartilage intermediate layer protein, highlighted in yellow, were selected for immunoblot analysis.](image-url)
### Table 1. Proteins Differentially Expressed in MMP-9 Null Mice (all \(P<0.05\) and At Least 2-Fold Difference), Ordered by NR (null/WT; Normalized to Total Ion Current by SIEVE) to Evaluate Substrate Accumulation in the Absence of MMP-9

| Accession No.   | Protein Description                                      | Cellular Component       | Glycopeptides                      | NR   | N-P Value |
|-----------------|----------------------------------------------------------|--------------------------|------------------------------------|------|-----------|
| 27734196        | Cartilage intermediate layer protein (CILP)              | Extracellular matrix     | EQRPGOACSNYTVR                      | 13.13| 9.90E-07  |
| 568933000       | Platelet glycoprotein 4 (CD36)                           | Plasma membrane          | QFWFODVONPDVAKANSSK                 | 9.35 | 2.00E-02  |
| 158341636       | Latent-transforming growth factor \(\beta\)-binding protein 2 | Extracellular region     | DSSPGAAV/NHLSSPWWGLNL7EK            | 7.12 | 1.60E-02  |
| 6753138         | Sodium/potassium-transporting ATPase subunit \(\beta\)-1   | Plasma membrane          | LDWLNACSLNDNDSGYSR                 | 6.74 | 8.60E-03  |
| 156616286       | Collagen \(\alpha\)-6 (VI)                              | Extracellular matrix     | LNANLSSSLWDTFQ/M                    | 6.37 | 4.00E-02  |
| 6753484         | Collagen \(\alpha\)-1 (VI)                              | Extracellular matrix     | RFW7ADWGHSR                        | 5.25 | 3.70E-02  |
| 568920137       | Laminin subunit \(\alpha\)-5                            | Extracellular matrix     | ELOGINSASSAWAR                      | 5.09 | 4.80E-02  |
| 61651673        | Dolichyl-diphospholigosaccharide protein glycosyltransferase subunit STT3B | Endoplasmic reticulum membrane | TTLVNDNTWAWSHALVGBK                 | 4.81 | 2.70E-02  |
| 170784829       | Procollagen galactosyltransferase 1                      | Unknown                  | TALWVATDHNTD/NTSAILR                | 4.34 | 2.30E-02  |
| 238637279        | 4F2 cell-surface antigen heavy chain                     | Plasma membrane          | APLMPWNESSFHIIPP/PSL/MTVK           | 4.33 | 2.00E-02  |
| 75549868        | Fibrillin-1                                               | Extracellular matrix     | AWGTCPCELCPSW/TSEYK                 | 4.29 | 3.20E-02  |
| 568911077       | Nicastrin                                                 | Plasma membrane          | CDSGFALDSEER/NC7DIDECR              | 0.45 | 4.50E-02  |
| 755537649        | Platelet endothelial cell adhesion molecule              | Plasma membrane          | ANWWSWFLSILK                       | 4.14 | 3.20E-02  |
| 568939471       | Serum paraoxonase/arylesterase 1                         | Extracellular region     | EKDRPFFQAVN/D7DAFWHNK               | 4.1  | 1.20E-02  |
| 568926396       | Tenascin                                                  | Extracellular matrix     | ASTEEPSLENL/TEAGWGDLR               | 3.89 | 2.50E-02  |
| 31982236        | Integrin \(\alpha\)-6                                   | Plasma membrane          | LWNSTFLEEYSK                       | 3.76 | 5.00E-03  |
| 755537859       | Solute carrier family 2, facilitated glucose transporter member 4 | Plasma membrane          | VIEOSYNA7WLGR                      | 2.71 | 8.70E-03  |
| 112494256       | Prolow-density lipoprotein-receptor–related protein 1   | Plasma membrane          | WTHGWNTWQR                         | 3.62 | 2.40E-02  |
| 148747128       | Dolichyl-diphospholigosaccharide protein glycosyltransferase subunit STT3A | Membrane                | TILVDNNTWAWTHISR                    | 3.52 | 2.20E-02  |
| 6755863         | Endoplasmian                                              | Plasma membrane          | HNN/D7DHWESDSNF/SVADPR              | 3.36 | 3.60E-02  |
| 121674797       | Palmitoyl-protein thioesterase                           | Golgi apparatus          | FFADSDVPDS/SEWGFYR                  | 3.29 | 4.40E-02  |
| 225543173       | Cartilage-associated protein                              | Extracellular matrix     | DKGGLSDEHOPR/PRPEAV/G/N/TLQK        | 3.05 | 3.20E-02  |
| 569003077       | Catherin-2                                                | Plasma membrane          | RW7/1NR                            | 3.03 | 1.00E-02  |
| 755537859       | Solute carrier family 2, facilitated glucose transporter member 4 | Plasma membrane          | VIEOSYNA7WLGR                      | 2.71 | 8.70E-03  |
| 31542891        | \(\gamma\)-glutamyltransferase 5                        | Membrane                 | LDWDPSSHPGQ/WSR                    | 2.65 | 2.50E-02  |
| 121583481       | Inhibitor of nuclear factor \(\kappa\)-B kinase–interacting protein, isoform 1 | Endoplasmic reticulum membrane | FQ/1/DFWK                          | 2.52 | 4.70E-02  |
| 8850219         | Haptoglobin                                               | Extracellular region     | VU/L/HWS/DV/IGLU                   | 2.36 | 1.00E-02  |
| 568972029       | C-type mannose receptor 2                                | Cell surface             | ASN/AKPGTLEР                       | 2.18 | 3.80E-02  |
| 568962784       | Laminin subunit \(\beta\)-2                             | Extracellular matrix     | AAALDKAASR                         | 2.14 | 2.10E-02  |
| 73050399        | Transhyretin                                              | Extracellular region     | TLGISPHEFA/DVFT/ANDSGHR             | 2.08 | 1.70E-02  |
| 147904569       | Carboxypeptidase N subunit 2                             | Extracellular region     | LDSLDSN/TAHL/PALFN/LSR             | 2.08 | 4.80E-02  |
| 31982712        | Carboxypeptidase B2                                      | Extracellular region     | IPFNLNMMVEDL/EQU/T/ND/VSPR          | 2.07 | 2.50E-02  |
| 219521935       | MHC class I-like protein GS10                            | Plasma membrane          | TLLLSYQAGSH/HTISOW/SGE/GSDGR        | 2.02 | 2.70E-03  |
| 45504394        | Integrin \(\beta\)-1                                    | Plasma membrane          | KDI/CAEOCHS/FL7K                    | 0.5  | 3.70E-02  |
| 755522163       | Integrin \(\alpha\)-M                                   | Plasma membrane          | TPLV/LNCSVA/CK                    | 0.49 | 5.20E-03  |
| 449083336       | Fibronectin                                               | Extracellular matrix     | WTP/LNST/SI/YR                      | 3.65 | 4.10E-02  |

(Continued)
STRING Analysis Revealed That MMP-9–Dependent Changes Work in Concert as a Network

To obtain an overview of the interconnections among the differentially expressed proteins, the protein list was submitted to the STRING protein–protein interaction database and filtered for interactions of high confidence (score >0.7).23 In Figure 1B, the upregulated proteins are shown in red, the downregulated proteins are shown in blue, and proteins associated with the changed proteins but not identified in this study are shown in gray. Stronger associations are represented by thick black lines, whereas weaker associations are indicated by thin gray lines. MMP-9 is highlighted in green. The analysis revealed that 20 of the 45 proteins (45%) had established interconnections. For example, platelet glycoprotein 4 (CD36) is connected with Thbs1, and the interaction between CD36 and Thbs1 stimulates proapoptotic signals.30,31 In addition, platelet glycoprotein 4 (CD36) is connected with the STRING protein–protein interaction database and filtered for interactions of high confidence (score >0.7).23 In Figure 1B, the upregulated proteins are shown in red, the downregulated proteins are shown in blue, and proteins associated with the changed proteins but not identified in this study are shown in gray. Stronger associations are represented by thick black lines, whereas weaker associations are indicated by thin gray lines. MMP-9 is highlighted in green. The analysis revealed that 20 of the 45 proteins (45%) had established interconnections. For example, platelet glycoprotein 4 (CD36) is connected with Thbs1, and the interaction between CD36 and Thbs1 stimulates proapoptotic signals.30,31 In addition, several proteins coregulated a common protein. For example, Tnc, Thbs1, Fn1, CD36, and Cdh2 directly or indirectly sur-

| Table 1. Continued |
|---------------------|
| Accession No. | Protein Description | Cellular Component | Glycopeptides | NR | N-P Value |
|-----------------|---------------------|--------------------|---------------|-----|-----------|
| 269315863 | Collagen α-5 (VI) | Unknown | SANDVLEPAK | 0.49 | 4.60E-02 |
| 166064058 | Slit homolog 2 protein | Unknown | LEDVMFAMGIEAANQTELEDIVSVPSR | 0.44 | 4.20E-02 |
| 218931165 | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 | Endoplasmic reticulum membrane | YNCIESPR | 0.47 | 1.50E-02 |
| 568940646 | Fibulin-2 | Extracellular matrix | EGTPCAEGNTCGVSLIK | 0.46 | 4.30E-03 |
| 568930638 | Basement membrane-specific heparan sulfate proteoglycan core protein | Extracellular matrix | DLEVTLVHNIWTR | 0.48 | 8.90E-03 |
| 47059073 | Thrombospondin-1 | Extracellular region | GCSSSATVLTTDNHNUGSSPAIR | 0.39 | 2.30E-02 |
| 145966840 | Eosinophil peroxidase | Cytoplasmic granule | ALLPDONHEDPOLLNIR | 0.28 | 7.50E-03 |
| 35803288 | Extracellular matrix protein 1 | Extracellular matrix | QIPGLIOAMWTR | 0.0 | 8.10E-06 |

Glycosylation motif is indicated in italics. MHC indicates major histocompatibility complex; MMP, matrix metalloproteinase; N-P value, normalized P value; NR, normalized ratio; and WT, wild-type.

CILP and CD36 Had the Highest Fold Increases in MMP-9 Null Mice

Glycoproteomics results identified CILP and CD36 as having the highest fold increase in MMP-9 null mice (Table 1). Because direct associations with MMP-9 had not been previously assigned, CD36 and CILP were selected for validation (highlighted in yellow in Figure 1B). Immunoblotting was performed on an independent set of mouse tissues and showed agreement with the proteomic results (Figure 2).

Because ECM remodeling post-MI is time and space dependent, we also investigated the protein expression of CILP and CD36 in the LVI or remote control (LVC) regions during the post-MI time course.2 CILP continuously increased post-MI in both WT and MMP-9 null mice (Figure V in the Data Supplement). This pattern is not what we would expect if CILP was a substrate of MMP-9. For this reason, further analysis of CILP was not performed in this study. CD36 was robustly expressed at day 0, consistent with past reports of endothelial expression.32 CD36 showed a pattern consistent with being an MMP-9 substrate; namely, it decreased over time in the WT LVI, and this decrease was attenuated by MMP-9 deletion. Surprisingly, a similar pattern was found in the remote LVC (Figure 3A). The fact that CD36 decreased in the remote region indicates additional MMPs may also cleave CD36.

MMP-9 Regulates CD36 Levels Through Proteolytic Degradation

To assess in vitro proteolytic degradation, CD36-recombinant protein was incubated with active MMP-9. Because we found CD36 decreased over time in the remote region, we also incubated CD36 with active MMP-2 or MMP-12—2 MMPs that increase in the remote region post-MI (Figure 3B). Fragments were visible with MMP-9 and MMP-12 incubation but not with MMP-2 incubation (Figure 3C and 3D). This demonstrates CD36 is cleaved by MMP-9 and MMP-12 in vitro, which may explain the decrease in CD36 levels observed in both the LVC and LVI samples. Interestingly, there were multiple bands generated with MMP-9 at molecular weights ranging from 50 to 10 kDa, illustrating MMP-9 not only generated a CD36 fragment but can further degrade it, whereas MMP-12 only generated 1 major fragment. To confirm whether CD36 was cleaved by MMP-9 in vivo, immunoblotting was performed on the soluble fraction of homogenized LV tissue of WT and MMP-9 null mice. The results showed a 35-kDa fragment detected only in WT mice and not in MMP-9 null mice, confirming CD36 is an in vivo substrate of MMP-9 (Figure 3E).

MMP-9 Decreases Macrophage Phagocytosis

CD36 is known to be expressed by multiple cell sources, including macrophages and endothelial cells.32,33 At day 7 post-MI, dual immunofluorescence revealed no significant difference in the number of macrophages present in the LVI in WT and MMP-9 null mice as previously shown.11 CD36 was significantly higher in MMP-9 null mice, consistent with glycoproteomic analysis.
In addition, CD36 was colocalized with the macrophage marker Mac-3 (Figure 4). MMP-9 null mice showed higher levels of CD36+ macrophages compared with the WT mice at day 7 post-MI consistent with CD36 being reduced in WT.

CD36 expression is required for macrophage-mediated phagocytosis, thus we proposed MMP-9 cleavage of CD36 would decrease phagocytosis in WT mice. Macrophages isolated from the infarct of MMP-9 null mice exhibited a higher phagocytic index compared with WT macrophages (Figure 5). Stimulation with MMP-9 at concentrations similar to what is observed physiologically reduced phagocytosis by 30% in WT and 50% in MMP-9 null, indicating MMP-9 regulates phagocytosis. MMP-9 null mice had higher levels of CD36+ macrophages, which explain the greater reduction in phagocytic index. To test if this decrease was because of CD36 degradation, macrophages were incubated with CD36-blocking peptide (CD36i) or a mixture of MMP-9 and CD36i. Phagocytic potential decreased to a similar extent after incubation with CD36i or the mixture indicating MMP-9 regulates phagocytosis through CD36.

MMP-9 Decreased Apoptotic Neutrophils in Infarct Region at Day 7 Post-MI

CD36 is required for the induction of macrophage-mediated phagocytosis of apoptotic neutrophils. If not removed in a timely manner, apoptotic neutrophils can continue to release granule components prolonging the inflammatory response. We showed MMP-9 can degrade CD36 post-MI leading to a decrease in phagocytosis but whether this leads to sustained neutrophil inflammation is unknown. To address this, the LVs of WT and MMP-9 null mice at day 7 post-MI were stained for neutrophils. MMP-9 null had lower neutrophil numbers in the infarct region compared with WT at day 7 post-MI (Figure 6A and 6C). In addition to facilitating the removal of apoptotic cells, CD36 also initiates the rapid activation of caspase-3 resulting in neutrophil apoptosis. MMP-9 null mice had higher levels of caspase-3 and transferase deoxyuridine triphosphate (dUTP) nick end labeling staining compared with WT mice at day 7 post-MI (Figure 6B and 6D). Caspase-3-mediated spontaneous death in neutrophils is critical for modulating inflammatory responses. To confirm the higher levels of cleaved caspase-3 were because of neutrophil apoptosis, dual immunofluorescence of neutrophils and transferase dUTP nick end labeling was performed (Figure 6E). Overlay analysis showed higher numbers of apoptotic neutrophils with MMP-9 deletion. During active inflammation, neutrophil apoptosis is delayed leading to increased tissue damage.

We hypothesized that a lack of CD36 degradation was the cause of increased apoptotic neutrophils observed in the MMP-9 null mice and tested this using an in vitro assay. Because neutrophils are known to undergo spontaneous apoptosis in the absence of extracellular stimuli, we used this natural process to induce apoptosis to limit exogenous influences. Incubating neutrophils with MMP-9 decreased the gene expression of the apoptotic marker caspase-9 by 2-fold and increased caspase-3, with no effect on Bax or Xiap. Caspase-9 is a critical upstream activator of the caspase pathway, and these results indicate that MMP-9 regulates apoptosis in vitro. CD36i increased caspase-3 and Xiap expression but did not affect caspase-9 or Bax. Xiap is an inhibitor of the caspase pathway. Our data indicate that although MMP-9 directly regulated neutrophil apoptosis, this effect was independent of CD36 degradation.
Excessive inflammation post-MI has been shown to impair infarct healing contributing to LV dysfunction. At day 7 post-MI, WT mice showed increased LV dilation (Table 2), indicated by elevated diastolic dimensions and volumes compared with baseline day 0 controls. This increase was attenuated in the absence of MMP-9, consistent with previous findings. Regression analysis showed decreased CD36 levels were linked to increases in end-diastolic dimensions ($R^2=0.48$; $P<0.05$). MMP-9 deletion removed the link between CD36 and LV dilation.

**Discussion**

The goal of this study was to identify MMP-9–dependent signaling mechanisms in the post-MI LV. Our results...
showed that of 541 N-glycosylated proteins quantified, 45 proteins were at least 2-fold upregulated or downregulated with MMP-9 deletion; and CILP and CD36 were identified as having the highest fold increases in MMP-9 null mice.

MMP-9 regulated CD36 levels through proteolytic degradation, decreasing macrophage phagocytosis and prolonging neutrophil inflammation. In addition, regression analysis showed decreased CD36 levels were linked to increases in

Figure 5. Matrix metalloproteinase (MMP)-9 regulates phagocytosis through CD36. Cells were stimulated in vivo and then isolated at day 7 post-myocardial infarction (MI) from the left ventricle. MMP-9 deletion increased the macrophage phagocytic index compared to wild-type (WT) MI macrophages. MMP-9 incubation decreased phagocytosis in both WT and MMP-9 null. MMP-9, CD36-blocking peptide (CD36i) and the combination of MMP-9 and CD36i decreased phagocytosis to similar degrees, indicating MMP-9 regulates macrophage phagocytic potential through CD36. Quantification of phagocytosis was performed using the SpectraMax M3 Plate Reader. Data are represented as mean±SEM. Representative images are at 40× magnification. Fluorescent bioparticles are shown in green. 4′,6-Diamidino-2-phenylindole staining is shown in blue. *P<0.05 vs WT; †P<0.05 vs null MI; n=5 per group.

Figure 6. Matrix metalloproteinase (MMP)-9 decreases removal of apoptotic neutrophils post myocardial infarction (MI). A and C, MMP-9 null mice had lower numbers of neutrophils in the infarct at day 7 post-MI compared with wild-type (WT). This decrease was coupled with an increase in the apoptotic markers (B) cleaved caspase-3 (pooled samples) and (D) terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) at day 7 post-MI. E, Overlay analysis indicated lower numbers of apoptotic neutrophils in WT animals indicating prolonged neutrophil-mediated inflammation at day 7 post-MI. Data are represented as mean±SEM. *P<0.05 vs respective day 0; †P<0.05 vs WT; n=8 per group. DAPI indicates 4′,6-diamidino-2-phenylindole.
end-diastolic dimensions. Combined, our results reveal a role for MMP-9 in regulating CD36-mediated aspects of post-MI LV remodeling.

The proteins identified in this study are potential downstream mediators of MMP-9. Of the 45 differentially expressed proteins, at least 5 are known MMP-9 substrates, including fibronectin, collagen XIV, tenasin, laminin α5, and laminin β2.10 Future studies identifying the role of the other key proteins identified, such as CILP and latent-transforming growth factor–β-binding protein–2, will be necessary to fully delineate MMP-9 post-MI mechanisms. This study validated CD36 as a novel in vivo MMP-9 substrate. CD36 degradation delayed inflammation resolution by decreasing macrophage-mediated phagocytosis. On the basis of our in vitro results, MMP-9 also regulates neutrophil apoptosis through a pathway that is not dependent on CD36 degradation.

In addition to MMP-9, CD36 was also cleaved by MMP-12. Inhibition of MMP-12 post-MI is known to suppress neutrophil apoptosis, leading to prolonged inflammation and worsened LV function.27 Interestingly, the in vitro cleavage assay showed multiple bands generated with MMP-9, whereas MMP-12 generated only 1 major fragment. This suggests that although MMP-12 cleaves CD36, MMP-9 degrades it further preventing CD36 biological activity. Although MMP-9 increases only in the infarct area, MMP-12 increases in both the remote and the infarct area. This is one explanation for why we see such a dramatic decrease in CD36 in the LVC. Lower levels of CD36 in the LVC could reduce fatty acid supply to the surviving myocytes, leading to a reduction in energy supply and contributing to infarct expansion.43

MMP-9 increases dramatically during the first week post-MI, and this lead to CD36 degradation. We focused our evaluation on day 7 post-MI, because this is a time when the early peak in MMP-9 concentrations is waning, as is the inflammatory process.44 This time point, therefore, allows us to monitor the end points of the inflammatory process and the beginning of the scar formation process. Our study identified macrophages as a source of CD36 in the infarct. CD36 is a multifunctional plasma membrane protein that plays a role in fatty acid transport, cell apoptosis, and inflammation.45 CD36 binds Thbs1-initiating apoptosis and the release of additional Thbs1 as a signal to recruit macrophages. The CD36/Thbs1 complex on the cell surface of the apoptotic cell acts as a ligand, which
interacts with the CD36/αvβ complex on macrophages initiating phagocytosis of the apoptotic cell. Macrophage CD36 recognition and internalization of apoptotic cells inhibits the release of proinflammatory cytokines, such as tumor necrosis factor-α, IL-12, IL-1β, and IL-8 and initiates the anti-inflammatory response, which is mediated by the release of IL-10 and transforming growth factor-β.

In our study, we showed CD36 levels decreased post-MI in WT only. This decrease was because of MMP-9–mediated degradation. Degradation of CD36 led to decreased macrophage phagocytosis at day 7 post-MI implicating MMP-9 as a key player in the persistence of the inflammatory response by mediating neutrophil removal.

### Future Directions

MMP-9 deletion has been proven to be beneficial post-MI in mice. However, the use of a nonspecific inhibitor in clinical trials has shown to be inconclusive. In our study, we showed CD36 levels decreased post-MI in WT only. This decrease was because of MMP-9–mediated degradation. Degradation of CD36 led to decreased macrophage phagocytosis at day 7 post-MI implicating MMP-9 as a key player in the persistence of the inflammatory response by mediating neutrophil removal.

| WT | MMP-9 null |
|----|------------|
| Myocardial Infarction | Myocardial Infarction |
| proteolytic cleavage | No MMP-9 |
| CD36 | CD36 |
| phagocytosis | phagocytosis |
| removal of neutrophils | removal of neutrophils |
| persistent inflammation | resolution of inflammation |
| ↑ LV dilation | ↓ LV dilation |

![Figure 8. Diagram depicting the roles of matrix metalloproteinase (MMP)-9–mediated CD36 degradation in left ventricular (LV) remodeling post-myocardial infarction (MI). After MI, MMP-9 degradation of CD36 resulted in decreased phagocytic index and prolonged neutrophil inflammation, leading to LV dysfunction. WT indicates wild-type.](image-url)

In summary, this study identified CD36 as an in vivo MMP-9 substrate. In the clinical setting, heart failure is marked by the persistent presence and activation of neutrophils because of their reduced clearance. Our study demonstrated that degradation of CD36 decreased macrophage phagocytosis and prolonged neutrophil inflammation leading to an enlarged LV post-MI (Figure 8). These results are the first to implicate MMP-generated CD36 proteolysis as a possible marker for sustained inflammation and the development of heart failure.

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DeLeon-Pennell et al  CD36 as a Novel In Vivo MMP-9 Substrate

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**CLINICAL PERSPECTIVE**

In the United States, >40% of all deaths are attributed to cardiovascular disease, and more than half of these deaths are due to myocardial infarction. After myocardial infarction, the cardiac extracellular matrix is proteolytically cleaved by matrix metalloproteinases (MMPs). MMPs are involved in multiple biological processes, including cell-surface receptor cleavage and release, cytokine and chemokine activation and inactivation, and extracellular matrix turnover. MMP-9 deletion has been shown to be beneficial post myocardial infarction in mice; however, the use of nonspecific MMP inhibitors have been inconclusive in clinical trials. The current article used a proteomic strategy to identify novel MMP-9 substrates. Platelet glycoprotein 4, CD36, was validated as a novel in vivo MMP-9 substrate. Degradation of CD36 by MMP-9 decreased macrophage phagocytosis and prolonged neutrophil inflammation. This study revealed that MMP-9 deletion promotes resolution of the inflammatory response. Identifying the effects of MMP-9 cleavage products may provide novel therapeutic insight for post-myocardial infarction patients.