



Modification of Extracellular Matrix by the Product of DHA Oxidation Switches Macrophage Adhesion Patterns and Promotes Retention of Macrophages During Chronic Inflammation

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Oxidation of polyunsaturated fatty acids contributes to different aspects of the inflammatory response due to the variety of products generated. Specifically, the oxidation of DHA produces the end-product, carboxyethylpyrrole (CEP), which forms a covalent adduct with proteins via an ϵ -amino group of lysines. Previously, we found that CEP formation is dramatically increased in inflamed tissue and CEP-modified albumin and fibrinogen became ligands for $\alpha_D\beta_2$ (CD11d/CD18) and $\alpha_M\beta_2$ (CD11b/CD18) integrins. In this study, we evaluated the effect of extracellular matrix (ECM) modification with CEP on the adhesive properties of M1-polarized macrophages, particularly during chronic inflammation. Using digested atherosclerotic lesions and *in vitro* oxidation assays, we demonstrated the ability of ECM proteins to form adducts with CEP, particularly, DHA oxidation leads to the formation of CEP adducts with collagen IV and laminin, but not with collagen I. Using integrin $\alpha_D\beta_2$ -transfected HEK293 cells, WT and $\alpha_D^{-/-}$ mouse M1-polarized macrophages, we revealed that CEP-modified proteins support stronger cell adhesion and spreading when compared with natural ECM ligands such as collagen IV, laminin, and fibrinogen. Integrin $\alpha_D\beta_2$ is critical for M1 macrophage adhesion to CEP. Based on biolayer interferometry results, the isolated α_D I-domain demonstrates markedly higher binding affinity to CEP compared to the “natural” $\alpha_D\beta_2$ ligand fibrinogen. Finally, the

presence of CEP-modified proteins in a 3D fibrin matrix significantly increased M1 macrophage retention. Therefore, CEP modification converts ECM proteins to $\alpha_D\beta_2$ -recognition ligands by changing a positively charged lysine to negatively charged CEP, which increases M1 macrophage adhesion to ECM and promotes macrophage retention during detrimental inflammation, autoimmunity, and chronic inflammation.

Keywords: macrophage, adhesion, migration, integrin $\alpha_D\beta_2$, CD11d/CD18, carboxyethylpyrrole (CEP), ECM, inflammation

INTRODUCTION

Low-grade chronic inflammation is a key component in the development of metabolic and autoimmune diseases such as diabetes, atherosclerosis, obesity, and rheumatoid arthritis (1–3). A critical step in the progression of the disease is the accumulation of classically activated pro-inflammatory (M1-like) macrophages in the extracellular matrix (ECM) of inflamed tissues (4, 5). The failure of pro-inflammatory macrophages to emigrate from the inflamed tissue leads to excessive leukocyte recruitment, metabolic imbalance, and damage of healthy tissue, which all together promote the development of chronic inflammation (1, 6). The mechanism and key components of macrophage retention within the site of inflammation are not yet well understood, although this information can improve the treatment of a variety of different pathologies. Enhanced adhesion that prevents macrophage migration is a potential central mechanism of chronic inflammation development. The cell adhesion force is regulated by the density of adhesive receptors, ligand availability, and receptor-ligand affinity (7). The major adhesive receptors on macrophages are integrins. β_1 integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, etc.) have a moderate expression on macrophages (8, 9) and support adhesion/migration to major ECM proteins, such as collagens and laminins. The role of high-density expressed β_2 integrins ($\alpha_M\beta_2$, $\alpha_D\beta_2$, etc.) in macrophage migration/retention in tissue is thought to be limited by lack of binding to major ECM proteins (10, 11).

Nevertheless, we recently demonstrated that macrophage receptor integrin $\alpha_D\beta_2$ (CD11d/CD18) contributes to the development of atherosclerosis and diabetes due to the generation of strong adhesion of pro-inflammatory macrophages to the inflamed ECM, thereby promoting the retention of macrophages at the site of chronic inflammation (12). Integrin $\alpha_D\beta_2$ is the most recently discovered leukocyte integrin (13, 14), that contributes to an inflammatory response (12, 15–17) and infection in different pathological conditions (18–21). Interestingly, integrin $\alpha_D\beta_2$ is upregulated on pro-inflammatory macrophages, which makes it potentially important for macrophage retention (12). However, the critical $\alpha_D\beta_2$ ligand in the inflamed tissue is not yet defined.

We recently discovered a potential mechanism that could explain the presence of inflammation-specific ligands in the

ECM for integrin $\alpha_D\beta_2$. We revealed that during acute inflammation, the oxidation of docosahexaenoic acid (DHA) leads to the generation of the end-product carboxyethylpyrrole (CEP), which forms an adduct with proteins *via* an ϵ -amino group of lysines (22, 23). *In vitro* analysis with BSA demonstrated that 10–20 lysines can be modified by CEP in one protein molecule. Notably, positively charged lysines are substituted to the pyrroles with exposed negatively charged carboxyl groups. (**Figure 1**). Based on our recent data, a carboxyl group in the CEP structure is specifically responsible for the binding to integrins $\alpha_D\beta_2$ and $\alpha_M\beta_2$ (23). Accordingly, CEP-modification significantly changes the surface charge of the targeted protein and its binding properties.

In our previous study, we found that during peritoneal inflammation at least two proteins are modified with CEP in the peritoneal cavity - fibrinogen and albumin. Most importantly, we found that CEP-modified proteins support the migration of non-polarized macrophages to the site of acute inflammation due to interaction with macrophage integrins $\alpha_D\beta_2$ and $\alpha_M\beta_2$ (23). Integrins $\alpha_D\beta_2$ and $\alpha_M\beta_2$ are adhesive receptors, which interact with many ligands and transduce a signal to the actin cytoskeleton that regulates cell adhesion, migration, and other cell responses (24, 25). $\alpha_D\beta_2$ possesses a high homology and similar ligand-binding specificity to $\alpha_M\beta_2$ (11); however, these two integrins demonstrate different roles in chronic inflammatory diseases. As discussed above, α_D supports the development of atherosclerosis (12) and diabetes (26), while integrin α_M protects against these metabolic diseases (27, 28). Apparently, the different surface densities of these integrins on primary monocytes (29) and distinct subsets of macrophages (12, 18, 26), determine their diverse roles in cell migration and chronic inflammation. Interestingly, the analysis of $\alpha_D\beta_2$ and $\alpha_M\beta_2$ binding to CEP demonstrated that α_D has a 5-folds higher affinity to CEP (23), which may have a physiological importance for macrophage adhesion.

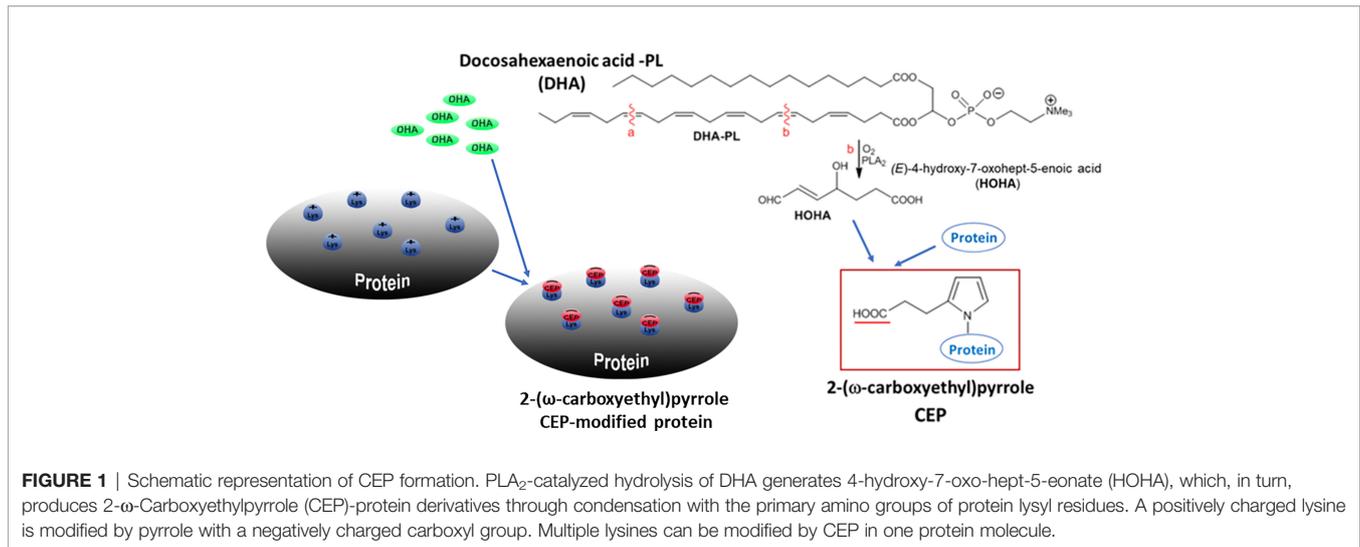
The goal of the current project is to evaluate how the modification of ECM proteins with CEP affects their binding properties and the strength of macrophage adhesion, which is critical for macrophage retention during chronic inflammation.

MATERIAL AND METHODS

Reagents and Antibodies

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA). Recombinant mouse IFN γ , CCL2, and CCL5 were purchased from

Abbreviations: CEP, carboxyethylpyrrole; ECM, Extracellular matrix; EDTA, Ethylenediaminetetraacetic acid; FACS, Fluorescence-activated cell sorting; IFN γ , interferon- γ ; CCL2, C-C motif chemokine ligand 2 (MCP-1); CCL5, C-C motif chemokine ligand 5 (RANTES); TG, thioglycollate; WT, wide type; 2D, 2 dimensional; 3D, 3 dimensional.



Invitrogen Corporation (Carlsbad, CA). Human fibrinogen and thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Collagen IV, collagen I, and laminin were purchased from Corning (Corning, NY). CEP-BSA (21:1 molar ratio) and CEP-Fg (29:1 molar ratio) were prepared through a Paar-Knorr pyrrole synthesis with (9H-fluoren-9-ylmethyl ester 4,7-dioxo-heptanoic acid) DOHA-Fm as described previously (28). Anti-CD68 mAb was from eBioscience. Polyclonal antibody against the α_D I-domain was described previously (11). The antibody recognizes both human and mouse α_D I-domains and has no cross-reactivity with recombinant human and mouse α_M , α_X , and α_L I-domains. The antibody was isolated from rabbit serum by affinity chromatography using α_D I-domain-Sepharose. Anti-macrophage antibody F4/80 was from eBioscience (San Diego, CA). A polyclonal antibody against CEP and monoclonal IgM anti-CEP antibody were described previously (30, 31). Blocking IgG anti-CEP antibody (Clone 3C9) was generated in Dr. Tatiana Byzova's laboratory (23).

Animals

Wild type (C57BL/6J, stock # 000664) and integrin α_D -deficient (B6.129S7-*Itgad*^{tm1Bl/J}, stock # 005258) mice were bought from Jackson Laboratory (Bar Harbor, ME). α_D -deficient have been backcrossed to C57BL/6 for at least ten generations. All procedures were performed according to animal protocols approved by East Tennessee State University IACUC.

Generation of Classically Activated (M1) Macrophages

Notably, the terms M1 and M2 macrophages are artificial and do not represent all variabilities of macrophage subsets *in vivo*. We used the term M1 in our manuscript to refer to *in vitro* polarized macrophages activated by IFN- γ which serve as a model for pro-inflammatory macrophages *in vivo*. Therefore, peritoneal macrophages from 8-12-week-old mice (WT and $\alpha_D^{-/-}$) were harvested by lavage of the peritoneal cavity with 5 ml of sterile PBS 3 days after intraperitoneal (IP) injection of 4% thioglycollate (TG; 0.5ml). The cells were washed twice with

PBS and resuspended in RPMI media supplemented with 10% FBS, 0.1 mg/ml streptomycin, and 0.1 unit/ml penicillin. The cell suspension was transferred into 100mm Petri dishes and incubated for 2h at 37°C in humidified air containing a 5% CO₂ atmosphere. Nonadherent cells were washed out with PBS, and the adherent macrophages were replenished with complete RPMI media. The macrophages were differentiated to M1 phenotypes by treatment with recombinant mouse interferon- γ (IFN- γ) (100 U/ml, Thermo Fisher) for 4 days. Medium with IFN- γ was changed every 2 days or as required. Macrophages were dissociated from the plates using 5mM EDTA in PBS and used for the experiments thereafter.

Generation of $\alpha_D\beta_2$ -Transfected HEK293 Cells

The HEK293 cells stably expressing human integrin $\alpha_D\beta_2$ were generated as described previously (11) and maintained in DMEM/F-12 (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 15 mM HEPES, 0.1 mg/ml streptomycin, and 0.1 unit/ml penicillin.

Isolation of Recombinant α_D I-Domain in an Active Conformation

The construct for the α_D I-domain was generated and recombinant protein was isolated as described in our previous paper (23). Briefly, α_D in the active conformation (Pro¹²⁸-Lys³¹⁴) was inserted in the pET15b vector, expressed in E. Coli as a His-tag fusion protein, and purified using affinity chromatography on Ni-chelating agarose (Qiagen Inc., Valencia, CA).

Flow Cytometry Analysis

Flow cytometry analysis was performed to assess the expression of integrin α_D on mouse peritoneal macrophages and $\alpha_D\beta_2$ -transfected HEK293 cells. Cells were harvested and pre-incubated with 4% normal goat serum for 30 min at 4°C, then 2x10⁶ cells were incubated with a specific antibody for 30 min at 4°C. Non-conjugated antibodies required additional incubation with Alexa 488 or Alexa 647-donkey anti-mouse IgG (at a 1:1000

dilution) for 30 min at 4°C. Finally, the cells were washed and analyzed using a Fortessa X-20 (Becton Dickinson).

Cell Adhesion Assay

The adhesion assay was performed as described previously (23) with modifications. Briefly, 96-well plates (Immulon 2HB, Cambridge, MA) were coated with different concentrations of fibrinogen, collagen IV, collagen I, or laminin for 3 h at 37°C. The wells were post-coated with 0.5% polyvinyl alcohol for 1 h at room temperature. Mouse peritoneal macrophages or HEK293 cells transfected with integrin $\alpha_D\beta_2$ were labeled with 10 μ M Calcein AM (Molecular Probes, Eugene, OR) for 30 min at 37°C and washed with HBSS and resuspended in the same medium at a concentration of 1×10^6 cells/mL. $\alpha_D\beta_2$ -transfected HEK 293 cells were also supplemented with 2mM MnCl₂. Aliquots (50 μ L) of the labeled cells were added to each well. After 30 minutes of incubation at 37°C in a 5% CO₂ humidified atmosphere, the nonadherent cells were removed by washing with PBS. The fluorescence was measured in a Synergy H1 fluorescence plate reader (BioTek, Winooski, VT), and the number of adherent cells was determined from a labeled control.

Cell Spreading Assay

Glass coverslips were coated with fibrinogen (20 μ g/ml), Collagen IV (5 μ g/ml) or CEP-BSA (750 μ M) for 3 hours at 37°C and post-coated with 0.5% polyvinylpyrrolidone for 1 hour at 37°C. To determine cell spreading, inflammatory macrophages isolated from the WT and $\alpha_D^{-/-}$ mice were allowed to adhere to coverslips for 1 hour at 37°C. Macrophages were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with Phalloidin-iFluor 555 and DAPI. Fluorescent images were obtained with EVOS FL auto cell fluorescent imaging system using 20x objective with multi-frame automatic setup (9-fields). The cell areas were calculated using Imaris 8.0 software.

Migration of Macrophages in 3D Fibrin Gel

Migration assay was performed as described previously (26). Polarized M1 macrophages were labeled with PKH26 red fluorescent dye. Cell migration assay was performed for 48 hours at 37°C in 5% CO₂ in a sterile condition. An equal number of WT macrophages was evaluated by cytopspin of mixed cells before the experiment and at the starting point before migration. Labeled WT (1.5×10^5) activated macrophages were plated on the membranes of transwell inserts with a pore size of 8 μ m and 6.5 mm in diameter (Costar, Corning, NY) precoated with fibrinogen (Fg). Fibrin gel (100 μ L/sample) was generated by mixing 0.75mg/ml Fg containing 1% FBS and 1% P/S and 0.5 U/ml thrombin. In some samples 9 μ M CEP was incorporated into the gel during polymerization. 30 nM of MCP-1 was added on top of the gel to initiate the migration. Migrating cells were detected by Leica Confocal microscope (Leica-TCS SP8), and the results were analyzed and reconstructed using IMARIS 8.0 software.

Biolayer Interferometry

The binding parameters of the interaction between the α_D I-domain with CEP or fibrinogen were determined using an Octet

K2 instrument (FortéBio, Sartorius Group). CEP-BSA or fibrinogen was immobilized on the Amine Reactive Second-generation (AR2G) biosensor using the amine coupling kit according to the manufacturer's protocol. Different concentrations of the active forms of α_D I-domain (50-1000 nM) were applied in the mobile phase, and the association between the immobilized and flowing proteins was detected. Experiments were performed in 20 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.02% (v/v) Tween-20, 0.02% BSA, pH 7.5. The protein-coated biosensors were regenerated with 25 mM NaOH and 2M NaCl. Analyses of the binding kinetics were performed using DataAnalysis HT11.0 software (FortéBio). The value of the equilibrium dissociation constant (K_D) was obtained by fitting a plot of response at equilibrium (Req) against the concentration.

Immunostaining

Cryosections (10 μ m) of aorta roots (from WT and ApoE^{-/-} mice after five weeks on a western diet) were warmed to room temperature for 30 minutes prior to immunofluorescence staining. Tissue sections were fixed in ice-cold acetone for 10 minutes followed by permeabilizing with 0.2% Tween-20 for 10 min to increase the signal of intracellular binding sites. Tissue sections were washed in PBS and incubated with SuperBlock (PBS) Blocking buffer (Thermo Scientific, Rockford, IL, USA) for 45 min to block nonspecific binding. Tissues were then incubated at 4°C overnight with the primary antibody (rabbit polyclonal anti-CEP and rat anti-mouse CD68 (macrophage marker). After washing several times with PBS, the sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Alexa Fluor 568-conjugated donkey anti-rat for 1 hour at room temperature. The sections were subsequently washed and sealed. The tissue sections were examined by Leica Confocal microscope (Leica-TCS SP8). Control sections without the primary antibody were also performed at the same time.

Analysis of CEP Formation in Atherosclerotic Lesions of ApoE^{-/-} Mice by Western Blot

Male ApoE-deficient mice were kept on a Western diet for 16 weeks. After that, ApoE^{-/-} and the same age WT C57BL/6 mice (as the control) were euthanized, their vascular system was perfused with 10 ml PBS, and the aortas were cleaned from the outer layers of connective tissue and removed. The isolated aortas were digested with Collagenase type I, type XI, Hyaluronidase, and DHAase I according to the published protocol (12). The cells were separated by centrifugation. The non-cellular supernatant was high speed centrifuged to exclude aggregates and cell traces, and used for Western Blot analysis.

The same concentration of proteins isolated from ApoE^{-/-} and WT aortas was loaded on SDS-electrophoresis analyzed by 7% SDS-PAGE electrophoresis and Western blotting. The Immobilon-P membranes (Millipore) were incubated with rabbit polyclonal anti-CEP antibody, followed by incubation with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase and developed using enhanced SuperSignal Chemiluminescent Substrate (Pierce).

Generation of CEP-Modified ECM Proteins

The evaluation of CEP-modified proteins was performed as described previously (23). Collagen I, collagen IV, and laminin were coated on a 96-well plate at a concentration of 20 $\mu\text{g}/\text{ml}$ overnight at 4°C. Wells were post-coated with 0.5% polyvinyl alcohol for 1 h at 37°C. 20 μM DHA in 20mM Hepes, 150 mM NaCl, 0.01% H_2O_2 , and 1mM CaCl_2 were added to the wells and incubated in an oxygen-free environment (under argon) for 18 hours at 37°C. After incubation, the plate was washed out with PBS supplemented with 0.05% Tween-20 3 times and incubated with anti-CEP polyclonal antibody (0.9 $\mu\text{g}/\text{ml}$) for 2 hours at 37°C. After washing, wells were incubated with goat-anti-rabbit HRP conjugated antibody for 1 h at 37°C and the binding was developed using TMB-ELISA substrate solution (Pierce). The result was detected by a plate reader using a wavelength of 450 nm.

Statistical Analysis

Statistical analyses were performed using a Student's t-test or Student's paired t-tests where indicated in the text using SigmaPlot 13. A value of $p < 0.05$ was considered significant. Error bars represent the SEM of experiments.

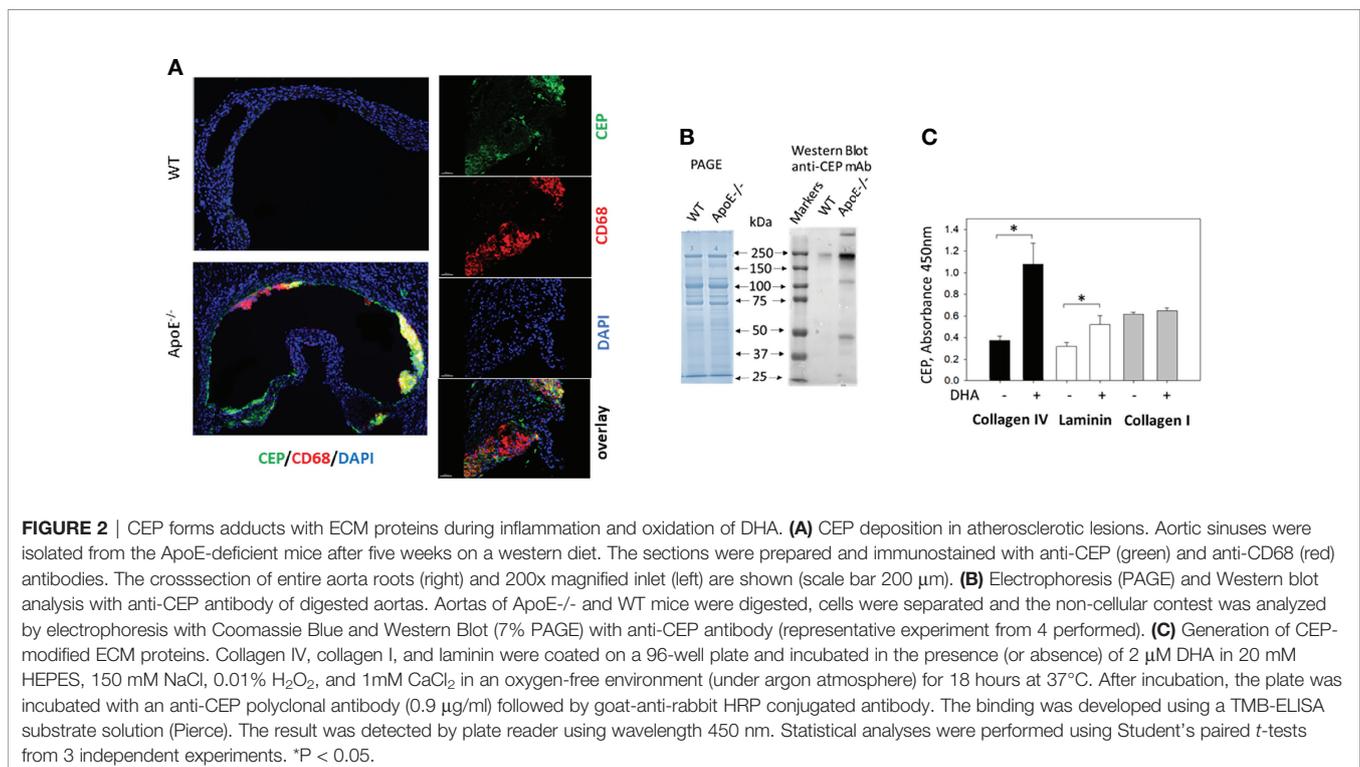
RESULTS

DHA Oxidation Generates CEP Adducts With ECM Proteins in Atherosclerotic Lesions and *In Vitro*

We and others have demonstrated the presence of CEP in different inflammatory tissues using immunohistochemistry (23, 30–34).

Particularly, anti-CEP antibody detected strong immunostaining in the sections of atherosclerotic lesions of $\text{ApoE}^{-/-}$ mice on a Western diet (Figure 2A). However, the direct formation of CEP-protein adducts was shown previously only in the peritoneal cavity, but not in the tissue (23). To demonstrate the link between CEP immunostaining and the formation of CEP-modified proteins in the inflamed tissue during chronic inflammation, we evaluated CEP-protein adduct formation in atherosclerotic lesions (Figure 2B). Male ApoE -deficient mice were kept on a Western diet for 16 weeks ($n=3$). After that, $\text{ApoE}^{-/-}$ and the same age WT C57BL/6 mice (as the control) were euthanized, their vascular system was perfused and aortas were removed. The isolated aortas were digested according to the published protocol (12) and cells were separated by centrifugation. The non-cellular supernatant was high speed centrifuged to exclude aggregates and cell traces, and used for Western Blot analysis.

The same concentration of proteins isolated from $\text{ApoE}^{-/-}$ and WT aortas was loaded on SDS-electrophoresis (Figure 2B, left panel) and analyzed using an anti-CEP antibody (Figure 2B, right panel). We found a dramatic difference in anti-CEP staining of proteins isolated from the atherosclerotic lesions of ApoE -deficient and WT mice. While only one weak band was spotted in the sample isolated from WT mice, several intensive bands were detected in the $\text{ApoE}^{-/-}$ mice samples that represent several CEP-modified proteins. Notably, the faded CEP signal from the WT mice corresponds to the concept that the natural oxidation of PUFA in a healthy organism can lead to a low level of adduct formation. We detected such signal previously in commercial fibrinogen that we used in our experiments as a control (23).



To confirm the ability of CEP to form the covalent adduct with ECM proteins, we tested the formation of CEP modification in collagen I, laminin, and collagen IV using *in vitro* DHA oxidation assay that we developed previously (23). ECM proteins were incubated with DHA and 0.01% H₂O₂ for 18 h at 37°C. After incubation, CEP formation was detected by ELISA with an anti-CEP antibody (Figure 2C). We found that DHA oxidation led to the formation of CEP-collagen IV adduct and CEP-laminin adduct, while collagen I was not modified.

These experiments clearly demonstrated that ECM proteins are modified by CEP, and inflamed tissue (e.g. atherosclerotic lesions) can be a source of specific adhesive substrate for macrophages.

M1 Macrophages Demonstrate Stronger Adhesion and Spreading to CEP-Modified Proteins

Pro-inflammatory, M1-like macrophages, are the major source of inflammatory stimuli during chronic inflammation. We tested how protein modification with CEP affects M1 macrophage adhesion to ECM proteins such as collagen IV, laminin, and fibrinogen (which leaks from blood to the ECM through the damaged endothelial monolayer during inflammation). Different concentrations of collagen IV, laminin, CEP-modified BSA, and BSA alone as a control were immobilized on the 96-well plate and tested as substrates for M1 macrophage adhesion (Figure 3A). We found that the adhesion of pro-inflammatory macrophages to CEP-modified BSA dramatically overreached the adhesion of macrophages to natural ECM ligands. Notably, BSA alone does not support the adhesion of macrophages. In a separate experiment, we compared the adhesion of fibrinogen-

modified by CEP and native fibrinogen. Clearly, CEP modification dramatically increased macrophage adhesion to fibrinogen (Figure 3B).

To verify these results macrophage spreading on CEP, collagen IV, and fibrinogen was evaluated. Cells were labeled with phalloidin-PE, examined by fluorescent microscopy, and analyzed using the ImarisCell module (Imaris 8.0). Corresponding to the adhesion data, the results demonstrate stronger spreading to CEP with larger cell areas and focal adhesion complexes (Figures 3C, D).

The Presence of CEP in a 3D Matrix Inhibits M1 Macrophage Migration

To evaluate the effect of CEP modification in ECM on cell motility we performed a 3D migration assay by comparing M1 macrophage migration in a polymerized fibrin matrix and polymerized fibrin matrix supplemented with 9 μM CEP. Based on our previous result the migration of M1 macrophages in a fibrin matrix is lower compared to non-activated or M2 macrophages (26). The goal of this experiment was to evaluate the additional effect of CEP on this migration. Cells were plated on one side of the gel and migration was initiated by CCL2 and CCL5 added to the other side of the gel (Figure 4A). We found that migration of M1 macrophages was dramatically reduced in fibrin gel supplemented with CEP (Figures 4B–D), which corresponds to strong adhesion events and promotion of macrophage retention at the site of chronic inflammation.

To more critically evaluate these results, we tested the morphology of macrophages in a 3D matrix supplemented with CEP. 3D gels were generated using polymerized fibrin and polymerized fibrin/9 μM CEP. The same macrophages

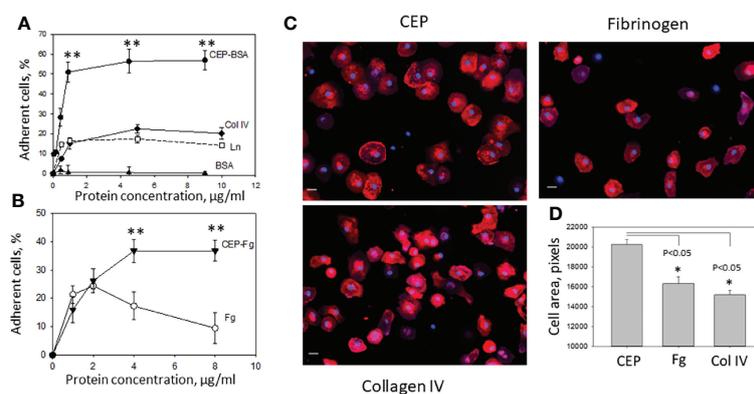


FIGURE 3 | Adhesion of M1-polarized macrophages to CEP and ECM proteins. **(A)** Different concentrations of CEP-BSA, collagen IV (Col IV), laminin (Ln) and BSA were immobilized on a 96-well plate for 3 h at 37°C. Fluorescently labeled macrophages were added to the wells and cell adhesion was determined after 30 min in a fluorescence plate reader. CEP-BSA is presented based on the concentration of BSA. 1 μg BSA contains 250 nM CEP. A representative experiment from 4 performed. Data are presented as mean ± SEM ***p* < 0.01. **(B)** Different concentrations of CEP-fibrinogen (CEP-Fg) and fibrinogen (Fg) were immobilized and tested in an adhesion assay as described above. A summary result from 3 experiments. CEP-Fg is shown based on the concentration of Fg. 1 μg Fg contains 87 nM CEP Data are presented as mean ± SEM ***p* < 0.01. **(C)** Macrophages were allowed to adhere to glass coverslips coated with fibrinogen, Collagen IV or CEP-BSA for 1 hour at 37°C. Macrophages were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with Alexa Fluor 555-conjugated phalloidin and DAPI. Fluorescent images were obtained with EVOS FL auto cell fluorescent imaging system using 20x objective with mosaic 9-fields setup. **(D)** The cell area was calculated using Imaris 8.0 software. Data are presented as mean ± SEM **p* < 0.05.

were incorporated into both matrixes during polymerization and migration was stimulated with a gradient of CCL2. After 18 hours, the cells inside the gel (at least 80 μM from the gel surface) were analyzed using the confocal microscope. We found that cells in the fibrin matrix present a more elongated shape when compared with the more rounded cells in the matrix with supplemented CEP (Figure 4E). The difference between the length-to-diameter ratio was extremely significant ($P < 0.0001$, $n = 30\text{--}35/\text{group}$) (Figure 4F). According to published data, elongated cell morphology in a 3D matrix corresponds to migrating cells.

Therefore, our results demonstrate that modification with CEP affects M1 macrophage adhesion, spreading, and migration-dependent morphology.

Integrin $\alpha_D\beta_2$ Is Critical for M1 Macrophage Adhesion and Spreading to CEP-Modified Proteins

The major adhesive receptors for CEP on macrophages are integrins $\alpha_D\beta_2$ and $\alpha_M\beta_2$. Integrin α_D is upregulated on M1 macrophages (12), (Figure 5A), while α_M maintains the same level of expression. This difference determines the distinct roles of these β_2 integrins in inflammation. While $\alpha_M\beta_2$ supports macrophage migration and demonstrates some protective function during chronic inflammation (27, 35), integrin $\alpha_D\beta_2$ inhibits M1 macrophage migration and contributes to the development of chronic inflammation (12, 26). We hypothesized that integrin $\alpha_D\beta_2$ is critical for M1 macrophage adhesion to CEP-modified proteins. To test this hypothesis, we

compared the adhesion of M1 macrophages isolated from the WT and α_D -deficient mice to CEP. The adhesion assay demonstrated a dramatic reduction in macrophage adhesion in α_D -deficient mice (Figure 5B). Moreover, macrophage spreading to CEP was significantly affected in $\alpha_D^{-/-}$ M1 macrophages, the total cell areas were reduced as well as cell shapes were irregular (Figures 5C, D).

Therefore, our data demonstrate that α_D -deficiency significantly reduced adhesion and spreading of M1 macrophages on CEP, which confirmed the critical role of $\alpha_D\beta_2$ in macrophage adhesion and potential retention in CEP-modified substrate.

The Binding Properties of CEP Exceed the Binding Properties of Natural $\alpha_D\beta_2$ Ligands

Macrophages are a complex system with a variety of adhesive receptors that have overlapping ligand binding properties. To verify the role of CEP as a strong $\alpha_D\beta_2$ ligand, we tested $\alpha_D\beta_2$ -transfected HEK293 cells (Figure 6A). HEK293 cells do not express any receptors that interact with CEP such as integrin $\alpha_M\beta_2$, CD36, or TLRs (23, 34). Adhesion of $\alpha_D\beta_2$ -transfected HEK293 cells was tested to CEP-BSA versus BSA and CEP-fibrinogen versus fibrinogen. As we expected $\alpha_D\beta_2$ -cells do not adhere to BSA, but demonstrate the strong concentration-dependent adhesion to CEP-BSA. (Figure 6B, upper).

Fibrinogen is a natural ligand for integrin $\alpha_D\beta_2$ and a common element of the ECM during inflammation. Markedly, CEP modification significantly improves the adhesive properties of fibrinogen to $\alpha_D\beta_2$ -HEK293 transfected

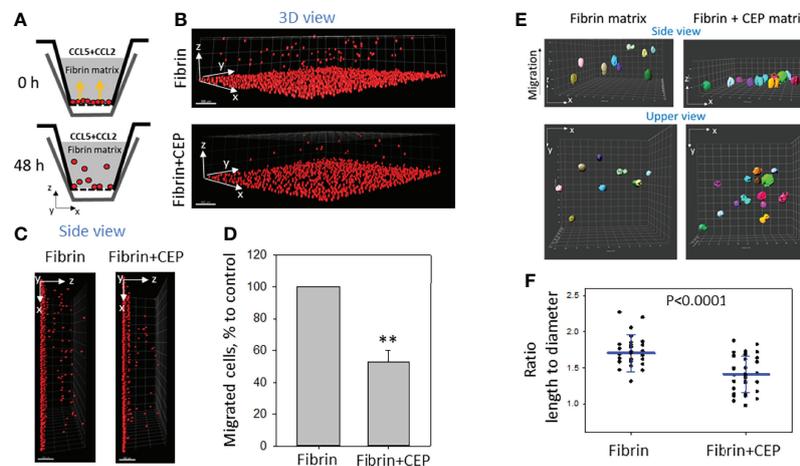


FIGURE 4 | The presence of CEP in the matrix inhibits the 3D migration of M1 macrophages. **(A)** Sketch diagram of the migrating cells in Boyden transwell chamber. Before migration (upper panel) and after 48h migration (lower panel). **(B)** WT M1 macrophages were labeled with red PKH26 fluorescent dye. Labeled WT (1.5×10^5) activated macrophages were plated on the membranes of transwell inserts (Boyden chamber) with a pore size of 8 μm and 6.5 mm in diameter (Costar, Corning, NY) precoated with fibrinogen (Fg). Fibrin gel (100 $\mu\text{l}/\text{sample}$) was generated by mixing 0.75mg/ml Fg containing 1% FBS and 1% P/S and 0.5 U/ml thrombin with or without 90 μM CEP. Migration of macrophages was stimulated by 30 nM CCL2 and 100nM CCL5 added to the top of the gel. After 48 hours, migrating cells were detected by a Leica Confocal microscope **(B, C)**. **(D)** The results were analyzed by IMARIS 8.0 software, and statistical analyses were performed using Student's paired t-tests ($n = 5$ per group). Scale bar = 300 μm . Data are presented as mean \pm SEM. ** $P < 0.01$. **(E)** The morphology of non-labeled macrophages was evaluated after gel staining with CytoPainet Phalloidin-iFluor 555 in fibrin matrix (left panel) and Fibrin matrix supplemented with CEP (right panel). The ratio length/diameter was calculated using the ImarisCell module (IMARIS 8.0). **(F)** Statistical analyses were performed using Student's t-tests, $n = 30$ (fibrin gel) 36 (fibrin+CEP gel). $P < 0.0001$.

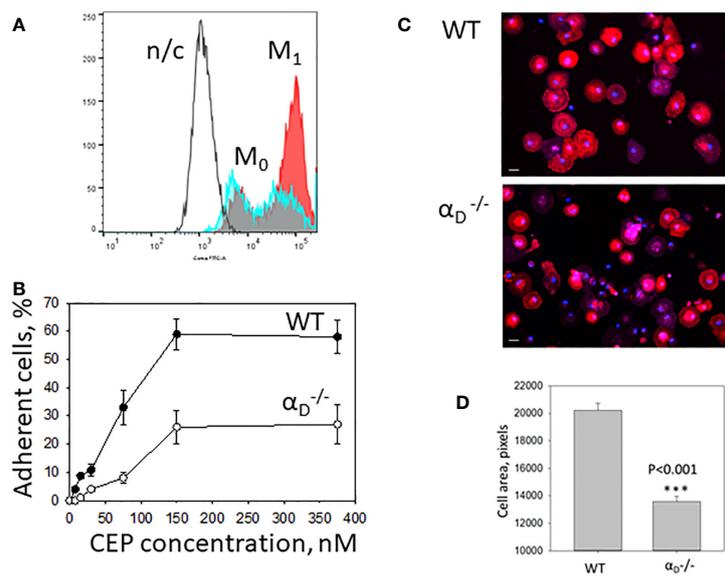


FIGURE 5 | Integrin $\alpha_D\beta_2$ is critical for M1 macrophage adhesion to CEP-modified proteins. **(A)** The expression of integrin α_D is upregulated on M1 macrophages. Peritoneal macrophages were isolated and plated for 4 days in the presence of IFN γ in complete RPMI media (Red). M1 macrophages were collected using cell dissociation buffer and stain with anti- α_D polyclonal antibody, followed by staining with donkey-anti-rabbit Alexa 647 secondary antibody. Freshly isolated peritoneal macrophages were used as a control (M0)(Blue). Integrin α_D expression was tested using flow cytometry (Fortessa X-20). N/c-negative control. **(B)** Adhesion of WT and $\alpha_D^{-/-}$ M1 macrophages to CEP-BSA. Isolated M1 macrophages were fluorescently labeled with Calcein AM and added to the well coated with CEP-BSA. Cell adhesion was determined after 30 min in a fluorescence plate reader. **(C)** Macrophages were allowed to adhere to glass coverslips coated with CEP-BSA for 1 hour at 37°C. After fixation with 2% paraformaldehyde and permeabilization with 0.1% Triton X-100, cells were incubated with CytoPainet Phalloidin-iFluor 555 and DAPI. Fluorescent images were obtained with EVOS FL auto cell fluorescent imaging system using 20x objective with mosaic 9-fields setup. **(D)** The cell area was calculated using Imaris 8.0 software. Data are presented as mean \pm SEM ***p < 0.001.

cells similar to the result, which was obtained for M1 macrophages (**Figure 3B**). Notably, it has been shown previously that cell adhesion to fibrinogen achieved maximum at fibrinogen concentration 2-3 $\mu\text{g/ml}$ and is significantly reduced at higher concentrations (10, 36). The mechanism is not known but may depend on the surface-induced lateral aggregation of fibrinogen (37). This result demonstrated that CEP modification in different proteins provides a similar augmentation of cell adhesive properties.

To evaluate a difference in the binding of $\alpha_D\beta_2$ to CEP and fibrinogen, we isolated $\alpha_D\beta_2$ ligand-binding motif – I-domain (**Figure 6C**) and examined its binding properties using BioLayer Interferometry. CEP and fibrinogen were immobilized on amino coupling biosensors and tested with different concentrations of α_D I-domain using two approaches such as kinetic titration (single cycle kinetics) (**Figure 6D**) and equilibrium analysis (steady-state analysis) (**Figures 6E, F**). The binding affinity was analyzed using DataAnalysis HT11.0 software (ForteBio). We found that the affinity of α_D binding to CEP ($K_D=1.0\times 10^{-7}\text{M}$) is 2-fold stronger than the α_D interaction with fibrinogen ($K_D=2.2\times 10^{-7}$).

Therefore, $\alpha_D\beta_2$ generates a more powerful adhesion to CEP-modified proteins due to a higher affinity mediated by a negative charge of a carboxyl group in CEP's structure. This result suggests the unique role of CEP-modified ECM proteins in

macrophage adhesion that generates strong $\alpha_D\beta_2$ -dependent adhesion *via* CEP that can be critical for macrophage retention.

DISCUSSION

In our previous project, we found that CEP, the product of DHA oxidation, is a ligand for integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$ (23). In the current study, we tested how the modification of the ECM proteins with CEP affects macrophage adhesion. Now we found that 1) Polyunsaturated fatty acid oxidation generates the formation of adducts between CEP and ECM proteins *in vitro*. 2) The development of atherosclerosis promotes a CEP formation in proteins within the atherosclerotic lesions. 3) Pro-inflammatory M1 macrophages demonstrate a stronger adhesion to CEP-modified proteins compared to natural ECM ligands. 4) M1 macrophage adhesion to CEP inhibits cell migration. 5) The adhesion of M1 macrophages to CEP-modified proteins is driven by integrin $\alpha_D\beta_2$.

The previous experiments using immunostaining with the anti-CEP antibody demonstrated the deposition of CEP in tissue during inflammation including myocardial infarction (38), peritoneal inflammation (23), atherosclerosis (38), and macular degeneration (32, 39). Our published data revealed that neutrophil-mediated oxidation led to the formation of CEP-

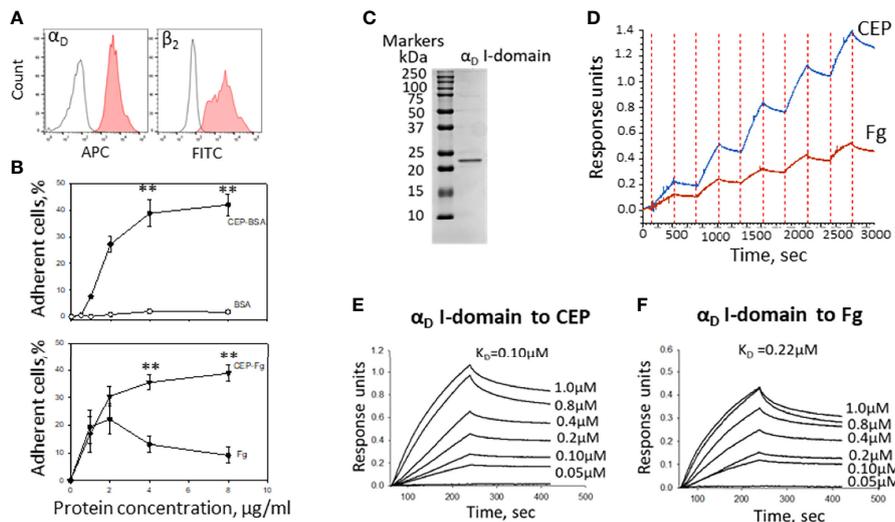


FIGURE 6 | The binding properties of CEP exceed the binding properties of natural $\alpha_D\beta_2$ ligands. **(A)** The expression of integrins α_D and β_2 on the surface of HEK293-transfected cells. After isolation, cells were stained with anti- α_D polyclonal antibody and anti- β_2 monoclonal antibody, followed by staining with donkey-anti-rabbit Alexa 647 and donkey-anti-mouse Alexa 488 secondary antibodies. Control is shown in open histogram. **(B)** Different concentrations of CEP-BSA and BSA (Upper panel) or CEP-fibrinogen (CEP-Fg) and Fg (lower panel) were immobilized on a 96-well plate for 3 h at 37°C. Fluorescently labeled $\alpha_D\beta_2$ HEK293-transfected cells were added to the wells and cell adhesion was determined after 30 min in a fluorescence plate reader. A summary result from 4-6 experiments. Data are presented as mean \pm SEM ** $p < 0.01$. **(C)** Isolated α_D I-domain binds to CEP in bilayer interferometry assays. SDS-PAGE of generated I-domain. α_D active (D¹²²-K³¹⁴) I-domain was isolated from soluble fractions of **(E)** coli lysates and purified using affinity chromatography. The protein purity was assessed by SDS-PAGE on 4-15% gradient gel under non-reducing conditions followed by staining with Coomassie Blue. Representative profiles of the bilayer interferometry responses for α_D binding to the immobilized CEP-BSA and Fibrinogen (Fg). **(D)** Single cycle kinetic of α_D I-domain binding (concentrations ranging from 24-750 nM) to CEP-BSA and Fg coupled to AR2G biosensor. **(E)** Steady state analysis of α_D I-domain binding to CEP **(E)** and Fg **(F)** (concentrations ranging from 50-1000 nM). The K_D of binding was calculated using DataAnalysis HT11.0 software (ForteBio).

fibrinogen and CEP-BSA adducts (23). We hypothesize that PUFA oxidation can lead to the modification of different ECM proteins that contain an exposed lysine on a protein's surface. We do expect some limitations, which can depend on special tertiary structures, post-translational modifications, or cross-linkage through lysines. Our concern was primarily based on the absence of CEP adduct formation with the E-fragment of fibrin (23), which has a strong triple-coiled helix structure. (The triple-coiled helix is also a major component of laminin and collagen fibrils). In addition to a triple-coiled structure, collagens IV, V, and VI contain flexible globular domains, which are absent in collagens I and III (40). In our current experiments, we found that *in vitro* oxidation of DHA generates CEP-collagen IV adduct formation, while collagen I is not modified by CEP, which confirms our hypothesis regarding the potential role of the globular domains in CEP adducts formation.

Previously we demonstrated that non-activated peritoneal macrophages can bind CEP *via* $\alpha_M\beta_2$ and $\alpha_D\beta_2$. However, we did not compare the level of macrophage adhesion to natural adhesive ligands and CEP-modified ligands. Moreover, we did not test the effect of CEP modification on the adhesion and migration of M1 macrophages, which is critical for the development of inflammatory response. In this paper, we are particularly focused on integrin $\alpha_D\beta_2$ since it is upregulated on pro-inflammatory M1-like macrophages and our previous data demonstrated that the α_D I-domain showed 5 folds stronger

affinity to CEP compared to the α_M I domain (23, 41). This difference most likely is mediated by clusters of positively charged amino acids on the surface of the α_D I-domain. The increased binding activity to negatively charged ligands was described previously for integrin $\alpha_X\beta_2$ which has a significantly lower expression on macrophages but has a high level of homology with integrin $\alpha_D\beta_2$ (42).

In our previous project, we found that $\alpha_D\beta_2$ on the surface of pro-inflammatory M1 macrophages contributed to the retention of macrophages at the site of atherosclerotic lesions due to strong cell adhesion (12). It has been shown that $\alpha_D\beta_2$ is a multiligand protein that can bind to ICAM-3, VCAM-1, fibrinogen, fibronectin, and vitronectin (11). However, these proteins are cell surface receptors or plasma proteins, which are sporadic in the interstitium, while the major ECM proteins such as collagens and laminins do not support $\alpha_D\beta_2$ -mediated adhesion (26). This raises the question regarding the potential ECM ligands for $\alpha_D\beta_2$ -mediated macrophage retention. The modification of collagen IV and laminin with CEP (**Figure 2C**) demonstrates the protein modification in ECM and resolves this issue. This mechanism presents the formation of the universal ligand for $\alpha_D\beta_2$ -mediated adhesion. Notably, in our previous project, we found that a carboxyl group in CEP's structure is critical for integrin binding since another product of DHA oxidation, ethylpyrole, has a similar structure, but does not have a carboxyl group and loses the ability to interact with integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$ (23).

Therefore, the positively charged lysines in different proteins can be modified with negatively charged CEP, which converts these proteins to the $\alpha_D\beta_2$ -dependent substrate.

We found that adhesion and spreading of M1 macrophages to the CEP-modified proteins is much stronger compared to native collagen IV, laminin, or fibrinogen (**Figure 3**). The adhesion to collagens and laminins is mediated *via* β_1 integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_2$, etc. The level of β_1 integrins is lower on macrophages compared to β_2 integrins (8, 9). Therefore, the switch of binding from classical collagen/ β_1 -dependent to CEP-modified collagen/ β_2 -dependent increased cell adhesion. Moreover, we demonstrated that M1 macrophage adhesion to CEP is integrin $\alpha_D\beta_2$ -dependent since it is dramatically reduced in α_D -deficient macrophages (**Figure 5B**). Notably, α_D -deficiency does not affect the expression of other major adhesive receptors on the macrophage surface (26). We verified this result on several systems including $\alpha_D\beta_2$ -transfected cells and isolated α_D I-domain. $\alpha_D\beta_2$ -transfected cells generated lower adhesion compared to M1 macrophages (**Figures 5B, 6B**) since the expression of $\alpha_D\beta_2$ on these cells is lower (**Figures 5A, 6A**). Nevertheless, these cells demonstrate a specific effect of integrin $\alpha_D\beta_2$ on cell adhesion. The results of the adhesion assay are confirmed by BioLayer Interferometry, which measures the direct protein-protein interaction between α_D I-domain and CEP or fibrinogen.

I-domain is a region of $\alpha_D\beta_2$ that contains most of the ligand-binding sites for β_2 integrins (43). We previously showed that binding sites for CEP and fibrinogen are located within the I-domain (23). In our earlier study regarding the ligand binding properties of integrin $\alpha_D\beta_2$, we compared α_D I-domain binding to all previously identified ligands including fibrinogen, fibronectin, ICAM-3, VCAM-1, plasminogen, vitronectin, and CYR61 using the surface plasmon resonance (Biacore 3000) (11). These studies demonstrated a K_D for α_D I-domain/fibrinogen binding 7.6×10^{-7} M. In another project we tested α_D I-domain binding to CEP by Biacore and calculated constant dissociation for this binding as K_D 1.8×10^{-7} M (23). However, we did not compare α_D I-domain binding to fibrinogen and CEP in the same experiment. Previously, the Biacore results for α_D binding to fibrinogen and to CEP were obtained from different α_D I-domain isolations, using different ligand densities on sensor chips and were performed several years apart (11, 23). Therefore, it was critical to analyze these interactions in one experiment to verify this result. Based on Biolayer Interferometry results, which were performed on one α_D I-domain isolation using the same I-domain concentrations, we found that the calculated affinity of α_D I-domain/CEP interaction (1.0×10^{-7} M) is 2 folds stronger compared to the α_D I-domain/fibrinogen binding (2.2×10^{-7} M). These results are slightly different from our previous Biacore results. We explain this difference by the difference in the experimental platforms between surface plasmon resonance (Biacore) and biolayer interferometry (Octet). Nevertheless, both methods provides a similar result and demonstrates the stronger binding of α_D I-domain to CEP compared to fibrinogen.

All α_D ligands analyzed by Biacore previously (11) showed a K_D for α_D I-domain binding in the range 0.2 – 8×10^{-6} M. The strongest binding was found for α_D I-domain interaction with the rare ECM

protein CYR61 (CCN1) with the K_D 2.9×10^{-7} M. This affinity is still weaker than α_D I-domain/CEP binding that we detected. Most importantly, the binding to “classical” $\alpha_D\beta_2$ ligands such as ICAM-3 (1.89×10^{-6} M), VCAM-1 (8.13×10^{-6} M), and fibrinogen (0.76×10^{-6} M) (11) are significantly less compared to CEP. These data together demonstrate that CEP modification generates preferential ligands for $\alpha_D\beta_2$ -mediated macrophage adhesion during inflammation. Therefore, inflammation and oxidation generate the improved conditions for $\alpha_D\beta_2$ -dependent macrophage adhesion.

The theory of mesenchymal cell migration postulates that optimal migration occurs with an intermediate level of receptor expression and substrate density, while the high density of receptors and corresponding substrates generate very strong adhesion, preventing cell motility (7, 26, 44–46).

Previously we found that non-activated macrophages demonstrated an improved migration in a CEP-supplemented matrix (23). Non-activated macrophages express an intermediate level of $\alpha_D\beta_2$ and $\alpha_M\beta_2$ integrins (26). We and others have shown experimentally that intermediate levels of integrin expression support cell migration (7, 26, 47, 48).

We propose that upregulation of $\alpha_D\beta_2$ on M1 macrophages and modification of ECM proteins with CEP promotes the conditions for the formation of an excessive number of receptor-ligand pairs with a high binding affinity. Accordingly, this high level of macrophage adhesion can prevent macrophage migration. In agreement with this concept, we found that migration of M1 macrophages in a 3D fibrin matrix supplemented with CEP is significantly reduced compared to migration in a pure fibrin matrix. Interestingly, we found that the morphology of M1 macrophages in a 3D matrix is affected by the presence of CEP (**Figure 4D**). Particularly, we revealed, that M1 macrophages in CEP supplemented matrix have a more rounded shape compared to macrophages in fibrin gel. It has been shown previously that migrated macrophages have an elongated shape and accordingly a greater length-to-diameter ratio (49, 50). The length-to-diameter ratio of macrophages in a CEP supplemented matrix is statistically significantly lower compared to control, which is in agreement with the reduced macrophage migration that we detected (**Figure 4A**).

To summarize, we propose a potential mechanism that affects macrophage adhesion and migration in inflamed tissue. Oxidation of DHA, which is a common structural component of cell membranes, leads to the formation of adducts between ECM proteins and CEP. Experimental data demonstrated that 20–30 modifications can occur in one protein molecule (23, 30, 32, 51). These modifications substitute a lysine-mediated positive charge for a CEP-mediated negative charge, which affects the conformation and ligand-binding properties of modified proteins. CEP is recognized by integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$. Accordingly, CEP modification can transform random proteins in integrin-related ligands. Freshly differentiated non-activated macrophages possess an intermediate level of $\alpha_M\beta_2$ and $\alpha_D\beta_2$ expression that supports CEP-mediated migration to the site of inflammation. Polarized pro-inflammatory (M1-like) macrophages express a high level of $\alpha_D\beta_2$, generating a high adhesion to CEP-modified proteins in the inflamed tissue that prevents M1 macrophage migration and leads to the development

of chronic inflammation. The high affinity between $\alpha_D\beta_2$ and CEP additionally intensifies the potential effect. This model proposes that targeting the binding between $\alpha_D\beta_2$ and CEP can provide benefits against macrophage retention in the inflamed tissue and the development of chronic inflammation during metabolic and autoimmune diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by East Tennessee State University IACUC.

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AUTHOR CONTRIBUTIONS

JC performed the experiments, analyzed the data, and edited the manuscript; KK, DG, CA, performed the experiments and analyzed the data; DW, EP, TB analyzed the data and edited the manuscript; VY designed the research, performed the experiments, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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