Inhibition of integrin $\alpha_{D}\beta_2$–mediated macrophage adhesion to end product of docosahexaenoic acid (DHA) oxidation prevents macrophage accumulation during inflammation

A critical step in the development of chronic inflammatory diseases is the accumulation of proinflammatory macrophages in the extracellular matrix (ECM) of peripheral tissues. The adhesion receptor integrin $\alpha_{D}\beta_2$ promotes the development of atherosclerosis and diabetes by supporting macrophage retention in inflamed tissue. We recently found that the end product of docosahexaenoic acid (DHA) oxidation, 2-((ω-carboxyethyl)-pyrrole (CEP), serves as a ligand for $\alpha_{D}\beta_2$. CEP adduct with ECM is generated during inflammation-mediated lipid peroxidation. The goal of this project was to identify a specific inhibitor for $\alpha_{D}\beta_2$–CEP interaction that can prevent macrophage accumulation. Using a specially designed peptide library, Biocore-detected protein–protein interaction, and adhesion of integrin-transfected HEK 293 cells, we identified a sequence (called P5 peptide) that significantly and specifically inhibited $\alpha_{D}$–CEP binding. In the model of thioglycollate-induced peritoneal inflammation, the injection of cyclic P5 peptide reduced 3-fold the macrophage accumulation in WT mice but had no effect in $\alpha_{D}$-deficient mice. The tracking of adoptively transferred, fluorescently labeled WT and $\alpha_{D}^{-/-}$ monocytes in the model of peritoneal inflammation and in vitro two-dimensional and three-dimensional migration assays demonstrated that P5 peptide does not affect monocyte transendothelial migration or macrophage eflux from the peritoneal cavity but regulates macrophage migration through the ECM. Moreover, the injection of P5 peptide into WT mice on a high-fat diet prevents macrophage accumulation in adipose tissue in an $\alpha_{D}\beta_2$-dependent manner. Taken together, these results demonstrate the importance of $\alpha_{D}\beta_2$-mediated macrophage adhesion for the accumulation of infiltrating macrophages in the inflamed ECM and propose P5 peptide as a potential inhibitor of atherogenesis and diabetes.

Chronic inflammation is an essential mechanism during the development of cardiovascular and metabolic diseases. Monocyte recruitment and subsequent macrophage accumulation in the damaged tissue are critical steps that regulate inflammatory response and disease progression (1, 2). Although monocyte recruitment during acute inflammatory response may have a protective effect, the excessive accumulation of macrophages at the site of inflammation can lead to strong proinflammatory signaling, damage to healthy tissue, and development of chronic inflammation (3). Leukocyte integrins are adhesive receptors that significantly contribute to the monocyte/macrophage migration and accumulation (4). Integrin $\alpha_{D}\beta_2$ (CD11d/CD18) is the most recently discovered leukocyte integrin (5) with a unique expression pattern and specific role in inflammation. Recently, we and others demonstrated that $\alpha_{D}\beta_2$ has a relatively low expression on neutrophils and monocytes in circulation (6, 7) but is up-regulated on tissue macrophages, particularly in atherosclerotic lesions and adipose tissue during diabetes (8–10) (Table S1). We revealed that high expression of $\alpha_{D}\beta_2$ on the cell surface promotes a strong adhesion to ECM proteins that leads to the retention of proinflammatory macrophages in inflamed tissue and supports atherogenesis and insulin resistance (11, 12).

Interestingly, $\alpha_{D}\beta_2$ shares a high level of homology and ligand binding properties with related integrin $\alpha_{M}\beta_2$ (CD11b/CD18; Mac-1) (13). $\alpha_{M}\beta_2$ is a well-studied leukocyte receptor, which is involved in the regulation of many acute and chronic inflammatory diseases (14–17). $\alpha_{M}\beta_2$ and $\alpha_{D}\beta_2$ shares many extracellular matrix ligands such as fibronectin, fibrinogen, and vitronectin; however, the expression of these integrins is markedly different on distinct subsets of macrophages (11). Particularly, $\alpha_{M}\beta_2$ has a low expression on resident and alternatively activated (M2) macrophages but is dramatically up-regulated on classically activated (M1) macrophages. $\alpha_{M}\beta_2$ demonstrates a high expression on resident macrophages but is expressed moderately on M1 and M2 macrophages. This difference determines the distinct role of $\alpha_{D}\beta_2$ and $\alpha_{M}\beta_2$ in macrophage...
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Figure 1. Screening the peptide library for binding to \( \alpha_\text{D} \) and \( \alpha_\text{M} \)-domains. The peptide library was synthesized on the cellulose membrane spanning the sequence of the \( \gamma \)-module of fibrinogen. The library was incubated with \(^{125}\text{I}\)-labeled \( \alpha_\text{D} \)-I-domain or \( \alpha_\text{M} \)-I-domain, and binding was visualized by autoradiography. The numbers on the left and above each panel indicate the peptide (spot) numbers. The peptide numbers correspond to the numbering of spots in the panel. Spot analysis indicated three peptides, called P3, P4, and P5, as unique sequences that bind to \( \alpha_\text{D}\beta_\text{2} \) (shown in red boxes).

Figure S1 illustrates the sequence of the \( \gamma \)-module of fibrinogen (Fig. S1). It has been shown that the \( \gamma \)-module of fibrinogen contains multiple binding sites for \( \alpha_\text{D}\beta_\text{2} \)-integrin and is critical for \( \alpha_\text{D}\beta_\text{2} \)-binding to fibrinogen (13). The peptide library consisting of 9-mer peptides with a 3-residue offset was tested for binding of \(^{125}\text{I}\)-labeled active \( \alpha_\text{D} \)-I-domain and \( \alpha_\text{M} \)-I-domains and have no cross-reactivity with \( \alpha_\text{M}\beta_\text{2} \)-binding, we synthesized a peptide library on the cellulose membrane spanning the sequence of the \( \gamma \)-module of fibrinogen (Fig. S1). We detected three sequences, which are specific only for the binding of \( \alpha_\text{D} \)-I-domain (spots 27–29, 49–51, and 67–70). The identified sequences, AGHLNVYQQGTYSKAS, TGTTEFWLGNEKHL, and GDAFDGFDGDPDS, were synthesized as soluble peptides and named P3, P4, and P5, correspondingly.

**Results**

Screening the peptide library for binding to \( \alpha_\text{D} \) and \( \alpha_\text{M} \)-domains

To identify the sequences that are unique for \( \alpha_\text{D}\beta_\text{2} \) binding and have no cross-reactivity with \( \alpha_\text{M}\beta_\text{2} \) binding, we synthesized a peptide library on the cellulose membrane spanning the sequence of the \( \gamma \)-module of fibrinogen (Fig. S1). It has been shown that the \( \gamma \)-module of fibrinogen contains multiple binding sites for \( \alpha_\text{D}\beta_\text{2} \)-integrin (25) and is critical for \( \alpha_\text{D}\beta_\text{2} \)-binding to fibrinogen (13). The peptide library consisting of 9-mer peptides with a 3-residue offset was tested for binding of \(^{125}\text{I}\)-labeled active \( \alpha_\text{D} \)-I-domain as described previously for \( \alpha_\text{M} \)-I-domain (25). We detected three sequences, which are specific only for the binding of \( \alpha_\text{D} \)-I-domain (spots 27–29, 49–51, and 67–70). The identified sequences, AGHLNVYQQGTYSKAS, TGTTEFWLGNEKHL, and GDAFDGFDGDPDS, were synthesized as soluble peptides and named P3, P4, and P5, correspondingly.

**Evaluation of inhibitory abilities of identified sequences by surface plasmon resonance and adhesion assay**

The abilities of detected peptides to inhibit \( \alpha_\text{D} \)-I-domain binding to CEP were tested by applying surface plasmon resonance (Biacore 3000) (Fig. 2, A and B). \( \alpha_\text{D} \)-I-domain and \( \alpha_\text{M} \)-I-domain were preincubated with 200 \( \mu \)g/ml P3 (106.8 \( \mu \)M), P4 (114.6 \( \mu \)M), and P5 (126.8 \( \mu \)M) peptides and added to the immobilized CEP using previously detected concentrations (19). Two peptides (P4 and P5) demonstrated marked inhibition of \( \alpha_\text{D} \)-I-domain binding, whereas inhibition of \( \alpha_\text{M} \)-I-domain was not significant. To extend this result, we tested peptides in the adhesion assay using \( \alpha_\text{D}\beta_\text{2} \)-transfected HEK 293 cells (Fig. 2C). CEP was immobilized on the 96-well plate, and integrin-transfected cell lines were preincubated with 200 g/ml antianti-inflammatory effects on leukocyte migration/retention and contribution to the development of inflammatory diseases (12). Particularly, recent data have demonstrated that \( \alpha_\text{M}\beta_\text{2} \) has a protective effect on the development of atherosclerosis and diabetes (16, 18), which is opposite to the pathological role of \( \alpha_\text{D}\beta_\text{2} \) in chronic inflammation.

Ligand recognition, followed by specific intracellular signaling, is a critical step that determines integrin-mediated leukocyte migration and cellular responses. Most recently, we found that the end product of DHA oxidation, 2-(\( \omega \)-carboxyethyl)pyrrole (CEP), serves as a specific inflammatory ligand for integrins \( \alpha_\text{D}\beta_\text{2} \) and \( \alpha_\text{M}\beta_\text{2} \) (19). CEP is formed during the oxidation of DHA that leads to the formation of CEP adds with ECM proteins (20, 21). These CEP-modified proteins support \( \alpha_\text{M}\beta_\text{2} \) and \( \alpha_\text{D}\beta_\text{2} \)-mediated macrophage migration to the site of inflammation. CEP is formed mostly during inflammation and was abundantly detected in atherosclerotic lesions and adipose tissue during diabetes (22, 23). Based on the \( \alpha_\text{D}\beta_\text{2} \)-specific pattern of expression on M1 macrophages, we hypothesized that CEP can be a critical ligand for \( \alpha_\text{D}\beta_\text{2} \)-mediated macrophage retention at the site of inflammation, particularly because the affinity of \( \alpha_\text{D} \) to CEP surpasses the affinity to natural ECM proteins (13, 19).

Therefore, the inhibition of \( \alpha_\text{D}\beta_\text{2} \)-mediated adhesion of macrophages to CEP-modified proteins in the ECM may have a strong anti-inflammatory effect. However, the overlapping ligand binding properties of \( \alpha_\text{M}\beta_\text{2} \) and \( \alpha_\text{D}\beta_\text{2} \) complicate the development of an effective inhibitor (13, 24).

In this project, we developed a strategy to identify the amino acid sequences that are specific only for integrin \( \alpha_\text{D}\beta_\text{2} \) and have no effect on \( \alpha_\text{M}\beta_\text{2} \). Using *in vitro* approaches, we selected the peptide, called P5, with strong blocking ability against \( \alpha_\text{D}\beta_\text{2} \)-CEP interaction. Applying the model of peritoneal inflammation, we demonstrated that P5 peptide significantly reduced the accumulation of macrophages in the peritoneal cavity, and this effect was directly related to the \( \alpha_\text{D}\beta_\text{2} \)-dependent migration via ECM. Moreover, P5 does not interfere with monocyte transmigration through endothelium or macrophage efflux from the peritoneal cavity. Finally, using the model of diet-induced diabetes, we demonstrated that P5 peptide markedly inhibits the accumulation of macrophages in the adipose tissue of mice, which demonstrates the effect of P5 on the development of chronic inflammation. Taken together, these data confirm the significant role of integrin \( \alpha_\text{D}\beta_\text{2} \) during an inflammatory response, support the concept of \( \alpha_\text{D}\beta_\text{2} \) as an important anti-inflammatory target, and propose the P5 sequence as a potential inhibitor of inflammation.

**Evaluation of inhibitory abilities of identified sequences by surface plasmon resonance and adhesion assay**

The abilities of detected peptides to inhibit \( \alpha_\text{D} \)-I-domain binding to CEP were tested by applying surface plasmon resonance (Biacore 3000) (Fig. 2, A and B). \( \alpha_\text{D} \)-I-domain and \( \alpha_\text{M} \)-I-domain were preincubated with 200 \( \mu \)g/ml P3 (106.8 \( \mu \)M), P4 (114.6 \( \mu \)M), and P5 (126.8 \( \mu \)M) peptides and added to the immobilized CEP using previously detected concentrations (19). Two peptides (P4 and P5) demonstrated marked inhibition of \( \alpha_\text{D} \)-I-domain binding, whereas inhibition of \( \alpha_\text{M} \)-I-domain was not significant. To extend this result, we tested peptides in the adhesion assay using \( \alpha_\text{D}\beta_\text{2} \)-transfected HEK 293 cells (Fig. 2C). CEP was immobilized on the 96-well plate, and integrin-transfected cell lines were preincubated with 200
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Figure 2. P5 peptide is a specific inhibitor for integrin $\alpha_D\beta_2$. A and B, representative profiles of the surface plasmon resonance measured by Biacore for $\alpha_D$ (A) and $\alpha_M$ (B) binding to CEP-BSA coupled to the CM5 chip in the presence of 200 $\mu$g/ml P3 (106.8 $\mu$M), P4 (114.6 $\mu$M), and P5 (126.8 $\mu$M) peptides. C and D, adhesion assay of $\alpha_D\beta_2$- and $\alpha_M\beta_2$-transfected HEK 293 cells in the presence of inhibitory peptides. C, a 96-well plate was coated with CEP for 3 h at 37°C. Calcein AM–labeled HEK 293 cells transfected with $\alpha_D\beta_2$ and $\alpha_M\beta_2$ were added to the wells, and cell adhesion was determined after 30 min in a fluorescence plate reader. Some samples were preincubated with P3, P4, or P5 peptide for 20 min before the adhesion assay. Data are presented as mean ± S.E. *, $p < 0.05$. D, adhesion of HEK 293 cells transfected with $\alpha_D\beta_2$ and $\alpha_M\beta_2$ to CEP in the presence of different concentrations of P5 peptide. Data are presented as mean ± S.E. *, $p < 0.05$. N/S, not significant.

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$\mu$g/ml peptides. Similar to direct protein–protein assay, P3 peptide did not have a blocking effect. However, the inhibitory ability of P4 peptide was reduced compared with direct protein–protein interaction assay (Fig. 2A). Apparently, the binding region for the P4 peptide is only exposed on isolated I-domain, but it is partially blocked on $\alpha_{12}\beta_2$ heterodimer, which is expressed on the cell surface. Therefore, P4-binding site is not a natural region for the $\alpha_{12}\beta_2$–CEP interaction. In contrast to these data, P5 peptide inhibited 50% of $\alpha_D\beta_2$ adhesion to CEP, which was similar to the Biacore results. The effect of P5 peptide on adhesion of $\alpha_M\beta_2$ cells was not significant. We tested different concentrations of P5 peptide in adhesion assays and found concentration-dependent inhibition of $\alpha_{12}\beta_2$ binding to CEP (Fig. 2D).

Integrins $\alpha_{12}\beta_2$ and $\alpha_{15}\beta_2$ are multiligand receptors (13, 24). It has been shown that several integrin ligands have overlapping binding sites within the I-domain (24, 27–29). Based on this information, we tested whether P5 peptide can inhibit $\alpha_{12}\beta_2$-mediated cell adhesion to other ligands. First, we evaluated the adhesion of $\alpha_{12}\beta_2$- and $\alpha_{15}\beta_2$-transfected HEK 293 cells to fibrinogen in the presence of P5 peptide. We found that P5 peptide blocked only the adhesion of $\alpha_{12}\beta_2$ (Fig. 2E) in a concentration-dependent manner (Fig. 2A). Because integrin $\alpha_{15}\beta_2$, which is also expressed on macrophages, has high homology with $\alpha_D$, and interacts with fibrinogen (Fg), we tested this receptor in an inhibition assay. The adhesion of $\alpha_{15}\beta_2$-transfected cells to immobilized fibrinogen was not affected in the presence of P5 peptide (Fig. S2B), which confirmed the specificity of selected inhibitor for integrin $\alpha_D$.

We also tested the ability of P5 to block the adhesion of $\alpha_D\beta_2$ and $\alpha_{15}\beta_2$ to another ligand, vitronectin, and received a similar result. Namely, P5 inhibits the adhesion of $\alpha_{12}\beta_2$-transfected cells but has no effect on adhesion of $\alpha_{15}\beta_2$-transfected cells (Fig. 2F). Taken together, these data demonstrated that P5 peptide can prevent the binding of $\alpha_{12}\beta_2$ to different ECM ligands without affecting the function of other macrophage integrins.

P5 peptide supports direct adhesion of $\alpha_D\beta_2$ cells and prevents receptor activation on the cell surface

The blocking peptide can bind directly to the binding site within $\alpha_D$ or may have an allosteric effect. To detect the mechanism of P5 inhibition, we tested the direct binding of $\alpha_D\beta_2$ to P5 peptide. Using immobilized P5 in an adhesion assay (Fig. 3A), we found that P5 peptide can support direct binding to $\alpha_D\beta_2$, whereas $\alpha_{15}\beta_2$ does not have this ability. The adhesion of both cell lines to Fg was used as a positive control (Fig. 3A).

The role of $\alpha_D$ I-domain conformation for the binding to P5 peptide was assessed using biolayer interferometry. Particularly, we tested the interaction of $\alpha_D$ I-domain in active and nonactive conformations to the biotinylated P5 peptide, which was immobilized on a streptavidin biosensor. We found that active form of $\alpha_D$ I-domain has a similar binding to P5 in the presence of 1 mM Mg$^{2+}$ and 5 mM EDTA. At the same time, a
nonactive conformation of \( \alpha_D \)-l-domain could not interact with P5 (Fig. 3B).

In a parallel experiment, we tested how binding of P5 peptide affected the change in the conformation of the entire \( \alpha_D \beta_2 \) heterodimer on the cell surface. Using activation-dependent antibody mAb24, we found that preincubation with P5 peptide significantly reduced \( \alpha_D \beta_2 \) activation (Fig. 3C). Therefore, the binding of P5 peptide does not require a fully active conformation of \( \alpha_D \beta_2 \) and can prevent a conformational change from the intermediate to the active stage. In agreement with our other data, P5 peptide did not have an effect on the activation of \( \alpha_N \beta_2 \) cells.

**Effect of P5 peptide on macrophage accumulation in the peritoneal cavities of WT, \( \alpha_D^{-/-} \), and \( \alpha_N^{-/-} \) mice**

The blocking effect of P5 peptide on \( \alpha_D \beta_2 \)-mediated cell adhesion might interfere with macrophage migration *in vivo*. We used the model of thioglycollate-induced peritoneal inflammation to evaluate changes in macrophage migration after P5 treatment. WT mice were injected intraperitoneally with P5 peptide or control peptide 30 min before the injection of thioglycollate, and the number of peritoneal macrophages was detected after 72 h. We selected a control peptide from the same \( \gamma \)-module sequence based on the absence of binding to \( \alpha_D \beta_2 \) and \( \alpha_N \beta_2 \), and presence of negatively and positively charged amino acids. Accordingly, the sequence (WNGRTSTADYAMFKV), which corresponds to spots 37–40, was synthetized and tested. The adhesion assay in the presence of the control peptide confirmed the lack of its effect on \( \alpha_D \beta_2 \)-mediated adhesion (Fig. 4A). The injection of cyclic P5 peptide to WT mice reduced 3-fold the accumulation of macrophages in the peritoneal cavity, whereas the treatment with the control peptide or PBS had no effect (Fig. 4B). Interestingly, the injection of P5 to \( \alpha_N \)-deficient mice demonstrated a reduction of macrophages in the peritoneal cavity similar to WT mice, whereas \( \alpha_D \) deficiency completely eliminated the blocking effect of P5 peptide (Fig. 4C). These results demonstrate the specificity of P5 peptide *in vivo*.

**Mechanism of P5 peptide inhibition during peritoneal inflammation**

The model of peritoneal inflammation is a well-described model of acute inflammation that is commonly used to evaluate monocyte/macrophage recruitment. Macrophage accumulation in the peritoneal cavity depends on several factors, including monocyte progenitor translocation to the blood stream, monocyte transmigration via the endothelium monolayer, macrophage migration through the interstitium to the peritoneal cavity, and efflux from the cavity to the lymphatics. We sought to detect the step of macrophage accumulation with which P5 peptide interferes.

To clarify this question, we developed several assays. First, we isolated monocyte progenitors from WT mice, labeled cells with PKH26 red fluorescent dye, and injected them intravenously to the mice with initiated peritoneal inflammation (Fig. 5A). One group of mice was treated with P5 peptide; the second was treated with the control. After 72 h, cells were isolated from the peritoneal cavity, and the number of red fluorescent, adoptively transferred macrophages was evaluated by FACS (Fig. 5B). We found that according to our previous observations (Fig. 4B) the total number of macrophages was significantly reduced after P5 treatment (Fig. 5C, left panel). More interestingly, the number of labeled macrophages was also significantly decreased (Fig. 5C, right panel). This result demonstrated the effect of P5 peptide on macrophage recruitment, but clearly P5 does not affect translocation from bone marrow because labeled cells were injected to the blood stream. Also, this result shows that the effect of P5 is mediated by monocyte-derived macrophages and is not related to the proliferation of resident macrophages.

Second, we tested the potential role of P5 in macrophage efflux from the peritoneal cavity (Fig. 6A). Macrophages were isolated at 72 h after thioglycollate injection and labeled with PKH26 fluorescent dye. The labeled macrophages were injected intraperitoneally to the mice at 48 h after thioglycollate-induced inflammation. One group was treated immediately to the mice at 48 h after thioglycollate-induced inflammation. One group was treated immediately to the mice at 48 h after thioglycollate-induced inflammation.
ately with P5 peptide; another group was treated with the control. After an additional 24 h, cells from the peritoneal cavity were collected, and the number of labeled macrophages was compared in both groups using FACS (Fig. 6B) and cytospin (Fig. S3). Again, the number of recipient macrophages was affected by P5 peptide (Fig. 6C). However, the amount of fluorescently labeled macrophages in the peritoneal cavity was not changed in the presence of P5 peptide, which demonstrates that P5 treatment did not affect efflux of macrophages during peritoneal inflammation (Fig. 6C).

Based on these experiments, we concluded that P5 interferes with the recruitment of monocytes/macrophages from the bloodstream to the peritoneal cavity. Therefore, the contribution of P5 peptide may affect endothelial transmigration or migration through the ECM.

**P5 peptide has no effect on 2D transendothelial migration but inhibits 3D migration in the matrix**

Accordingly, we tested the role of P5 in monocyte transmigration via endothelial monolayer *in vitro*. A Boyden chamber was coated overnight with human umbilical vein endothelial cells (HUVECs), which were labeled with green PKH67 fluorescent dye. Monocytes, labeled with red fluorescence (PKH26), were added to the upper chamber (Fig. 7A). Monocyte migra-
tion was stimulated with MCP-1 added to the lower chamber. One group of monocytes was pretreated with P5 peptide 20 min before the experiment. Transmigration was evaluated after 3 h by confocal microscopy and analyzed by IMARIS software (Fig. 7B). We did not detect an effect of P5 on transmigration that corresponds to the relatively low level of integrin α6β2 on the circulatory monocytes (Fig. 7C).

To test a contribution of P5 peptide to macrophage migration in the matrix, we used an in vitro 3D migration assay in fibrin gel (Fig. 8A). Thioglycollate-induced peritoneal macrophages were isolated from WT and α13−/− mice and labeled with green (PKH67) or red (PKH26) fluorescent dyes, respectively. An equal number of cells was loaded on one side of a 3D fibrin gel, and MCP-1 was added to the opposite side to stimulate the migration. One group of samples was pretreated with P5 peptide. P5 was also added to the fibrin matrix. Migration was evaluated after 48 h by confocal microscopy (Fig. 8, B and C). Preincubation with P5 peptide markedly reduced migration of nonpolarized macrophages. Therefore, this experiment confirmed that P5 peptide affects migration of macrophages through ECM during acute peritoneal inflammation.

Usually, further development of inflammation promotes polarization of macrophages to the proinflammatory M1 phenotype. We recently showed that expression of α6β2 is up-regulated on M1-polarized macrophages and α6β2’s high expression generates a strong adhesion followed by macrophage retention (11, 12). Therefore, we hypothesized that P5 peptide treatment may have the opposite effect on the migration of M1-activated macrophages. WT and α6β2−/− peritoneal macrophages were stimulated with interferon-γ (IFNγ) for 4 days and tested in a 3D migration assay in the fibrin matrix. As we have shown previously, M1-polarized WT macrophages demonstrate significantly lower migration compared with nonactivated macrophages; however, α6β2−/− M1 macrophages demon-
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Figure 8. 3D migration of macrophages was regulated by P5 peptide. A, schematic representation of the experiment. Labeled cells were mixed in equal amounts and added to the Transwell. Before the initiation of migration, the background was verified by scanning samples with a confocal microscope. The migration was stimulated by adding 30 nM MCP-1 to the opposite side of the fibrin gel. B, after 48 h, the migration was evaluated using a Leica confocal microscope. A 3D view of the migrating cells in fibrin matrix is shown. C and D, side view of the migration of nonactivated (C) and M1-activated (D) macrophages. The results were analyzed and reconstructed by IMARIS 8.0 software. Statistical analyses were performed using Student's paired t tests (n = 4 per group). Scale bars, 500 μm. Data are presented as mean ± S.E. (error bars). *, p < 0.05. Con, control.

strate enhanced migration compared with WT (Fig. 8D, left panel). Accordingly, the addition of P5 peptide improved migratory properties of WT M1 macrophages (Fig. 8D, right panel). Apparently, P5-mediated inhibition of α\(_{\text{D}}\)β\(_{2}\) adhesion induces macrophage migration. Notably, the migration of α\(_{\text{D}}\)−/− macrophages is not significantly changed after P5 peptide treatment, which is in agreement with our previous observations (Fig. 4C). Based on these results, we can predict that the effect of P5 peptide on the development of chronic inflammation would be more complex and would include inhibition of macrophage migration to the site of inflammation and inhibition of macrophage retention at the site of inflammation.

Inhibition of macrophage accumulation in the adipose tissue of diabetic mice by P5 peptide

To test P5 effects on chronic inflammation, we analyzed an accumulation of macrophages in adipose tissue of prediabetic mice. Mice after 8 weeks on a high-fat diet were injected with fluorescently labeled WT (PKH26 red) and α\(_{\text{D}}\)-labeled (PKH67 green) monocytes. One group was injected with cyclic P5 peptide; another group was injected with the control. After 48 h, the number of red- and green-labeled macrophages in the adipose tissue was evaluated using classical FACS (Fig. 9A) and imaging flow cytometry (Fig. 9B). We have previously shown that α\(_{\text{D}}\) deficiency reduced macrophage accumulation in the adipose tissue. Now, we demonstrate that P5 peptide possesses a similar effect on WT macrophages. The accumulation of P5-treated WT macrophages was reduced by 2.5-fold. Interestingly, the migration of α\(_{\text{D}}\)−/− macrophages was not affected. Specifically, the ratio of WT to α\(_{\text{D}}\)−/− macrophages in adipose tissue of control mice was 3-fold, whereas this ratio was reduced to 1 after P5 peptide treatment (Fig. 9, C and D).

Discussion

Our previous results demonstrated that modification of ECM proteins with the product of DHA oxidation, CEP, generates new inflammation-specific substrates in the tissue (19). We found that CEP is a ligand for α\(_{\text{D}}\)β\(_{2}\) and α\(_{\text{M}}\)β\(_{2}\)-mediated macrophage adhesion and migration (19). Importantly, we and others detected CEP-modified proteins in different inflamed tissues such as atherosclerotic lesions, pathological angiogenesis, adipose tissue during diabetes, and peritoneal tissue during sterile inflammation (19, 22, 30, 31). Our other recent results demonstrated that the up-regulation of integrin α\(_{\text{D}}\)β\(_{2}\) at the site of inflammation promotes strong adhesion of macrophages to the substrate, related macrophage retention, and disease progression (11).

The proposed study was designed to develop an inhibitor of α\(_{\text{D}}\)β\(_{2}\)-mediated adhesion of macrophages to the inflamed ECM, focusing on CEP as an inflammation-specific ligand. Because α\(_{\text{M}}\)β\(_{2}\) and α\(_{\text{D}}\)β\(_{2}\) have different, rather opposite roles during chronic inflammation (11, 12, 16, 18), our goal was to identify an inhibitor that will work specifically only with integrin α\(_{\text{D}}\)β\(_{2}\). The lack of commercially available monoclonal antibodies against α\(_{\text{D}}\)β\(_{2}\) as well as a focus on specific α\(_{\text{D}}\)β\(_{2}\) ligand led us to search for a peptide-based inhibitor. Based on different affinities between CEP–α\(_{\text{D}}\) I-domain (\(K_{\text{D}}\) 1.81 × 10\(^{-7}\)) and CEP–α\(_{\text{M}}\) I-domain (\(K_{\text{D}}\) 2.1 × 10\(^{-6}\)) (19), we hypothesized that ligand-
Figure 9. P5 peptide inhibited accumulation of macrophages in adipose tissue of mice during diet-induced diabetes. Isolated WT and α5β2−/− bone marrow monocytes were labeled with red PKH26 (WT) or green PKH67 (α5β2−/−) fluorescent dyes, respectively; mixed in an equal amount; and injected into the tail veins of WT mice fed a high-fat diet (45% kcal from fat) for 8 weeks. Experimental groups were intraperitoneally injected with 100 μg/mouse P5 (63.45 μM) peptide 20 min before the injection of labeled cells. After 3 days, visceral adipose tissue was isolated, digested, and analyzed using flow cytometry. A, Q1 and Q4 quadrants represent the labeled cells in digested adipose tissue. B, Imaging flow cytometry. The upper panels represent the injected monocytes, isolated from WT and α5β2−/− mice, labeled with red and green fluorescent dyes, respectively. The lower panels represent macrophages isolated from adipose tissue. The population of single, alive cells was analyzed on red and green channels. Channel 1 (Ch01), brightfield (BF); channel 2 (Ch02), 488-nm wavelength (PKH67); channel 3 (Ch03), 566-nm wavelength (PKH26); channel 6 (Ch06), side scattering (SSC); channel 11 (Ch11), F4/80 represents macrophage staining. C, macrophage number was calculated based on flow cytometry data and presented as mean ± S.E. (error bars), *, p < 0.05. D, the ratio of WT and α5β2−/− macrophages in each mouse was calculated and presented as mean ± S.E. (error bars), *, p < 0.05. Con, control; PE, phycocerythrin.
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Mouse and human integrins $\alpha_D$ have a high level of homology (identity, 71%). CEP formation is similar in human and mouse tissues (35). P5 peptide inhibited the binding of CEP to human $\alpha_D$-I-domain, human $\alpha_D\beta_2$-transfected HEK 293 cells, and mouse macrophages in vitro and in vivo. Therefore, P5 peptide represents the common inhibitor for human and mouse systems.

To evaluate the effect of P5 peptide in vivo, a mouse peritoneal model of inflammation was applied. Thioglycollate-induced peritoneal inflammation represents a sterile acute inflammation. In contrast to chronic inflammatory diseases, the expression of integrin $\alpha_D\beta_2$ on peritoneal macrophages is intermediate (10). However, this model is commonly used to study the mechanism of neutrophil and macrophage migration and provided important information regarding the effect of P5 peptide inhibition in vivo. Macrophage accumulation in the peritoneal cavity at 72 h after injection of sterile thioglycollate allows tracking monocyte recruitment and macrophage efflux during inflammation (36–38). We demonstrated the specificity of P5 peptide–mediated inhibition because P5 peptide significantly blocked accumulation of WT and $\alpha_M^{-/ -}$ macrophages but had no effect on the accumulation of $\alpha_D$-deficient macrophages in the peritoneal cavity (Fig. 4).

The monocyte/macrophage recruitment to and efflux from the peritoneal cavity is a complex process that can be divided into several stages: translocation of monocytes from bone marrow/spleen, monocyte transmigration through the endothelium, migration via ECM, and efflux from the cavity to lymphatics. Because each step is regulated by leukocyte integrins, we tested a potential role of P5 in these processes. Using adoptively transferred macrophages, we found that P5 peptide has no effect on macrophage efflux from the peritoneal cavity (Fig. 6). This corresponds to the previous results that macrophage efflux is regulated by integrin $\alpha_D\beta_2$ (37, 38) and $\alpha_M\beta_2$ (36). In contrast, the injection of fluorescently labeled monocytes to the bloodstream in the presence of P5 peptide significantly reduced the accumulation of labeled macrophages in the peritoneal cavity (Fig. 5). This result demonstrated that P5 peptide inhibits monocyte endothelial transmigration and/or migration via ECM (peritoneal wall) toward the cavity. Also, this result indicated that the P5 effect is not related to monocyte translocation from the bone marrow. These data are in agreement with the fact that $\alpha_D\beta_2$ has a low expression on monocyte progenitors that reduces the potential contribution of $\alpha_D\beta_2$ to this step (39).

To further determine the role of P5 peptide in the recruitment, we tested P5 in monocyte transmigration (Fig. 7) and migration through the extracellular matrix in vitro (Fig. 8). We did not detect a difference in monocyte transmigration via the endothelial monolayer in the presence of P5 peptide. This corresponds to the previous results that monocyte diapedesis depends on integrins $\alpha_D\beta_2$, $\alpha_M\beta_1$, and to some extent $\alpha_M\beta_2$ (40–42). It also in agreement with our previous data that $\alpha_D$ deficiency does not change transmigration of monocytes during atherogenesis (11).

In contrast to these data, P5 had a strong effect on the migration of WT macrophages in a 3D matrix. Macrophages can apply a mesenchymal (adhesion-dependent) or amoeboid (adhesion-independent) migration mode in the 3D environment (43–47). We recently found that integrin $\alpha_D\beta_2$ can regulate mesenchymal migration (12), and the density of $\alpha_D\beta_2$ on macrophage surfaces is important for this outcome. The interplay between integrin density and cell migration is based on the theory of cell migration, which postulates that intermediate adhesion supports migration, whereas very strong adhesion will inhibit cell locomotion (48, 49). In our current experiments, we used nonactivated peritoneal macrophages, which have a moderate level of $\alpha_D\beta_2$ expression (10). Clearly, $\alpha_D$ deficiency reduced migration of nonactivated macrophages (Fig. 8C, left panel [$\alpha_D^{-/ -}$ green fluorescence versus WT red fluorescence]), which confirmed a supportive role of $\alpha_D\beta_2$ in migration. Accordingly, P5 peptide reduced the migration of WT nonactivated macrophages (Fig. 8C, right panel) but did not have an effect on migration of $\alpha_D$-deficient macrophages.

In our previous project, we found that high expression of $\alpha_D\beta_2$ on M1 macrophages serves to inhibit cell migration due to strong adhesion (11, 12). We verified this result by demonstrating a reduced migration of $\alpha_D$-deficient M1-activated macrophages (green fluorescence) (Fig. 8D, left panel). Accordingly, the migration of WT M1-activated macrophages in the presence of P5 peptide was improved because $\alpha_D\beta_2$-mediated adhesion was reduced (Fig. 8D, right panel). The migration of $\alpha_D$-deficient macrophages (green fluorescence) surpassed WT (red fluorescence) in the control sample but had a similar level after P5 treatment (Fig. 8C, C and D). These data demonstrate that P5 peptide may differently affect macrophage migration depending on the subset of macrophages and level of $\alpha_D\beta_2$ expression on the surface. The obtained result is in agreement with our previous data that integrin $\alpha_D\beta_2$ has a different role during migration depending on receptor density on the cell surface (10, 12). The intermediate expression of $\alpha_D\beta_2$ during acute inflammation supports macrophage migration to the site of inflammation (50), whereas up-regulation of $\alpha_D\beta_2$ on proinflammatory macrophages promotes macrophage retention at the site of chronic inflammation.

To further confirm that P5 may affect macrophage accumulation during chronic inflammation, we applied the model of diet-induced insulin resistance. Recently, we demonstrated that $\alpha_D$ deficiency improved glucose tolerance and reduced insulin resistance in C57BL6 mice (12). Using adoptive transfer of fluorescently labeled WT and $\alpha_D^{-/-}$ monocytes, we demonstrated that the ratio of WT to $\alpha_D^{-/-}$ macrophages in the adipose tissue was reduced after P5 peptide treatment (Fig. 9). Macrophage accumulation in adipose tissue is a critical marker of inflammation and development of diabetes. This result confirmed the important role of integrin $\alpha_D\beta_2$ in the development of inflammation and proposes P5 peptide as a potential tool for the development of an anti-inflammatory treatment that can prevent macrophage accumulation and the development of different inflammatory diseases, particularly type 2 diabetes.

Materials and methods

Reagents

Reagents were purchased from Sigma-Aldrich and Thermo Fisher Scientific (Waltham, MA). Human fibrinogen and thrombin were obtained from Enzyme Research Laboratories.
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(South Bend, IN). The synthesis of peptides (P-con, WNGRT-STADYAMFKV; P3, ACHLGNVYQGGTYSKAS; P4, TGT-TEFWLGNEKIHL; P5, GDAFGDFGDPPDS; and cyclic P5) was carried out by Peptide 2.0 Inc. (Chantilly, VA). The cyclization was done by amide cyclization from N to C terminus. The schematic sequence of cyclic peptide is shown in Fig. S1. Recombinant mouse IFNγ was purchased from Thermo Fisher Scientific. Phorbol 12-myristate 13-acetate was purchased from Sigma. Recombinant murine IFNγ M1-1 (CCL2) was purchased from PeproTech (Rocky Hill, NJ). Anti-human α<sub>5</sub>, mAb (clone 240I) was generously provided by Eli Lilly Corp. Mouse FITC- and allophycocyanin-conjugated anti-α<sub>k5</sub>mAb (clone M1/70) and F4/80 mAbs were from eBioscience (San Diego, CA). The conformation-dependent antibody mAb24 against β<sub>2</sub> integrin was from Hy cultura Biotechnology (The Netherlands). mAb44a directed against the human α<sub>M</sub> integrin subunit was purified from the conditioned media of the hybridoma cell line obtained from American Type Culture Collection (ATCC, Manassas, VA) using protein A-agarose (GE Healthcare). PKH26 (red) and PKH67 (green) fluorescent dyes were purchased from Sigma.

Animals

WT (C57BL/6), integrin α<sub>5</sub>-deficient (B6.129S7-Itgad<sup>tm1Bll</sup>/J), and integrin α<sub>k5</sub>-deficient (B6.129S4-Itgam<sup>tm1Mvyd</sup>/J) mice were bought from The Jackson Laboratory (Bar Harbor, ME). α<sub>5</sub>-deficient and α<sub>k5</sub>-deficient mice were backcrossed to C57BL/6 for at least 10 generations. To develop insulin-resistant mice, C57BL/6 WT mice were fed a high-fat diet with 45% kcal from fat (TD08811, Envigo) for 8 weeks. All procedures were performed according to animal protocols approved by the East Tennessee State University Institutional Animal Care and Use Committee.

Expression and isolation of recombinant α<sub>5</sub> and α<sub>M</sub> I-domains in active and nonactive conformations

The constructs for α<sub>5</sub>, I-domain and α<sub>M</sub> I-domains were generated, and recombinant proteins were isolated as described in our previous studies (13, 19). Briefly, α<sub>5</sub> in the nonactive conformation (Pro<sup>128</sup>–Ala<sup>122</sup>) and α<sub>M</sub> in the active conformation (Glu<sup>123</sup>–Lys<sup>315</sup>) were inserted into pGEX4T-1 vector. In “active” α<sub>M</sub> I-domains, the unpaired Cys<sup>128</sup> was substituted to Ser to prevent I-domain dimerization. Proteins were expressed in Escherichia coli and purified using affinity chromatography on GSH-agarose, and its fusion part was removed by thrombin. α<sub>5</sub> in the active conformation (Pro<sup>128</sup>–Lys<sup>314</sup>) was inserted in 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).

Analyses of the α<sub>5</sub> I-domain binding to CEP, Fg, and P5 peptide by surface plasmon resonance and biolayer interferometry

The interaction between I-domains and CEP or fibrinogen in the presence of P3, P4, and P5 peptides was measured using surface plasmon resonance (Biacore 3000 instrument, Biacore, Uppsala, Sweden) as described previously (13, 22). Fibrinogen and CEP conjugated to albumin were immobilized on the CM5 biosensor chip using standard amine coupling chemistry (1000 response units/flow cell). Steady-state experiments were performed at room temperature in 10 mM HEPES (pH 7.4) buffer containing 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 0.005% surfactant P20 at a flow rate of 20 μl/min. Surface plasmon resonance sensograms were obtained by injecting various concentrations of α<sub>5</sub> and α<sub>M</sub> I-domains. In some samples, analytes were preincubated with blocking peptides for 15 min at room temperature. All data were corrected for the response obtained using a blank reference flow cell that was activated with N-ethyl-N”-(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide and then blocked with ethanolamine. Non-specific binding to the blank flow cell was subtracted. The chip surfaces were regenerated by injecting a short pulse of 25 mM NaOH. The resulting sensograms were analyzed in overlay plots using BLAevaluation software (version 4.01, GE Healthcare).

The interaction between the α<sub>5</sub>, I-domain (in active and non-active conformations) and P5 peptide was measured using biolayer interferometry (ForteBio, Fremont, CA). N-terminally biotinylated P5 peptide was immobilized on a streptavidin biosensor. Different concentrations of the I-domains in 20 mM HEPES (pH 7.4) buffer containing 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 0.05% Tween 20 were added to immobilized P5 peptide. For some experiments, Mg<sup>2+</sup> and Ca<sup>2+</sup> were exchanged for 5 mM EDTA. All data were corrected for the response obtained using a blank reference biosensor. The biosensor surface was regenerated using 2 mM NaCl and 25 mM NaOH. Data were analyzed using the ForteBio Data Analysis 11.0 program (ForteBio).

Synthesis of cellulose-bound peptide library

The fibrinogen γ-module–derived peptide library assembled on a single cellulose membrane support was prepared by parallel spot synthesis as described previously (25, 26). The libraries were synthesized as 9-mer overlapping peptides with a 3-amino-acid offset. Peptides were C-terminally attached to the cellulose via a (β-Ala)<sub>2</sub> spacer and were acetylated N-terminally. The membrane-bound peptides were tested for their ability to bind the α<sub>5</sub> I-domain and α<sub>M</sub> I-domain. In brief, membranes were blocked with 1% BSA and incubated with 5 μg/ml [125]I-labeled α<sub>M</sub> I-domain or α<sub>M</sub> I-domain in 20 mM Tris buffer solution containing 1 mM MgCl<sub>2</sub>, 0.1% BSA, and 2 mM DTT. Membranes were washed with Tris buffer solution containing 0.05% Tween 20 and dried, and α<sub>M</sub> and α<sub>M</sub> I-domain binding was visualized by autoradiography and analyzed by densitometry.

Flow cytometry analysis

Flow cytometry analysis was performed to assess the expression and activation of receptors on the surface of cells transfected with α<sub>5</sub>β<sub>2</sub>, α<sub>M</sub>β<sub>2</sub>, and α<sub>5</sub>β<sub>2</sub> integrins and to evaluate the number of fluoroscopically labeled mouse macrophages isolated from the peritoneal cavity or adipose tissue. Transfected HEK 293 cells were incubated with anti-α<sub>5</sub> (clone 240I), anti-α<sub>M</sub> (clone M1/70), and anti-β<sub>2</sub> (clone IB4) antibodies and analyzed using Fortessa X-20 (BD Biosciences) as described (13, 24). The isolated prelabeled WT (red PKH26) bone marrow–derived macrophages, peritoneal macrophages, or adipose tissue macrophages (WT, red; α<sub>5</sub>−/−, green) were washed with PBS,
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counted, and analyzed by flow cytometry (Fortessa X-20) and imaging flow cytometry (ImageStream Mark II, Amnis). Macrophage numbers were calculated based on the percentage of F4/80-positive population in flow cytometry.

Cell adhesion assay

The adhesion assay was performed as described previously with modifications (13, 24). Briefly, 96-well plates (Immulon 2HB, Cambridge, MA) were coated with fibrinogen, CEP, P5, or vitronectin for 3 h at 37 °C. The wells were postcoated with 0.5% polyvinyl alcohol for 1 h at 37 °C. HEK 293 cells transfected with $\alpha_{M}\beta_{2}$, $\alpha_{x}\beta_{2}$, or $\alpha_{D}\beta_{2}$ integrins were labeled with 10 μM Calcein AM (Molecular Probes, Eugene, OR) for 20 min at 37 °C, washed with Dulbecco’s modified Eagle’s medium, and resuspended in the same medium at a concentration of 1 × 10^6 cells/ml. Aliquots (50 μl) of the labeled cells were added to each well. For inhibition experiments, cells were mixed with various concentration of peptides (P3, P4, and P5) and incubated for 20 min at 37 °C before they were added to the ligand-coated wells. After 30 min of adhesion at 37 °C in a 5% CO2 humidified atmosphere, the nonadherent cells were removed by washing with Hanks’ balanced salt solution. 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.

Isolation of peritoneal macrophages and activation of M1 macrophages

WT and $\alpha_{D}^{-/-}$ 8–10-week-old mice were intraperitoneally injected with 1 ml of 4% thioglycollate, and 3 days later, peritoneal cells were harvested with 5 ml of sterile PBS by lavage of the peritoneal cavity. The cells were washed with PBS and resuspended in RPMI 1640 medium. The cell suspension was transferred into 100-mm Petri dishes and incubated for 2 h at 37 °C in humidified air containing 5% CO2 atmosphere. Nonadherent cells were washed out with RPMI 1640 medium, and the adherent macrophages were replenished with RPMI 1640 medium. The macrophages were differentiated to M1 phenotype by treatment with recombinant mouse IFNγ (100 units/ml) for 4 days. Medium with IFNγ was changed every 2 days or as required. The M1-phenotype macrophages from WT and $\alpha_{D}^{-/-}$ mice were labeled with red fluorescent marker PKH26 and green fluorescent marker PKH67, respectively, according to the manufacturer’s instructions (Sigma-Aldrich). The fluorescently labeled cells were dissociated from the plates using 5 mM EDTA in PBS and used for the experiments thereafter.

Adoptive transfer in the recruitment of macrophages to the peritoneal cavity

The approach is based on our previous publication with some modifications (11). Bone-marrow monocytes were isolated from WT mice using a magnetic bead separation kit (Miltenyi Biotec, Gaithersburg, MD). Monocytes were labeled with red PKH26 (WT) fluorescent dye. Recipient WT mice were intraperitoneally injected with 100 μg/mouse P5 (63.4 μM) peptide. After 20 min, 1 ml of 4% thioglycollate was intraperitoneally injected to all mice to induce inflammation. Then fluorescently labeled WT (red PKH26 dye) bone marrow monocytes were injected into the tail veins of the recipient mice. After 72 h, the peritoneal macrophages were harvested and assessed by fluorescence microscopy and flow cytometry (Fortessa X-20).

Adoptive transfer in macrophage efflux from the peritoneal cavity

The adoptive transfer was performed as described previously with some modifications (11). Briefly, recipient and donor WT mice were intraperitoneally injected with 4% thioglycollate. After 48 h, macrophages were isolated from the peritoneal cavity of donor mice, labeled with PKH26 red fluorescent dye, and injected into the peritoneal cavity of the recipient mice (1 × 10^6 cells/mouse). Immediately, the recipient mice were intraperitoneally injected with 100 μg/mouse P5 (63.4 μM) peptide or control. After an additional 24 h, macrophages were harvested from the peritoneal cavity and counted, and the number of fluorescently labeled macrophages was assessed by fluorescence microscopy and flow cytometry (Fortessa X-20).

Adoptive transfer in the model of diet-induced diabetes

The adoptive transfer was performed as described previously (12). Briefly, WT and $\alpha_{D}^{-/-}$ bone marrow monocytes were isolated and purified by a magnetic bead separation kit (Miltenyi Biotec); labeled with red PKH26 (WT) or green PKH67 ($\alpha_{D}^{-/-}$) fluorescent dye, respectively; mixed in an equal amount (1 × 10^6 cells/color/mouse); and injected into the tail veins of WT mice fed a high-fat diet (45% kcal from fat) for 8 weeks. Mice in the experimental group were intraperitoneally injected with 100 μg/mouse P5 (63.4 μM) peptide 20 min before the injection of labeled cells. After 3 days, visceral adipose tissue was isolated, digested, and analyzed using flow cytometry (Fortessa X-20) and imaging flow cytometry (ImageStream Mark II).

Transendothelial migration assay

HUVECs were seeded at 10^5 cells/well in the upper chamber of Transwell inserts (diameter, 6.5 mm; pore size, 5.0 μm; Corning), labeled with PKH67 green fluorescence, and cultured overnight in vascular cell basal medium with vascular endothelial growth factor (ATCC). On the next day, isolated bone marrow monocytes were labeled with PKH26 red fluorescent dye and added to the top of endothelial cells, and MCP-1 was added to the lower chamber of the wells to stimulate the migration of monocytes. In some experiments, the monocytes were preincubated with 200 μg/ml P5 (126.8 μM) peptide for 20 min. After 3-h incubation at 37 °C, the number of migrated cells was determined by a Leica confocal microscope, and the results were reconstructed and analyzed using IMARIS 8.0 software.

Migration of macrophages in 3D fibrin gel

The migration assay was performed as described previously (12, 19). WT and $\alpha_{D}^{-/-}$ peritoneal nonactivated macrophages were labeled with PKH26 red fluorescent dye and PKH67 green fluorescent dye, respectively. The cell migration assay was performed for 48 h at 37 °C in 5% CO2 in a sterile condition. An equal number of WT and $\alpha_{D}^{-/-}$ macrophages was evaluated by cytospin of mixed cells before the experiment and at the start-
ing point before migration. Labeled WT (1.5 × 10^5) and α5β1^−/− (1.5 × 10^5) activated macrophages were plated on the membranes of Transwell inserts with a pore size of 8 μm and 6.5 mm in diameter (Costar, Corning) precoated with Fg. Fibrin gel (100 μl/sample) was generated by mixing 0.75 mg/ml Fg containing 1% fetal bovine serum, 1% penicillin/streptomycin, and 0.5 unit/ml thrombin. 30 nm MCP-1 was added on the top of the gel to initiate the migration. Migrating cells were detected by a Leica TCS SP8 confocal microscope, and the results were analyzed and reconstructed using IMARIS 8.0 software.

**Statistical analysis**

Statistical analyses were performed using 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.

**References**

1. Alexandraki, K., Piperi, C., Kalofoutis, C., Singh, J., Alaveras, A., and Kalofoutis, A. (2006) Inflammatory process in type 2 diabetes: the role of cytokines. *Ann. N.Y. Acad. Sci.* 1084, 89–117 CrossRef Medline
2. Ouchi, N., Kihara, S., Funahashi, T., Matsuzawa, Y., and Walsh, K. (2003) Obesity, adiponectin and vascular inflammatory disease. *Curr. Opin. Lipidol.* 14, 561–566 CrossRef Medline
3. Subramanian, S., and Chait, A. (2009) The effect of dietary cholesterol on macrophage accumulation in adipose tissue: implications for systemic inflammation and atherosclerosis. *Curr. Opin. Lipidol.* 20, 39–44 CrossRef Medline
4. Schittenhelm, L., Hilken, C. M., and Morrison, V. L. (2017) β2 integrins as regulators of dendritic cell, monocyte, and macrophage function. *Front. Immunol.* 8, 1866 CrossRef Medline
5. Danilenko, D. M., Rossitto, P. V., Van der Vieren, M., Le Trong, H., McDonough, S. P., Afferter, V. K., and Moore, P. F. (1995) A novel canine leukointegrin, αβ2δ, is expressed by specific macrophage subpopulations in tissue and a minor CD8+ lymphocyte subpopulation in peripheral blood. *J. Immunol.* 155, 34–44 Medline
6. Grayson, M. H., Van der Vieren, M., Serbinski, S. A., Michael Gallatin, W., Hoffman, P. A., Staunton, D. E., and Bochner, B. S. (1998) αδβ2 integrin is expressed on human eosinophils and functions as an alternative ligand for vascular cell adhesion molecule 1 (VCAM-1). *J. Exp. Med.* 188, 2187–2191 CrossRef Medline
7. Miyazaki, Y., Vieira-de-Abreu, A., Harris, E. S., Shah, A. M., Weyrich, A. S., Castro-Faria-Neto, H. C., and Zimmerman, G. A. (2014) Integrin αδβ2 (CD11d/CD18) is expressed by human circulating and tissue myeloid leukocytes and mediates inflammatory signaling. *PLoS One* 9, e112770 CrossRef Medline
8. Thomas, A. P., Dunn, T. N., Oort, P. J., Grino, M., and Adams, S. H. (2011) Inflammatory phenotyping identifies CD11d as a gene markedly induced in white adipose tissue in obese rodents and women. *J. Nutr.* 141, 1172–1180 CrossRef Medline
9. Van der Vieren, M., Le Trong, H., Wood, C. L., Moore, P. F., St John, T., Staunton, D. E., and Gallatin, W. M. (1995) A novel leukointegrin, α5β1, binds preferentially to ICAM-3. *Immunity* 3, 683–690 CrossRef Medline
10. Yakubenko, V. P., Belevych, N., Mishchuk, D., Schurin, A., Lam, S. C., and Ugarova, T. P. (2008) The role of integrin αδβ2 (CD11d/CD18) in monocye/macrophage migration. *Exp. Cell Res.* 314, 2569–2578 CrossRef Medline
11. Aziz, M. H., Cui, K., Das, M., Brown, K. E., Ardell, C. L., Febbraio, M., Pluskota, E., Han, J., Wu, H., Ballantyne, C. M., Smith, J. D., Cathcart, M. K., and Yakubenko, V. P. (2017) The upregulation of integrin αδβ2 (CD11d/CD18) on inflammatory macrophages promotes macrophage re-tention in vascular lesions and development of atherosclerosis. *J. Immunol.* 198, 4855–4867 CrossRef Medline
12. Cui, K., Ardell, C. L., Podolnikova, N. P., and Yakubenko, V. P. (2018) Distinct migratory properties of M1, M2, and resident macrophages are regulated by αδβ2 and αδβ2 integrin-mediated adhesion. *Front. Immunol.* 9, 2650 CrossRef Medline
13. Yakubenko, V. P., Yadav, S. P., and Ugarova, T. P. (2006) Integrin αδβ2, an adhesion receptor up-regulated on macrophage foam cells, exhibits multiligand-binding properties. *Blood* 107, 1643–1650 CrossRef Medline
14. Wang, Y., Gao, H., Shi, C., Erhardt, P. W., Pavlovsky, A., Soloviev, D. A., Blełdzka, K., Ustinov, V., Zhu, L., Qin, J., Munday, A. D., Lopez, J., Plow, E., and Simon, D. I. (2017) Leukocyte integrin Mac-1 regulates thrombosis via interaction with platelet GPIba. *Nat. Commun.* 8, 15559 CrossRef Medline
15. Yakubenko, V. P., Bhattacharjee, A., Pluskota, E., and Cathcart, M. K. (2011) αδβ2 integrin activation prevents alternative activation of human and murine macrophages and impedes foam cell formation. *Circ. Res.* 108, 544–554 CrossRef Medline
16. Szpak, D., Izem, L., Verbovetskiy, D., Soloviev, D. A., Yakubenko, V. P., and Pluskota, E. (2018) αδβ2 is antiatherogenic in female but not male mice. *J. Immunol.* 200, 2426–2434 CrossRef Medline
17. Wolf, D., Anto-Michel, N., Blankenbach, H., Wiedemann, A., Busker, K., Hohmann, J. D., Lim, B., Bäuml, M., Marki, A., Maurer, M., Duerschmid, D., Fan, Z., Winkels, H., Sidler, D., Diehl, P., et al. (2018) A lipid-specific blockade of the integrin Mac-1 selectively targets pathologic inflammation while maintaining protective host-defense. *Nat. Commun.* 9, 525 CrossRef Medline
18. Wolf, D., Bukosza, N., Engel, D., Poggi, M., Jehle, F., Anto Michel, N., Chen, Y. C., Colberg, C., Hoppe, N., Dufner, B., Boon, L., Blankenbach, H., Hilgendorf, L., von Zur Muhlen, C., Reinöhl, J., et al. (2017) Inflammation, but not recruitment, of adipose tissue macrophages requires signaling through Mac-1 (CD11b/CD18) in diet-induced obesity (DIO). *Thromb. Haemost.* 117, 325–338 CrossRef Medline
19. Yakubenko, V. P., Cui, K., Ardell, C. L., Brown, K. E., West, X. Z., Gao, D., Stell, S., Salomon, R. G., Podrez, E. A., and Byzova, T. V. (2018) Oxidative modifications of extracellular matrix promote the second wave of inflammation via β2 integrins. *Blood* 132, 78–88 CrossRef Medline
20. Wang, H., Linetsy, M., Guo, J., Choi, J., Hong, L., Chamberlain, A. S., Howell, S. J., Howes, A. M., and Salomon, R. G. (2015) 4-Hydroxy-7-oxo-5-heptenoic Acid (HOHA) lactone is a biologically active precursor for the generation of (ω-carboxyethyl)pyrrole (CEP) derivatives of proteins and ethanolamine phospholipids. *Chem. Res. Toxicol.* 28, 967–977 CrossRef Medline
21. Gu, X., Meer, S. G., Miyagi, M., Rayborn, M. E., Hollyfield, J. G., Crabb, J. W., and Salomon, R. G. (2003) Carboxyethylpyrrole protein adducts and autoantibodies, biomarkers for age-related macular degeneration. *J. Biol. Chem.* 278, 42027–42035 CrossRef Medline
22. Kim, Y. W., Yakubenko, V. P., West, X. Z., Gugiu, G. B., Renganathan, K., Biswas, S., Gao, D., Crabb, J. W., Salomon, R. G., Podrez, E. A., and Byzova, T. V. (2015) Receptor-mediated mechanism controlling tissue levels of bioactive lipid oxidation products. *Circ. Res.* 117, 321–332 CrossRef Medline
23. Biswas, S., Xin, L., Panagiri, S., Zimmah, A., Wang, H., Yakubenko, V. P., Byzova, T. V., Salomon, R. G., and Podrez, E. A. (2016) Novel phosphatidyethanolamine derivatives accumulate in circulation in hyperlipidemic ApoE−/− mice and activate platelets via TLR2. *Blood* 127, 2618–2629 CrossRef Medline
24. Yakubenko, V. P., Lishtso, V. K., Lam, S. C., and Ugarova, T. P. (2002) A molecular basis for integrin α5β1 in binding ligand specificity. *J. Biol. Chem.* 277, 48635–48642 CrossRef Medline
25. Lishtso, V. K., Podolnikova, N. P., Yakubenko, V. P., Yakovlev, S., Medved, L., Yadav, S. P., and Ugarova, T. P. (2004) Multiple binding sites in fibrin-
Inhibition of integrin αDβ2-mediated macrophage accumulation

ogen for integrin αMβ2 (Mac-1). J. Biol. Chem. 279, 44897–44906

26. Podolnikova, N. P., Podolnikov, A. V., Haas, T. A., Lishko, V. K., and Ugurova, T. P. (2015) Ligand recognition specificity of leukocyte integrin αMβ2 (Mac-1, CD11b/CD18) and its functional consequences. Biochemistry 54, 1408–1420 CrossRef Medline

27. Yakubenko, V. P., Soloviov, D. A., Zhang, L., Yee, V. C., Plow, E. F., and Ugurova, T. P. (2001) Identification of the binding site for fibrinogen recognition peptide γ383–395 within the αM I-domain of integrin αMβ2. J. Biol. Chem. 276, 13995–14003 CrossRef Medline

28. Ustinov, V. A., and Plow, E. F. (2002) Delineation of the key amino acids involved in NIF binding to the I-domain supports a mosaic model for the capacity of integrin αMβ2 to recognize multiple ligands. J. Biol. Chem. 277, 18769–18776 CrossRef Medline

29. Zhang, L., and Plow, E. F. (1999) Amino acid sequences within the α subunit of integrin αMβ2 (Mac-1) critical for specific recognition of C3bi. Biochemistry 38, 8064–8071 CrossRef Medline

30. West, X. Z., Malinin, N. L., Merkulova, A. A., Tischenko, M., Kerr, B. A., Borden, E. C., Podrez, E. A., Salomon, R. G., and Byzova, T. V. (2010) Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. Nature 467, 972–976 CrossRef Medline

31. Panigrahi, S., Ma, Y., Hong, L., Gao, D., West, X. Z., Salomon, R. G., Byzova, T. V., and Podrez, E. A. (2013) Engagement of platelet toll-like receptor 9 by novel endogenous ligands promotes platelet hyperreactivity and thrombosis. Circ. Res. 112, 103–112 CrossRef Medline

32. Liu, R., Rieu, P., Griffith, D. L., Scott, D., and Arnaout, M. A. (1998) Two functional states of the CD11b A-domain: correlations with key features of two Mn²⁺-complexed crystal structures. J. Cell Biol. 143, 1523–1534 CrossRef Medline

33. Xiong, J.-P., Li, R., Essafi, M., Stehle, T., and Arnaout, M. A. (2000) An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b A-domain. J. Biol. Chem. 275, 38762–38767 CrossRef Medline

34. Zhu, J., Zhu, J., and Springer, T. A. (2013) Complete integrin headpiece opening in eight steps. J. Cell Biol. 201, 1053–1068 CrossRef Medline

35. Salomon, R. G. (2017) Carboxyethylpyrroles: from hypothesis to the discovery of biologically active natural products. Chem. Res. Toxicol. 30, 105–113 CrossRef Medline

36. Cao, C., Lawrence, D. A., Strickland, D. K., and Zhang, L. (2005) A specific role of integrin Mac-1 in accelerated macrophage efflux to the lymphatics. Blood 106, 3234–3241 CrossRef Medline

37. Bellingan, G. J., Caldwell, H., Howie, S. E., Dransfield, I., and Haslett, C. (1996) In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. J. Immunol. 157, 2577–2585 Medline

38. Bellingan, G. J., Xu, P., Cocksley, H., Cauldwell, H., Shock, A., Bottoms, S., Haslett, C., Mutsaers, S. E., and Laurent, G. J. (2002) Adhesion molecule-dependent mechanisms regulate the rate of macrophage clearance during the resolution of peritoneal inflammation. J. Exp. Med. 196, 1515–1521 CrossRef Medline

39. Noti, J. D. (2002) Expression of the myeloid-specific leukocyte integrin gene CD11d during macrophage foam cell differentiation and exposure to lipoproteins. Int. J. Mol. Med. 10, 721–727 Medline

40. Chuluyan, H. E., and Issekutz, A. C. (1993) VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. J. Clin. Investig. 92, 2768–2777 CrossRef Medline

41. Shang, X. Z., and Issekutz, A. C. (1998) Contribution of CD11a/CD18, CD11b/CD18, ICAM-1 (CD54) and -2 (CD102) to human monocyte migration through endothelium and connective tissue fibroblast barriers. Eur. J. Immunol. 28, 1970–1979 CrossRef Medline

42. Meerschaert, J., and Furie, M. B. (1994) Monocytes use either CD11D or VLA-4 to migrate across human endothelium in vitro. J. Immunol. 152, 1915–1926 Medline

43. Lämmermann, T., Bader, B. L., Monkley, S. J., Worbs, T., Wedlich-Söldner, R., Hirsch, K., Keller, M., Förster, R., Critchley, D. R., Fässler, R., and Sixt, M. (2008) Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature 453, 51–55 CrossRef Medline

44. Bouissou, A., Proag, A., Bourg, N., Pingris, K., Cabriel, C., Balor, S., Mangeat, T., Thibault, C., Vieu, C., Dupuis, G., Fort, E., Lévêque-Fort, S., Maridonneau-Parini, I., and Poincloux, R. (2017) Podosome force generation machinery: a local balance between protrusion at the core and traction at the ring. ACS Nano 11, 4028–4040 CrossRef Medline

45. Cougoule, C., Van Goethem, E., Le Cabec, V., Lafouresse, F., Dupré, L., Mehrjui, V., Mège, J. L., Lastrucci, C., and Maridonneau-Parini, I. (2012) Blood leukocytes and macrophages of various phenotypes have distinct abilities to form podosomes and to migrate in 3D environments. Eur. J. Cell Biol. 91, 938–949 CrossRef Medline

46. Maridonneau-Parini, I. (2014) Control of macrophage 3D migration: a therapeutic challenge to limit tissue infiltration. Immunol. Rev. 262, 216–231 CrossRef Medline

47. Wiesner, C., Le-Cabec, V., El Azouzi, K., Maridonneau-Parini, I., and Lindé, S. (2014) Podosomes in space: macrophage migration and matrix degradation in 2D and 3D settings. Cell Adh. Migr. 8, 179–191 CrossRef Medline

48. Palecek, S. P., Loftus, J. C., Ginsberg, M. H., Lauffenburger, D. A., and Horwitz, A. F. (1997) Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature 385, 537–540 CrossRef Medline

49. DíMilla, P. A., Stone, J. A., Quinn, J. A., Albelda, S. M., and Lauffenburger, D. A. (1993) Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. J. Cell Biol. 122, 729–737 CrossRef Medline

50. Mabon, P. J., Weaver, L. C., and Dekaban, G. A. (2000) Inhibition of monocyte/macrophage migration to a spinal cord injury site by an antibody to the integrin αD: a potential new anti-inflammatory treatment. Exp. Neurol. 166, 52–64 CrossRef Medline