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## Human Neutrophil Peptides Mediate Endothelial-Monocyte Interaction, Foam Cell Formation, and Platelet Activation

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**Objective**—Neutrophils are involved in the inflammatory responses during atherosclerosis. Human neutrophil peptides (HNPs) released from activated neutrophils exert immune modulating properties. We hypothesized that HNPs play an important role in neutrophil-mediated inflammatory cardiovascular responses in atherosclerosis.

**Methods and Results**—We examined the role of HNPs in endothelial-leukocyte interaction, platelet activation, and foam cell formation in vitro and in vivo. We demonstrated that stimulation of human coronary artery endothelial cells with clinically relevant concentrations of HNPs resulted in monocyte adhesion and transmigration; induction of oxidative stress in human macrophages, which accelerates foam cell formation; and activation and aggregation of human platelets. The administration of superoxide dismutase or anti-CD36 antibody reduced foam cell formation and cholesterol efflux. Mice deficient in double genes of low-density lipoprotein receptor and low-density lipoprotein receptor–related protein (LRP), and mice deficient in a single gene of LRP8, the only LRP phenotype expressed in platelets, showed reduced leukocyte rolling and decreased platelet aggregation and thrombus formation in response to HNP stimulation.

**Conclusion**—HNPs exert proatherosclerotic properties that appear to be mediated through LRP8 signaling pathways, suggesting an important role for HNPs in the development of inflammatory cardiovascular diseases. (*Arterioscler Thromb Vasc Biol.* 2011;31:2070-2079.)

**Key Words:** atherosclerosis ■ leukocytes ■ transgenic models

Recent studies have revealed that polymorphonuclear neutrophils (PMNs) participate in the development of atherosclerotic lesions.<sup>1–5</sup> Indeed, PMNs can be detected in human tissue specimens at sites of plaque rupture and erosion or in thrombi from patients with acute coronary syndrome.<sup>6</sup> The number of PMNs in blood and the levels of elastase and myeloperoxidase (MPO) released from PMNs have been reported to be associated with atherosclerosis.<sup>6–8</sup> A study in more than 350 000 patients with atherosclerosis demonstrated that the subjects with higher leukocyte counts have higher relative and absolute acute and chronic mortality rates than those with lower leukocyte counts.<sup>9</sup> A positive correlation between peripheral PMN count and myocardial infarction has also been reported.<sup>10,11</sup> In patients with chronic stable angina, PMN count and activation are an independent predictor of the presence of multiple complex stenoses irrespective of the extent of coronary artery disease.<sup>7,12</sup> A recent study demonstrated that peripheral blood PMN counts are higher in patients with rheumatoid arthritis implicated with premature atherosclerosis than

in control subjects.<sup>13</sup> The increased PMN count is associated with high concentration of inflammatory mediators, including interleukin-6, intercellular adhesion molecule-1 (ICAM-1), E-selectin, and tumor necrosis factor- $\alpha$  in the blood.<sup>13</sup>

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Using apolipoprotein E-deficient mice with fluorescent PMNs to study specifically PMN presence and recruitment in atherosclerotic lesions, Rotzius et al have recently shown that a majority of leukocytes interacting with the endothelium on lesion shoulders are PMNs, suggesting a significant recruitment of these cells to the plaque.<sup>14</sup> The functional importance of PMNs in the formation of the atherosclerotic plaque was demonstrated by Zerneck et al,<sup>3</sup> who reported that the size of murine atherosclerotic plaques has been shown to be closely correlated with the number of PMNs in the peripheral blood, and depletion of circulating PMNs resulted in reduction of plaque formation.

Evidence has shown that several members of PMN-specific proteins, including human neutrophil peptides

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(HNPs) and azurocidin (heparin-binding protein) are found at the luminal site of the endothelium,<sup>15</sup> suggesting their deposition by PMNs. During inflammation, large amounts of intracellular proteins are released from the activated PMNs into the extracellular milieu as a consequence of PMN degranulation, leakage during phagosome formation, and cell death. The highly homologous HNP-1, -2, and -3, also known as  $\alpha$ -defensins, make up more than 50% of the total protein content within the azurophilic granules of PMNs.<sup>16</sup> HNPs have the capacity to kill a wide spectrum of Gram-positive and Gram-negative bacteria, fungi, and some enveloped viruses *in vitro*.<sup>17</sup> Recent studies have demonstrated that other leukocytes, including monocytes, macrophages, T cells, and immature dendritic cells, also express HNPs in particular when stimulated with cytokines.<sup>18–20</sup> Therefore HNPs appear to participate in both innate and acquired immunity.

In healthy volunteers, plasma levels of HNPs range from undetectable to 50 to 100 ng/mL. However, mean HNP levels are 2- to 4-fold greater at the onset of bacterial infection and during nonbacterial infection compared with healthy volunteers.<sup>21</sup> Under septic conditions, plasma HNP levels range from 900 to 170 000 ng/mL, compared with a mean of  $42 \pm 53$  ng/mL in healthy controls.<sup>22</sup> An excellent correlation exists between the concentration of HNPs and the number of PMNs in the blood of patients with inflammatory diseases.<sup>21</sup> We speculate that these proteins released from PMNs and other leukocytes are not bystanders but play an active role in the development of atherosclerosis.

Indeed, HNPs are found in the luminal site of human atherosclerotic coronary arteries in both large- and small-vessel endothelium.<sup>23</sup> HNPs are also found in intimal smooth muscle cells of atherosclerotic human cerebral arteries.<sup>24</sup> Moreover, a significant correlation is observed in men between the amount of HNP deposition in skin and severity of coronary artery disease.<sup>25</sup> HNP stimulation increased adhesion of the cultured human endothelial ECV-304 cells in plastic plate coated with gelatin.<sup>26</sup> In porcine coronary artery, endothelium-dependent relaxation in response to bradykinin was significantly reduced after treatment with HNPs associated with an increased production of superoxide anion and a decreased expression of endothelial nitric oxide synthase at both levels of mRNA and protein.<sup>27</sup> We observed that stimulation of human umbilical vein endothelial cells (HUVECs) with HNPs resulted in enhanced formation of peroxynitrite that led to endothelin-1 production, which was largely attenuated by the treatment with the reactive oxygen species scavenger *N*-acetyl-L-cysteine.<sup>28</sup> This observation is of interest because endothelin-1 has been found at increased levels in endothelial cells, smooth muscle cells, and macrophages within atherosclerotic plaques.<sup>25</sup>

Retention of lipoproteins within the vasculature is an important event in the pathogenesis of atherosclerosis. HNPs have been reported to form stable multivalent complexes with lipoprotein(a).<sup>29</sup> Whereas HNPs and lipoprotein(a) individually can readily traverse through the endothelial cell membranes, HNP/lipoprotein(a) complexes are lodged onto the cell surface of endothelial and smooth muscle cells, resulting in a marked increase in the total amount of cell-associated lipoprotein.<sup>30</sup> Furthermore, HNPs can stimulate the binding

of <sup>125</sup>I-low-density lipoprotein (LDL) to cultured HUVECs, smooth muscle cells, and fibroblasts in a dose-dependent and saturated manner.<sup>30</sup> An antibody against the LDL receptor (LDLR) is able to inhibit the binding of <sup>125</sup>I-HNPs to HUVECs.<sup>30</sup> Moreover, HNPs bind directly to isolated  $\alpha$ 2-macroglobulin receptor (LDLR-related protein [LRP]) in a dose-dependent manner, and the binding is inhibited by anti-LRP antibodies and LDLR-associated protein<sup>31</sup> to prevent binding of LRP ligands.

Given its biological nature as an inflammatory marker, the ability to promote cellular oxidation, and the interacting capacity with lipoproteins described above, we hypothesized that HNPs function as an effector in the pathogenesis of cardiovascular diseases. Specifically, HNPs increase monocyte-endothelial cell interaction, accelerate foam cell formation, and trigger the activation of platelets through LRP receptors. This hypothesis was tested *in vitro* with cultured primary human coronary endothelial cells, human acute monocytic leukemia cell line (THP-1)-derived macrophages, and human primary platelets, and *in vivo* with mice with LDLR and LRP double-gene or LRP8 single-gene deficiency, respectively.

## Materials and Methods

The study protocol was approved by the institutional Research Ethics Board for isolation of HNPs from sputum of patients with cystic fibrosis and for human monocyte and platelet isolation from healthy nonsmoking blood donors. The animal experiments were approved by the institutional Animal Care and Use Committee.

### HNP Isolation

HNPs were purified from pooled sputum of patients with cystic fibrosis as previously described<sup>32</sup> (see supplemental material, available online at <http://atvb.ahajournals.org>).

### Cell Activation, Adhesion, and Transmigration Assays

Primary human coronary artery endothelial cells (HCAECs) (Cell Applications, San Diego, CA) were used within 4 passages. Expression of CD11b was measured to evaluate human monocyte activation after HNP stimulation, and monocyte adhesion and transmigration assays were performed as previously described<sup>33</sup> (see supplemental material).

### THP-1-Derived Macrophages Expressing CD36 and CD68

The expression of surface markers CD36 and CD68 was used to identify THP-1 monocyte-derived macrophages.<sup>34</sup> Briefly, THP-1 cells were stimulated with 5 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 72 hours, and adherent cells were cultured for additional 24 hours. The cells were then incubated with mouse anti-human CD36- (Invitrogen, Carlsbad, CA) or CD68-FITC antibody (AbD Serotec, Oxford, United Kingdom) for 30 minutes followed by flow cytometric analysis (FACSCanto flow cytometer, Becton Dickinson, Franklin Lakes, NJ).

### Foam Cell Assay

The macrophages described above were incubated with 10  $\mu$ g/mL HNPs, 50  $\mu$ g/mL native LDL (Calbiochem, La Jolla, CA), or HNPs and LDL together in serum-free medium for 16 hours. As for the positive control condition, macrophages were incubated with 50  $\mu$ g/mL oxidized LDL (oxLDL) (Kalen Biomedical, Savage, MD) or in combination with HNPs for 16 hours. To investigate the effects of HNPs on LDL oxidation in macrophages, additional cells were treated with the O<sub>2</sub><sup>-</sup> scavenger superoxide dismutase (200 U/mL,

Sigma-Aldrich) 5 minutes before the incubation with HNPs and LDL. The foam cell formation was evaluated as previously described.<sup>35</sup>

### Cholesterol Efflux Assay

To label the intracellular cholesterol pool, the THP-1 monocyte-derived macrophages were loaded with HNPs, LDL, oxLDL, anti-CD36 antibody and fluorescent Pennsylvania Green/*N*-alkyl-3 $\beta$ -cholesterylamine-derived molecular probe (F-Ch, 10  $\mu$ mol/L from a 10 mmol/L dimethyl sulfoxide stock solution, provided by Dr B. Peterson, University of Kansas). After 24 hours, cells were washed and incubated in 1% FBS medium for another 12 hours to allow the labeled cholesterol to be distributed to various intracellular compartments. After washing, efflux was induced by addition of high-density lipoprotein (100  $\mu$ g/mL) for 1 hour. The medium was collected, and cells were lysed and centrifuged at 13 200g for 10 minutes. The F-Ch in the medium and cell lysates was quantified relative to individual standard curves using excitation and emission wavelengths of 485 and 535 nm, respectively.<sup>36</sup>

### Murine Platelet Isolation

Male B6129SF2-Lrp8<sup>tm1Her/J</sup> mice (6 to 8 weeks, Jackson Laboratory, Bar Harbor, ME) and heterozygotes were mated to generate LRP8<sup>+/+</sup> and LRP8<sup>-/-</sup> littermates. LRP8<sup>-/-</sup> mice have 2 exons deleted from the cytoplasmic domain of the LRP8 receptor, resulting in a DNA sequence that is 100 base pairs shorter than full-length gene sequence in wild-type mice. Platelets from mice were isolated as previously described to obtain platelet-rich plasma (PRP) and platelet-poor plasma.<sup>37</sup> Gel filtration of PRP on columns of Sepharose 2B was performed, and 2 $\times$ 10<sup>8</sup>/mL of platelets was used in experiments.

### Competitive Binding Assay Between HNPs and Recombinant Human LRP8

HNPs were preincubated with recombinant human LRP8 (R&D Systems Inc, Minneapolis, MN) at a 10:1 molar ratio for 30 minutes at 37°C. Murine and human platelets were stimulated at room temperature for 5 minutes with HNPs (10  $\mu$ g/mL), vehicle, or the mixture of HNPs and recombinant human LRP8. Platelets were stained with anti-mouse CD62P-FITC antibody (BD Biosciences, Mississauga, ON) or anti-human CD62P-FITC antibody (R&D Systems), respectively, for 25 minutes and fixed for analysis by flow cytometry.

### Platelet Aggregation Assay

Human and murine gel-filtered platelets and PRP were stimulated with HNPs, and aggregation was assessed at 37°C using an aggregometer. For priming experiments, murine and human PRP were incubated with HNPs or vehicle for 2 minutes before the addition of ADP. At the end of the aggregation experiment, an aliquot of 30  $\mu$ L of PRP from each condition was placed on coverslips for examination of thrombus formation under a confocal microscope.

### Murine Pulmonary Endothelial Cell Isolation

Male LRP8<sup>-/-</sup> and age (2 to 3 weeks) and background strain-matched mice (Jackson Laboratory) were used for endothelial cell isolation by using rat anti-mouse CD31 antibody (PECAM-1, Biolegend, San Diego, CA) followed by a magnetic separation (Dyna MPC-S, Invitrogen Dynal AS, Oslo, Norway) (see supplemental material for details). FITC rat anti-mouse CD54/ICAM-1 monoclonal antibody (1:50, Biolegend), PE rat anti-mouse CD106/vascular cell adhesion molecule-1 monoclonal antibody (1:50, GenWay Biotech, Inc, San Diego, CA), and DAPI (Molecular Probe, Molecular Probes, Inc, Eugene, OR) were used for immunostaining, followed by quantitative analysis using ImageJ software (NIH, <http://imagej.nih.gov/ij/download.html>).

### Mouse Monocyte Isolation and Flow Cytometry

Male LRP8<sup>-/-</sup> and age (2 to 3 weeks) and background strain-matched mice (Jackson Laboratory) were euthanized by overdose anesthesia for monocyte isolation from bone marrow (Mouse Monocyte Enrichment Kit, Stemcell Technologies Inc, Vancouver, British Columbia, Canada). Monocytes (2 $\times$ 10<sup>5</sup>) were suspended in RPMI-1640 in 24-well plates for 2 hours and then incubated without or with HNPs 10  $\mu$ g/mL for 1 hour. The cells were stained with unlabeled or PE rat anti-mouse CD11b monoclonal antibody (Biolegend) for 30 minutes at room temperature and then were analyzed using a BD FACSCanto with BD FACSDiva software (BD Biosciences, Mountain View, CA).

### Measurements

Concentrations of reactive nitrogen species, HNPs, MPO, chemokines, and adhesion molecules were measured (see supplemental material).

### Intravital Microscopy

#### Leukocyte Adhesion

Male B6129SF2 and age-matched (6 to 8 weeks) and background strain-matched *Ldlr*<sup>tm1Her/J</sup>/*Lrpap1*<sup>tm1Her/J</sup>, and LRP8<sup>-/-</sup> mice (Jackson Laboratory) were anesthetized and received intravenously HNPs (10 mg/kg body weight corresponding to approximately 10  $\mu$ g/mL)<sup>38</sup> or vehicle control solution. In additional experiments, platelets were depleted in mice who received intraperitoneal injection of rat anti-mouse CD41 monoclonal antibody at 2  $\mu$ g per mouse (from a 500  $\mu$ g/mL PBS stock solution provided by Dr A. Lazarus, the University of Toronto) 24 hours before HNP injection. This dose and timing of application of anti-CD41 resulted in 80% platelet depletion in the whole blood. An equal volume of PBS was used as for vehicle control.

To label circulating leukocytes,<sup>39</sup> rhodamine 6G at 200  $\mu$ L (20  $\mu$ g/ $\mu$ L, Sigma-Aldrich, St. Louis, MO) was injected intravenously. The carotid artery was exposed and monitored under an upright microscope (Axiovert 100HD, Zeiss, Jena, Germany) on a custom-built, computer-controlled warmed stage. The percentage of rolling leukocytes was calculated as previously described.<sup>39</sup>

#### Platelet Aggregation

Male LRP8<sup>-/-</sup> and age (6 to 8 weeks) and background strain-matched mice (Jackson Laboratory) were anesthetized and received intravenously HNPs (10 mg/kg body weight)<sup>38</sup> or vehicle control solution. Ex vivo-labeled platelets (250 $\times$ 10<sup>6</sup> to 300 $\times$ 10<sup>6</sup>) were infused intravenously into the tail vein for real-time visualization by using the intravital fluorescence microscope setup described above. The thrombus formation was recorded during the first 15 minutes after FeCl<sub>3</sub> administration to determine the time required for reaching maximal vessel occlusion.

### Statistical Analysis

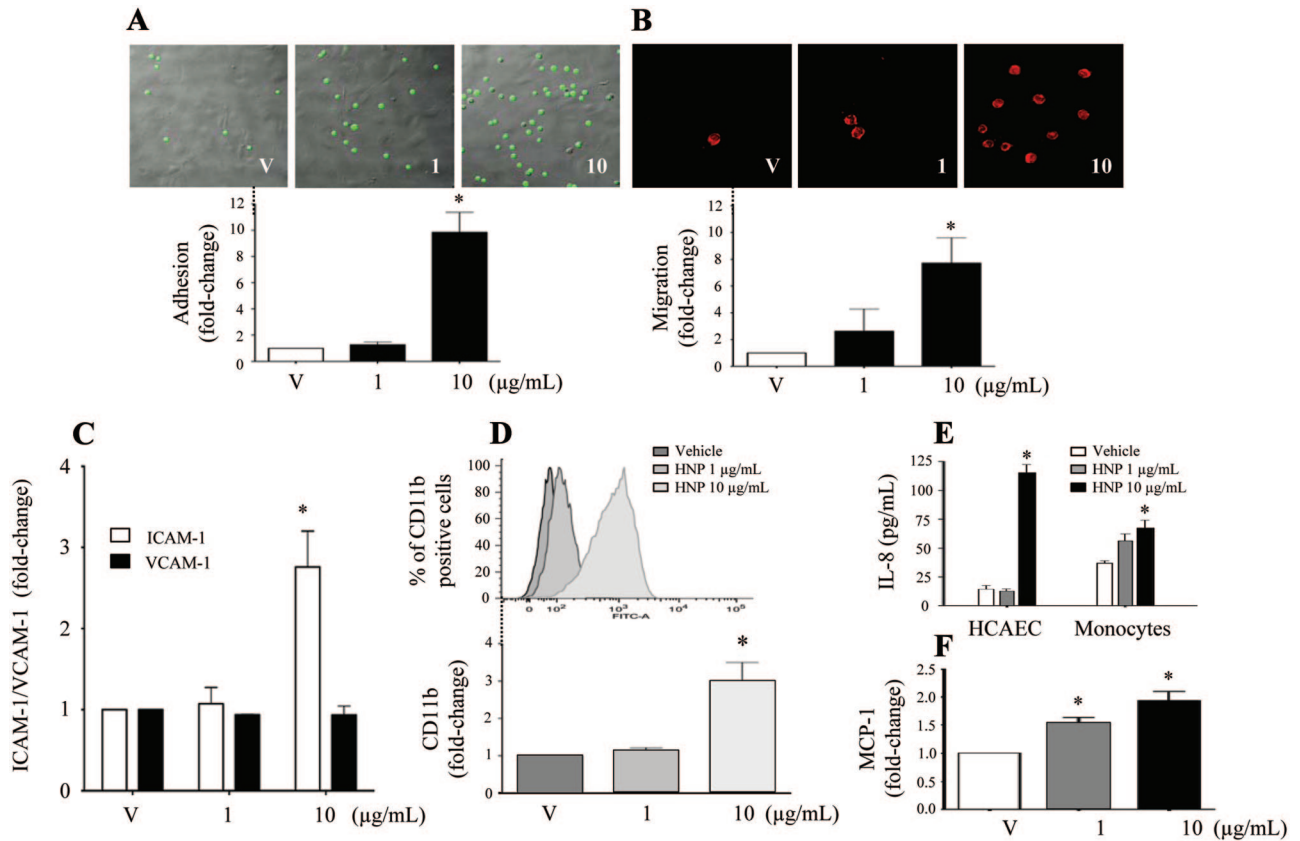
Data are presented as a means $\pm$ SE. Statistical significance was determined using a 1-way ANOVA followed by a Dunnett comparison. Analyses were performed using GraphPad Prism, version 5.00c (GraphPad Software Inc, San Diego, CA). A probability value <0.05 was considered statistically significant.

## Results

### HNPs Induce Monocyte-Endothelial Interaction and Chemokine Production

When the HCAECs were incubated with HNPs, monocyte adhesion to HCAECs increased at 10  $\mu$ g/mL compared with the vehicle control group (Figure 1A). In addition, HNPs induced significant monocyte transmigration through the HCAEC monolayer in a dose-dependent manner (Figure 1B). The expression of ICAM-1 but not vascular cell adhesion molecule-1 increased in HCAECs, and CD11b increased in human monocytes after stimulation with HNPs, compared





**Figure 1.** Human neutrophil peptides (HNPs) induce leukocyte-monocyte interaction. A, Human monocyte adhesion on human coronary artery endothelial cells (HCAECs) after stimulation with HNPs for 4 hours. V indicates vehicle. B, Monocyte (defined by CD33 as surface marker) transmigration through HCAECs after stimulation with HNPs. C, Expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in HCAECs after stimulation with HNPs for 4 hours. D, CD11 expression 1 hour after HNP stimulation in primary human monocytes. E, Interleukin-8 (IL-8) production in coculture supernatants of HCAECs and monocytes stimulated with HNPs for 4 hours (n=5 experiments). F, Monocyte chemotactic protein-1 (MCP-1) production in HCAECs stimulated with HNPs for 4 hours (n=5 experiments). \**P*<0.05 vs V under identical conditions.

with the vehicle control groups (Figure 1C and 1D). An increase in the production of interleukin-8 was observed in the cultural supernatants of both HCAECs and monocytes after HNP stimulation at 10  $\mu\text{g/mL}$  (Figure 1E) and monocyte chemotactic protein-1 in HCAECs at both 1  $\mu\text{g/mL}$  and 10  $\mu\text{g/mL}$  (Figure 1F).

### HNPs Increase Oxidative Stress and Accelerate Foam Cell Formation

The macrophage scavenger receptors CD36 and CD68 are major receptors responsible for uptake of modified LDL contributing to foam cell formation. We observed an increase in macrophage surface expression of CD36 and CD68 after stimulation with HNPs, as compared with the vehicle control group (Figure 2A and 2B). An enhanced oxidative stress as reflected by nitrotyrosine formation was observed in macrophages after HNP stimulation at 10  $\mu\text{g/mL}$  (Figure 2C).

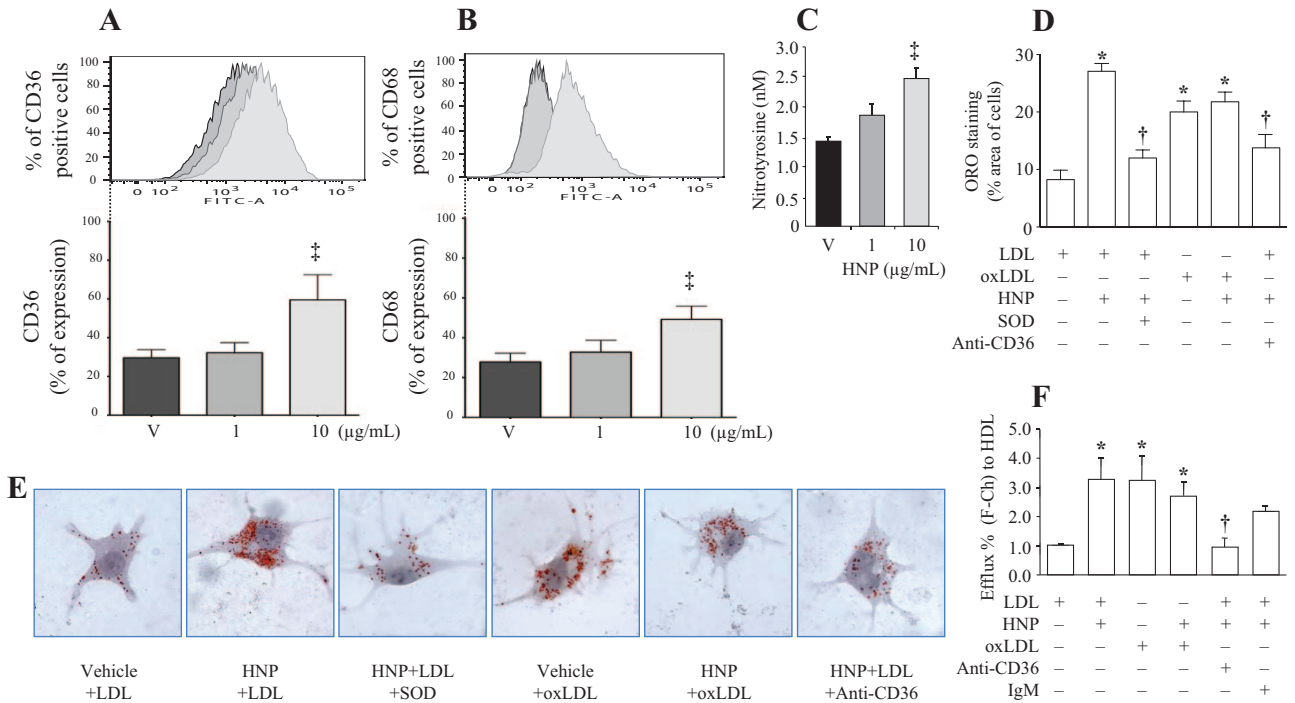
Incubation of macrophages with oxLDL resulted in increased Oil Red O staining, indicative of foam cell formation (Figure 2D and 2E). A combined stimulation of oxLDL and HNPs did not result in any further increase in Oil Red O staining. A similar increase in intracellular Oil Red O staining as seen with oxLDL incubation was observed after macrophages were incubated together with LDL and HNPs but not

with HNP vehicle control buffer (Figure 2D and 2E). The HNP-induced foam cell formation (Figure 2D and 2E) and cholesterol efflux (Figure 2F) were largely attenuated by the treatment with superoxide dismutase and anti-CD36 blocking antibody, respectively.

### HNPs Activate Platelets

Incubation of platelets with HNPs induced a dose-dependent increase in CD62P expression in human PRP (Figure 3A). Similar results were seen in murine PRP (Figure 3B). The HNP-induced platelet activation was associated with an increase in aggregation in human platelets (Figure 3C), but not in murine platelets (data not shown). The HNP-induced human platelet aggregation resulted in thrombus formation detected in culture supernatants (Figure 3D).

We then examined whether HNPs sensitize the weak agonist ADP for platelet aggregation. When human and murine platelets were primed with HNPs or vehicle control followed 2 minutes later by incubation with ADP (1.25  $\mu\text{mol/L}$  for human, 5  $\mu\text{mol/L}$  for murine cells), a significantly prolonged aggregation was observed in both human (Figure 3E) and murine (Figure 3G) platelets as compared with a transient aggregation after ADP stimulation alone. The increased platelet aggregation by HNP priming



**Figure 2.** Human neutrophil peptides (HNPs) increase oxidative stress and foam cell formation. A and B, Surface expression of CD36 and CD68 on human acute monocytic leukemia cell line (THP-1) derived macrophages stimulated with HNPs or vehicle (V) control for 8 hours. C, Concentration of nitrotyrosine measured in human THP-1 derived macrophage lysates after HNP stimulation. n=5 experiments. D, Foam cell formation, quantified by Oil Red O (ORO) staining. Human macrophages were incubated with HNPs (10 μg/mL) in the presence of low-density lipoprotein (LDL) or oxidized LDL (oxLDL) for 16 hours. In additional experiments, superoxide dismutase (SOD) or anti-CD36 blocking antibody was added 30 minutes before the stimulation with HNPs. n=10 experiments. E, Representative cells with ORO staining in different groups described in D. F, Cholesterol efflux. ‡P<0.05 vs V, \*P<0.05 vs LDL alone, †P<0.05 vs the LDL+HNP group.

was associated with greater thrombus formation as compared with ADP alone (Figure 3F and 3H).

### HNPs Mediate Cellular Inflammatory Responses Through LRP Signaling

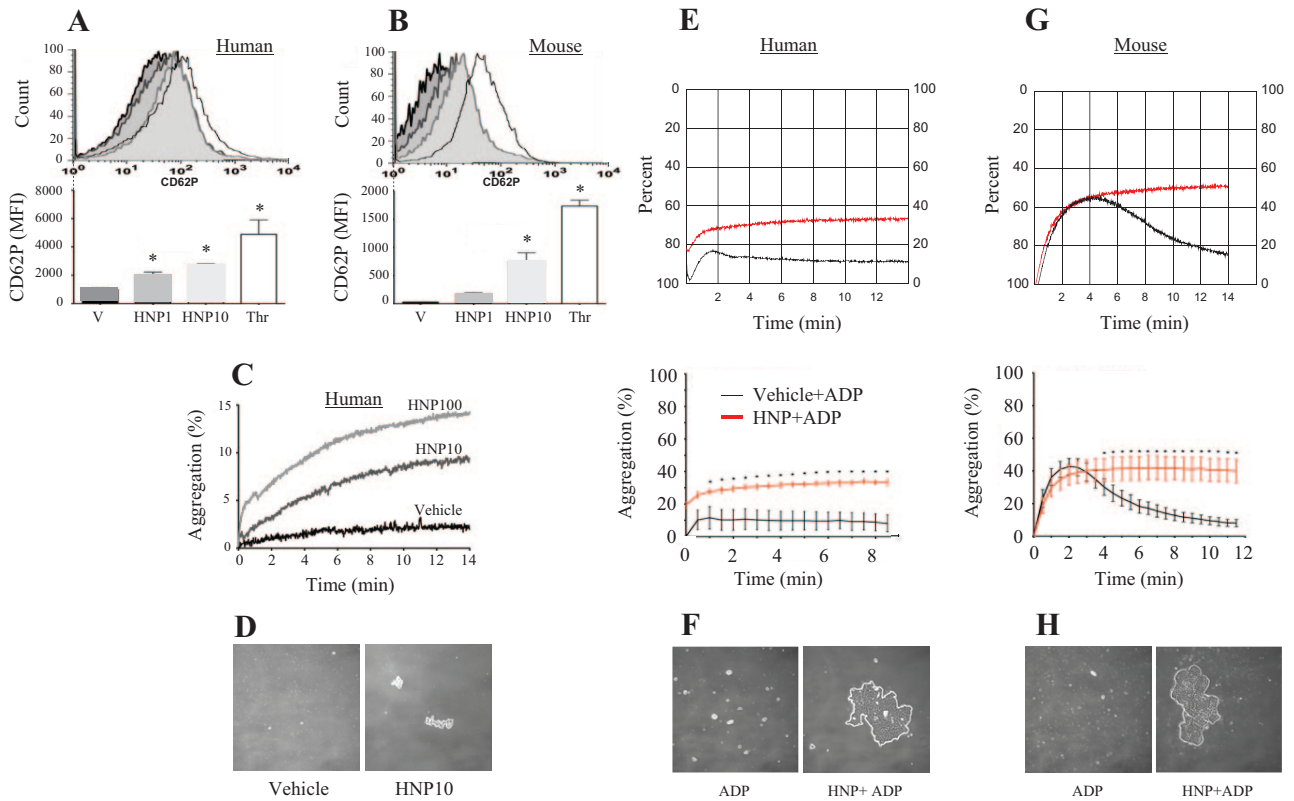
To understand the signaling mechanisms by which HNPs induced cell adhesion, we repeated the leukocyte adhesion experiments in wild-type and the LDLR and LRP double-gene knockout mice by using an intravital microscopy setup. We observed a dramatic increase in leukocyte rolling in carotid artery 4 hours after HNP challenge in wild-type control mice (Figure 4A and 4B). In contrast, there was a significant decrease in leukocyte rolling in the LDLR<sup>-/-</sup>/LRP<sup>-/-</sup> mice (Figure 4C) despite the observation that the plasma level of HNPs and MPO activity were identical in both the wild-type and the gene knockout mice (Figure 4D and 4E). These results suggest a role of LRP signaling in mediating the HNP-induced leukocyte adhesion in vivo.

To identify the specific phenotype of LRP in mediating the HNP-induced inflammation, we went further, to examine the role of LRP8 in leukocyte rolling and platelet activation, because LRP8 is the only LRP family member expressed on platelets.<sup>40</sup> We first observed a significant decrease in leukocyte rolling in the LRP8<sup>-/-</sup> mice as compared with the wild-type mice (Figure 4C) given that the plasma level of HNPs and MPO activity were identical in both the wild-type and the gene knockout mice (Figure 4D and 4E).

We then conducted a competitive binding assay in which recombinant human LRP8 was incubated with HNPs at a 1:10 molar ratio 30 minutes before stimulation of human platelets. HNPs alone induced an increase in CD62P expression that was dramatically reduced in the presence of recombinant human LRP8 (Figure 5A). We observed a decrease in CD62P expression in platelets isolated from LRP8<sup>-/-</sup> mice after HNP stimulation as compared with the wild-type mice, whereas stimulation with thrombin (5 U/mL) resulted in a similar increase in CD62P expression in both groups (Figure 5B). This phenomenon suggests a specific role for LRP8 in mediating the HNP-induced platelet activation.

Under in vivo conditions in which mice received intravenous injection of HNPs, the platelet intensity/area representing platelet aggregation was significantly greater in the wild-type mice but remained low in the LRP8<sup>-/-</sup> mice (Figure 5C and 5D). The time to reach maximal occlusion of carotid artery prolonged significantly in the LRP8<sup>-/-</sup> mice compared with the wild-type mice (Figure 5E).

To verify whether endothelial, monocytic, or platelet LRP8 is required for increased monocyte adhesion in vivo, we examined leukocyte rolling under intravital microscope in the presence and absence of platelets after HNP stimulation in LRP8<sup>-/-</sup> mice. Our results show that the absence of platelets slightly reduced leukocyte rolling in the wild-type mice, whereas platelet LRP8 is not required for leukocyte adhesion (Figure 5F).



**Figure 3.** Human neutrophil peptides (HNPs) induce platelet activation. A and B, Surface expression of CD62P in human and murine platelet-rich plasma (PRP) stimulated with either HNPs (1, 10  $\mu\text{g}/\text{mL}$ ), vehicle control (V), or human  $\alpha$ -thrombin (Thr) (5 U/mL). C, Dose-dependent increase in platelet aggregation in human PRP after stimulation with HNPs at 10 or 100  $\mu\text{g}/\text{mL}$ . D, Thrombus formation after the HNP stimulation shown in C. E, HNP-induced human platelet aggregation and synergy with ADP. Human PRP was primed with the HNP stimulation shown in E. G, HNP-induced murine platelet aggregation and synergy with ADP. Murine PRP was primed with HNPs (10  $\mu\text{g}/\text{mL}$ ) or vehicle control followed 2 minutes later by incubation with ADP (1.25  $\mu\text{mol}/\text{L}$ ). Platelet aggregation is illustrated by representative actual traces (top) and by average traces (bottom,  $n=7$  experiments). F, Thrombus formation after the HNP stimulation shown in E. H, Thrombus formation after the HNP stimulation shown in G. \* $P<0.05$  vs V group under identical conditions.

To examine the exact role of endothelial and monocytic LRP8 in contributing to leukocyte rolling, we went further, to measure cell surface markers of activation in vitro endothelial cells and monocytes isolated from wild-type and LRP8<sup>-/-</sup> mice. We demonstrated that the expression of vascular cell adhesion molecule-1 and ICAM-1 by endothelial cells decreased (Figure 5G), whereas CD11b expression was not significantly altered (Figure 5H) in the absence of LRP8 after HNP stimulation.

### Discussion

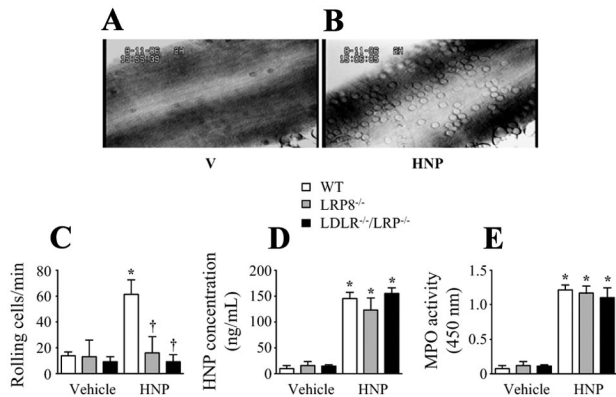
Several recent advances have suggested that PMNs play a functional role in atherosclerotic progression. By using a small-molecule antagonist to increase peripheral PMNs, Zerneck et al demonstrated a close correlation between murine PMN counts and atherosclerotic lesion size.<sup>3</sup> Furthermore, depletion of PMNs significantly reduced plaque formation, confirming a role for PMNs in their formation.<sup>3,41</sup> Rotzius et al have shown substantial numbers of PMNs in aortic plaques, especially in the shoulder regions.<sup>14</sup>

One of the criticisms against the involvement of PMNs in atherosclerosis has been their lack of direct detection within lesions in human atherosclerosis. It has been suggested that

the short life span and high turnover of emigrated PMNs coupled with technical limitations in accurately identifying them could account for this discrepancy. Hence, even low numbers of plaque PMNs may represent significant recruitment of cells that have undergone decay, leaving behind only “footprints” revealing their presence. Indeed, it has been shown that the abundant content of HNPs released from activated PMNs are located in luminal site of human atherosclerotic coronary arteries<sup>23</sup> and around intimal and medial smooth muscle cells within human atherosclerotic carotid and coronary arteries.<sup>20,23</sup> HNPs were found in lesions in which intimal thickening was minimal, suggesting that HNP deposition occurs early in the disease process.<sup>20,23</sup> Furthermore, a significant correlation was revealed between HNP skin deposition and the severity of coronary artery diseases, as evaluated by the number of blood vessels associated with focal lesions and stenosis.<sup>25</sup>

We demonstrated that HNPs may act not only as a biomarker like MPO to predict coronary diseases, as previously reported,<sup>42</sup> but also function as an effector to induce or deteriorate inflammatory cardiac events by inducing endothelium-monocyte interaction through increasing the conventional receptor-ligand interaction between ICAM-1 and





**Figure 4.** Human neutrophil peptides (HNPs) induce leukocyte adhesion mediated by low-density lipoprotein receptor (LDLR)-related protein 8 (LRP8) in vivo. A and B, Leukocyte rolling in carotid artery was monitored by intravital microscopy in wild-type (WT) mice 4 hours after receiving intravenous injection of vehicle (V) or HNPs (10 mg/kg). C, Mean values of leukocyte rolling in WT, LRP8<sup>-/-</sup>, or LDLR<sup>-/-</sup>/LRP<sup>-/-</sup> mice 4 hours after injection with HNPs. D and E, Mean plasma levels of HNPs and myeloperoxidase (MPO) activity measured in WT, LRP8<sup>-/-</sup>, or LDLR<sup>-/-</sup>/LRP<sup>-/-</sup> mice 4 hours after injection with HNPs. \**P*<0.05 vs V under identical conditions, †*P*<0.05 vs WT under identical conditions.

CD11b, oxidative stress and foam cell formation. In a previous study, we also showed that the stimulation of HUVECs with HNPs dose-dependently increases COX-2 expression.<sup>28</sup> The latter is required for integrin-dependent endothelial cell activation and migration.<sup>43</sup>

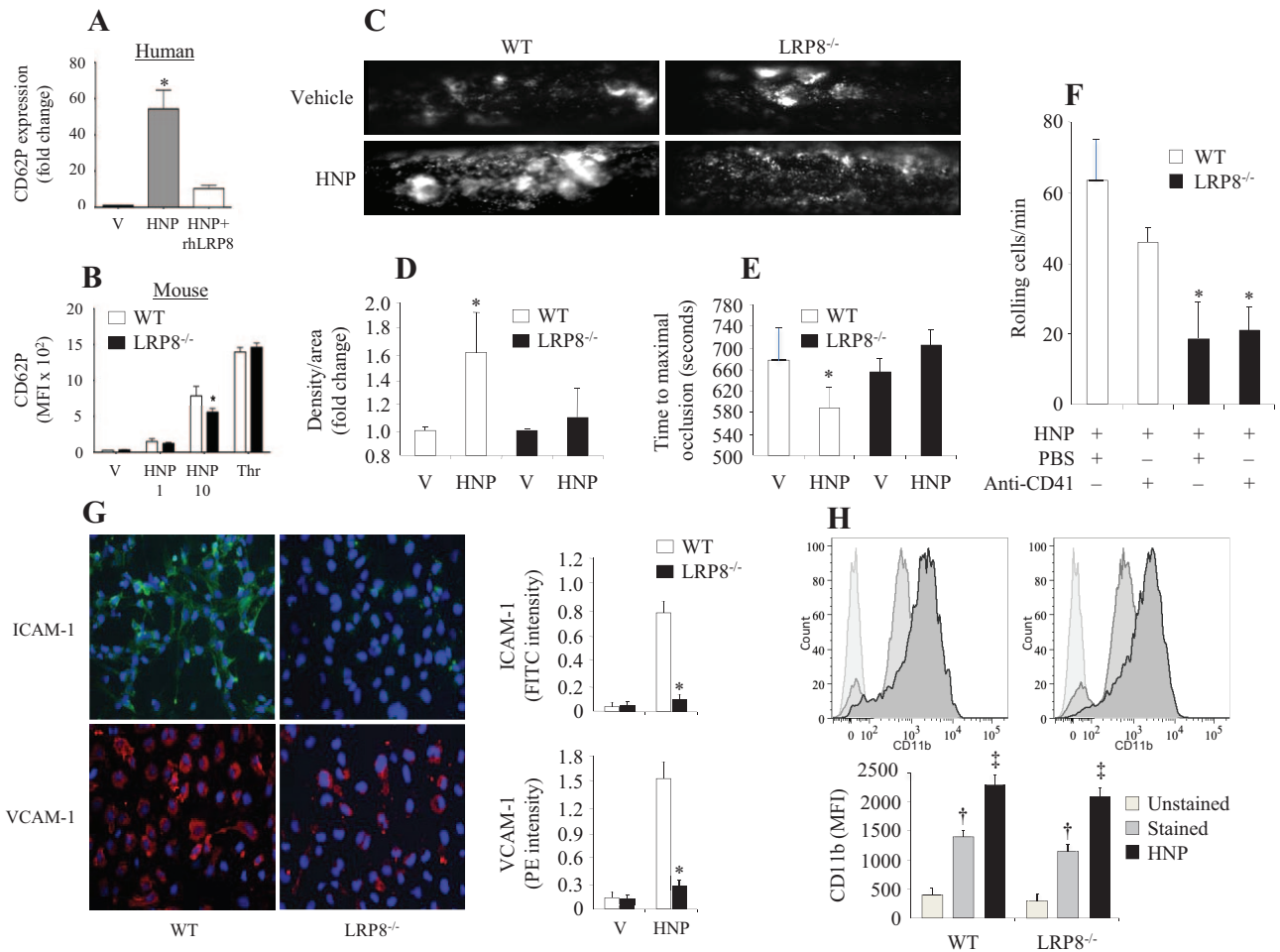
On adhesion to the endothelial wall, the monocytes being recruited to the lesion-prone sites play a critical role in the development of atherosclerosis. This process is governed by inflammatory molecules, such as monocyte chemoattractant protein-1, endothelin-1, and interleukin-8.<sup>44</sup> We demonstrate that HNPs can activate endothelial cells to increase the production of monocyte chemoattractant protein-1, a powerful inducer of monocyte chemotaxis. The presence of monocyte chemoattractant protein-1 is likely responsible for potentiating monocyte chemotaxis through the endothelium in our in vitro assays. However, there may be other mechanisms contributing to the observed monocyte chemotaxis. We have previously shown in primary human endothelial cells that HNPs increase the production of endothelin-1, another known monocyte chemoattractant,<sup>28</sup> and induce the expression of leukocyte function-associated antigen-1 in lymphocytes.<sup>33</sup> Besides the chemoattractive properties of HNPs through secondary production of chemokines, HNPs have also demonstrated direct chemotactic activity toward monocytes and dendritic cells at nanomolar concentrations.<sup>45</sup>

Once trapped in the arterial wall, monocytes can undergo phenotypic differentiation into macrophages and contribute to atherosclerosis-associated inflammation through formation of foam cells. The oxidation of LDL by reactive oxygen species is a well-known mechanism of the formation of foam cells, a characteristic feature of atherosclerosis at all stages of disease progression. We and others have previously demonstrated the production of reactive oxygen species and reactive nitrogen species, including superoxide anion, H<sub>2</sub>O<sub>2</sub>, and nitrotyrosine,<sup>27,28</sup> on HNP stimulation in endothelial and epithelial

cells. In the present study, we observed a significant production of nitrotyrosine, reflecting peroxynitrite formation when macrophages are stimulated with HNPs. Nitrotyrosine is a known molecule contributing to the oxidation of LDL and thus is likely to contribute to increased foam cell formation in the presence of HNPs. This concept is further supported by the observation that the use of the reactive oxygen species scavenger superoxide dismutase significantly decreases the foam cell formation. In addition, macrophage scavenger receptors CD36 and CD68 are the major receptors for macrophages to internalize oxLDL to become lipid engorged foam cells. Our results suggest that the HNP-induced increase in the surface expression of CD36 has contributed to the further enhancement in foam cell formation.

Platelets play a central role in physiological hemostasis but also contribute to atherothrombosis in pathological conditions. We have identified the ability of HNPs to dose-dependently enhance CD62P expression on human and murine platelets. CD62 is not only a marker of platelet  $\alpha$ -degranulation but also is responsible for mediating various inflammatory responses through interactions with P-selectin glycoprotein ligand-1 on monocytes and endothelial cells.<sup>46</sup> Furthermore, CD62P is elevated in patients with acute coronary syndrome<sup>47</sup> and has been found to promote atherosclerosis in *apolipoprotein E*<sup>-/-</sup> mouse models.<sup>48</sup> The functional consequence of HNP-mediated platelet activation is demonstrated by the ability of HNPs to independently induce human platelet aggregation and sensitize both murine and human aggregation in response to ADP. The relatively modest response in human platelets, compared with murine platelets, in response to HNP stimulation may be donor specific, and a larger cohort of carefully screened subjects may be required to define maximal response.

We examined the role of LRP in initiating the HNP-induced cell-cell interaction for several reasons: (1) LRP has been shown to regulate integrin CD11b/CD18-mediated adhesion between monocytes and the endothelium<sup>49</sup>; (2) inhibition of LRP decreases leukocyte pulmonary infiltration and the acid-induced lung injury in a mouse model overexpressing HNPs<sup>50</sup>; (3) HNPs have been reported to bind to LRP, resulting in HNP internalization by smooth muscle cells<sup>31</sup>; and (4) the HNP-induced inhibition of aortic contractility was abolished by the use of anti-LRP antibodies and the LRP antagonist receptor-associated protein.<sup>31</sup> Furthermore, HNPs and LDL formed stable complexes in solution and on the endothelial cell surface,<sup>30</sup> the interaction between HNPs and LDL resulted in LDL retention and slowed LDL degradation in endothelium,<sup>24,30</sup> and anti-LDLR antibodies that block LDL binding inhibited the binding of HNPs to HUVECs.<sup>30</sup> We thus chose the LRP and LDLR double-gene knockout mice to keep the potential binding background as clean as possible. Our results suggest that LRP is required for HNP signaling by demonstrating a reduced leukocyte flux after HNP stimulation in LRP<sup>-/-</sup>/LDLR<sup>-/-</sup> mice and decreased foam cell formation in the macrophages isolated from LRP<sup>-/-</sup>/LDLR<sup>-/-</sup> mice. This observation suggests that the LRP family plays an important role in the HNP-induced inflammatory events.



**Figure 5.** Low-density lipoprotein receptor–related protein 8 (LRP8) plays a role in the human neutrophil peptide (HNP)–induced platelet activation and leukocyte rolling. **A**, Surface expression of CD62P in human platelet-rich plasma (PRP) after stimulation with vehicle control (V) or HNPs (10  $\mu$ g/mL) in the presence or absence of recombinant human LRP8 (rhLRP8) (n=7 experiments). **B**, Surface expression of CD62P in murine PRP isolated from wild-type (WT) or LRP8<sup>-/-</sup> mice after incubation with HNPs (1 or 10  $\mu$ g/mL) or thrombin (5 U/mL) (n=7 experiments). **C**, Representative images of platelet aggregation under fluorescence intravital microscope in WT and LRP8<sup>-/-</sup> mice 4 hours after intravenous injection of HNPs. Low-dose FeCl<sub>3</sub> was topically dropped on carotid artery to stimulate platelet aggregation. **D**, Mean values of fluorescent density of platelets deposited on the vessel wall 5 minutes after FeCl<sub>3</sub>. **E**, Time required reaching maximal vessel occlusion after FeCl<sub>3</sub> stimulation in mice treated with HNPs. **F**, Platelet LRP8 is not required for increasing leukocyte rolling. Platelets were depleted by intraperitoneal injection of rat anti-mouse CD41 monoclonal antibody (2  $\mu$ g per mouse) in WT and LRP8<sup>-/-</sup> mice 24 hours before intravenous injection of HNPs. Leukocyte rolling in carotid artery was monitored by intravital microscopy 4 hours after receiving HNPs. **G**, Expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in response to HNP stimulation (10  $\mu$ g/mL) for 4 hours by primary endothelial cells isolated from WT and LRP8<sup>-/-</sup> mice (n=5). **H**, Expression of CD11b in response to HNP stimulation (10  $\mu$ g/mL) for 1 hour by primary monocytes isolated from bone marrow of WT and LRP8<sup>-/-</sup> mice (n=5). \*P<0.05 vs WT under identical conditions, †P<0.05 vs unstained, ‡P<0.05 vs stained.

LRP is a large family consisting of several members.<sup>49</sup> To identify the specific phenotype of LRP in mediating the HNP-induced inflammation, we examined the effects of HNP8 in leukocyte rolling and platelet activation because only LRP8 is expressed in platelets.<sup>40</sup> HNPs alone induce an increase in CD62P expression that was dramatically reduced in the presence of recombinant human LRP8, indicating competitive inhibitory effects between the recombinant human LRP8 and HNPs in platelet activation. Our results also suggest that the HNP-induced platelet activation is independent of the thrombin pathway. The requirement of LRP8 signaling in mediating the HNP-induced inflammatory responses is further supported by using LRP8<sup>-/-</sup> mice, where reduced leukocyte rolling, platelet activation, and thrombus

formation are seen after stimulation with HNPs. The endothelial but not monocyte LRP8 appears to play a major role in mediating the HNP-induced cell-cell interaction, which is supported by our in vitro data following direct stimulation of HNPs on these cell types isolated from wild-type and LRP8<sup>-/-</sup> mice. Interestingly, our data also suggest that platelet LRP8 is not required for leukocyte adhesion, although platelets have been reported to contribute to leukocyte rolling.<sup>51,52</sup>

It is noteworthy that LRP8<sup>-/-</sup> mice do not experience hypercholesterolemia as LDLR knockout mice do<sup>53</sup>; thus, the HNP-induced platelet activation is likely independent of modification of lipid metabolism, although this issue is not addressed in the present study. Furthermore, LRP8<sup>-/-</sup> mice

have been shown to have decreased platelet activation and prolonged carotid artery occlusion time in response to ADP and thrombin stimulation, compared with littermate controls.<sup>54</sup> Our study examining platelet responses to HNPs in LRP8<sup>-/-</sup> may provide novel ligand-membrane protein interaction in addition to conventional ADP and thrombin pathways.

In summary, HNPs can initiate the interaction between the endothelium and monocytes, induce leukocyte transendothelial migration, and increase foam cell formation and platelet activation and aggregation through LRP8 signaling pathways. The role of HNPs in the pathogenesis of inflammatory cardiovascular diseases warrants further investigation to confirm their potential as a therapeutic target.

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### Disclosures

None.

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