Neutrophils Cast NETs in Atherosclerosis
Employing Peptidylarginine Deiminase as a Therapeutic Target

Yvonne Döring, Oliver Soehnlein, Christian Weber

Atherosclerosis, an inflammatory response in large arteries starting with a subclinical endothelial dysfunction, can turn into a life-threatening disease manifesting as myocardial infarction or stroke. Throughout the past decades, it has become apparent that atherosclerosis is not solely based on an imbalanced lipid metabolism but is mainly driven by a chronic inflammation of the vessel wall involving numerous cell types. Regardless of intensive research in the past 30 years, gold standard atherosclerosis-targeted therapies are still restricted to the control of primary risk factors such as hypertension and hyperlipidemia. Despite the proven efficacy of these drugs, cardiovascular diseases remain the leading cause of mortality in Western societies, underlining the need for new therapeutic approaches. In this issue of Circulation Research, Knight et al. show that the inhibition of peptidylarginine deiminase (PAD) by CI-amidine treatment prevents neutrophil extracellular trap (NET) formation and thereby decreases atherosclerotic lesion size and delays carotid artery thrombosis in Apoe−/− mice receiving a cholesterol-rich diet. Specific inhibition of NETosis was accompanied by a decrease in arterial interferon (IFN)-α expression, less neutrophil recruitment to the arterial wall, and a decline in intimal macrophages. Notably, all these findings could not be repeated when CI-amidine was administered into Apoe−/− mice treated with a neutrophil-depleting antibody or into mice lacking a functional type I interferon receptor. These data suggest an important role for NET formation and the subsequently instigated type I interferon response in atherogenesis. In contrast to studies on early lesion formation, a treatment regimen targeting advanced stages of atherosclerosis was without success, underscoring the importance of neutrophil-driven proatherosclerotic processes during atherogenesis only.

NETs and Type I Interferons in Cardiovascular Disease

Neutrophils have just recently emerged as important contributors to atherosclerosis. Depletion studies revealed that neutrophils primarily orchestrate the early stages of atherosclerosis by mechanisms involving the release of alarmins such as cathelicidin (LL37 in humans, CRAMP in mice), which promotes arterial recruitment of classical monocytes. In addition, NETs were identified in luminal location in murine and human atherosclerotic lesions, but their pathophysiological relevance in atherosclerosis remained initially unclear. The importance of these structures has been described in various diseases, including infection-associated thrombosis, small vessel vasculitis, systemic lupus erythematosus (SLE), and skin inflammation (Table). A current study has shown that NET fragments may serve as indicators of cardiovascular inflammation, while we could elucidate a mechanism of NET-driven atherogenesis involving the autoimmune activation of plasmacytoid dendritic cells. Mechanistically, complexes of self-DNA (presumably NET-borne DNA, but also self-DNA from dying cells) and neutrophil-derived granule proteins (eg, cathelicidin) stimulate plasmacytoid dendritic cells in the vessel wall, resulting in a strong type I interferon response, which drives atherogenesis. In line, the depletion of plasmacytoid dendritic cells has been shown to reduce plaque burden and type I interferon response. Moreover, NETs have also been implicated in direct activation and damage of endothelial cells (Figure). The importance of a type I interferon response in atherosclerosis has been reported earlier, when long-term treatment of Ldlr−/− mice with IFN-α was found to significantly increase atherosclerotic lesion development. Moreover, in human plaques, IFN-α was shown to trigger the activation of IFN-γ producing CD4+ T-cells, which kill stressed vascular smooth muscle cells in a tumor necrosis factor-related apoptosis-inducing ligand–dependent way, thereby decreasing plaque stability. The same group extended these findings by demonstrating that IFN-α upregulates Toll-like receptor 4 expression on and increases proinflammatory cytokine production of conventional dendritic cells (Figure). In addition, a central role for IFN-β in aggravating lesion formation in Apoe−/− and Ldlr−/− mice on high-fat diet has more recently been identified. Herein, IFN-β initiated a chemokine-dependent augmented endothelial cell activation and leukocyte attraction to atherosclerosis-prone sites along with an increased macrophage accumulation within plaques. Notably, patients with SLE exhibit chronically enhanced type I interferon levels and show a strong tendency to develop atherosclerosis. Generally, it is proposed that type I IFNs (and in particular IFN-α) alter the phenotype and function of endothelial progenitor cells resulting in endothelial dysfunction and defective vascular repair, thereby explaining an increased cardiovascular risk.

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From Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Germany (Y.D., O.S., C.W.); Academic Medical Center, Department of Pathology, Amsterdam University, the Netherlands (O.S.); and DZHK (German Centre for Cardiovascular Research), partner site Munich Heart Alliance, Germany (O.S., C.W.).
Correspondence to Drs Yvonne Döring, Oliver Soehnlein, or Christian Weber, Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Pettenkoferstr 9, 80356 Munich, Germany. E-mail ydoering@med.lmu.de, oliver.soehnlein@gmail.com, or chweber@med.lmu.de

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and premature atherosclerosis in SLE patients.\textsuperscript{25,26} Additionally, IFN-\(\alpha\) enhances the expression of scavenger receptor on monocytes/macrophages in SLE.\textsuperscript{27}

At later stages of atherosclerosis, a NET-instructed vicious circle involving platelets and neutrophils may lead to atherothrombosis. Heteromers of platelet-derived chemokines activate neutrophils to release NETs.\textsuperscript{28} These NETs may, in turn, further propagate platelet activation and induce thrombus formation as was shown in the models of venous thrombosis\textsuperscript{29,30} or arterial thrombosis.\textsuperscript{32}

### Reducing Hypercitrullination by PAD Inhibition—A Therapeutic Option in Chronic Diseases?

Although mechanisms underlying the pathophysiology of NET-driven atherogenesis and atherothrombosis have been elucidated, the present study provides a possible therapeutic link. Chromatin decondensation plays an important role in NET formation and is highly associated with histone citrullination. The latter is catalyzed by PAD4, a neutrophil-enriched nuclear enzyme. Li et al\textsuperscript{31} showed that PAD4 is crucial for NET formation and bacterial killing because PAD4-knockout mice were not able to generate NETs and were strongly susceptible to bacterial infections. The authors concluded that NET formation depends on histone hypercitrullination mediated via PAD4. Dysregulation of PAD activity has been described for several chronic (autoimmune) diseases, including rheumatoid arthritis (see the Table). The emergence of PAD4 as a possible therapeutic target has led to the development of specific inhibitors of which Cl-amidine, which was used in the present study, has been shown to be the most potent one.\textsuperscript{32}

Citrullinated synovial proteins, generated by PAD, are known to develop in murine collagen-induced arthritis, a model of inflammatory arthritis. Here, Cl-amidine treatment

### Table. Pathophysiological Relevance of Neutrophil Extracellular Traps (NETs) and Inhibition of NETosis in Disease

<table>
<thead>
<tr>
<th>Diseases With a Pathophysiological Relevance of NETs</th>
<th>Reference</th>
<th>PAD4 Inhibition Reduces Disease Severity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerosis</td>
<td>2,9,17</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>12–14</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Infection-associated thrombosis</td>
<td>10</td>
<td>Not investigated</td>
<td></td>
</tr>
<tr>
<td>Venous thrombosis</td>
<td>29,30,35</td>
<td>P\textsuperscript{AD4}−/−</td>
<td>35</td>
</tr>
<tr>
<td>Arterial thrombosis</td>
<td>2,12</td>
<td>Yes</td>
<td>2,12</td>
</tr>
<tr>
<td>Small vessel vasculitis</td>
<td>11</td>
<td>Not investigated</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>33</td>
<td>Yes</td>
<td>33</td>
</tr>
<tr>
<td>Colitis</td>
<td>34</td>
<td>Yes</td>
<td>34</td>
</tr>
<tr>
<td>Skin inflammation (psoriasis)</td>
<td>15</td>
<td>Not investigated</td>
<td></td>
</tr>
</tbody>
</table>

PAD indicates peptidylarginine deiminase.

![Figure. Pathophysiological alliance of neutrophil extracellular traps (NETs) and type I interferon response in atherogenesis.](https://circres.ahajournals.org/doi/fig/10.1161/CIRCRESAHA.113.303091)
resulted in a decreased generation of synovial citrulline, a diminished antibody response to citrullinated proteins and other autoantigens, and a substantially reduced arthritis severity. Furthermore, elevated PAD levels can also be measured in human and mouse colitis, and CI-amidine treatment reduces the clinical signs of colitis along with increased apoptosis of inflammatory cells in a dextran sulfate sodium mouse model. In addition, the previous work of Knight et al revealed enhanced NET formation in New Zealand mixed mice, a model of SLE driven by type I IFNs and characterized by accelerated vascular dysfunction and prothrombotic risk. In contrast, CI-amidine–treated New Zealand mixed mice in this study showed reduced NET formation, improved endothelial-dependent vasorelaxation, and prominently delayed time to arterial thrombosis. Interestingly, PAD4 and netting neutrophils also play an important role in deep vein thrombosis. The absence of NETosis in PAD4−/− knockout mice resulted in the formation of fewer thrombi, and PAD4 deficiency did not influence initial vessel wall activation and platelet–leukocyte adhesion. Moreover, PAD4−/− platelets were fully functional and able to produce platelet plugs comparable with wild-type platelets. The authors concluded that a low incidence of deep vein thrombosis in PAD4−/− mice is largely caused by a lack of PAD4 in neutrophils, resulting in decreased NET formation. Other functions of neutrophils seem not to be affected because PAD4−/− neutrophils interact properly with the vessel wall and are present in the rare thrombi that form in PAD4−/− veins.

Concluding Remarks

Taken together, the ill alliance of NET formation and a type I interferon response seems to be a pathophysiologically relevant concept in many chronic (autoimmune) diseases, and preventive or therapeutic targeting seems as a promising approach. Interfering with hypercitrullination by PAD inhibition, resulting in the inhibition of NETosis, has recently emerged as a therapeutic tool with surprisingly low side effects over long time of treatment and even at high doses. However, not many studies have been published to date, and all were in mice. Moreover, the efficacy of PAD4 inhibition and also the impact of NETosis seems to be disease-dependent. CI-amidine treatment reduced colitis both before and after the onset of disease, whereas athroprogression could not be prevented. One should also keep in mind that NETs represent an important first defense against invading bacteria and their (long-term) inhibition may come at the cost of debilitated host defense.

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Disclosures

None.

References


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