Editorial

Neutrophil Extracellular Traps as Therapeutic Targets for Inflammatory Disease

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Neutrophilic granulocytes, or neutrophils are the most abundant circulating white blood cell and constitute a first line of defense against pathogens. They are characterized by a large polymorphic lobular nucleus and the presence of many secretory granules in their cytoplasm. Neutrophils are essential for a functional host defense, as is evidenced by the occurrence of severe infections in individuals with decreased neutrophil numbers or neutrophil function. Neutrophils bind to and subsequently phagocytize bacteria, which are effectively destroyed by reactive oxygen species and digestive enzymes in the neutrophil's interior. Being secretory cells, neutrophils also release numerous factors with antimicrobial or immunomodulatory activity (Brinkmann *et al*., 2004; Soehnlein and Lindbom, 2010; Kolaczkowska and Kubes, 2013; Nauseef and Borregaard, 2014).

A decade ago, a further mode of the neutrophil's antimicrobial action was discovered, the release of its nuclear DNA content to the extracellular space (Brinkmann *et al*., 2004). The resulting extracellular DNA exists in complex with histones and with a variety of neutrophil effector proteins, e.g., Neutrophil Elastase (NE), Myeloperoxidase (MPO), LL37 and proteinase 3. This structure was named" neutrophil extracellular trap (NET)"and the process of NET-release was termed "NETosis" (Brinkmann *et al*., 2004; Soehnlein and Lindbom, 2010; Kolaczkowska and Kubes, 2013; Nauseef and Borregaard, 2014). A variety of pathogenic bacteria can induce the release of NETs from neutrophils and the predominant opinion is that NETs function to entrap bacteria, which are immobilized and subsequently killed by the antimicrobial factors that populate the NET (Brinkmann and Zychlinsky, 2012; Yipp and Kubes, 2013).

Interestingly, the potential to induce NET release in neutrophils is not restricted to pathogens. For example, activated platelets have also been shown to induce NETosis when bound to neutrophils (Clark *et al*., 2007; Caudrillier *et al*., 2012). This finding has important implications for our understanding of (auto-) immune-related disorders, as this would mean that NET formation can also be detrimental to the host organism. Indeed since their discovery, an adverse action of NET release has been implicated in many auto-immune and inflammatory diseases e.g., vasculitis, lupus, rheumatoid arthritis and atherosclerosis (Kolaczkowska and Kubes, 2013), but also in acute lung injury (Rossaint *et al*., 2014), venous thrombosis (Martinod and Wagner, 2014) and myocardial ischemia/reperfusion injury (Savchenko *et al*., 2014). In these disorders, NETs may both constitute a source of auto-antigens as well as being a propagator of inflammation. Thus, counteracting the formation of NETs, or their effects, would be an attractive approach for the prevention or treatment of these diseases.

A number of recent studies focused on the potential usefulness of NET-targeted approaches for the treatment of a number of disorders. One of these approaches is the inhibition of Peptidylarginine Deiminase 4 (PAD4), a histone-modifying enzyme that catalyzes the conversion of arginine to citrulline that was identified to play an important role in NET release by neutrophils (Wang *et al*., 2009). Mice with a genetic deletion of PAD4 showed impaired host defense during a model of bacterial necrotizing fasciitis, which was explained by the inability of PAD4$^{-/-}$ neutrophils to release the NETs needed to inactivate the pathogen (Li *et al*., 2010). In addition, the development of venous thrombosis, which was recently described to be a NET-driven pathologic event (von Brühl *et al*., 2012; Fuchs *et al*., 2012), was significantly reduced in PAD4$^{-/-}$ mice. The potential of PAD as a therapeutic target was further highlighted by a recently described synthetic inhibitor, termed Cl-amidine, which irreversibly inactivates PAD by covalently binding to a cysteine residue in the active site. A mouse model of lupus was shown to depend on the formation of NETs by neutrophils, as neutrophils derived from diseased mice showed increased NET formation and sera from diseased mice could induce NETosis in healthy control mice (Knight *et al*., 2013). Mice with lupus also showed measurable circulating antibodies directed against neutrophil intracellular components. Treatment of neutrophils with Cl-amidine resulted in decreased histone citrullination and reduced release of NETs from neutrophils. In addition, administration of Cl-amidine to mice with lupus reduced the deposition of MPO-containing immune complexes in the kidney and ameliorated endothelial dysfunction (Knight *et al*., 2013).

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Although these findings indicated that pharmacologic inhibition of PAD (and thus NET formation) might be an attractive target for the treatment of lupus, the study contained no data about the clinical severity of the lupus in mice without or with Cl-amidine treatment.

The potential benefit of a pharmacologic inhibition of PAD wasalso investigated in a mouse model of atherosclerosis (Knight *et al*., 2014). An increase of NET formation was observed in the sera of hyperlipidemic ApoE$^{-/-}$ mice during the administration of a high-fat diet and the sera of atherosclerotic mice were able to induce NETosis in isolated neutrophils. Advanced atherosclerotic lesions (after 11 weeks of diet) showed increased histologic staining of citrullinated histone 3, compared to those of 8 week oldApoE$^{-/-}$ mice. Repeated injection of Cl-amidine reduced atherosclerotic lesion formation in the aortic trees of ApoE$^{-/-}$ mice after 11 weeks of high-fat diet and decreased both neutrophil and macrophage content in the lesions. Interestingly, treatment with Cl-amidine appeared to be ineffective after depleting neutrophils in the ApoE$^{-/-}$ mice, indicating that the primary target of Cl-amidine was indeed the NET-forming neutrophil. However, it must be mentioned that neutrophil-depletion alone almost completely abrogated atherosclerotic lesion formation in those ApoE$^{-/-}$ mice (Knight *et al*., 2014), which would leave little room for a further prevention of atherosclerosis by Cl-amidine.

A different approach for preventing NET formation was investigated by (Rossaint *et al*., 2014) and colleagues in a mouse model of Ventilator-Induced Acute Lung Injury (VILI). Given that activated platelets can induce NET-formation, the authors investigated the effect of platelet-depletion on the extent of VILI in mice. Depletion of platelets resulted in improved clinical parameters and less circulating platelet-neutrophil complexes after VILI in mice. Moreover, a decreased content of NETs was observed in lung sections of platelet-depleted mice after VILI, compared with non-depleted mice. Concomitant integrin-mediated contact with the extracellular matrix of adhesion molecules along with physical binding to activated platelets was found to be necessary to induce NET release by neutrophils. In addition, the chemokines CCL5 and CXCL4 were identified as platelet-derived soluble factors required to stimulate the release of NETs (Rossaint *et al*., 2014). However, neither CCL5 nor CXCL4 alone was able to induce NET-formation, only the combination of CCL5 and CXCL4 triggered NETosis. Previous studies have demonstrated that these chemokines exist as a particularly potent heteromeric complex (von Hundelshausen *et al*., 2005) and a peptide, termed MKEY, that disrupts the CCL5-CXCL4 complex was shown to reduce atherosclerosis in ApoE$^{-/-}$ mice (Koenen *et al*., 2009). Interestingly, MKEY was also able to prevent NET-release by platelets. Administration of MKEY in mice during the course of VILI reduced the number of circulating platelet-neutrophil complexes, decreased alveolar neutrophil accumulation and prevented NET-formation, at least when applied early after the onset of VILI (Rossaint *et al*., 2014). The above findings are in agreement with those from previous studies, where platelet depletion or pharmacologic platelet inhibition protected mice from experimental lung injury (Caudrillier *et al*., 2012; Looney *et al*., 2009). Thus, alsofunctional inhibition of platelet-secreted chemokines might prevent NET formation and might be a beneficial therapeutic approach for the treatment of auto-immune or inflammatory diseases.

Taken together, NETs appear to be an attractive therapeutic target, as they are implicated in the pathophysiology of a variety of inflammatory disorders. However, most of the observations were done using experimental models in mice, whose neutrophil counts and physiology differ from those in humans (Kolaczkowska and Kubes, 2013; Nauseef and Borregaard, 2014). In addition, a recent study presented a patient with an almost complete deficiency in several neutrophil granule proteins (e.g., NE, LL37 and protease 3) and with impaired NET-formation, yet who suffered only from mild clinical immunodeficiency (Sørensen *et al*., 2014). Thus, clinical studies in humans are required to establish the potential benefits of preventing NET-release in neutrophils.

Conflict of Interest Statement

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