Neutrophil extracellular traps as potentiation mediators of reparative vascular regeneration

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Neutrophils mediate immune responses and inflammation via three distinct mechanisms: phagocytosis, degranulation, and neutrophil extracellular trap (NET) formation. The latter is a tightly regulated process mediated by the citrullinating enzyme peptidylarginine deiminase 4 (PAD4), wherein neutrophils release intricate webs of unraveled DNA, histones, and granular protein capable of trapping pathogens¹. Their immunomodulatory capacities, although critical to proper innate immune function, have been shown to contribute to sterile inflammation and consequent NET-associated host damage in disease. NET-induced vascular damage, resulting from increased endothelial cell (EC) apoptosis and activation, has been reported in cardiovascular and inflammatory autoimmune diseases². Contrastingly, Binet et al. report that, by promoting EC apoptosis, NETs could prove to be critical in the regression of pathological vessels in retinopathy, allowing for proper vascular remodeling³. This study showed that neutrophil infiltration and sterile inflammation (characteristic of the late stages of retinopathy) allow for NET release at sites of neovascularization and is facilitated by the senescence-associated secretory phenotype (SASP) of ECs.

Mouse pups were exposed to 75% oxygen from postnatal day 7 to 12 to induce vaso-obliteration, after which hypoxia-driven neovascularization occurs (Oxygen-Induced Retinopathy, OIR). Retinas were collected for transcriptome analysis by bulk and droplet-based single cell RNA sequencing. Gene-set variation analysis (GSVA) revealed enrichment in transcripts related to innate immune system activation, more specifically for neutrophil-associated genes, at time of maximal pathological angiogenesis. Neutrophil presence was further validated using fluorescent-activated cell sorting (FACS) and found adjacent to neovascular tufts, as observed by ex-vivo live confocal-imaging microscopy.

Immunostaining of retinas for citrullinated histone H3 (CitH3) and myeloperoxidase (MPO) was used to detect NETs, which were observed at neovascular tufts but absent in normoxic controls. Similar neutrophil infiltration and NET release was observed in retinas from proliferative diabetic retinopathy (PDR) patients. To identify potential NETosis stimuli, GSVA was used on single-cell transcriptomic data for gene sets related to cellular senescence and secretory processes. Transcripts related to cellular senescence, namely RAS signaling-associated genes, were found to be up-regulated in ECs and triggered SASP. Human Umbilical Vein Endothelial Cells (HUVECs) were rendered senescent through sustained RAS activation by transduction with a RAS-expressing plasmid retrovirus. Exposing neutrophils to senescent HUVECs and/or their conditioned media triggered significant DNA release. Stable isotope labeling using amino acids in cell culture (SILAC) was used to identify senescent EC secretome, which was primarily enriched in proteins related to immune pathways, namely IL-1b and CXCL1. Downregulation of both proteins through lentiviral transduction of short hairpin RNAs lead to significant reduction in NET release. In OIR mice, DNase I injection at time of maximal neovascularization and deficiency in myeloid-resident PAD4 both stalled clearance of senescent ECs, which allowed for persistence of pathological vessels, and restricted vascular regeneration.

This study thoroughly investigated the role of NETs at all stages of OIR, leading into functional impacts on vascular regeneration. Murine OIR presents three distinct stages (i.e. neovascularization, vascular remodeling, and vascular regression), characteristic of the pathology in humans, and thus serves as an appropriate model for investigating mechanisms of reparative vascular regeneration. SASP induction is observed in various cell types in PDR patients, leading to SASP-associated

cytokine release in the vitreous humour⁴. Binet et al. highlighted the role of SASP-associated cytokines (i.e. IL-1B and CXCL1) in neutrophil recruitment and NETosis and, more specifically, the contribution of EC SASP. Reciprocally, EC activation, brought on by NET-induced cytokine signalling, leads to expression of leukocyte adhesion molecules and increased EC-immune cell interactions⁵. Through induction of EC apoptosis, NETs were shown to be vital in vascular remodeling, as to allow for vascular regression of pathological vessels. Such clearance of senescent vessels and enhanced vascular repair has not been achieved with currently used anti-VEGF treatment, which only shows inhibition of pathological neovascularization⁴. Despite the therapeutic promise of NETs in retinopathy, the impact of sustained NETosis on retinal health and its potential to exacerbate vascular damage through microvascular occlusions has yet to be investigated. Still, this paper established a novel intrinsic reparative mechanism of NETs and contributes to further understanding on their dichotomous role in disease.

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