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Comment on Massena et al, page 2016

Hypoxia-recruited angiogenic neutrophils

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In this issue of *Blood*, Massena et al identify a novel CD49d^{+}CXCR4^{high}VEGFR1^{high} population of neutrophils that specifically migrate to sites of hypoxia and enhance angiogenesis.[1]

Tissue hypoxia results from an imbalance between oxygen supply and demand. Adaptation to this metabolic stress includes a switch to glycolytic metabolism and angiogenesis. Best studied in tumor biology, the hypoxic core of a tumor secretes proangiogenic vascular endothelial growth factor A (VEGF-A) to mitigate the metabolic burden and ultimately gain access to circulation. Inflammation and hypoxia are now appreciated to be inextricably linked, with hypoxia occurring at sites of inflammation and inflammation incited as a result of tissue hypoxia. In particular, neutrophils have long been known to migrate to ischemic tissues and mitigate the deleterious effects of reperfusion through lipid peroxidation.[2] Moreover, upon recruitment to inflamed tissues, neutrophils can themselves elicit tissue hypoxia through the action of the respiratory burst.[3] Myeloid cells, including neutrophils and monocytes, have been observed at sites of angiogenesis and are known to secrete VEGF-A and matrix metalloproteinase 9 (MMP9), likely contributing to angiogenesis.[4] However, the mechanisms of their recruitment and subsequent impact on neovascularization are largely unknown.

In the present study, Massena and colleagues used transgenic mice deficient in VEGF receptors 1 (VEGFR1; *Flt-1 tk*$^{-/-}$) and 2 (VEGFR2; *tsad*$^{-/-}$) and generated bone marrow chimeric mice to differentiate between neutrophil-dependent and endothelial-dependent responses to VEGF-A. The authors demonstrate that VEGFR1, expressed on a defined subset of circulating neutrophils, and VEGFR2, expressed on endothelia, are both required for efficient trafficking and diapedesis of neutrophils at sites of hypoxia.

Neutrophils responding to inflammatory stimuli typically are recruited to and migrate across endothelia following engagement of lymphocyte-associated antigen 1 (LFA-1; CD11a/CD18) or macrophage antigen 1 (Mac-1; CD11b/CD18) integrins. Massena and colleagues demonstrate that recruited neutrophils, migrating in response to VEGF-A, preferentially used very late antigen-4 (VLA-4) integrin (CD49d/CD29) over LFA-1 or Mac-1. Interestingly, CD29 (ITGB1) is known to be induced by hypoxia[5] in fibroblasts, which may partially explain the selective recruitment of this CD49d^{+} population of neutrophils to sites of hypoxia. The authors subsequently identified a subset of circulating neutrophils in both mice and humans that coexpresses CD49d, VEGFR1, and CXC chemokine receptor 4 (CXCR4). Stromal cell–derived factor-1 (SDF-1), the primary ligand for CXCR4, is a known hypoxia-dependent chemokine.[6] Chemokines, such as SDF-1, are known to bind glycosaminoglycans on the surface of endothelia to promote leukocyte homing and chemotaxis. Taken together, these findings suggest that a specific subpopulation of neutrophils present in circulation are primed to respond to and preferentially track to sites of localized hypoxia.

Under basal conditions, the majority of neutrophils reside in bone marrow. Following stimulation, neutrophils enter circulation and exhibit a short (8-16 hours) half-life. However, following recruitment to tissues, tumors, or sites of infection with subsequent exposure to inflammatory stimuli, such as granulocyte macrophage colony-stimulating factor,[7] neutrophil longevity is significantly extended compared with their circulating counterparts. To maintain homeostasis, aging neutrophils are thought to be efferocytosed by macrophages in the spleen, liver, or return to bone marrow. Given the relatively small percentage of circulating neutrophils identified in the current study that express this triple-positive phenotype, it is unclear what stage of differentiation such a population represents. The authors acknowledge that CD49d expression is downregulated following maturation, which may indicate an immature neutrophil population. Somewhat paradoxically, CXCR4 expression on neutrophils has been characterized as a retention signal for bone marrow neutrophils,[8] a regulator of stress granulopoiesis,[9] and a means to remove senescent neutrophils from circulation by returning to the bone marrow.[10] Whether this newly defined CD49d^{+} CXCR4^{high}VEGFR1^{high} subpopulation represents a less differentiated, activated, functionally distinct, or longer-lived neutrophil has yet to be established. In terms of functionality, the authors begin to address these questions, demonstrating that CD49d^{+} neutrophils exhibit enhanced chemokinesis in response to VEGF-A. Furthermore, in supplemental Figure 9, the authors demonstrate that human CD49d^{+} neutrophils exhibit a higher ability to degrade basement membrane than CD49d^{-} neutrophils, likely through the action of MMP9 release, supporting the suggestion that this newly identified triple-positive population of neutrophils is proangiogenic.

To summarize, the authors Massena et al describe a novel mechanism to support the role of a specific subpopulation of neutrophils that specifically traffic to sites of hypoxia. This process is dependent on neutrophil VEGFR1 and endothelial VEGFR2 expression. Recruited neutrophils coexpress CD49d, CXCR4, and VEGFR1 and use VLA-4 integrin to facilitate extravasation. This study sheds new light on the immune contribution to angiogenesis and has important ramifications for potential targeted therapies in the future.

Conflict-of-interest disclosure: The author declares no competing financial interests.

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● ● ● PLATELETS AND THROMBOPOIESIS

Comment on Stevenson et al, page 2027

Paris-Trousseau: evidence keeps pointing to *FLI1*

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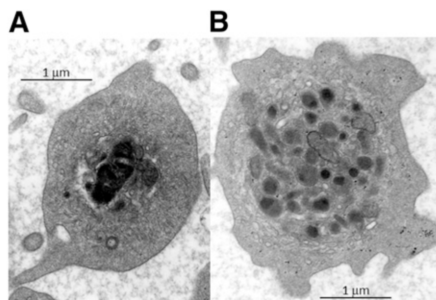
In this issue of *Blood*, Stevenson et al describe a family with a homozygous missense mutation in FLI1 that is associated with a platelet phenotype identical to the one observed in Paris-Trousseau syndrome, supporting existing evidence that FLI1 is directly involved in the mechanism of thrombocytopenia observed in this disease.[1]

Patients with a terminal deletion of the long arm of chromosome 11 exhibit several developmental abnormalities and distinctive facial features. This rare disorder is known as Jacobsen syndrome. A large majority of these individuals (>90%) have a bleeding diathesis called Paris-Trousseau thrombocytopenia characterized by autosomal dominant thrombocytopenia and a subpopulation of platelets that exhibit abnormal responses to thrombin and contain giant α-granules.[2] The thrombocytopenia can be severe but often resolves during adolescence. The bleeding phenotype is variable with reports of excessive bleeding even after normalization of the platelet counts, indicating an intrinsic platelet defect.[3] One of the characteristic findings in the bone marrow of patients with this disorder is the presence of 2 populations of megakaryocytes: 1 population of normal appearing megakaryocytes and the other one represented by micromegakaryocytes.



Electron micrographs of platelets from a patient homozygous for the *FLI1* c.970C>T mutation show large electron-dense fused α-granules (A) when compared with a normal platelet electron micrograph (B). See Figure 1E in the article by Stevenson et al that begins on page 2027.

The 11q deletions can span up to 16 megabases and 300 genes with several of them being potential candidates for the platelet phenotype. Among these, *FLI1* (a member of the ETS [E-twenty-six] family of transcription factors), is a natural candidate because it has been identified as a key regulator of megakaryopoiesis and its hemizygous deficiency has been previously associated with defective megakaryocyte development in a murine model.[4,5] Additionally, all individuals with Paris-Trousseau thrombocytopenia have deletions that are centromeric to *FLI1*. Interestingly, it has been proposed that transient monoallelic expression of FLI1 is critical for megakaryocyte development. Therefore, when the nonaffected allele is expressed, normal megakaryocyte development occurs; but when the deleted allele is (the one expected to be) expressed, megakaryocyte development is restricted, giving rise to a subpopulation of small megakaryocytes.[6] Recently, the role of FLI1 and other ETS transcription factors in megakaryopoiesis and platelet function have been underscored by reports of enrichment of *FLI1* mutations in individuals with functional platelet defects as well as the recent discovery that mutations in the gene *ETV6* also cause thrombocytopenia.[7-9]

In their Brief Report, Stevenson et al describe a family with 2 siblings, affected by a lifelong history of moderate thrombocytopenia and excessive bleeding with seemingly unaffected parents. The patients' platelets exhibit abnormal response to traditional agonists such as collagen and adenosine diphosphate, and platelet electron micrographs show large, fused electron-dense α-granules characteristic of Paris-Trousseau thrombocytopenia (see figure). Sequencing of *FLI1* revealed a homozygous missense mutation at position 970 (c.970C>T) that predicts an arginine to tryptophan substitution in the conserved DNA-binding domain of FLI1.[1]

Functional analysis of the c.970C>T mutation by a luciferase reporter assay demonstrated decreased transcriptional activity of known gene targets of FLI1, including *GP6*, *GP9*, and *ITGA2B*, with a concomitant reduction of the respective protein expression in the cell lines as well as in platelets of affected individuals, strongly indicating a role of the *FLI1* mutation in the platelet phenotype. As the parents do not show a platelet defect despite being heterozygotes for the c.970C>T mutation, the mechanisms that govern the autosomal dominant thrombocytopenia associated with *FLI1* hemizygous deletions are still unclear.

Overall, despite robust cumulative genetic and biological evidence pointing to the role of *FLI1* in Paris-Trousseau thrombocytopenia, the involvement of other genes in the 11q deletion syndrome could not be ruled out. The report by Stevenson et al, in which a point mutation in *FLI1* is associated with the same phenotype, adds strong evidence to the case for FLI1 as the main driver of the Paris-Trousseau thrombocytopenia.

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