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THROMBOSIS AND HEMOSTASIS

Comment on Zelaya et al, page 845

PAR2 biased signaling on the move

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In this issue of *Blood*, Zelaya et al show that macrophage infiltration into the lungs in a model of viral infection is mediated through biased signaling of protease-activated receptor 2 (PAR2) initiated by factor VIIa (FVIIa) in complex with tissue factor (TF).[1]

G protein–coupled receptors (GPCRs) initiate complex signaling cascades depending on the cellular contexts. The immediate downstream effectors are G proteins and arrestins. In the study, the role of signaling mediated by these 2 effectors was explored in a physiologic context using novel mouse model. Biased agonism refers to the functional selectivity of an agonist to signal through specific pathways.[2] Structural studies with a panel of agonists for β-adrenergic receptors reveal multiple conformations of active receptors.[3] The functional selectivity of agonists is likely tied to how they impact the structure of the receptors and subsequent interactions with partner proteins, including whether the signaling is mediated through G proteins or arrestin. In addition to the agonist itself, the local membrane environment, cofactors, and posttranslational modification can also influence which signaling pathways are initiated.[4]

Protease-activated receptors (PARs) are GPCRs that mediate biased signaling.[5] In recent years, there has been much work describing previously unrecognized cleavage sites in the N-terminus of PARs. These alternative cleavage sites result in the generation of a unique set of tethered ligands, each with their own biochemical properties.[6] This creates a natural mechanism to have a library of ligands that induce distinct conformations and potentially influence the interactions with intracellular signaling molecules. This rather straightforward hypothesis has largely been worked out in cellular models. These simplified models allow for mechanistic studies that provide a great level of detail and insights into the cellular machinery involved in mediating the signaling. However, they do not capture the complexities and contributions of the specific cellular environments and how they impact physiologic responses in vivo.

To fully understand protease-mediated signaling in vivo requires identifying the interplay between the (1) activating protease, (2) receptor, (3) cellular response, (4) specific signaling pathways, and (5) cells involved. Further, this all needs to be done with a physiologically relevant readout. Now that gene editing has become commonplace, cleavage-resistant mutations can be introduced into mice that allow these parameters to be assessed in vivo. Zelaya and colleagues used the synthetic viral RNA polyinosinic:polycytidylic acid (poly I:C) as an in vivo model of viral infection to examine the role of TF-FVIIa-PAR2 signaling on lung inflammation by measuring monocyte and macrophage recruitment to lung exudates.

TF-FVIIa can activate PAR2 by 2 mechanisms. The first is mediated by FXa in an endothelial protein C receptor–dependent manner. The second is independent of FXa but requires integrins. The initial experiments set out to determine whether the activating protease in this model was FXa or FVIIa. Floxed mice were used to determine that FVIIa is required, whereas FXa is dispensable. These data also show that monocytic cells were the primary source of the FVIIa. Importantly, the monocytic FVII is directly responsible for the phenotype in a cell-autonomous manner after acute stimulus.

The next set of experiments explored the role of PAR2 as the receptor mediating the phenotype. The authors took advantage of a mouse line expressing a PAR2 mutant (PAR2-R38E) that is resistant to cleavage by all proteases. These mice also have decreased leukocytes in the bronchoalveolar lavage (BAL), as seen in mice with FVII deleted in the macrophages. These experiments were complemented using PAR2 mutants resistant to FXa activation (PAR2-G37I). There was no impact on the cellular components of the BAL, which mimicked the mice with FX deleted.

In previous studies, Zelaya et al demonstrated that TF-FVIIa-integrin complexes regulate cell migration in a PAR2-dependent manner.[7,8] Here, the authors provide direct evidence that monocytes and macrophages from mice treated with poly(I:C) have increased migration in vitro. This correlates with the in vivo phenotype in Figures 1 and 2 in the article by Zelaya et al. Using their panel of mouse models and pharmacologic approaches, they show that it is dependent on TF and PAR2. Importantly, the increased migration was specific to fibrinogen, leading to the hypothesis that it is in response to the increased coagulation from the viral infection.

The authors next linked the TF-FVIIa–PAR2-dependent migration to specific signaling pathways. There are 2 primary mechanisms by which cells sense poly(I:C), extracellularly through toll-like

receptor 3 (TLR3) or intracellularly through retinoic acid–inducible gene and melanoma differentiation–associated protein 5. The latter ultimately induces mitochondrial antiviral signaling protein (MAVS)-dependent inflammatory signaling. The migration of isolated peritoneal macrophages was dependent on MAVS and monocytic FVII expression, but not TLR3. The authors also took advantage of TF antibodies that specifically block TF signaling, but not TF procoagulant activities, to demonstrate that the promigratory phenotype is dependent on TF-FVIIa signaling to extracellular signal–regulated kinase (ERK). The expression of proinflammatory markers induced by poly(I:C) was not affected by deletion of FVII nor by blocking the TF-FVIIa signaling complex, indicating that the phenotype is not merely due an induction of proinflammatory signaling.

PAR2, like many GPCRs, activates both G protein and arrestin signaling pathways. Having demonstrated that migration of macrophages was mediated by ERK signaling, the authors next demonstrated that this was downstream of PAR2-mediated arrestin signaling. To accomplish this, they generated a new mouse model that prevents PAR2 arrestin signaling by mutating a conserved protein kinase C phosphorylation site in the C-terminal tail of PAR2 (Ser365/Thr368). These mice expressing a PAR2 devoid of arrestin signaling have decreased leukocytes in the BAL. This perfectly phenocopies the macrophage-specific deletion of FVII and the cleavage-resistant PAR2 mice shown in Figures 1 and 2 in the article by Zelaya et al.

This long journey through multiple mutant mice meticulously demonstrates the in vivo consequences of PAR2 biased signaling through TF-FVIIa activation. The tools used by Zelaya and colleagues provide a roadmap for defining cell-specific biased signaling pathways in the complex environment in vivo. Going forward, it will be important to consider how proteases mediate specific signaling in the context of other disease models and in other tissues.

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CLINICAL TRIALS AND OBSERVATIONS

Comment on Böhm et al, page 872

Old is gold: frontline etoposide for pHLH

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In this issue of *Blood*, Böhm et al[1] provide contemporary real-world data on the use of etoposide for frontline therapy for pediatric patients with primary hemophagocytic lymphohistiocytosis (pHLH). The improved outcomes reported in this study relative to the HLH-94 and HLH-2004 trials provide a new benchmark to which novel, and often more expensive, treatment regimens should be compared.

HLH is syndrome initiated by common infectious, autoimmune, or malignant triggers. Abnormal cytotoxic lymphocyte function results in an inability to clear the trigger, with subsequent pathologic hyperinflammation. Diagnosis is based on criteria established by the Histiocyte Society in 2004. It includes fulfillment of 5 of 8 clinical and laboratory parameters or presence of a family history or genetic mutation consistent with HLH.[2] Historically, HLH has been divided into "primary" or "secondary" forms. Patients with inherited defects in genes regulating cytotoxicity or associated with inherited immunodeficiency or immune dysregulation syndromes are categorized as having pHLH. Patients who develop HLH in association with a strong immunologic stimulus but lacking known familial mutation are categorized as having secondary HLH. Irrespective of the category, failure to promptly initiate immune suppression is associated with high mortality rates. Allogeneic hematopoietic stem cell transplant (HSCT) is the only curative option for patients with pHLH, isolated central nervous system (CNS) HLH, or recurrent disease.

The current standard of care for pHLH, on the basis of the HLH-94 and HLH-2004 protocols, consists of dexamethasone- and etoposide-based therapy and results in a 5-year overall survival (OS) rate of 50% to 59%.[2-4] Alternative approaches, including anti-thymocyte globulin (ATG),[5] emapalumab (monoclonal antibody directed against interferon-gamma that the US Food and Drug Administration approved for refractory, recurrent, or progressive HLH),[6] alemtuzumab (monoclonal antibody against CD52),[7] and ruxolitinib (Janus kinase inhibitor),[8] are being studied and used as frontline therapy. To better understand the efficacy and role of these novel agents, Böhm et al set out to provide more contemporary data on the real-world performance of etoposide as a