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Advances in understanding the interplay between adaptive and innate immunity in experimental venous thrombus resolution

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Much has been learned over the last 2 decades about experimental venous thrombosis (VT) pathophysiology. The role of innate immune cells are central to both thrombogenesis and resolution[1, 2]. In murine VT, peak thrombus size is achieved 24–48 hours post VT induction [3, 4]. The process of VT resolution closely mimics sterile tissue injury: neutrophils infiltrate the vein wall and thrombus during early VT and monocytes/macrophages (Mos/Mφs) predominate throughout the course of prolonged thrombus resolution [5, 6], with the thrombus and vein wall, ultimately forming a collagen-rich fibrotic scar [7, 8]. While leukocyte inhibition has been identified as a strategy for thromboprophylaxis [9, 10], no strategy exists to harness the role of the leukocyte in thrombus clearance once a DVT has already occurred. Mos/Mφs have been implicated directly as playing predominant roles in thrombus clearance by several mechanisms: (1) as the major source of urokinase plasminogen activator (uPA), accelerating fibrinolysis (2) as the major source of matrix metalloproteases (predominantly MMP-2) in chronic thrombus resolution and (3) by directing the formation of endothelial lined channels during thrombus recanalization [1, 11-16]. Mos/Mφ phenotype (pro-healing or pro-inflammatory), can play a critical role in the timing and amount of recanalization [16]. Until recently, little was understood about the role, if any in adaptive immune cells played in thrombus clearance.

The mainstay of therapies for the clinical treatment of DVT rely on treating only the thrombotic component via inhibition of coagulation or exogenous fibrinolysis [17]. The incompletely understood cellular and molecular determinants of thrombus resolution

and vein wall injury, have limited additional therapeutic options to improve thrombus clearance and limit venous inflammation[18]. For this reason, despite a clear role of cell mediated inflammation in the pathogenesis of DVT, no immune targeted therapy exists today. In this review, we explore the newly described concept of adaptive and innate immune cell crosstalk in thrombus resolution, first described in a seminal publication from the Becker lab. In this manuscript, antigen independent activation of T effector memory cells is show to delay thrombus resolution via altering monocyte phenotype, a key determinant in thrombus recanalization [19]. A more nuanced understanding between T cells, Mos/Mφs, and the complex process of thrombus resolution is revealed in the highlighted study.

Profs Shahneh and colleagues, in a series of sophisticated experiments, elaborate on another previously understudied adaptive immune cell in venous thrombus resolution: the regulatory T cell (Treg).[20] Tregs, identified as CD4+, CD25+ cells with selective expression of Foxp3, function as immunomodulators, essential for preventing exuberant inflammation. Utilizing a diphtheria toxin FoxP3 depletion transgenic reporter mouse (Foxp3-DTR/eGFP) and Treg expansion utilizing IL-2/anti-IL-2 complexes, they demonstrate a direct correlation between thrombus Treg presence, post thrombotic monocyte recruitment, monocyte MMP activity and thrombus clearance. Transcriptomic Treg analysis revealed production of ostenectin (SPARC) transcripts, not previously described in Treg cells. In a series of experiments utilizing both *ex vivo* whole IVC thrombus culture and adoptive transfer of SPARC+ and – Tregs into *Rag*$^{-/-}$ mice they demonstrate two mechanisms by which Treg SPARC directly modulates the monocyte response: (1) via direct activation of monocyte MMP activity and (2) via alterations in monocyte differention, with SPARC- Tregs recruiting a higher proportion of Ly6C^{Hi} monocytes.

This work builds upon previously limited knowledge of the relative importance of T cells and monocyte cooperation in intravascular thrombus clearance, in addition to the monocyte functions described above. Global CD4+/CD8+ depletion has been previously linked to late impairment of thrombus clearance, characterized by marked reduction of CD68+ monocyte infiltrate, uPA and MMP9 activity.[21] Antigen independent activation of

T effector memory cells results in recruitment or differentiation of monocytes to Ly6C^{hi} subtype.[19] In these previous experiments, global T cell depletion strategies precluded the selective study of Tregs, and the role they may play in monocyte recruitment. From this most recent study, we can infer that the Treg is the "yin" to the T effector memory cell "yang:" shifting monocyte differentiation preferentially to Ly6C^{lo} subtype, a phenotype which has been shown to be essential to normal thrombus clearance.[22] To date, the exact mechanism by which the Ly6C^{lo}/Ly6C^{hi} balance directs clot lysis is somewhat unclear but likely involves MMP activation, chemokine/cytokine release and intrinsic uPA expression.[19, 22]

A technical issue of this study does bear discussion. The way to measure thrombus resolution is usually by at harvest thrombus weight, length, or both.[23] However, Shahneh and coworkers evaluate VT resolution in a slightly different way than others have, using sequential high resolution duplex ultrasound over time. It would be important to correlate this method with actual thrombus weights and lengths, as well as confirm with histological area measurements. They used a stenosis IVC model of thrombus generation that provides a narrow flow channel right below the renal veins. This is important because the stenosis model and its variance in thrombus development may confer a different impact on the vein wall and thrombus resolution as compared to a complete IVC stasis model, as well as compared with an electrolytic non-stenotic VT model.[23] Finally, the recurrent VT model was created with a vicryl tie, which is braided and has the potential to induce peri-venous inflammation independently from inflammation induced by the process of thrombosis. The issue of VT models are important because we really don't know in humans what the exact nidus or tipping point factor is in manifesting a subclinical to clinical DVT, outside of the fact that the valve sulcus is where the thrombi most likely begin, and is due to flow and local endothelial changes.[24] Nonetheless, this stenosis model is well accepted, and has been shown to be a neutrophil extracellular trap (NET) dependent model, in contrast to the complete IVC ligation model.[25] This factor may also play a role in how Treg lymphocytes interact with monocyte/macrophages and their role in thrombus resolution.

While over half of the top 10 grossing global pharmaceuticals currently belonging to immunomodulating therapies, no such immune therapy yet exists for DVT.[26] In the final set of experiments, Prof Shahneh and colleagues utilize IL-2 complex therapy to expand Tregs post thrombosis in an effort to more effectively clear the thrombus. This strategy is successful when precisely administered within a narrow therapeutic window: 8-12 days' post thrombosis. Earlier administration delays thrombus resolution and prior treatments provide no advantage when a second DVT is formed. Such therapies have been gaining interest translationally in other disease states. For example, CD4+ T cell inflammation is a pathological hallmark of abdominal aortic aneurysms and immunomodulation via injection of Tregs as a strategy is currently being pursued in the Aortic Aneurysm Repression with Mesenchymal Stem Cells (ARREST) phase 1 clinical trial [NCT02846883]. Prior to initiation in humans, important further details of the influence of Treg lymphocytes in the post thrombotic state must be elucidated. For example, *ex vivo* analysis of intrathrombus Tregs identified TGF-β as essential to SPARC production. In turn, SPARC production was the determinant of optimal monocyte differentiation and increased MMP activity. However, TGF-β and MMP activity (as is the result of SPARC production) are both implicated in vein wall fibrosis which can lead to suboptimal valvular function and post thrombotic symptoms.[14, 27] Therefore, a thorough evaluation of Treg and IL2 influence on vein wall injury and post-thrombotic fibrosis would represent a critical area of further investigation. Further studies, such as this series of investigations are essential to gain mechanistic insight into new immune-based therapeutic strategies for DVT.

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