Leukocytes and the natural history of deep vein thrombosis: current concepts and future directions

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Abstract

Observational studies have shown that inflammatory cells accumulate within the thrombus and surrounding vein wall during the natural history of venous thrombosis. More recent studies have begun to unravel the mechanisms that regulate this interaction and have confirmed that thrombosis and inflammation are intimately linked. This review outlines our current knowledge of the complex relationship between inflammatory cell activity and venous thrombosis and highlights new areas of research in this field. A better understanding of this relationship could lead to the development of novel therapeutic targets that inhibit thrombus formation or promote its resolution.

Keywords

venous thrombosis; inflammation; leukocytes; resolution

Introduction

Deep Vein Thrombosis (DVT) is a common condition affecting 1-2% of the population with an annual incidence of 1 in 500[1]. DVT can lead to death through pulmonary embolism (PE) and many patients subsequently suffer from venous reflux, which can lead to the post-thrombotic syndrome (PTS). This condition is characterised by pain, swelling and chronic leg ulceration. Around one quarter of patients develop PTS within 1 year of the episode of thrombosis[2]. DVT is therefore potentially fatal, can cause significant patient morbidity and has become an economic burden for health care services in the developed world.

Treatments for DVT such as anticoagulation prevent thrombus propagation and extension, but have little effect on existing thrombi, which resolve naturally through a process of organisation and vein recanalisation[3]. Rapid natural resolution is associated with less valvular damage, reduced venous hypertension, and fewer post thrombotic complications[4-6]. Treatments that remove thrombus rapidly such as thrombolysis and mechanical removal are, however, associated with increased morbidity, have significant haemorrhagic side effects and increase the risk of re-thrombosis[7]. An organised thrombus (usually those which have been present for more than 14 days), a history of stroke, or a recent operation, are contraindications to the use of thrombolytic therapy[8]. Alternative forms of treatment, which

prevent thrombosis or accelerate natural thrombus resolution without haemorrhagic side effects, would therefore be attractive. It is likely that these will only be developed from a better understanding of the mechanisms that regulate thrombus formation and its resolution.

There is increasing evidence that inflammatory processes and DVT are intimately linked. This review outlines our current understanding of the complex relationship between inflammatory cell activity and venous thrombosis and highlights new areas of research in this field.

Venous thrombus formation

A triad of vessel wall injury, venous stasis and blood hypercoagulability have historically been considered to predispose to venous thrombosis[9]. Venous thrombi arise in both the vein valve pockets and dilated sinuses of the lower limbs, are fibrin and red cell rich, and form on the surface of the endothelium[10]-[12]. They have a laminar structure consisting of layers of platelets, leukocytes and fibrin ('lines of Zahn') that encompass the main erythrocyte mass. This is unlike the amorphous structure of a 'blood clot', which consists predominantly of erythrocytes within a fine fibrin mesh[3].

Studies in the 1970s using radiolabeled leukocytes have shown uptake of white blood cells into venous thrombi[13], while accumulation of polymorphonuclear neutrophils (PMNs) on the abluminal side of the endothelium following occlusion of canine veins, first led to the speculation that 'white-cell' induced damage to the endothelium may be a contributing factor to venous thrombosis in man[14] (Fig 1i). Exposure of the collagen rich wall is said to lead to platelet aggregation and further leukocyte sequestration, which results in a nidus for thrombus propagation[15], [16]. C-reactive protein (CRP), an inflammatory marker, has been shown to increase in patients suffering with DVT[17]. Inflammation is therefore considered an important mechanism for venous thrombus formation.

Inflammation and the endothelium

The formation, propagation, and dissolution of venous thrombi represents a balance between coagulation and innate protective mechanisms, specifically the circulating inhibitors of coagulation (e.g. tissue factor pathway inhibitor, thrombomodulin, protein C, plasminogen activator inhibitors) and promoters of fibrinolysis (e.g. plasminogen activators)[9].

Endothelial 'microtears' containing leukocytes have been demonstrated by electron microscopy in the deep veins following hip surgery in dogs[18], leading to the suggestion that these may be the nidus for venous thrombus formation. Others have, however, found no major overt damage to the endothelium at sites of thrombosis in man[10]. Crushing the vein to cause endothelial damage does not lead to the formation of experimental venous thrombi[19] and scanning electron microscopy reveals minimal endothelial damage immediately after thrombus formation in the rat[20]. Disturbance of the endothelium by mechanical (e.g. stretch or surgery) or chemical means (e.g. sepsis) can, however, cause activation of the endothelium resulting in increased expression of procoagulant proteins such as tissue factor (TF)[21], cytokines and surface adhesion of molecules that promote leukocyte adhesion and initiate thrombosis[22] (Fig 1i). Genetic knock-out of the adhesion molecules E- and P-selectin results in reduced thrombus size, and this is associated with altered leukocyte accumulation in the surrounding vein wall[23]. Neutralising P-selectin glycoprotein ligand-1 (PSGL-1) also reduces local inflammation and thrombus size, and could be a potential treatment for the prevention of DVT in the future[24].

Circulating TF, in the form of microparticles (MPs) released by activated leukocytes, accumulate in areas of stasis such as the vein valve pockets[21]. Leukocyte MPs that express

PSGL-1, bind to P-selectin both on platelets and activated endothelial cells[25], [26], and this source of TF could sustain the production of thrombin on the forming thrombus, promoting its propagation[27]. The relative contribution of leukocyte MPs to venous thrombosis is, however, not clear, as adoptive transfer of bone marrow from mice expressing low levels of TF, into wild types does not inhibit thrombus formation[28]. It appears that the vessel wall is the most important source of TF that promotes thrombogenesis[28], and therefore activation of the endothelium may be pivotal in the inflammatory processes that lead to thrombosis.

Polymorphonuclear neutrophils (PMNs)

PMNs are found in large numbers within the early venous thrombus[20], [29]. Recent studies on the mechanisms of PMN recruitment to sites of sterile inflammation have revealed that intravascular danger signals, including the activation of the Nlrp3 inflammasome, generation of a chemokine gradient and release of formyl-peptide signals, act as a guide to these sites[30]. PMN adhesion to endothelium is mediated by interactions between the integrin $\alpha M\beta 2$ (Mac1) and its endothelial ligand intracellular adhesion molecule-1 (ICAM-1)[30] (Fig 1ii). Whether a similar mechanism is involved in PMN accumulation during venous thrombosis, remains to be determined though it appears that the thrombogenic effects of antiphospholipid antibodies are mediated in part by ICAM-1[31].

Aside from the formation of a nidus for thrombus propagation, recent data have emerged which suggest that recruited PMNs may initiate thrombosis through the formation of neutrophil extracellular traps (NETs)[32] (Fig 1iii). These extracellular DNA fibres, which comprise of histones and neutrophil antimicrobial proteins, form following a cell death programme in response to inflammatory stimuli (e.g. IL-8, reactive oxygen species) from cells in vitro[33-35], and have been linked to small vessel vasculitis[36] and pre-eclampsia[37]. DNA traps appear in the plasma and thrombus following induction of DVT in a baboon model and could provide a scaffold for thrombus formation, though their precise mechanism of action remains to be elucidated[32].

The role of PMNs in the natural history of the venous thrombus is however complex. Selective antibody depletion of PMNs in a rat stasis model, led to larger venous thrombi suggesting that PMNs are not required for thrombus formation, but may be important in removal of forming thrombus[38] (Fig 2). A similar finding was not, however, demonstrated in a murine model and CXCR2-dependent thrombus resolution appeared independent of the CXCR2 primary effector leukocyte, the PMN[39]. PMNs also show both pro and antifibrinolytic activity in an *ex vivo* thrombosis model[40]. These paradoxical results could, at least in part, be explained from functional heterogeneity in the neutrophil compartment. Circulating PMNs, express a variety of membrane receptors including CD11b, CD16, CXCR1, C5aR, FcγRII and TLR4. Differential expression of these receptors is known to confer functionally distinct roles for PMNs following LPS-induced inflammation in man; activated, differentiated PMNs mediate tissue damage[41]. Future studies into the role of the PMNs in venous thrombosis should consider the functional heterogeneity of this cell type. It is possible that given certain environmental cues, specific subsets of PMNs contribute to thrombus formation, while other distinct PMNs may be more important for its resolution.

Red blood cells, inflammation and thrombosis

The contribution of red blood cells (RBCs) to venous thrombosis remains poorly understood, despite their abundance in the early venous thrombus and their contribution to platelet rich arterial thrombi[42]. The cytoplasm of RBCs is rich in iron, which when released into the circulation is highly inflammatory because of its oxidative effects on the endothelium. It has been hypothesised that reactive oxygen species, produced by leukocytes and vessel wall at the nidus of thrombosis, oxidises haemoglobin in RBCs that become

trapped by cross-linked fibrin forming at this point. This results in the formation of methaemoglobin (metHb) containing Fe^{3+}. The release of Fe^{3+} leads to a cascade of RBC lysis that results in further endothelial dysfunction[43] and thrombus propagation (Fig 1iv). Natural antioxidants, such as hemeoxygenase-1 (HO-1)-derived carbon monoxide and superoxide dismutase, produced by cells within the vessel wall and thrombus, may limit these inflammatory effects. This putative mechanism by which RBCs may influence the development of venous thrombi is supported by the finding that propagation of venous thrombosis is enhanced in *HO-1*^{−/−} mice[44], while specific genetic variants (long GT-repeat alleles) in the HO-1 gene appear to confer increased risk of recurrent venous thromboembolism in man[45].

The inflammatory nature of the early, oxidised, RBC-rich thrombus has been exploited in the development of imaging methods to identify acute venous thrombi in man[46]. MetHb exerts paramagnetic properties resulting from its five unpaired electrons, which shortens the nuclear magnetic resonance longitudinal relaxation time (T_1) of the thrombus. It is therefore possible to accurately demonstrate contrast between young thrombi and surrounding tissues using MRI[47]. Advances in technology have allowed the rapid quantification of T_1 relaxation time, suitable for a clinical setting[48]. When scanning thrombus in the same patient, T_1 relaxation times are short in the young thrombus (800ms), but return to that of blood (1300ms) by six months, presumably because of the phagocytic action of cells that accumulate within the thrombus as it resolves and take up iron[49]. The in vivo characterisation of DVT in man, without the need for a contrast agent, could be used to predict clinical outcome following DVT and guide management. MRI identification of fresh thrombi, could help stratify patients into whom thrombolysis has the greatest potential. It could also act as a surrogate outcome measure when testing the efficacy of novel treatments in clinical trials. Validation of the relationship between T_1 relaxation times and thrombus structure by comparing MR images with histology is, however, still required.

Venous thrombus resolution

Natural thrombus resolution is characterised by tissue organisational processes (including neovascularisation) reminiscent of those seen during wound healing[3, 9]. Organisation ultimately leads to varying degrees of thrombus resolution with consequent recanalisation of the vein lumen[10, 11]. As in wound healing, there is a temporal change in the leukocyte composition of the thrombus both in terms of cell types and their numbers during resolution[20, 29] (Fig 2). The majority of the leukocytes in the early thrombus appear to be PMNs, which may have a role in vein wall remodeling[50], while macrophages predominate in the later stages of resolution and are likely to be the most important effector cells of this process[51-53]. The chemotactic proteins interleukin-8 (IL-8) and Monocyte chemotactic protein-1 (MCP-1) that are produced within the thrombus, may be important stimuli for the recruitment of these cells[54, 55]. Our studies of thrombosis in mice with severe combined immunodeficiency (SCID) suggest that lymphocytes have no role in either thrombus formation or its resolution[56].

Putative functions of inflammatory cells during thrombus resolution

The role of inflammation, and specifically leukocytes such as PMNs and the monocyte/ macrophage, during thrombus resolution is not completely understood (Fig2). Studies in mice lacking the *ets* transcription factor *Pu.1* suggest that inflammatory cell activity may not be a prerequisite for tissue repair[57]. Nevertheless, leukocytes comprise a significant proportion of the cells in the thrombus, and, interventions that lead to alterations in their accumulation lead to significant effects on subsequent thrombus resolution.

There is increased plasminogen activator content, both tissue-type (tPA) and urokinase-type (uPA) that co-localises with macrophages in thrombus formed in the rat[58], [59]. This has led to the speculation that fibrinolysis is important for thrombus resolution. Deletion of the tissue-type plasminogen activator gene (*tPA*$^{-/-}$*)*, however, has no effect on this process[51]. By contrast, deletion of the urokinase-type plasminogen activator gene (*uPA*$^{-/-}$*)*, prevents resolution and is associated with reduced macrophage numbers in the thrombus[51]. Adoptive transfer of wild-type bone marrow into *uPA*$^{-/-}$ mice rescues normal resolution[51, 60-62], while upregulation of uPA in macrophages enhances this process[56]. Urokinase - urokinase receptor (uPAR) interaction is commonly thought to be a major regulatory mechanism for cell migration. Plasmin generation at the cell surface activates other proteases such as matrix metalloproteinases (MMPs, including MMP-2 and MMP-9) that degrade extracellular matrix, facilitating cell migration[60-62]. These data lead us to speculate that monocyte-associated urokinase-activity is important for venous thrombus resolution.

Neovascular channels appear around the thrombus wall junction and within the thrombus as resolution proceeds[3], [10], [11]. Histological studies in rodent models suggest that these channels are derived from the vein wall[3], and they may also be derived from cells residing in the thrombus[63]. We have found that enhancing the levels of either VEGF alone, or simultaneously with a number of other angiogenic factors through upregulation of HIF1a within the thrombus, enhances its resolution[64-67]. These outcomes are linked to macrophage accumulation within the thrombus, which perhaps act as 'cellular chaperones' as recently described in the development of the vascular network[68].

Leukocyte signaling during venous thrombus resolution

Fibrin(ogen) and its degradation products are present in abundance in the thrombus[51], [69]. These molecules are able to stimulate recruitment and activation of leukocytes, to produce cytokines (TNFα and IL1β) and chemokines (IL8 and MCP1) in inflammatory settings[70], [71] and promote phagocytosis and cell migration in vitro[72]. Interaction of the fibrinogen γ chain residue 390-396 with Mac1 is thought to be an important pathway by which these molecules influence leukocyte activity[72]. Mononuclear cells may also interact with fibrinogen to produce chemokines through a TLR4 dependent mechanism[73]. Examination of thrombus formation and resolution in mice that carry mutations such as Fibγ 390-396A, may provide new insights into the role of fibrin(ogen) and its degradation products in the natural history of venous thrombi.

More recently, data have emerged that have provided new insights into the signaling mechanisms that regulate the functions of leukocytes during venous thrombus resolution. Deletion of toll-like receptor 9 gene (*TLR9*$^{-/-}$*)* impairs resolution assessed at days 2 and 8 after induction, despite an increase in the numbers of both PMNs and Mac2$^+$ macrophages in the thrombus[74]. These data suggest that TLR9 is important for leukocyte function during thrombus resolution. This effect was independent of MyD88 signalling (a major TLR signaling pathway), and was related to NOTCH ligand delta-like 4 pathways. *TLR9*$^{-/-}$ mice have reduced thrombus neovascularisation and decreased levels of the Th1 inflammatory cytokines IFNα, IL1α and IL2 in the vein wall, which appears to be important for venous thrombus resolution[52]. Further investigation is required to elucidate the role of TLR9 and MyD88 in later phases of thrombus resolution (beyond 8 days) and whether other TLRs are involved.

Mononuclear phagocytes

Macrophage accumulation within the thrombus is a hallmark of resolving thrombus in both man and experimental models[3], [9], [49]. Adoptive transfer experiments suggest that these cells are derived from the bone marrow (BM)[75], however macrophages that are not of BM origin,

may also have a role in thrombus resolution[51]. Macrophages that accumulate in the thrombus could be derived from cells that are resident within the vein wall, or may even arise from the 'splenic reservoir' that has recently been described[76]. The relative contribution of these sources for cells implicated in the resolution of venous thrombi remains unknown.

Although it is generally thought that the accumulation of macrophages is dependent on the recruitment of circulating monocytes, direct visualisation of these cells entering the thrombus has yet to be demonstrated. Renewal of certain resident mononuclear cells (microglia and Langerhans cells) in the steady state appears to be independent of BM[77, 78]. Adult LCs self renew in situ and proliferate during inflammation[79]. A demonstration that macrophages proliferate in the thrombus would change the current paradigm regarding the nature of their accumulation.

The function of mononuclear cells in the thrombus also remains to be fully elucidated. As they are phagocytic by definition, it seems reasonable to speculate that they contribute to the clearance of cells, nucleotides and matrix proteins within the thrombus. These cells may also promote fibrinolysis, are associated with angiogenesis and could regulate tissue remodeling[80-82] - processes seemingly beneficial for thrombus resolution. To add further complexity, both monocytes and macrophages consist of heterogeneous populations of cells, which appear to have distinct functions[83].

Circulating monocyte subsets can be distinguished on the basis of their expression of surface receptors[84]. Circulating 'inflammatory' monocytes, express Ly6C in the mouse, and are recruited into tissue where they undergo activation in a pathogen dependent response[84, 85]. In a model of spinal cord injury, recruitment of Ly6C$^+$ monocytes appears important for tissue repair[86] and these monocytes may also contribute to the fraction of myeloid derived suppressor cells (MDSCs) that promote tumour driven angiogenesis[87]. The contribution of Ly6C$^+$ monocytes in the formation and resolution of venous thrombosis has yet to be established, but our previous studies suggest that recruitment of this subset may be important for thrombus resolution. Impaired resolution occurs in *Ccr2$^{-/-}$* mice[52, 53], and CCR2 is required for the exit of Ly6C$^+$ monocytes from bone marrow[88].

Ly6C$^-$ monocytes exhibit long range crawling over the endothelium of both arteries and veins[89]. It has been hypothesised that they are involved in the surveying of the vasculature and sensing of tissue damage such as dying or infected cells[78, 90]. In a model of myocardial infarction, Ly6C$^+$ monocytes initially accumulate in the healing myocardium and may digest damaged tissue[81]. In a later reparative phase, Ly6C$^-$ monocytes predominate and are suggested to be involved in tissue repair by inducing myofibroblast accumulation, angiogenesis and collagen deposition[81]. The human equivalent of Ly6C$^-$ murine monocytes (CD14^{dim} monocytes), have recently been implicated in the pathogenesis of autoimmune diseases such as lupus, and respond to viruses and nucleic acid-containing immune complexes via a pro-in?ammatory TLR7-TLR8-MyD88-MEK pathway[90]. Whether these patrolling cells have a role in venous thrombosis (initiation or resolution) remains to be determined, although we speculate that their patrolling function makes them an ideal candidate for the detection of endothelial dysfunction and possible initiation of coagulation.

When monocytes enter tissue they differentiate into macrophages[91]. Based on *in vitro* studies, these cells have been tentatively classified by some into two main phenotypes: those that promote inflammatory responses (M1 or classically activated – expressing inflammatory mediators such as TNFα and NOS2); and those that attenuate inflammatory responses (M2a-c or alternatively activated – expressing arginase, mannose receptor [MR] and the transcription factors - Fizz1 and Ym1/2). Others consider dividing macrophage

populations based on their immunological or trophic roles in response to granulocyte/ macrophage CSF (also known as CSF2) or macrophage CSF (also known as CSF1) respectively[80]. A rigid classification of macrophages probably represents the extremes of a continuous spectrum and may be too simplistic as these cells may exhibit characteristics of more than one phenotype[81]. M2-like macrophages have, however, been reported in the healing myocardium and injured skeletal muscle, where they are considered to be involved in tissue repair and wound resolution[92, 93].

Clinical trials involving the therapeutic targeting of macrophages in other vascular diseases such as atherosclerosis has been largely unsuccessful[94]. This in part is because of a lack of understanding of their function[95]. Their roles in venous thrombosis require investigation, especially because different monocyte and macrophage phenotypes may have complimentary and contrasting functions[83]. This could be achieved through the use of functional reporter mice that express fluorescent proteins linked to cell specific genes[96]. We are currently developing these tools to examine cellular functions in thrombus resolution.

Inflammation is a central mechanism in both the genesis and resolution of venous thrombi. The temporal accumulation of leukocytes in the forming (PMNs) and resolving thrombus (macrophages) is part of a dynamic 'intravascular wound healing process' that results in either the early lysis of the thrombus, or its stabilisation and subsequent resolution. Enhancing our understanding of the cellular and molecular pathways that mediate sterile inflammation in the context of venous thrombosis, could lead to the development of novel therapeutic targets for: i) prevention of deep vein thrombosis in a manner that does not promote pathological bleeding; and ii) acceleration of natural thrombus resolution to reduce the incidence of post thrombotic complications. These may be achieved through developments in molecular and cellular imaging capable of delineating specific inflammatory processes that are currently on the horizon.

Acknowledgments

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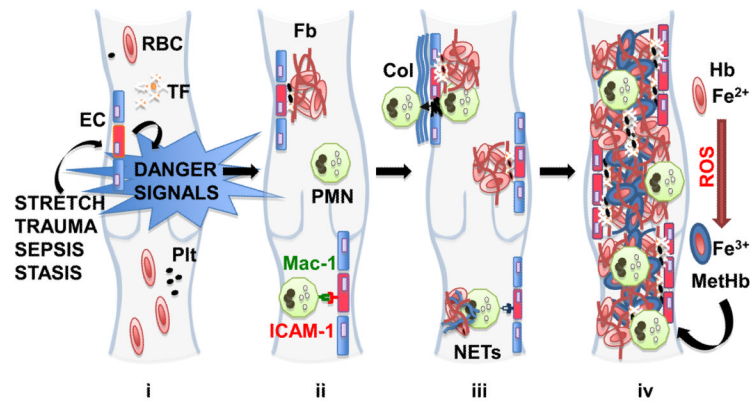
Fig 1. Inflammation in venous thrombogenesis

(i) Activation of the endothelium (EC - red cell) generates intravascular danger signals, which guide leukocytes to areas of inflammation and induces tissue factor (TF) production. (ii) Upregulation of adhesion molecules on endothelium mediate PMN recruitment while tissue factor generates thrombin, activating platelet (Plt) deposition and converting fibrinogen to cross-linked fibrin (Fb) that entraps the main red blood cell (RBC) mass. (iii) PMN accumulation in the subendothelial layer and subsequent exposure of collagen (Col) causes platelet aggregation and further PMN sequestration establishing a nidus for thrombus formation (iii). PMN apoptosis in response to inflammatory stimuli releases neutrophil extracellular traps (NETs, blue strands) that provide a scaffold for further RBC capture (iii). Reactive oxygen species (ROS), released from the vessel wall and leukocytes, oxidise haemoglobin (Hb) to methaemoglobin (MetHb, blue RBC). As trapped RBCs lyse, Fe3+ contained in metHb is released and induces further RBC lysis. This leads to a positive feedback loop with increased areas of endothelial dysfunction resulting in thrombus propagation (iv).
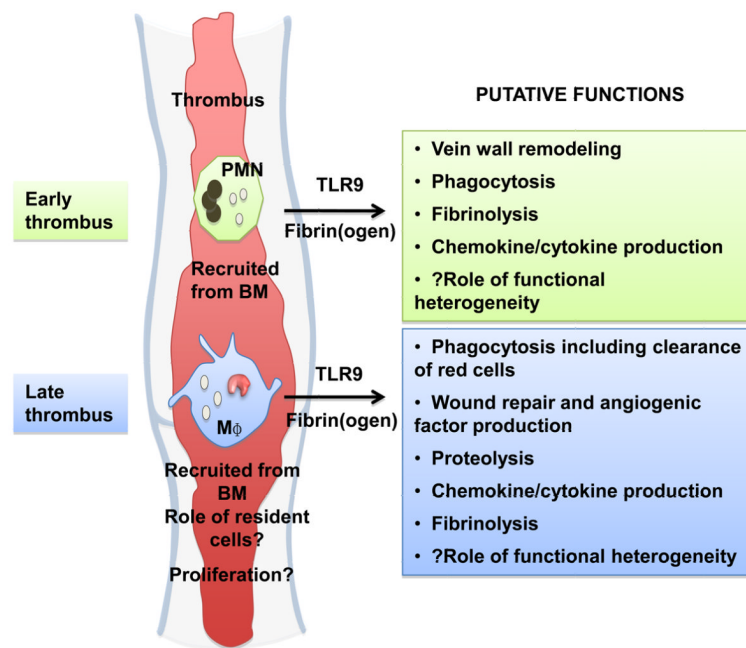
PUTATIVE FUNCTIONS

Fig 2. Putative functions of leukocytes in venous thrombus resolution
Leukocytes accumulate in the venous thrombus during its resolution. Polymorphonuclear neutrophils (PMN) predominate in the early stages of resolution with mononuclear phagocytes (Mϕ) predominating later. The origin of these cells appears to be from the bone marrow (BM), however the contribution of tissue resident macrophages or cells derived from the 'splenic reservoir', remains unknown. Leukocytes signal through a TLR9 mechanism and may be stimulated by fibrin(ogen) and its degradation products. They are speculated to have a number of functions important for thrombus resolution.