Innate Effector-Memory T-Cell Activation Regulates Post-Thrombotic Vein Wall Inflammation and Thrombus Resolution


Rationale: Immune cells play an important role during the generation and resolution of thrombosis. T cells are powerful regulators of immune and nonimmune cell function, however, their role in sterile inflammation in venous thrombosis has not been systematically examined.

Objective: This study investigated the recruitment, activation, and inflammatory activity of T cells in deep vein thrombosis and its consequences for venous thrombus resolution.

Methods and Results: CD4+ and CD8+ T cells infiltrate the thrombus and vein wall rapidly on deep vein thrombosis induction and remain in the tissue throughout the thrombus resolution. In the vein wall, recruited T cells largely consist of effector-memory T (TEM) cells. Using T-cell receptor transgenic reporter mice, we demonstrate that deep vein thrombosis–recruited TEM receive an immediate antigen-independent activation and produce IFN-γ (interferon) in situ. Mapping inflammatory conditions in the thrombotic vein, we identify a set of deep vein thrombosis upregulated cytokines and chemokines that synergize to induce antigen-independent IFN-γ production in CD4+ and CD8+ TEM cells. Reducing the number of TEM cells through a depletion recovery procedure, we show that intravenous TEM activation determines neutrophil and monocyte recruitment and delays thrombus neovascularization and resolution. Examining T-cell recruitment in human venous stasis, we show that superficial varicose veins preferentially contain activated memory T cells.

Conclusions: TEM orchestrate the inflammatory response in venous thrombosis affecting thrombus resolution.

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Key Words: chemokines ■ cytokines ■ immune system ■ lymphocytes ■ venous thrombosis

Deep vein thrombosis (DVT) is the most common type of venous thromboembolism. Patients with DVT are at risk of developing potentially life-threatening complications, such as pulmonary embolism, but also long-term complications, including post-thrombotic syndrome and chronic thromboembolic pulmonary hypertension. Current treatments for DVT focus on preventing clot expansion with anticoagulants and vasoactive drugs and by increasing venous blood flow through physical methods. However, anticoagulation cannot accelerate the natural thrombus resolution and reciprocally increases the risk of bleeding. Despite initial management of symptomatic acute events, patients remain at high risk for recurrence and predisposed to developing long-term complications. Transition of thromboembolic events to chronic disease is essentially caused by persistent inflammation resulting in delayed thrombus resolution, fibrosis, and vessel wall damage. T cells are powerful regulators of immune and nonimmune cell function, and their recruitment and activation represents a critical step in either resolution or dysregulation of inflammatory immune responses. Investigating their role in DVT in the inferior vena cava (IVC) stenosis model in mice, we observed that T cells rapidly infiltrate the vein wall on DVT induction and remain in the vein wall and thrombus throughout the thrombus resolution. Assessing their mode of activation and inflammatory activity in state-of-the-art reporter mice in combination with depletion procedures, we show that recruited effector-memory T cells (TEM) experience an immediate antigen-independent activation and produce IFN-γ (interferon) in situ. We further show that innate T-cell activation is critical for...
DVT because it determines neutrophil and monocyte recruitment and delays thrombus neovascularization and resolution.

Methods

Animals and Animal Experiments

C57BL/6, B6.129S4-Ijfgm1Mly/j (interferon-gamma reporter with endogenous poly A transcript), C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J (Nur77GFP),7 and B6.129S4-Tcratm1MomTg(TcrLCMV)327Sdz (45.1) (P14) mice were once intravenously injected with a monoclonal anti-CD3 antibody (30 µg/animal, clone 145-2C11).

To investigate the functional role of T cells in post-thrombotic inflammation, we induced DVT in mice, GFP (green fluorescent protein) is expressed under the control of the orphan nuclear receptor Nr4a1 (Nur77) transiently upregulated in T cells by antigen receptor stimulation with maximum expression between 12 and 24 hours.7 In addition, we measured the expression levels of the earliest surface activation marker CD69, upregulated either by antigen receptor stimulation10 or, independently, by inflammatory cytokines alone.11 To confirm simultaneous regulation of both markers (GFP and CD69), by antigen (T-cell receptor [TCR]-mediated) activation in vivo, an additional group of ligated Nur77GFP mice was injected with a T-cell stimulatory anti-CD3 monoclonal antibody.

Flow Cytometry and Flow-Assisted Cell Sorting

Flow cytometry procedures and antibodies used for flow cytometry are described in the Online Data Supplement. For polyclonal activation of T cells, mice were once intravenously injected with a monoclonal anti-CD3 antibody (30 µg/animal, clone 145-2C11).

Fluorescence-activated cell sorting with a BD FACS (fluorescence-activated cell scanning) Aria II. Cells were sorted with a BD FACs (fluorescence-activated cell scanning) Aria II.

Immunohistochemistry

IVC and Varizose veins were digested, stained, and fixed as outlined in the Material in Online Data Supplement. Results were photographed on a Olympus BX51 microscope. All images were quantified by morphometric analyses using image analysis software (Image ProPlus, version 7.0; Media Cybernetics).

Varicose Veins

Ethical approval was obtained from the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Germany. All individuals provided written informed consent before being included in the study.

Reverse Transcription Polymerase Chain Reaction

Tissue digestion procedures and antibodies used for flow cytometry are listed in the Online Data Supplement. Data were acquired on a BD LSRII and analyzed with FlowJo (version 9.6.4). Cells were sorted with a BD FACS (fluorescence-activated cell scanning) Aria II.

Varicose Veins

Ethical approval was obtained from the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Germany. All individuals provided written informed consent before being included in the study.

Results

Venous Blood Flow Restriction Recruits T Cells Into the Vein Wall

To investigate the functional role of T cells in post-thrombotic inflammation, we induced DVT in mice by 80% flow reduction in the IVC.4,5 On thrombogenesis, CD4+ and CD8+ T cells rapidly infiltrated the thrombotic vein wall and thrombus and remained in the tissue throughout the period of thrombus resolution (Figure 1A). In contrast, vena cava samples in sham-operated and untreated animals contained almost no T cells.

T cells exhibit distinct migration patterns depending on their differentiation. On the basis of differential expression of CD44 and CD62L, adhesion/homing receptors, IVC-recruited CD4+ and CD8+ T cells at day 2 mainly consisted of (CD44highCD62Llow) TEM (Figure 1B), reflecting the particular ability of these cells to enter inflamed tissue.9 In contrast, T-cell populations in day 2 thrombi showed a similar composition as in blood, suggesting nonselective inclusion at thrombus formation.

DVT-Recruited T_EM Cells Receive an Immediate Antigen-Independent Activation and Produce IFN-γ In Situ

T cells are obligatory participants of cognate immunity, and effector T cells develop on antigen-mediated activation within 2 to 4 days. To precisely follow their activation on recruitment into the thrombotic IVC, we induced DVT in Nur77GFP mice. In these mice, GFP (green fluorescent protein) is expressed under the control of the orphan nuclear receptor Nr4a1 (Nur77) transiently upregulated in T cells by antigen receptor stimulation with maximum expression between 12 and 24 hours. In addition, we measured the expression levels of the earliest surface activation marker CD69, upregulated either by antigen receptor stimulation or, independently, by inflammatory cytokines alone.

To confirm simultaneous regulation of both markers (GFP and CD69), by antigen (T-cell receptor [TCR]-mediated) activation in vivo, an additional group of ligated Nur77GFP mice was injected with a T-cell stimulatory anti-CD3 monoclonal antibody.

Activation of T cells by anti-CD3 injection upregulated GFP and CD69 expression of all T cells within 24 hours simultaneously, including those in the thrombotic vein wall. In contrast, T cells in the vein wall of thrombotic mice upregulated only their CD69 expression within 24 hours of recruitment (and became entirely CD69 positive afterward), whereas their GFP expression levels remained unaltered (Figure 2). GFP and CD69 expression remained unaltered in the blood and lymphoid organs of ligated compared with unligated reporter mice (Online Figure I), demonstrating that the activation occurs only in the vein. No changes in GFP levels were observed in T cells in thrombi. Supposedly reflecting their heterogeneous composition also, only few T cells in thrombi increased their CD69 expression at day 7, whereas at day 2 no signs of activation could be detected.

Although these observations suggested that T cells exclusively respond to inflammatory signals in the thrombotic IVC, they could not exclude an occasional activation of a few T cells by antigen, particularly at later time points. To gather further evidence whether or not antigens are involved in T-cell activation in the vein wall, we turned to TCR transgenic Nur77GFP reporter mice expressing an LCMV (lymphocyte choriomeningitis virus)-specific TCR (P14) in the CD8+ compartment. To generate virus-specific TEM cells, double transgenic T cells were adoptively transferred into B6 mice, and the recipients were infected with LCMV virus on transfer. After the mice had cleared the virus (at day 20 after infection) and P14 T cells had become TEM cells (Online Figure II), we followed their
recruitment and potential activation on DVT induction in the vein wall. Like TCR nontransgenic T cells, transgenic CD8+ TEM cells are readily recruited into thrombotic vein walls and upregulate their CD69 expression, ultimately demonstrating that T cells receive an immediate antigen-independent local activation in the early thrombotic vein (Online Figure II).

Inflammatory cytokines play a key role in post-thrombotic inflammation. Through its capacity to stimulate endothelial and vascular smooth muscle cells and prime inflammatory cytokine genes in monocytes, IFN-γ is of particular importance for inflammatory disease pathogenesis. IFN-γ produced by T cells, NK cells, and innate lymphoid cells has been shown to delay thrombus resolution by suppressing matrix metalloproteinase-9 (MMP-9) production in macrophages. We, therefore, investigated IFN-γ production in DVT-recruited intravenous T cells. To reliably detect live IFN-γ-expressing T cells in vivo, we performed IVC ligation in interferon-gamma reporter with endogenous poly A transcript mice. In these mice, eYFP (enhanced yellow fluorescent protein) expression faithfully reflects IFN-γ protein production (Online Figure II).

Collectively, these results demonstrate that T cells experience an immediate, antigen-independent activation in the thrombotic vein wall and contribute to post-thrombotic vascular inflammation.

DVT Induces an Upregulation of Cytokines/Chemokines in the Thrombotic Vein That Triggers IFN-γ Secretion in TEM Cells

Although GFP expression in Nur77GFP T cells strictly depends on cognate stimulation, CD69 expression is regulated by numerous inflammatory stimuli. We, therefore, set out to identify the inflammatory signals triggering intravenous T-cell activation on blood flow restriction. Consistent with rapid innate immune cell recruitment and activation, we observed an upregulation of mRNA encoding for several cytokines and chemokines in thrombotic veins compared with unaffected veins of sham-operated mice (Figure 3A). The most significantly upregulated genes included IL-1, IL-6, IL-18, the alarmin IL-33, and the T-cell–activating chemokines CCL3 (Macrophage inflammatory protein-1α [MIP-1α]) and CCL8.
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We also found IL-12, IL-27, and IFN-β mRNA to be increased. Expression of the IL-12 subunit p40 shared by IL-12 and IL-23 was representative of IL-12 regulation because IL-23 expression remained unaltered 24 and 48 hours after DVT. We subsequently analyzed DVT-induced cytokines and chemokines in regard to their ability to trigger IFN-γ production in isolated T EM cells. Flow-sorted (CD44 highCD62Llow) CD4+ and CD8+ TEM cells (Online Figure III) were, therefore, stimulated for 48 hours with recombinant IL-1β, IL-6, IL-12, IL-18, IL-27, IFN-β, CCL3, and CCL8 (termed hereafter venous cytokine cocktail), followed by intracellular analysis of IFN-γ production. Stimulation with the venous cytokine cocktail induced IFN-γ production in both CD4+ and CD8+ TEM, and the frequency of IFN-γ–secreting cells peaked after ≈24 hours (Online Figure III). In accordance with our observations in vivo, CD8+ TEM contained more IFN-γ producers compared with CD4+ TEM. Mapping the contribution of individual cytokines by measuring their individual and combined IFN-γ–stimulating potential and by removing them from the cocktail confirmed IL-12 and IL-18 as important IFN-γ inducers in CD4+ and CD8+ TEM (Figure 3B; Online Figure IV).17,18 Corroborating similar observations in CD8+ T cells, IFN-β also contributed to IFN-γ induction in CD4+ and CD8+ TEM.19 The strongest IFN-γ–stimulating potential was, however, observed with the complete venous cytokine cocktail, surpassing the prototypical IL-12/IL-18 combination.

Because our observations in Nur77GFP mice indicated that DVT-recruited T cells experience an antigen-independent activation in the venous tissue, we examined whether the venous cytokine cocktail affects GFP and CD69 expression in Nur77GFP TEM cells. In accordance with our in vivo observations, isolated Nur77GFP TEM cells upregulated CD69 but not GFP expression on stimulation with the venous cytokine cocktail (Online Figure III).

TEM Cells Regulate Post-Thrombotic Vein Wall Inflammation and Thrombus Resolution

Thrombus resolution is known to be an inflammatory process, and T cells are obligate effectors in inflammatory responses. Given their ability to rapidly generate inflammatory cytokines, tissue-infiltrating TEM cells are perfectly poised to orchestrate immediate action on sterile tissue injury. To understand the importance of innate TEM activation in DVT, we set out to compare the course of post-thrombotic sterile inflammation in the vein wall in the presence and absence of this population. TEM cells, however, cannot be selectively depleted, and their adoptive transfer into T-cell–deficient mice significantly alters their functional state.20,21 To generate mice that selectively lack a TEM compartment, we performed antibody-mediated T-cell depletion and let depleted mice refill their T-cell compartment with newly generated naive T cells, including regulatory T cells, for several days before ligation (Online Figure VI). Of note, T-cell depletion experiments were performed in age-matched mice of at least 30 weeks of age.

TEM deficiency did not affect initial thrombus sizes but significantly increased thrombus resolution leading to reduced thrombus sizes and weights in TEM-depleted mice at day 14 (Figure 4A). In accordance with Nosaka et al.,4 we also observed increased thrombus resolution in IFN-γ−/− mice (Online Figure 2. A, Flow cytometric analysis of GFP (green fluorescent protein) and CD69 expression in T cells in the blood and vein wall of inferior vena cava (IVC)–ligated Nur77GFP mice 24 h and 7 d after treatment. Ligated Nur77GFP mice additionally injected with an anti-CD3 mAb served as control. Data are representative of 3 independent experiments. Results are shown as means±SEM. **P≤0.005, ***P≤0.0005. B, Flow cytometric analysis of YFP (yellow fluorescent protein; IFN-γ)–expressing T cells in the blood and IVCs of ligated IFN-γ reporter mice 72 h after treatment. YFP gates set using a wild-type mouse as control. Left panels: representative flow cytometric plots shown from 1 of 3 comparable experiments, each including 3 to 5 mice. Right panels: percentages of YFP–expressing venous CD4+ and CD8+ T cells in the blood and IVCs preparations of ligated mice. Each symbol represents an individual mouse; horizontal lines indicate means. ***P≤0.0005, n=5 per group.)
Figure VII), emphasizing the notion, that IFN-γ production by TEM suppresses thrombus resolution.

Because thrombus resolution involves the plasmin and the MMP system, we measured intrathrombotic gene expression of tissue-type plasminogen activator, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 as well as MMP-2 and MMP-9. TEM deficiency did not affect urokinase-type plasminogen activator expression, slightly reduced tissue-type plasminogen activator, plasminogen activator inhibitor-1, and MMP-2 expression but increased MMP-9 expression in thrombi on day 14 (Figure 4B). To address potential differences in the cellular content, thrombi were also subjected to histochemical analysis. Using CD31 as marker for immature endothelial cells, which can be found on newly formed vessels lining neovascular channels, we observed higher numbers of CD31⁺ endothelial cells in thrombi of TEM-deficient mice at day 14 (Figure 4C), indicating enhanced intrathrombotic recanalization.

One of the key processes of acute inflammation consists in a defined sequence of leukocyte recruitment: an initial wave of polymorphonuclear neutrophils (PMN) precedes a second PMN-driven wave of inflammatory Ly6Chigh monocytes followed by local conversion of recruited monocytes to a reparatory Ly6Clow/neg state. To address the role of innate TEM activation in DVT-induced PMN and monocyte recruitment into the vein wall, we returned to Nur77 GFP mice. In monocytes, expression of the transcription factor Nur77 is regulated by growth factors and inflammatory stimuli and inversely coupled to their inflammatory state.

Impressively, TEM deficiency almost completely blunted PMN and inflammatory monocyte recruitment into the vein wall in the first few days on IVC ligation (Figure 5). Consistent with local differentiation and Nur77 upregulation in recruited monocytes, higher numbers of reparatory Nur77highLy6Clow/neg monocytes/macrophages were found in the IVCs of untreated compared with TEM-deficient mice within the first 7 days after DVT induction. Presumably, reflecting the advanced healing process, late thrombi in TEM-deficient mice also contained significantly lower numbers of cells expressing Mac2 (Figure 4C), a marker selectively upregulated in M2 macrophages.

TEM Recruitment Into Inflamed Venous Tissue Increases With Age

Because in both mice and humans, the overall frequency of memory T cells increases with age, we compared peripheral
TEM pool sizes and numbers of DVT-recruited intravenous TEM cells in thrombotic mice of different ages. Plotting both parameters against each other individually revealed that the number of DVT-recruited intravenous TEM nonlinearly increases with age and the size of the peripheral TEM population (Figure 6).

Comparing thrombus sizes in young (10 weeks) and old (>30 weeks) animals confirmed the previously published observation that older animals develop significantly larger venous thrombi.29,30 However, thrombi in young and old animals resolved with a similar speed (not shown), whereas TEM deficiency did not affect initial thrombus sizes but increased thrombus resolution (Figure 4A). Together these observations show that T cells do not influence thrombus formation but affect thrombus resolution by shaping the early inflammatory response.

Human Varicose Veins Predominantly Contain TEM Cells

Because our observations pertain to mice, the obvious question was raised as to how they translate to humans. Patients with DVT rarely require surgical assistance; consequently, human thrombotic veins remain largely inaccessible. Varicose veins, caused by reduced venous blood flow, affect a different part of the venous system but represent a DVT-associated disease, that, at least in its severe form, presents a risk factor for DVT.31 Similar to thrombotic deep veins, human varicose veins often contain a greater number of inflammatory cells, including T cells.12 To examine the impact of blood flow reduction on T-cell recruitment in the human venous system, we analyzed the composition of the T cell infiltrate in varicose veins compared with peripheral blood. Histochemical analysis of the walls of varicose great saphenous veins identified T cells in the tunica adventitia (Figure 7A). Flow cytometric analysis of their phenotype revealed a preferential expression of TEM markers and of the activation marker CD69 on a large part of these cells (Figure 7B). These observations show that inflamed human veins (similar to thrombotic veins in the model) attract T cells that become activated and remain in the tissue for an unknown time.

Discussion

T cells play a key role in initiating and perpetuating inflammation. In comparison to inflammatory processes associated with pathogen clearance, little is known about their role in inflammation under sterile settings, such as in cardiovascular diseases. Herein, we provide first evidence for a fundamental importance of T cells in DVT-induced venous inflammation and thrombus resolution and offer mechanistic insight into their intravascular activation mode and inflammatory activity.

The immune system is traditionally divided into innate and adaptive components with distinct roles and functions. T cells are major components of the adaptive immune system, and their activation and inflammatory activity depends on cognate stimulation by antigen-presenting cells. More recently, however, it has been recognized that antigen-experienced
CD4+ and CD8+ memory T cells are not limited to cognate antigen for their activation and produce signature cytokines in response to cytokine stimulation alone. In TEM cells, the ability to respond to inflammatory signals is coupled to an increased ability to migrate into extralymphoid tissue. In contrast to naive T cells and central memory T cells, which are largely restricted to secondary lymphoid organs and blood, TEM cells lack lymph node homing signals and recirculate between lymphoid organs and tissues. While showing some tissue selectivity in the steady state, TEM recruitment becomes a common feature in inflamed tissue.

Although antigen-independent T-cell cytokine production by TEM cells remains incompletely understood, it is expected to play a key role in inflammatory responses for many reasons. Most important, immediate retrieval of imprinted effector functions in tissue-patrolling cells omits the expense of antigen sensitization, clonal expansion and differentiation of antigen-specific populations. At the single-cell level, cytokine-induced cytokine production in T cells surpasses their antigen-induced cytokine production in both amount and duration. With respect to the total amount of cytokine formed, a small population of TEM cells may thus be more effective than a similar sized population of antigen-specific T cells. Because the size of the TEM population continually increases with antigen exposure, an ever-greater number of responder cells accumulates over the lifetime of an individual. Together innate cytokine production extends the role of the T cell from functioning exclusively as a component of the adaptive immune

**Figure 5.** Absence of effector-memory T (TEM) cells reduces the early inflammatory response in the inferior vena cava (IVC). Flow cytometric enumeration of polymorphonuclear neutrophils (PMN) and monocytes in the vein walls of untreated and TEM-depleted Nur77GFP mice 1 to 7 d after IVC ligation. Monocytes and neutrophils were gated as live, single, CD45+ cells. In separate stainings, PMN were further identified as Lin(CD3ε, B220, NK1.1)−CD11b+Ly6G− cells. Monocytes were stained and gated as Lin(CD3ε, B220, NK1.1, Ly6G)−CD11b+CD115+, further assessed for GFP (green fluorescent protein; Nur77) and Ly6C expression and subgated into Ly6ChighNur77− and Ly6Cneg/lowNur77+ cells. Results shown as mean±SEM, n≥4 to 5 per group and time point. **P≤0.005, ***P≤0.0005. Numbers indicate percentages of gated cells.

**Figure 6.** Deep vein thrombosis (DVT)–induced effector-memory T (TEM) recruitment increases with age/TEM frequency. Individual frequencies of TEM cells in spleens and thrombotic inferior vena cava (IVCs) of 8- to 50-wk-old male C57BL/6 mice 48 h after IVC ligation. Comparisons in panels were made using 2-way analysis of variance. A best-fit curve was calculated using Prism’s nonlinear regression analysis using the 1-site binding (hyperbola) model and plotted with 95% confidence bands.
response toward participating as an innate component in the early inflammatory response.

Tracking GFP regulation in Nur77GFP mice as a strict and sensitive reporter of TCR-mediated activation and CD69 expression as a marker of TCR or inflammation-induced activation, we provide in vivo evidence that T cells perceive an immediate innate activation in the thrombotic vein. Correspondingly, IVC-recruited T cells in the thrombus and vein wall begin to produce IFN-γ in situ. Mapping inflammatory conditions in the early thrombotic vein, we show that DVT induction upregulates a distinct set of cytokines and chemokines that synergize to induce IFN-γ secretion and upregulate CD69 but not GFP expression in CD4+ and CD8+ TEM cells from reporter mice. Interestingly, CD69 upregulation was not restricted to the IFN-γ–producing fraction of T EM cells, suggesting that nonproducers also become activated. Whether and how activated TEM cells that do not produce IFN-γ contribute to post-thrombotic inflammation in vein walls remains to be determined. Although we only mapped a small segment of the inflammatory course after DVT, our study suggests that the early phase after DVT preferentially drives a Th1 TEM-cell response. In contrast to cognate activation that triggers simultaneous production of numerous cytokines in effector T cells, cytokine-mediated stimulation recalls only hallmark signature cytokines in TEM cells. In line, TEM neither produced TNF-α nor GM-CSF in the presence of the venous cytokine cocktail. We also observed no IL-9, IL-17, or IL-22 production, suggesting that the early inflammatory conditions in the vein wall represented by the cytokine cocktail favor IFN-γ production by TEM cells. Increased recanalization of thrombi in the absence of TEM cells underlines the role of TEM-cell–derived IFN-γ as a similar increase in intrathrombotic angiogenesis has been observed in IFN-γ−/− mice.14

Concerning the functional state of monocytes, we show that monocytes/macrophages mainly consist of Ly6Chigh Nur77neg cells in the early thrombotic vein wall and turn to a Ly6ClowNur77+ reparatory phenotype from day 3 onward. Ly6Chigh monocyte immigration coincides with an increased IFN-γ production by immigrant TEM cells, suggesting that monocytes and T cells undergo a reciprocal stimulation. In the absence of TEM cells, the early PMN and inflammatory monocyte recruitment is greatly reduced, showing that T cells determine the extent of early inflammatory damage. In this context, IFN-γ signaling to Ly6Ch high monocytes and macrophages directly controls their capacity to secrete key mediators.22

However, whereas TEM-cell deficiency reduced the initial monocyte influx, it did not seem to influence their transition from inflammatory to reparatory phenotype. Interestingly, Ly6Cneg monocytes/macrophages produce anti-inflammatory cytokines some of which have been recently shown to inhibit antigen-independent T-cell IFN-γ secretion.38 It is, thus, tempting to speculate that acquisition of anti-inflammatory cytokine production by monocytes in the late thrombotic vein limits innate TEM-driven pathology.

Whether T-cell effector function in the thrombotic vein is restricted to IFN-γ secretion is not clear. IFN-γ has, however, been shown to represent a key factor in thrombolysis. Nosaka et al14 observed that IFN-γ accumulates in venous thrombi and suppresses thrombus resolution by inhibiting MMP-9 production by monocytes. Accordingly, thrombus
resolution was accelerated in Ifng−/− compared with wild-type mice, although thrombus formation remained unaffected. In line with a key role for TEM-cell-derived IFN-γ, we show here that MMP-9 expression and thrombus resolution increase in the absence of TEM cells, whereas initial thrombus sizes remain unaffected.

Both the number of DVT cases and the incidence of long-term complications increase dramatically with age.39,40 Among known age-dependent changes in the immune system, the dynamic of the memory T-cell compartment seems particularly striking. After an initial marked increase in the first decade of life, memory T-cell frequencies reach a plateau and remain stable throughout the adulthood. Eventually, after decades of homeostasis, frequencies of memory T cells in the blood increase again at ages over 65 years.41 Changes in T-cell subset composition in the last third of lifespan are accompanied by drastically reduced primary adaptive immune responsiveness.42 One of the most intriguing findings of our study is that the number of TEM cells infiltrating the thrombotic IVC wall increases with the size of the peripheral TEM pool. This observation points to a correlation between the age-dependent increase in circulating TEM cells and the age-dependent increase of long-term complications. Suggestive of an age-dependent role of innate TEM-cell activation in vascular inflammation increased circulating TEM cell numbers have been associated with aggravated atherosclerosis and coronary artery disease in humans and animal models.43 With regard to the role of T cells in venous inflammation in humans, we show here that human varicose veins preferentially contain TEM cells that in large parts express an activated phenotype. It seems that, similar to our observations in the murine model, sterile vascular inflammation in man induces memory T-cell recruitment and activation.

Decades of work explored the role of innate immune cells in thrombogenesis and thrombus resolution. Although representing key protagonists in the promotion or resolution of inflammation, adaptive immune cells received almost no attention in this context. This study has shed new light on the role of T cells in thrombosis by showing that TEM cells receive an immediate innate activation in the thrombotic vein and orchestrate the inflammatory response in venous thrombosis affecting thrombus resolution.

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Disclosures

None.

References

Venous thrombosis recruits TEM cells into the vein wall and thrombus.

What New Information Does This Article Contribute?

- Venous thrombosis recruits TEM cells into the vein wall and thrombus.
- Recruited TEM cells experience an immediate antigen-independent activation and produce IFN-γ (interferon) in situ.
- TEM cell-derived IFN-γ determines neutrophil and monocyte recruitment and delays thrombus neovascularization and resolution.
Innate Effector-Memory T-Cell Activation Regulates Post-Thrombotic Vein Wall Inflammation and Thrombus Resolution

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Detailed Methods

Mice

C57BL/6, B6.129S4-Ifng\textsuperscript{tn3.1Lky}/J (Jackson, Stock No. 017581, abbreviated GREAT),\textsuperscript{1} B6.129S7-Ifng\textsuperscript{m1Agt}/J (Jackson, Stock No. 003288, abbreviated IFN-\gamma\textsuperscript{-/-}), C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J (Jackson, Stock No. 018974, abbreviated Nur77\textsuperscript{GFP})\textsuperscript{2} and TCR327 CD45.1 mice\textsuperscript{3} (abbreviated P14) mice were bred in the central animal facility of the Johannes Gutenberg-University Mainz. P14 and Nur77\textsuperscript{GFP} mice were crossed to generate double transgenics (abbreviated P14-Nur77\textsuperscript{GFP}). All mice were on a C57BL/6 background. All animal procedures were carried out in accordance with relevant laws, permission of the Landesuntersuchungsamt Rheinland-Pfalz, Germany (authorization G12-1-087), current institutional guidelines and the Helsinki convention for the use and care of animals.

IVC flow restriction

IVC flow restriction was performed as previously described.\textsuperscript{4, 5} Briefly, mice were anesthetized by intraperitoneal injection of midazolam (5 mg/kg; Ratiopharm GmbH, Ulm, Germany), medetomidine (0.5 mg/kg; Pfizer Deutschland GmbH, Berlin, Germany), and fentanyl (0.05 mg/kg; Janssen-Cilag GmbH, Neuss, Germany). The animals were fixed on a custom built stage and maintained at physiological temperature. Upon median laparotomy the IVC was exposed by atraumatic surgery and a space holder (Asahi Fielder XT Guide Wire 0.014 in [0.36 mm]; Abbot Vascular, Abbot Park, USA) was positioned on the outside of the vessel. A permanent narrowing ligature (7.0 monofil polypropylene filament, Premilene; Braun, Melsungen, Germany) was placed below the left renal vein and the space holder wire removed to avoid complete vessel occlusion. In order to rule out endothelial injury as a trigger for venous thrombosis, all mice with IVC injury were excluded from further analysis. Median laparotomy was immediately closed by a 8.0 polypropylene suture (Ethicon). Sham operation consisted in preparation of the IVC and placement of the filament under the vessel without ligation. Mice received BORGAL (1 mg/mL sulfadoxin and 0.2 mg/mL trimethoprim) in the drinking water from the start of the experiment for 3 weeks. At different time points after IVC-ligation mice were anaesthetized using isoflurane and killed by exsanguination. Blood was collected by right ventricular puncture and the vessel system was flushed with PBS to remove the blood. The IVC was isolated caudal to the ligation around the thrombus. Upon removal of perivascular fat the vein was opened longitudinally and the thrombus dissected. Adhesive blood was flushed with PBS and the luminal surface gently scraped with a scalpel to remove attaching blood-born cells.
High frequency ultrasound (HFUS)
Ultrasound was performed with the Vevo 770 System and a 40 MHz mouse scanhead (VisualSonics). Mice were anesthetized by isoflurane inhalation. Anaesthesia was maintained by mask inhalation of isoflurane vaporized at 2-4% isoflurane mixed with 0.2 liters per minute (L/min) 100% O₂ concentration in the induction phase, and at 0.8-1.3% (0.5-1.5% isoflurane with 0.05-0.1 L/min 100% O₂) during observation. During observations the body temperature was kept at 37 degrees. Heart rates were monitored and kept at 500 beats per minute. Upon depilation of the abdomen and application of ultrasound transmission gel a long axis view was used to visualize the IVC, the ligation or the injury site and the formed thrombus. An optimal freeze-frame image was taken manually and the cross-sectional area of the clot traced using the Vevo 770 software to obtain the measurement. The length and width of clots were measured applying B-mode.

Immunohistochemistry
Sections were deparaffinized and quenched in 3% H₂O₂ (in methanol; Roth), followed by blocking of unspecific antigen binding sites using 10% normal serum (in PBS; Abcam) and heat-induced epitope retrieval (0.01 M citrate buffer, pH 6.0; 800 W for 10 min). Endothelial cells, smooth muscle cells and macrophages were visualized using monoclonal antibodies against murine CD31 (dilution, 1:20; Dianova), SMA (dilution, 1:800; Sigma) or Mac2 (dilution, 1:400; Biozol), respectively, followed by incubation with peroxidase-conjugated secondary antibodies (dilution, 1:1000; Molecular Probes), avidin biotin-link (VectorLabs) and peroxidase substrate (AEC; VectorLabs) until color development. Sections were briefly counterstained with Gill’s hematoxyline (Sigma) and mounted in ImmuMount (ThermoScientific). Results were photographed on a Olympus BX51 microscope. The number of positive cells was determined by automatically quantifying the immunopositive area per total vessel area. All morphometric analyses were performed using image analysis software (Image ProPlus, version 7.0; Media Cybernetics).

Morphometric analysis of venous thrombi
Inferior Vena cava (IVC) samples were harvested, fixed in 4% zinc formalin (Thermo Scientific) and subsequently embedded in paraffin (ParaPlast Plus®; Leica). For the morphometric analysis of venous thrombus size and composition, 5 µm-thick serial sections were cut through the injured IVC segment beginning from the site of ligation to its distal end. From those, 3 cross-sections, 200 µm apart were stained and analyzed and the results averaged.
Reverse Transcription Polymerase Chain Reaction

mRNA expression was analyzed by quantitative real-time RT-PCR using the CFX96™ Real-Time PCR System (Biorad, Hercules, CA, USA). Briefly, total RNA from mouse IVCs/thrombi was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. 0.5 µg of total RNA was utilized for real-time RT-PCR analysis using the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for TATA-box binding protein (TBP; Mm01229177_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-33 (Mm00505403_m1), IL-18 (Mm00434225_m1), IL-12p40 (Mm00434174_m1), IL-27 (Mm00461162_m1), IFN-β (Mm00439552_s1), CCL8 (Mm0129783_m1), CCL3 (Mm00441259_m1), uPA (Mm00447054_m1), tPA (Mm00476931_m1), PAI-1 (Mm00435858_m1), MMP-2 (Mm00439498_m1), MMP-9 (Mm00442991-m1) were purchased as probe and primer sets. The comparative ΔΔCt method was employed for relative mRNA quantification. Gene expression was normalized to the endogenous control, TBP mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of the control.

Flow cytometric analyses

Mouse IVCs were cut into small pieces and lyzed for 14 min at 37°C using a mixture of DNase I (50 µg/ml, Roche, Basel, Switzerland) and collagenase type II (1 mg/ml, Life Technologies, Carlsbad, CA, USA) for 14 min at 37°C. The digestion procedure did not affect any of the markers used (Online Figure VIII). The cell suspension was passed through a 70 µm cell strainer and incubated with unlabeled mAb against CD16/CD32 in order to block nonspecific Fc receptor-mediated binding of staining antibodies. All IVC single cell suspensions were stained for 30 min with APC-eFluor-780-labeled anti-mouse CD45 and Fixable Viability Dye eFluor506 (both eBioscience). T cells were identified with PE-Cy5-labeled anti-mouse TCRβ (BD Pharmingen) and eFluor 450-labeled anti-mouse CD4 (eBioscience). NK cells were gated out with PE-Cy7-labeled anti-mouse NK1.1 (Biolegend). Further phenotyping was performed with APC-labeled anti-mouse CD62L and PE-labeled anti-mouse CD44 (eBioscience). In reporter mice PE-labeled anti-mouse NK1.1 and PE-Cy7-labeled anti-mouse CD69 (eBioscience) were used. Regulatory T cells were stained with PE-labeled anti-mouse FoxP3 (eBioscience) upon fixation with Fixation/Permabilization solution (eBioscience) and gated out from analysis of T cells in Nur77GFP mice because of their two-fold higher basal GFP-expression. IFN-γ was stained with PE-labeled mAb (eBioscience). PMN were stained with APC-labeled anti-mouse CD11b (BD Pharmingen, San Diego, CA) and PE-labeled anti-mouse Ly6G (BD Pharmingen).
For lymphocyte exclusion T-, B- and NK cells were stained with PE-Cy7-labeled anti-B220, anti-CD3, anti-NK1.1 antibodies (all Biolegend). Monocytes in Nur77GFP mice were stained with APC-labeled anti-mouse CD11b, PE-labeled anti-mouse CD115 and PerCP-Cy5.5-labeled anti-mouse Ly6C (all Biolegend). T-, B-, NK cells and PMN were excluded by staining with PE-Cy7-labeled anti-B220, anti-CD3, anti-NK1.1 and Ly6G antibodies (eBioscience). A minimum of 100,000 events was acquired using the BD FACS Canto II or LSR II (Becton Dickinson, Heidelberg, Germany), and data analyzed with FlowJo 8.7.

**T cell depletion and isolation**

T cells were depleted by intraperitoneal injection of the anti-CD4 (clone: GK1.5) and anti-CD8 (clone: 2.43) mAbs (0.5 mg/animal) on three consecutive days. The mice were then allowed to recover from depletion for 7 days. Depletion was evaluated by flow cytometry in peripheral blood, isotype-injected or untreated animals served as control. For polyclonal activation of T cells mice were once intravenously injected with a monoclonal anti-CD3 antibody (30 µg/animal, clone 145-2C11).

**T cell stimulation and intracellular cytokine staining**

CD4+ and CD8+ T cell purification from single cell suspensions was performed by MACS in accordance with the manufacturer’s instructions (Miltenyi Biotec). CD4+ T cell preparations were subsequently depleted of regulatory T cells by anti-CD25 microbeads (Miltenyi Biotec). Effector memory T (TEM) cell subsets (CD62L−CD44+) were isolated from MACS-purified CD8+ and CD4+ T cells upon surface staining using a FACS Aria cell sorter (Becton Dickinson). Surface staining was performed using PE-Cy5-labeled anti-mouse TCRβ and eFluor 450-labeled anti-mouse CD4 APC-labeled anti-mouse CD62L and PE-labeled anti-mouse CD44. Sorted cells (average purity >98%) were immediately used for in vitro stimulation assays. Cytokines were purchased from R&D Systems (Minneapolis, MN, USA) and used at a final concentration of 10 ng/mL. For polyclonal activation T cells 24-well plates were pre-coated over night with 10 µg anti-CD3 at 4°C and 10^6 isolated T cells/well incubated on pre-coated plates at 37°C. 1 hour before the end of the stimulation brefeldin A (10 µg/ml; Sigma Aldrich) was added. After stimulation and surface staining, cells were fixed with the BD Cytofix/Cytoperm Kit as indicated in the manufacturer’s instructions (BD Pharmingen, San Diego, CA) and stained with FITC-labeled anti-mouse IFN-γ and PE-labeled anti-mouse TNF-α or matched isotype controls (eBioscience).

**LCMV**

LCMV strain WE (LCMV-WE) was propagated on L929 fibroblast cells at a low MOI. LCMV titers were determined in spleens 5 days after infection as described. 6 1 × 10^5 splenocytes from
P14-Nur77 mice were injected i.v. into B6 mice. Recipient mice were infected with 200 PFU LCMV i.v. and CD8+ T cell differentiation and activation analyzed 20 days later. As a positive control for T cell activation, cells were restimulated with $10^6$ M GP$_{33-41}$ peptide (KAVYNFATM) for 16h.

**Varicose veins**
Ethical approval was obtained from the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Germany. All individuals provided written informed consent before being included in the study. Specimens were taken from the proximal long saphenous vein at the level of the saphenofemoral junction by excision of the proximal 5-10 cm of vein prior to vein stripping.

Some samples were harvested, fixed in 4% zinc formalin (Thermo Scientific) and subsequently embedded in paraffin (ParaPlast Plus®; Leica).

Single cell suspensions for flow cytometry were generated by cutting specimens into small pieces and lysing them for 20 min at 37°C using a mixture of DNase I (50 µg/ml, Roche, Basel, Switzerland) and collagenase type II (1 mg/ml, Life Technologies, Carlsbad, CA, USA). The cell suspension was passed through a 70 µm cell strainer. Mononuclear cells were isolated from peripheral blood (PBMC) by mechanical Ficoll gradient centrifugation. Upon erythrocyte lysis, PBMC and venous single cell suspensions were stained for 30 min. with FITC-labeled anti-human CD45RA (Biolegend), Fixable Viability Dye eFluor506 (eBioscience), APC-labeled anti-human CD3 (BD Pharmingen), APC-eFluor 780-labeled anti-human CD4 (eBioscience), PE-Cy5-labeled anti-human CD62L and PE-labeled anti-human CD69 (eBioscience) monoclonal antibodies. The digestion procedure did not affect any of the markers used (Online Figure VI).

**Statistical analyses**
GraphPad Prism® data analysis software was used for statistical analysis (version 6.03; GraphPad Software Inc.). Normal distribution was checked using the D’Agostino & Pearson omnibus normality test. Quantitative data are presented as mean±SEM or median±interquartile range. Differences were tested by Student’s t test for unpaired means, if normal distributed, or Mann-Whitney test, if not. Differences between multiple groups were compared using 1-way ANOVA. Statistical significance was assumed if the P value <0.05.
Online Figures and Figure Legends

Online Figure I. DVT induction does not affect GFP and CD69 expression by T cells in lymphoid organs of Nur77GFP mice. (A) Antigen exposure simultaneously up-regulates GFP and CD69 expression in a fraction of lymph node T cells in Nur77GFP mice. Nur77GFP mice were either left untreated (Ctrl.) or immunized and challenged with irradiated tumor cells 24 h before analysis. (B) Comparative analysis of CD69 and GFP expression in lymph node and splenic T cells of untreated (Ctrl.) and ligated Nur77GFP mice 48 h after DVT induction (ns = non-significant).

Online Figure II. LCMV-specific P14-Nur77GFP CD8+ TEm cells are recruited and activated in the vein wall and thrombus. (A) Representative flow cytometric differentiation profiles of transgenic (CD45.1+) and non transgenic (CD45.1neg) T cells in the blood of B6 mice 20 days after adoptive transfer of naïve P14-Nur77GFP CD8+ T cells and LCMV infection. (B) Representative flow cytometric analysis of GFP and CD69 expression in splenic LCMV-specific P14-Nur77GFP CD8+ TEM cells re-isolated from adoptively transferred and LCMV-infected B6 mice 20 days after infection and either left unstimulated or stimulated with cognate (LCMV gp33) peptide for 16 h. C) Flow cytometric analysis of GFP and CD69 expression in P14-Nur77GFP CD8+ TEM cells in blood, vein walls and thrombi 2 and 7 days after ligation (22 and 27 days after infection). Data are representative of two independent experiments. Results are shown as mean±SEM * P≤0.05, ** P≤0.005, *** P≤0.0005.

Online Figure III. (A) YFP (IFN-γ) expression in “interferon-gamma reporter with endogenous polyA transcript” (GREAT) T cells. Th1 differentiation was induced for 96 h and YFP and intracellularly stained IFN-γ protein determined by flow cytometry after IL-12/IL-18 restimulation for 3 h. (B) Representative flow cytometric analysis of unsorted and sorted CD4+ TEM cells. (C) IFN-γ production kinetic measured by intracellular cytokine staining in sorted CD4+ and CD8+ TEM cells stimulated with the complete venous cytokine cocktail (each cytokine/chemokine 10 ng/ml). (D) CD69 but not GFP induction in Nur77GFP TEM cells by the venous cytokine/chemokine cocktail. FAC-sorted CD4+ and CD8+ Nur77GFP TEM cells were stimulated with the complete venous cytokine cocktail and CD69 and GFP expression analyzed by flow cytometry after 24 h. TEM cells stimulated with plate-bound anti-CD3 (1 µg/ml) served as control. Results in A, B and D representative of one out of three individual experiments.

Online Figure IV. Frequencies of IFN-γ-producing cells measured by intracellular cytokine staining in FAC-sorted CD8+ (A) or CD4+ (B) TEM cells, stimulated with the complete venous cytokine cocktail (up-regulated cytokines/chemokines shown in Figure 3) or indicated individual
cytokines/combinations (10 ng per ml each) for 24 h. Cumulative data from three individual experiments are shown as mean ± SEM.

**Online Figure V. CD62Lneg T cells in thrombi produce IFN-γ in situ.** Flow cytometric analysis of YFP (IFN-γ)-expressing T cells in the blood, spleen and thrombus of ligated IFN-γ reporter mice 72 h after treatment. Representative flow cytometric plots shown from 1 of 3 comparable experiments, each including 3-5 mice. Numbers indicate percentages of cells in quadrants.

**Online Figure VI. T cell depletion/recovery regimen.** Mice use in Experiments in Fig. 4 and Fig. 5 were depleted of CD4+ and CD8+ T cells by intraperitoneal injection of the anti-CD4 (clone: GK1.5) and anti-CD8 (clone: 2.43) mAbs (0.5 mg/animal each) on three consecutive days and subsequently allowed to recover from depletion for 7 days. Representative flow cytometric analyses of CD4+ T cells (left panel) in the blood and quantification of indicated CD4+ and CD8+ T cell subpopulations before and after treatment (n = 5 mice/group). Results are shown as mean±SEM. T_N: naïve (CD62L+CD44lo) T cells, T_CM: central memory (CD62L+CD44int−hi) T cells, T_EM: effector memory (CD62L−CD44hi) T cells and regulatory (CD4+Foxp3+) T cells (T_reg).

**Online Figure VII. Faster thrombus resolution in IFN-γ-deficient mice.** Sizes of thrombi (determined by high frequency ultrasound (HFUS)) in age-matched BL/6 and IFN-γ−/− mice at indicated time points after ligation, B6 n=5, IFN-γ−/− n= 6. All values represent mean ± SEM, *** P≤0.0005.

**Online Figure VIII. Short-term digestion used in the liberation of T cells from murine and human venous tissue does not affect T cell differentiation markers.** (A) CD45 and CD62L expression in murine vena cava tissue cells digested for 14 min with DNAse I / collagenase type II compared to undigested peripheral blood cells. (B) CD45 and CD62L expression in human PBMC digested for 20 min with DNAse I / collagenase type II compared to undigested cells.
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