Inflammation Modulates Murine Venous Thrombosis Resolution In Vivo
Assessment by Multimodal Fluorescence Molecular Imaging

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Objective—Assessment of thrombus inflammation in vivo could provide new insights into deep vein thrombosis (DVT) resolution. Here, we develop and evaluate 2 integrated fluorescence molecular-structural imaging strategies to quantify DVT-related inflammation and architecture and to assess the effect of thrombus inflammation on subsequent DVT resolution in vivo.

Methods and Results—Murine DVT was created with topical 5% FeCl₃ application to thigh or jugular veins (n=35). On day 3, mice received macrophage and matrix metalloproteinase activity fluorescence imaging agents. On day 4, integrated assessment of DVT inflammation and architecture was performed using confocal fluorescence intravital microscopy. Day 4 analyses showed robust relationships among in vivo thrombus macrophages, matrix metalloproteinase activity, and fluorescein isothiocyanate-dextran deposition (r=0.70; P<0.01). In a serial 2-time point study, mice with DVT underwent intravital microscopy at day 4 and day 6. Analyses revealed that the intensity of thrombus inflammation at day 4 predicted the magnitude of DVT resolution at day 6 (P<0.05). In a second approach, noninvasive fluorescence molecular tomography-computed tomography was used and detected macrophages within jugular DVT (P<0.05 versus sham controls).

Conclusion—Integrated fluorescence molecular-structural imaging demonstrates that the DVT-induced inflammatory response can be readily assessed in vivo and can inform the magnitude of thrombus resolution. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: deep vein thrombosis ■ inflammation ■ intravital microscopy ■ molecular imaging ■ post-thrombotic syndrome

Deep vein thrombosis (DVT) occurs in >350,000 patients annually in the United States. In addition to the acute risk of pulmonary embolism, up to 50% of all DVT patients will develop post-thrombotic syndrome (PTS), despite appropriate anticoagulant therapy. PTS symptoms include pain, heaviness, swelling, and cramping in the affected limb, which are frequently aggravated by standing and walking. In advanced cases, extensive varicose vein and acute venous ulcers can form. PTS occurs after incomplete DVT resolution and DVT-induced vein wall and valve damage, leading to venous hypertension, valvular incompetence, and chronic inflammation.

It is now established that venous thrombosis is an inflammatory process, with accumulating evidence demonstrating that inflammation orchestrates DVT resolution. At present, however, minimal data are available regarding in vivo spatiotemporal measures of leukocyte influx and inflammatory mediators and their subsequent effects on DVT resolution. Furthermore, it is not known whether the extent of inflammation can predict the magnitude of subsequent DVT resolution. Therefore, the development and application of in vivo inflammation molecular imaging approaches could provide new insights into DVT resolution and inform the future development of PTS.

In this experimental murine investigation, we harness multispectral and multimodal fluorescence molecular imaging to quantify the in vivo inflammatory response elicited by subacute DVT and then assess the impact of DVT inflammation on subsequent DVT resolution. We first validate and then use multiwavelength intravital microscopy (IVM) to simultaneously assess thrombus macrophages, matrix metalloproteinase (MMP) activity, and thrombus architecture.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.251983/-/DC1.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.112.251983
in resolving venous thrombi. We then apply serial, 2–time point IVM to investigate whether the initial intensity of the inflammatory response in FeCl₃-induced DVT predicts the magnitude of DVT resolution in vivo. In a translational study, we assess whether noninvasive integrated fluorescence molecular tomography (FMT)-computed tomography (CT) can detect inflammatory macrophages in subacute jugular DVT.

Methods

IVM imaging system and image analysis, IVM fluorescein isothiocyanate (FITC)-dextran time-course experiments, histological image analysis, correlational histopathology, and fluorescence microscopy are detailed in the online-only Data Supplement.

Experimental Venous Thrombosis

The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all animal studies. Male C57Bl/6 mice (n=35 total, 26 for thigh IVM studies and 9 for jugular FMT-CT/fluorescence reflectance imaging [FRI] studies) were obtained from Jackson Laboratory (Bar Harbor, ME) and were 12 to 16 weeks of age at the time of study (Figure 1). DVT was created using topical FeCl₃ to induce vessel wall injury and venous thrombosis in the thigh containing the distal femoral vein and the proximal saphenous vein. At day 0, mice were anesthetized with an intraperitoneal injection of ketamine and xylazine (80/12 mg/kg). For the thigh vein thrombosis model, a midline skin incision was made and vessels were gently exposed by blunt dissection. A 1x2 mm strip of number 1 filter paper (Whatman, Inc.) was soaked in 5% FeCl₃ and applied to the anterior surface of the femoral/saphenous vein for 5 minutes. After removal of the filter paper, the area was thoroughly rinsed with sterile saline, and the surgical incision was sutured closed.

A similar procedure was followed to create jugular vein thrombi. After blunt dissection of the jugular vein, a 1-mm-wide strip of filter paper soaked in 7.5% FeCl₃ was placed on the anterior surface of the vein for 3 minutes. The area was then rinsed with sterile saline, and the surgical incision was sutured closed. Sham surgery with topical saline was also performed on the contralateral jugular vein. In all mice, buprenorphine analgesia (0.05–0.1 mg/kg/12 hours) was administered 30 minutes before induction of anesthesia and every 12 hours after procedure.

Fluorescent Imaging Agents

Crosslinked dextran-coated magnetofluorescent nanoparticles were harnessed for imaging of macrophages. Briefly, crosslinked iron oxide (CLIO) nanoparticles were obtained from the Center for Systems Biology Chemistry Core at Massachusetts General Hospital. To 20 mg of CLIO (9.98 mg Fe/mL) was added 1 mg of AlexaFluor 555 (λ_max absorption=555 nm, λ_max emission=565 nm, AF555, Invitrogen, Carlsbad, CA) or, for FMT studies, 1 mg of VivoTag 680 (λ_max absorption=670 nm, λ_max emission=688 nm, VT680, PerkinElmer, Waltham, MA) in 200 µL dimethylsulfoxide. The reaction was allowed to proceed for 16 hours, at which time it was filtered through Sephadex G25 to yield the magnetofluorescent nanoparticles (CLIO-AF555, 3.6 mg Fe/mL, 1.8x10⁻⁴ M AF555, CLIO-VT680, 4.7 mg Fe/mL, 1.0x10⁻⁴ M VT680).

MMP activity within the thrombus was imaged using a protease-activatable near-infrared fluorescence reporter (MMPSense-680, excitation/emission 680/700 nm, PerkinElmer). MMPSense is optically silent upon injection and becomes highly fluorescent after gelatinase MMP-mediated activation. 

FITC-modified dextran (FITC-dextran, 0.5 mg in 100 µL PBS, λ_max absorption=490 nm, λ_max emission=520 nm, molecular weight 2 000 000, Sigma Chemical, St. Louis, MO), injected immediately before IVM, enabled vessel angiography and thrombus anatomical imaging. Tetramethylrhodamine-modified dextran (tetramethylrhodamine-dextran, 0.5 mg in 100 µL PBS, λ_max absorption=557 nm, λ_max emission=576 nm, molecular weight 2 000 000, Sigma Chemical) was also used in IVM validation experiments.

Confocal Fluorescence IVM

For fluorescence IVM studies, n=16 mice were injected on day 3 with the macrophage sensor CLIO-AF555 (10 mg Fe/kg). Twelve of the 16 CLIO-AF555–injected mice were additionally injected with MMPSense-680 (150 nmol/kg) dissolved in sterile PBS. Macrophage and MMP activity agents were injected 24 hours before imaging. Additional uninjected mice with DVT (n=3) served as IVM controls.

All mice were injected with the angiographic agent FITC-dextran as described above. To determine whether FITC-dextran would deposit into thrombi in vivo, a set of serial IVM time-course experiments was performed (see Methods in the online-only Data Supplement).

In the first IVM imaging study, mice underwent IVM once at day 4 (n=16, 10 injected with inflammation imaging agents and 6 uninjected), followed by euthanasia and histological assessment. In a second IVM imaging experiment, mice underwent serial IVM once on
day 4 and then once on day 6 (n=10, 6 for 2–time point inflammation imaging and 4 for FITC-dextran time-course imaging).

During the IVM procedure, the thighs of anesthetized mice were exposed, and the thrombus region was visually identified in the femoral/saphenous vein. The area was then bathed in sterile saline at room temperature, a coverglass was placed on the vessel surface, and the mouse was placed on the stage of a custom-built laser scanning intravital confocal microscope.21

See the online-only Data Supplement for details on the IVM imaging system and IVM image analysis methodology.

Fluorescence Reflectance Imaging
To image macroscopic DVT inflammation, n=9 mice with subacute jugular DVT underwent ex vivo FRI studies. At day 3 post-DVT, mice were injected via tail vein with the near-infrared macrophage reporter CLIO-VT680 (10 mg Fe/kg, Massachusetts General Hospital-Center for Systems Biology, n=6) or PBS (n=3). On day 4, mice were euthanized, and both jugular veins were carefully dissected, rinsed in sterile PBS, and placed on the stage of an FRI system (Kodak Image Station 4000MMPro, Carestream Health Inc, New Haven, CT). Optical images at 630/700 nm excitation/emission were collected with 42 x 42 micron spatial resolution and 8-second exposure time. For these images, the target-to-background ratio (TBR) was calculated as mean thrombus signal/mean sham signal, and therefore represents number-fold change over sham-operated veins.

Fluorescence Molecular Tomography-Computed Tomography
Before FRI, the 6 mice that received CLIO-VT680 underwent noninvasive in vivo FMT-CT imaging of jugular DVT on day 4. FMT was performed at 680 nm/700 nm excitation/emission wavelengths with 1-mm isotropic resolution using an FMT 2500 system (PerkinElmer), as previously described.22 Briefly, anesthetized mice were placed in a multimodal imaging cartridge that allows for coregistration of FMT and CT data through the use of multimodal fiducial markers. FMT imaging took ≈5 to 8 minutes. Immediately after FMT, CT angiography was performed to map the jugular veins (Inveon PET-CT, Siemens). The 3-dimensional CT spatial resolution was 80 µm. To visualize the lumen of jugular veins, mice were continuously infused with the radiopaque contrast agent Isovue-370 (Bracco Diagnostics) at 50 µL/min via the tail vein. CT imaging took ≈10 minutes. Reconstructed FMT and CT images were imported into OsiriX software to coregister and create fusion images. After FMT-CT, resected jugular veins were imaged by ex vivo FRI, as described above.

Statistical Analysis
All results are reported as mean±SD. Correlation coefficients were determined using linear regression and by calculating Pearson correlation coefficient (r). Differences between thrombus area and length at day 4 and day 6 were assessed using the paired Student t test. Differences in thrombus length and area measurements in the FITC-dextran time-course experiments were assessed using ANOVA. A P value <0.05 was considered statistically significant. To assess rate agreement for the IVM measurement of thrombus length, the intraclass correlation coefficient (1-way random effects model) was calculated using IBM SPSS Statistics version 21. All other statistical analyses were performed using Prism (v 5.0c, GraphPad, La Jolla, CA).

Results
In Vivo Assessment of Inflammation in Subacute DVT
Inflammatory macrophages and MMP activity in day 4 murine DVT were visualized via IVM and the spectrally distinct fluorescence imaging reporters CLIO-AF555 and MMPSense-680, respectively. Prior experimental work suggests the day 4 time point as the transition point of polymorphonuclear leukocyte to monocyte/macrophage influx.3,23 Vessel angiograms, obtained with FITC-dextran, provided high-resolution images of dark thrombi surrounded by bright luminal signal intensity (Figure 2). The average thrombus length was 1.09±0.27 mm. Macrophage and MMP activity IVM signals were detectable to a depth of ≈90 µm from the top of the vessel (Figure I in the online-only Data Supplement), with heterogeneous signal intensity noted throughout the thrombus. Elevated inflammatory signals were noted often along the thrombus edges (Figures 2 and 3).

The average macrophage and MMP activity TBR, calculated by encompassing the entire thrombus area, were 2.07±0.51 and 1.21±0.27, respectively. The CLIO-AF555 macrophage signal reflected the signal above the circulating background, which is typically low 24 hours after injection of 10 mg/kg CLIO.20 Although the MMP TBR was just slightly >1,
it was significantly greater than the TBR of uninjected mice in the 680-nm channel (TBR uninjected, 0.70±0.18; P=0.009), with a TBR <1.0 as a result of stronger light absorbance of thrombi.

Hematoxylin and eosin histopathology of the femoral/saphenous bundle demonstrated partially occlusive venous thrombi at the site of FeCl3 treatment in all animals (Figure II in the online-only Data Supplement). Fluorescence microscopy of CLIO-AF555 and MMPSense-680 signals corresponded with immunoreactive macrophages and MMP-9, consistent with prior investigations of CLIO18 and the MMP activity sensor19,20 (Figure 3A and 3B).

In Vivo Relationships Among Inflammation, FITC-Dextran, and Area in Resolving DVT

Substantial topographical heterogeneity of thrombus macrophages and MMP activity were noted along the thrombus length (Figures 2A). Quantitative IVM analyses revealed that whole-thrombus macrophage and MMP activity associated closely (r=0.84; P=0.001; Figure 4A). At day 4, measures of whole-thrombus inflammation and FITC-dextran deposition did not correlate with day 4 thrombus area (P>0.05; Figure 4D–4F).

Interestingly, 45 minutes after FITC-dextran injection, IVM images demonstrated augmented FITC-dextran signal within thrombus zones, often including thrombus–lumen edge interfaces (Figure 2A). To further understand whether the FITC signal within thrombi was a result of time-dependent uptake of FITC-dextran (as opposed to pores within the thrombus), a serial IVM time-course experiment was performed. Serial IVM images (Figure III in the online-only Data Supplement) demonstrated that thrombus regions enhanced over the 60-minute imaging period, with minimal thrombus interior signal noted at the 10-minute time point, in contrast to the 30-, 45-, and 60-minute time points.

In vivo measures of whole-thrombus inflammation (macrophage, MMP activity) were found to significantly associate with in vivo FITC deposition (r=0.75; P=0.001 and r=0.74; P=0.006, respectively; Figure 4B and 4C). This finding further suggested that FITC-dextran was depositing into the thrombus. This observation was confirmed on fluorescence microscopy (Figure 3C; Figures IV and V in the online-only Data Supplement).

To further understand mechanisms of FITC-dextran deposition into thrombi in vivo, a line-by-line segmental analysis of thrombus macrophages and FITC-dextran signal was performed across thrombi (Figure 1VA in the online-only Data Supplement). One-dimensional IVM line-by-line plots of the average signal intensity demonstrated a close relationship between thrombus macrophages and FITC-dextran deposition in vivo (average r=0.58±0.25; P=0.0001 for 8 thrombi analyzed). On ex vivo fluorescence microscopy analyses, FITC-dextran deposition and macrophage signals were also strongly correlated (average r=0.93±0.04; P<0.0001 for 3 thrombi analyzed; 103 426±59 229 pixels analyzed per thrombus; Figure IVB in the online-only Data Supplement). In addition, we evaluated the association between FITC-dextran deposition and von Willebrand Factor (vWF) thrombus staining, because vWF-positive areas may reflect thrombus neovessels and indicate thrombus resolution, as demonstrated previously.9,24,25

Analyses of microscopic sections (Figure 3C; Figure V in the online-only Data Supplement) demonstrated a good correlation between FITC-dextran deposition and vWF-positive area in thrombi (r=0.86; P=0.003). Fluorescence microscopy revealed that the FITC-dextran deposited into deeper thrombus zones (Figure 3C; Figures IV and V in the online-only Data Supplement), suggesting that diffusion did not exclusively govern FITC-dextran deposition into murine DVT.
To investigate whether the intensity of the DVT-associated inflammatory response could inform the future extent of DVT resolution, we performed a serial IVM 2-time point study in a separate group of mice (n=6). IVM was performed on day 4 for thrombus inflammation and architecture, and then again on day 6 for thrombus architecture. DVT resolution was then evaluated by quantifying the change in thrombus area and thrombus length from day 4 to day 6.

First, to confirm that the above IVM methodology could accurately measure thrombus length and area over both imaging sessions, IVM data from the time-course studies (n=4 mice) and additional mice (n=3) were analyzed. The time-course studies revealed that thrombus length and area measurements derived from FITC-dextran images were stable over the 60-minute IVM imaging session (Figure IIID and IIIE in the online-only Data Supplement). To evaluate the agreement of thrombus length measurement among 3 readers, the intra-class correlation coefficient was calculated from 12 measurements (4 per reader) of the coronal and sagittal IVM data sets (Figure IIIF–IIIG in the online-only Data Supplement) and was found to be 0.915.

IVM of day 4 femoral/saphenous DVT (Figure 5) demonstrated a range of whole-thrombus inflammation levels in both macrophages (TBR range, 1.47–3.18) and MMP activity (TBR range, 0.87–1.83). Serial IVM allowed calculation of DVT resolution, specifically the reduction in thrombus area and thrombus length during a 48-hour period from day 4 to day 6. Time-course experiments demonstrated that FITC-dextran from day 4 did not remain in the thrombus at day 6 (Figure IIC in the online-only Data Supplement) and therefore did not produce a day 6 thrombus measurement error. Quantification of day 4 to day 6 DVT resolution across all animals (Figure 6A and 6D) demonstrated a reduction in mean DVT length (1.21±0.22 mm to 0.941±0.27 mm; P=0.002) and a reduction in DVT area (0.18±0.07 mm^2 to 0.11±0.04 mm^2; P=0.002).

The IVM data demonstrated greater reductions in DVT burden in mice with higher degrees of DVT inflammation at day 4 (Figures 5 and 6). The intensity of the macrophage response (whole-thrombus macrophage TBR) correlated strongly with reductions in thrombus length and area (r=0.92 and r=0.93; Figure 6B and 6E, respectively; P<0.05). The intensity of the MMP activity (whole-thrombus MMP activity TBR) also correlated strongly with reductions in thrombus length (r=0.92; Figure 6C; P<0.05) and showed a trend with reduced thrombus area (r=0.54; Figure 6F; P>0.05).

**Noninvasive FMT-CT Imaging of Thrombus Inflammation**

Noninvasive imaging of thrombus macrophages may offer a translational approach to assess inflammation in DVT resolution and the future development of PTS. Therefore, integrated noninvasive FMT-CT\textsuperscript{22,26} was performed on day 4 mice with jugular DVT (Figure 7). To enable in vivo noninvasive fluorescence imaging, the CLIO-based macrophage sensor (CLIO-VT680, excitation/emission 670/688 nm) was red-shifted into the near-infrared window.\textsuperscript{14}
Noninvasive FMT-CT of the CLIO-VT680–injected mice demonstrated focal signal enhancement within thrombosed jugular veins, as localized by fiducial coregistered CT venograms showing the absence of intravascular contrast material within the thrombosed vein (Figure 7A). After FMT-CT, mice underwent macroscopic ex vivo FRI. FRI revealed that the near-infrared fluorescence signal from CLIO-VT680 in the DVT was significantly higher in the CLIO-treated group compared with a saline-treated group (CLIO-VT680 group n=6, thrombus TBR=1.65 ± 0.09 versus saline group n=3, thrombus TBR=1.04 ± 0.14, \( P < 0.006 \); Figure 7B and 7C).

Figure 5. Serial intravital microscopy micrographs at day 4 and day 6 of 2 deep vein thrombosis subjects. A, In a thrombus with relatively high inflammatory macrophage and matrix metalloproteinase (MMP) activity at day 4, the thrombus length and area were reduced by 37.0% and 50.5%, respectively, from day 4 to day 6. B, In a thrombus with relatively lower inflammatory macrophage and MMP, respectively, the thrombus length and area were reduced by 25.0% and 43.4%, respectively, from day 4 to day 6. Macrophage (MAC, green) and MMP activity (MMP, red) micrographs are overlaid with fluorescein isothiocyanate-dextran angiogram (ANGIO, grayscale). Yellow dashed lines demarcate the measured ROI of the thrombus at day 4 and day 6. Scale bar, 200 \( \mu \)m. TBR indicates target-to-background ratio.

Figure 6. Intensity of thrombus inflammation and the magnitude of subsequent deep vein thrombosis resolution after 48 hours. A, Thrombus length at day 4 and day 6, measured from intravital microscopy (IVM) fluorescein isothiocyanate (FITC)-dextran micrographs (\( P = 0.002 \)). Day 4 (B) macrophage accumulation and (C) matrix metalloproteinase (MMP) activity vs the percent decrease in thrombus length at day 6. D, Thrombus area at day 4 and day 6, measured from IVM FITC-dextran micrographs (\( P = 0.002 \)). Day 4 (E) macrophage accumulation and (F) MMP activity vs the percent decrease in thrombus area at day 6. \( * P < 0.05 \). NS indicates nonsignificant; CLIO, cross-linked iron oxide; TBR, target-to-background ratio.
Discussion

In this experimental murine venous thrombosis study, we investigated the in vivo spatiotemporal relationships of inflammatory cells and proteinase factors that mediate DVT resolution. We found that the inflammatory response can predict the extent of DVT resolution in an FeCl₃ DVT model. IVM and FMT, in conjunction with specialized macrophage and MMP activity fluorescent imaging reporters, enabled assessment of the inflammatory response in murine thigh and jugular DVT. Compared with histological studies of DVT resolution, multitarget IVM provided in vivo, whole-thrombus insights into venous thrombus inflammation, architecture, and resolution.

Recruited thrombus macrophages play a pivotal role in mediating DVT resolution. Landmark histological-based experimental studies demonstrated that deficiency of macrophages impaired DVT resolution⁸⁻¹⁰ and that heightened macrophage responses improved DVT resolution.⁶⁻²⁸ In day 4 FeCl₃-induced DVT, the present IVM data revealed substantial thrombus infiltration by macrophages that were heterogeneously distributed along the length of the thrombi. Architecturally, the in vivo thrombus resolution process, as measured by macrophage and MMP activity, demonstrated an outward-in pattern (Figures 2, 3, and 5), from the edges of the thrombus toward the middle, extending prior ex vivo observations.⁹ These findings suggest an active interface between perithrombus leukocytes and the thrombus itself.⁸⁻⁹ In addition, the developed IVM imaging method shows great potential for evaluating therapies that might increase monocyte entrance into the thrombus to accelerate resolution.⁹

Thrombus MMP activity was simultaneously imaged using a spectrally distinct MMP-activatable imaging agent that reports on MMP activity, in particular MMP-9 and MMP-2, in vivo.¹⁶⁻²⁰ MMPs, predominantly gelatinases MMP-2 and MMP-9, likely play an important role in thrombus resolution by promoting collagenolysis and neoangiogenesis, fibrinolysis, and facilitating leukocyte influx into thrombi.³¹ A robust correlation of whole-thrombus MMP activity and macrophages was found (r=0.84; P=0.001), supporting that macrophages are a predominant source of active gelatinases in day 4 subacute DVT.

FITC-dextran, a routine angiographic agent for IVM, was found to deposit into thrombi at time points after 30 minutes of injection. In vivo and histological assessments demonstrated an association of the FITC-dextran signal with macrophages,
MMP activity, and vWF+ regions within thrombi. Although vWF staining in thrombi was present, thrombus neovessels were not clearly visible, possibly because of the earlier day 4 time point used here, as opposed to later time points. Additional studies are required to determine whether specific mechanisms are responsible for FITC-dextran deposition into venous thrombi.

To provide new insights into the inflammatory response and subsequent magnitude of DVT resolution, serial IVM of femoral/saphenous DVT was performed in mice at day 4 and day 6 (Figures 5 and 6). We found that the intensity of day 4 inflammatory signals significantly predicted the magnitude of the reduction in DVT burden at day 6 (r values, 0.54–0.92). The overall IVM findings provide new in vivo evidence that local macrophage and MMP activity facilitate favorable DVT resolution, presumably by promoting plasminogen activation and fibrinolysis, as well as neoendothelialization and possibly early collagenolysis.8,80 In future studies, the developed IVM methodology can be used to evaluate the in vivo effects of inflammation-modulating therapies (genetic and pharmacological) on DVT resolution.

FMT-CT imaging enabled the detection of macrophages in subacute DVT (Figure 7) and thus offers a noninvasive molecular-structural approach to track the DVT inflammatory response. In addition, because the CLIO particle can also serve as a magnetic resonance imaging macrophage imaging agent,22 noninvasive magnetic resonance imaging of macrophages in DVT may also be possible (magnetic resonance imaging was not used in this study because of confounding signal changes from FeCl₃).33 Because CLIO-like superparamagentic nanoparticles have already been tested in patients, translational extension of this work may enable molecular imaging of inflammation in DVT at 2 time points: early after DVT (<1 month), where impaired inflammation is likely to identify poorly resolving thrombi and therefore patients at risk for PTS;4 and later after DVT (>3 months), where persistent inflammation indicates a high risk for PTS.35,36 Limitations are present in this study. Later time points beyond day 6 DVT were not amenable to IVM because of superficial scar formation induced by FeCl₃, which limited light penetration and thus IVM assessment of deeper vessels. Alternative murine thrombosis models such as ligation of venous stasis may overcome this limitation and be more clinically relevant but require greater technical development in the smaller femoral/saphenous vessels. Because thrombi extended deeper than 100 μm, confocal IVM did not permit full thrombus volume measurement; multiphoton IVM approaches may allow deeper imaging. Regarding delivery of the imaging agents to the thrombus, if restricted diffusion (ie, limited thrombus permeability) is present, it is possible that the CLIO and MMP signals may not reflect the full extent of macrophages and MMP activity, respectively, within the thrombus. Of note, MMP activity, although beneficial for thrombus resolution, may detrimentally affect the vein wall.37 The ability to resolve the vein wall from the thrombus was not feasible in the current IVM confocal imaging study but may become feasible using higher-resolution multiphoton imaging approaches coupled with motion compensation methods.

Acknowledgments
We acknowledge Yoshiko Iwamoto, BS, for assistance with histology; Peter Waterman, BS, and Brett Marrelli, BS, for assistance with fluorescence molecular tomography-computed tomography imaging and data analyses; and Tetsuya Harai, MD, PhD, and Jie Cui, MD, for assistance with area and length measurements.

Sources of Funding
This work was supported by American Heart Association Scientist Development grant number 0830352N (to F.A.J.), Howard Hughes Medical Institute Career Development Award (to F.A.J.), National Institutes of Health (NIH) R01 HL 108229-01A1 (to F.A.J.), NIH T32 HL07208 (to C.M.R.), and NIH U24CA092782 Small Animal Imaging Resource (to R.W.).

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol, published online September 20, 2012;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/09/20/ATVBAHA.112.251983

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SUPPLEMENTAL MATERIAL

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Supplemental Methods

IVM Imaging System

Excitation beams at wavelengths of 491/532 nm and 561 nm were generated by a single diode-pumped solid state lasers (Dual Calypso and Jive, respectively, Cobolt AB, Solna, Sweden). The 635 nm excitation light was generated by a He-Ne laser (Radius, Coherent Inc, Santa Clara, CA). These laser beams were deflected into a custom-designed x-y scanner (polygon, galvanometer) and then focused onto the femoral/saphenous veins with a 30x, N.A.=0.9, water-immersion microscope objective lens (EAF-30-1, LOMO, Northbrook, IL). Each fluorescence signal was collected by a corresponding photomultiplier tube (R3896, Hamamatsu Photonics, Bridgewater, NJ). The FITC-dextran fluorescence was collected through a 509-547 nm bandpass filter, CLIO-AF555 or TMR-dextran fluorescence was collected through a 573-613nm bandpass filter, and MMPSense-680 fluorescence was collected through a 667-722nm bandpass filter. The two-dimensional images in x-y plane (500x500 pixels) were acquired by a frame grabber (Snapper-8/24 PCI, Active Silicon, North Chelmsford, MA) installed on a Macintosh PC. The imaging speed was 30 frames/sec and each static image was an average of 30 frames. Multiplexed z-stacks were collected throughout the entire thrombus region with a step-size of 2 µm. For the serial IVM study, incisions were sutured closed after the day 4 IVM procedure. Mice were then placed on a warming blanket until ambulant and returned to the colony housing. On day 6 imaging, the animals were prepped similarly to day 4, and underwent IVM as above, followed by sacrifice.

IVM Image Acquisition and Analysis

Each intravital fluorescence microscopy (IVM) image within a single z-plane is the product of averaging 30 frames at that location. Acquisition speed was 30 frames/second. Thus, each z-plane took approximately 1 second to acquire. For each z-stack, a maximum of 100 slices were acquired. Accounting for approximately 0.5 seconds for stage movement between slices, this required ~2.5 minutes total acquisition time for each z-stack. For each thrombus, 3-4 z-stacks were stitched together to encompass the entire thrombus region. Therefore, total acquisition time was approximately 10 minutes.
IVM image stacks were manually aligned in the x-, y-, and z-dimensions and stitched together to create one large image stack for the entire thrombus (examples in Figure 2, 5). Typically, 3 to 4 long-axis image stacks were stitched longitudinally together to encompass the entire thrombus and surrounding healthy vessel. Fusion images were created in OsiriX software (Pixmeo, Switzerland) by overlaying a color image of either the CLIO-AF555 or MMPSense-680 channels onto a grayscale FITC angiogram image. Composite images were obtained by overlaying color images of all 3 channels by manually tracing the thrombus region (target) and neighboring healthy vessel (background) on the stitched image stacks. Thrombus regions of interest (ROIs) were chosen using the FITC-dextran angiogram channel. These thrombus ROIs were then copied onto the intrinsically co-registered CLIO-AF555 and MMPSense680 images. Specifically, these ROIs were traced at mid-luminal depth and were projected up and down (±20 µm) to each slice in the z-stack and the TBR was calculated at each z-plane. The average TBR throughout the mid-luminal region (±20 µm) was then calculated as the TBR for the thrombus. The macrophage and MMP TBRs are traditional TBR measurements in which the target is bright and the background is dim. However, for thrombus permeability measurements (FITC TBR), the healthy vessel (background) is bright due to the circulating luminal FITC-dextran signal, and the thrombus (target) is relatively dim, resulting in TBR values of < 1.0.

FITC-dextran thrombus deposition time course experiments
To determine the rate of FITC-dextran accumulation into thrombi and its uptake effects on thrombus length and area measurements, mice at day 4 post femoral/saphenous DVT (N=4) underwent serial IVM at 10, 30, 45, 60 minutes and 48 hours after FITC-dextran injection. IVM imaging datasets were generated and processed as described above to yield a mid-luminal angiogram of the venous thrombosis for each timepoint. To compare FITC-dextran based thrombus length/area measurements to a spectrally distinct angiographic agent that had not yet extravasated into the thrombi, tetramethylrhodamine (TMR) – dextran (TMR-dextran, 0.5 mg in 100 µL PBS, λ_{absorption} = 557 nm, λ_{emission} = 576 nm, MW: 2,000,000, Sigma Chemical, St. Louis, MO) was intravenously injected ten minutes prior to the 60-minute and 48 hour timepoints, to directly compare the differences in thrombus deposition of dextran at 10 and 60 minutes. For the 48-hour timepoint, TMR-dextran injection was required to visualize the venous thrombosis, due the lack of residual FITC-dextran in the circulation and thrombus.
Thrombus area and length measurements were compiled from all IVM timepoint images in a blinded fashion from 3 reviewers.

In vivo co-localization analysis from IVM datasets
Co-localization of macrophages and FITC signals from IVM images was performed for the entire thrombus area, as well as along the length of the thrombus in single IVM images. For the entire thrombus area, TBRs were calculated as above and plotted against other TBRs or thrombus area. Linear regression was performed (one data point per animal) and Pearson’s correlation coefficient was calculated, with data presented in Figure 4 and Figure 6. For co-localization analysis between macrophage and FITC-dextran signals along the length of the thrombus (Supplemental figure IIa), a rectangular ROI was defined around the thrombus in a mid-luminal slice of the stitched image stacks. The average vertical signal intensity was calculated at each horizontal pixel location and the permeability and macrophage signals were then plotted against each other to calculate a correlation coefficient (NIH ImageJ). As part of this analysis, a correlation coefficient was calculated for each thrombus.

Correlative Histopathology and Fluorescence Microscopy
Immediately after final imaging experiments, animals were euthanized and thrombosed vessels were identified, dissected, and embedded in Tissue-Tek O.C.T. compound (Ted Pella, Inc., Redding, CA) on dry ice. Ten µm-thick sections were cut and fluorescence microscopy was performed in multiple channels at 40x magnification. The adjacent tissue sections were stained with hematoxylin and eosin (H&E) to identify intravascular thrombi. In order to identify macrophages, MMPs, and von Willebrand factor (vWF), the sections were incubated with Mac-3 (BD Biosciences, San Jose, CA), MMP-9 (Abcam, Cambridge, MA), and vWF (vWF, Abcam, Cambridge, MA) primary antibodies, respectively. The appropriate biotinylated secondary antibodies were applied, and Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and AEC substrate (Dako, Carpinteria, CA) were used for color development. All sections were counterstained with Harris’ hematoxylin. In addition to axial histological sections, sagittal sections of day 4 DVT were also obtained and stained with H&E for comparison of the thrombus length measured in IVM imaging (N=3, Supplemental Figures IIIF-IIIQ).

Microscopic co-localization of macrophage and FITC-dextran signals
Co-localization of macrophages and FITC-dextran was also calculated from fluorescence microscopy images (Supplemental Figure IVb). As the FITC and CLIO-AF555 fluorescence
microscopy images were simultaneously obtained from the same vessel section, pixel-by-pixel co-localization was possible. The thrombus region was manually traced and the CLIO-AF555 and FITC signal intensities at each pixel location were plotted on the x- and y-axis, respectively for all pixels within the ROI. A correlation coefficient for each image was then calculated (ImageJ).

**Microscopic FITC-Dextran and von Willebrand Factor Colocalization Analyses**

Colocalization of FITC-dextran deposition into the thrombus interior and thrombus vWF expression was determined from digitized FITC microscopy images and from vWF immunohistochemistry images, using ImageJ software (Supplemental Figure V). Images of vessel sections were manually aligned and identical rectangular ROIs were defined to include the entire thrombus. The grayscale FITC images were thresholded using Yen’s thresholding plugin (ImageJ). The color vWF images were thresholded by excluding all hues greater than 27 (of 255) and only including those remaining pixels with saturation greater than 66 (of 255). The remaining pixels showed a deep red-brown typical of positive staining from the AEC reaction. The percent positive surface area (SA) was calculated as the number of pixels above threshold in each image divided by the total number of pixels in the ROI. The FITC-positive SA and vWF-positive SA were then plotted against each other for each vessel section to calculate a correlation coefficient for each pair of images.

**Supplemental Figure I.** Multichannel IVM of murine DVT as a function of depth below the surface. FITC-dextran angiograms (left column), macrophage fusion images (middle column), and MMP fusion images (right column) sampled throughout the depth of the femoral/saphenous veins. The $z = 0 \ \mu m$ image depicts the superior surface of the vein. Although the high concentration FITC-dextran angiogram is evident at $z = 90 \ \mu m$, the macrophage and MMP signals are relatively less detectable at this depth, in part due to light attenuation and scattering by the thrombus. Scale bar = 200 $\mu m$. 
Supplemental Figure II. Histological micrograph of femoral venous thrombosis four days post-FeCl₃ treatment. (A) Hematoxylin and eosin (H&E) stained axial section showing venous thrombosis and microthrombi in the adjacent artery. (B) Schema of the micrograph in (A) depicting the venous thrombus and arterial microthrombi, as well as other structures such as the nerve and muscle. Scale bars = 100 μm.
Supplemental Figure III

![Image of Supplemental Figure III](image_url)

### D

**Percent Change in Thrombus Length compared to 10 min**

- **p=N.S.**

### E

**Percent Change in Thrombus Area compared to 10 min**

- **p=N.S.**

Supplemental Figure III continued on next page
Supplemental Figure III. Time course and accuracy analysis of FITC-dextran as an angiographic reporter of thrombus length and area. Serial IVM datasets were captured at 10, 30, 45, and 60 minutes, and finally at 48 hours post-FITC-dextran injection. All FITC channel images were processed and windowed identically. (A-C) Areas of thrombus FITC-dextran deposition were seen at 30, 45, and 60 minutes (A, and left image in B). (B) At the 60-minute timepoint, the FITC-dextran (ex/em 488/510 nm) angiographic image shows a similar thrombus length and area as the angiographic image obtained 10 minutes after tetramethylrhodamine (TMR)-dextran (ex/em 557/576nm) injection. (C) IVM image at 48 hours shows no FITC-dextran signal retention in the thrombus (left image). TMR-dextran was then injected (right image) and shows the venous thrombus at day 6 post-DVT induction. (*) denotes injection of TMR-dextran 10 minutes prior to IVM image collection. Quantitative analysis of the percent change of thrombus length (D) and area (E) over the 60-minute time course following FITC-dextran injection was compiled. Compared to the 10 minute IVM images, there were no significant differences between the timepoints (p>0.05, D and E). (F) Thrombus length analyses
based on the coronal IVM, sagittal IVM, and sagittal H&E datasets, yellow dashed line denotes sagittal plane used to generate the IVM sagittal image. (G) No significant difference in thrombus length was found among the three different measures ($p > 0.05$, N.S.). Data for 3 mice are shown; each group shows the individual measurements from three blinded readers. The intraclass correlation coefficient (ICC) for the IVM measurements was 0.915 (12 measurements, 4 per each of 3 blinded readers). Scale bar = 200 µm for all subsets. Ms = mouse. N.S. = not significant.
Supplemental Figure IV. Relationship between thrombus macrophages and FITC-dextran deposition (A) *in vivo* and (B) *ex vivo*. (A) Segmental line-by-line evaluation of thrombus inflammation and FITC-dextran in thrombus niches. Top, IVM images of FITC-dextran, macrophage infiltration (CLIO-AF555), and a composite image. Scale bar, 200 µm. Middle, average signal intensity of FITC-dextran (blue) and macrophage activity (green, CLIO) in the thrombus region (yellow box) from left to right. Bottom, correlation between FITC dextran and macrophage activity within the thrombus. (B) Microscopic evaluation of thrombus inflammation and FITC dextran in DVT sections. Top, composite cross-sectional fluorescence microscopy image of thrombus FITC-dextran deposition (blue) and macrophage activity (green, CLIO). The thrombus is outlined with a dashed yellow line. Middle, fluorescence microscopy images of the isolated thrombus region showing individual FITC and CLIO signals. Scale bars, 40 µm. Bottom, pixel co-localization analysis of thrombus macrophages and FITC-dextran deposition.
**Supplemental Figure V.** FITC-dextran deposition and its relationship to von Willebrand Factor (vWF) expression in day 4 DVT. (A) Thrombus cross-section of vWF staining. (B) Fluorescence microscopy image of FITC-dextran leakage from an adjacent section. Scale bar, 40 µm. (C) FITC-positive surface area (SA) correlates well with vWF-positive SA.