Toll-Like Receptor 9 Signaling Is Critical for Early Experimental Deep Vein Thrombosis Resolution


Objective—Toll-like receptors (TLR) bridge innate immunity and host responses, including inflammation. Sterile inflammation such as a venous thrombus (VT) may involve TLR signaling, including TLR9.

Methods and Results—TLR9 signaling on thrombus resolution was investigated using a mouse model of stasis VT. VT were significantly larger in TLR9−/− mice compared with wild-type (WT) at 2 and 8 days, despite a 2-fold increase in thrombus polymorphonuclear neutrophils at 2 days and monocytes at 8 days, whereas thrombus collagen and neovascularization was 55% and 37% less, respectively, at 8 days. Coincidently, decreased fibrinogen and increased thrombin-antithrombin complex were observed in TLR9−/− mouse thrombi. Vein wall interferon-α, interleukin-1α, and interleukin-2 were significantly reduced in TLR9−/− mice compared with WT. Thrombus cell death pathway markers were not significantly altered at 2 days, but caspase-1 was reduced in TLR9−/− thrombi at 8 days. MyD88 confers TLR9 intracellular signaling, but MyD88−/− mice had VT resolution similar to that of WT. However, inhibition of the NOTCH ligand δ-like 4 was associated with larger VT. Finally, stimulation with a TLR9 agonist was associated with smaller VT.

Conclusion—TLR9 signaling is integral for early and mid VT resolution through modulation of sterile inflammation, maintaining a TH1 milieu, and effects on the thrombosis pathway. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: leukocytes ▪ thrombosis ▪ vascular biology ▪ venous thrombosis

Deep vein thrombosis (DVT) resolution is an inflammatory process.1 Well-known factors that predispose to a DVT include systemic infectious processes, such as pneumonia and urinary tract infection.2 Current therapy primarily involves anticoagulation with heparin, followed by a vitamin K antagonist.3 Although this therapy is effective, the risks of bleeding with anticoagulation are significant, and many patients have contraindications to anticoagulation.

Venous thrombosis (VT) pathophysiology is now better understood from experimental studies. Leukocytes, chemokines, and proinflammatory cytokines are all involved in the process of VT resolution and vein wall healing.4–6 This process resembles sterile wound healing in the distinct phases of polymorphonuclear neutrophil (PMN) and monocyte influx, followed by fibrosis. Similarly, sterile inflammation results in apoptosis and necrosis, with matrix breakdown and release of cellular products, such as nucleotides and proteins, which must be cleared by the organism.7–10 Although septic thrombophlebitis occurs, most DVT are sterile, and the pathways for clearance of the sterile thrombus have not been elucidated.

Recently, the role of toll-like receptor (TLR) signaling in modulating sterile inflammation has become better defined in experimental injury models.7–9,11 TLR signaling pathways bridge innate and adaptive immunity and are highly conserved, and most signal via the adaptor molecule MyD88.11 TLR9 is located on endosomes and binds viral DNA and also certain forms of self DNA.7,11 In particular, the hypomethylated CpG motif binds to TLR9, conferring type I interferon expression and promoting a Th1 milieu.12 The CpG containing oligodeoxynucleotides (ODN) are immune adjuvants and are beneficial for certain models of experimental disease.13,14 Neutrophils, monocytes, lymphocytes, and dendritic cells express TLR9,11 and TLR9 agonists can directly stimulate PMN function.15 In this study, the role of TLR9 signaling was examined in early and midtime VT resolution. We found that targeted TLR9 deletion or exogenous inhibition was associated with impaired VT resolution and altered the local Th1 cytokine milieu, as well as several measures of clotting. Furthermore, VT resolution was independent of MyD88, a primary intracellular signaling protein for TLR9, but dependent, in part, on the NOTCH ligand, δ-like ligand 4 (DLL4). Finally, using a TLR9-stimulatory ODN, we were able to accelerate early VT resolution.

Methods

Animal Model
Male mice (BALB/c, C57/BL6, 20 to 30 g, wild-type [WT]) and BALB/c TLR9−/− and C57/BL6 MyD88−/− mice were used for all studies.16 For certain experiments, the TLR9 inhibitor ODN-2088...
or the TLR9 agonist ODN-1826 and their respective control ODN (InvivoGen, San Diego, Calif), 200 μg IP, 72 hours preligation, were administered to BALB/c mice, with harvest at day 2. In other experiments, anti-DLL4 antisera16 or rabbit serum control, 0.5 mL IP, was given 1 day pre- and 1 day postligation to BALB/c mice, with harvest at day 2. For all surgical procedures, the mice underwent general anesthesia with isoflurane/O2. All animal studies were done with University of Michigan Animal Use Committee approval.

Stasis VT was induced by IVC ligation.4,6,17 Briefly, the model consists of a laparotomy with 6-0 prolene ligation of the IVC below the renal veins and division of all visible side branches. At euthanization, the thrombosed IVC was carefully dissected and removed for histological analysis or protein and molecular analysis after thrombus–vein wall separation. Mice were euthanized on days 2 and 8, representing the early and mid-time point resolution. The thrombus is easy to remove at or before 8 days. Thrombus resolution is measured by true weight in g (early) or by weight/length in mg/mm (late) time points. The remnant thrombus represents the balance of thrombosis and thrombolysis at a given time point of tissue harvest.

**Thrombus Bacterial Culture**

Swabs of thrombus and serum, under sterile conditions, were placed on blood and MacConkey agar plates (Remel, Lenexa, Kan) using a sterile 1-loop spreader. Positive control blood and MacConkey agar plates were swabbed as well. Plates were placed in a 37°C incubator and were checked for bacterial growth at 9 and 16 hours.

**Histological Analysis/Immunohistochemical Staining/Trichrome Staining**

Immunohistochemical staining was performed on the paraffin-embedded tissue sections (5 μm). The following antigens were stained for: anti-PMN (Accurate Chemical, Westbury, NY), anti–von Willebrand factor (1:100, Serotec, Oxford, United Kingdom), anti–TLR9 (Imgenex, San Diego, Calif), and anti-MAC 2 (1:200; Cedarlane, Hornby, Ontario, Canada). After processing, the slides were counterstained with hematoxylin and coverslipped. In a blinded fashion, positive cells in 5 high-power fields (magnification, ×1000) radially around the IVC wall were counted and totaled.4,6

Masson’s trichrome staining was done on each section taken from the same region of thrombosed IVC6 and was quantified by computerized analysis.

**Antigen Analysis by Bioplex and ELISA**

Thrombus or vein wall homogenate underwent quantification of peptide mediators (normalized to total protein in the sample) for mouse tumor necrosis factor-α, interleukin (IL)-4, IL-12, IL-13, IL-2, and interferon-α (IFNα), using the Bio-Plex multiantigen technology (Bio-Rad, Hercules, Calif).16,18 IL-1α was determined by a commercially available ELISA per the manufacturer’s instructions (R&D Systems, Minneapolis, Minn). Serum and thrombus homogenate was assessed for urokinase plasminogen activator and plasminogen activator inhibitor-1 by ELISA (Innovative Research, Novi, Mich).

**Quantitative (Real-Time) Polymerase Chain Reaction**

Expression of the genes of interest was determined as follows6,17: messenger RNA was isolated by treatment of vein wall segments with TRIzol reagent and reverse transcribed by incubating with oligo(dT) primer and M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, Calif) at 94°C for 3 minutes followed by 40°C for 70 minutes. The resultant cDNA was amplified by Taq polymerase (Promega, Madison, Wis) in the SmartCycler quantitative polymerase chain reaction system (Cepheid, Sunnyvale, Calif). SYBR Intercalating Dye (Roche, Indianapolis, Ind) was used to monitor levels of cDNA amplification for each gene. The genes included B-Actin, IL-1α, Jagged 1, Jagged 2, Notch1, Notch2, Notch3, Notch4, TLR9, TLR3, and TLR4.

**Analysis of Thrombosis Characteristics**

Using the thrombus tissue homogenate preparation, assessment of plasmin, factor X (FX), fibrinogen (all from Innovative Research), and thrombin-antithrombin (TAT) complex (Enzyme Research Laboratories, South Bend, Ind) was performed according to manufacturers’ instructions. Blood was also taken from the various genotypes of mice at baseline and subjected to thromboelastography.19

**Analysis of Thrombus Cell Death/Caspase-1/Double-Stranded DNA Content**

Thrombus homogenate was subjected to the cell death assay measuring histone-associated DNA fragments (Roche, Mannheim, Germany), caspase-1 activity (R&D Systems), and double-stranded DNA (Innitrogen, Carlsbad, Calif) according to the manufacturers’ instructions.

**Statistical Analysis**

All data are represented as mean ± SE. An unpaired Student t test and 1-way ANOVA were used as appropriate for comparison between the groups at their individual time points and appropriate controls (Sigma Stat, SPSS Inc., Chicago, Ill). A level of P < 0.05 was assigned significance.

**Results**

**TLR9−/− Is Associated With Impaired Thrombus Resolution and Increased Leukocyte Influx**

In our animal model, the thrombus tissue harvested represents the balance of thrombosis and thrombolysis. After IVC ligation to produce stasis, the overall thrombus size decreases over time.4,6,17 Compared with WT controls, the TLR9−/− mice had approximately 20% larger thrombi, (P = 0.01, n = 9), at both 2 days and 8 days (Figure 1A and 1B). This was consistent whether measured as thrombus weight or as measured by weight/length ratio. Additional time points of 4 days showed a 50% increase in size (P = 0.01, n = 6 to 8), whereas no significant difference was observed at 12 days. To confirm TLR9’s role in VT resolution, a specific ODN-2088 inhibitor was administered to WT mice.13 Compared with control, the TLR9 inhibitor was associated with significantly larger thrombi at 2 days (18±1 vs 22±1, P = 0.02, n = 6 to 7), consistent with the TLR9−/− phenotype.

TLR9-positive (+) cells in the WT thrombi remained relatively stable in number between 2 and 8 days (22±3 and 24±6 cells/5 high-power fields, respectively), but the cell nuclear characteristics changed from predominantly polymorphonuclear to mononuclear (Figure 1D and 1E). Of note, no TLR9+ cells were observed in the vein wall. Co-localization of PMN nuclear morphology with TLR9 staining was 93±3% at 2 days, and mononuclear morphology with TLR9 staining was 96±2% at 8 days.

Compared with WT, TLR9−/− mice had an ∼2-fold increase in thrombus PMN at 2 days (Figure 1C and 1F). As few PMN are present in the thrombus at 8 days,4 monocytes were evaluated. Compared with WT controls, TLR9−/− had a 2-fold increased number of thrombus Mac-2+ monocytes (P < 0.01, n = 3 to 4) (Figure 1G). In both cases, these cells were primarily in the thrombus periphery.

Thrombus fibrosis and neovascularization occurs as VT resolves and matures.1,4,6 By image assessment (trichrome percentage positive staining), there was no significant difference between groups at 2 days. At 8 days, the WT had...
approximately 45% greater fibrosis compared with TLR9−/− thrombi (P<0.001, n=3 to 4) (Figure 2A to 2D), suggesting impaired thrombus maturation. Consistent with impaired maturation at 8 days, TLR9−/− mice had 37% fewer von Willebrand factor–positive channels in the thrombus compared with WT (P=0.05, n=5 to 6) (Figure 2E and 2F).

**TLR9−/− Mice Have Altered In Vivo Clotting Parameters**

Ex vivo thrombogenesis was assessed in the 2 mouse genotypes by thromboelastography. Decay, α, and maximum amplitude parameters showed no significant differences between the WT and TLR9−/− mice, suggesting that the basic clotting mechanisms were not different between groups (data not shown).

In vivo thrombus parameters were assessed to determine differences between groups in the early 2 days thrombus milieu. Compared with WT controls, the TLR9−/− thrombi had ~3-fold increased TAT complex (P<0.001, n=5 to 7) (Figure 3). Free fibrinogen was ~50% less in the TLR9−/− thrombi compared with WT controls (P<0.001, n=4 to 6). However, FX, plasmin, and both serum and thrombus urokinase plasminogen activator:plasminogen activator inhibitor-1 balance were not significantly different between TLR9−/− and WT at 2 or 8 days (data not shown).

**TLR9−/− Mice Have Early Intact Sterile Cellular Processes**

As the TLR9 signaling pathway confers sterile inflammatory processing, several measures of this were ascertained. First, there was no significant difference between thrombus caspase-1 antigen at 2 days in TLR9−/− thrombi compared with WT. At 8 days, caspase-1 was reduced ~3-fold in the TLR9−/− thrombi compared with WT (0.07±0.01 versus 0.19±0.02, P<0.001, n=7 to 9). A cell death assay, reflecting histone-associated DNA fragments, showed no significant difference between the WT or the TLR9−/− groups at day 2 or 8. Assessment of double-stranded DNA, a ligand for TLR9 that is present within the sterile inflammatory milieu, was higher in the VT than in the serum, by approximately 100-fold (0.7±0.13 versus 0.03±0.09, ng/mL, P<0.01, n=3).

As TLR9 receptors are innate pattern recognition proteins for bacteria, we needed to confirm VT sterility. Importantly, there was no bacterial growth in either of the auger types from the VT sample (n=4), confirming the sterile nature of the inflammation.

**TLR9−/− Is Associated With Decreased Th1 Cytokine Levels**

To characterize the thrombus cytokine milieu, Th1- and Th2-type cytokines were assessed. No significant differences in WT or TLR9−/− thrombus or vein wall tumor necrosis factor-α, IL-4, IL-13, and IL-12 were found at 2 days, and most of these had low, nondetectable expression (data not shown). In contrast, vein wall IFNα was reduced 4-fold in TLR9−/− compared with WT (P<0.01, n=5 to 7) (Figure 4). Similarly, vein wall IL-1α was reduced 8-fold (P<0.01, n=4 to 5), and vein wall IL-2 was reduced 3.5-fold (P=0.02, n=5 to 8) in TLR9−/− compared with WT controls.

**Lack of MyD88 Signaling Does Not Affect VT Resolution**

To determine whether TLR9 signaling effects on VT resolution were dependent on the MyD88 signaling pathway, we used genetically identical WT and MYD88−/− mice and subjected them to both stasis thrombosis. There was no significant difference in thrombus size of MyD88−/− compared with WT at 2 day (19±7 versus 23±1 mg, P=0.18,
TLR9-Dependent VT Resolution Is Dependent in Part on the NOTCH Ligand, δ-Like 4

As MyD88 deletion did not impair VT resolution, recent studies have suggested that TLR9 signaling may require DLL4-Notch for activity. Using neutralizing anti-DLL4 antiserum, we found that the thrombus weight was increased by approximately 25% \( (P<0.01, n=8 \text{ to } 9) \) (Figure 5). Thrombus PMN counts with DLL4 inhibition was increased by 15%, but this did not reach statistical significance \( (62 \pm 8 \text{ versus } 42 \pm 8 \text{ cells/5 high-power fields, } P=0.12, n=5 \text{ to } 8) \). There was no significant difference in thrombus TLR9+ cell staining in the treated or control mice at 2 days \( (15 \pm 1 \text{ versus } 12 \pm 1 \text{ } P=0.10, n=4 \text{ to } 5) \).

Assessing thrombus characteristics, the TAT complex, plasmin, and FX levels were not significantly altered with the anti-DLL4, but free fibrinogen was reduced by 20% compared with Ig control \( (P=0.03, n=5) \).

Evaluating the cytokine milieu, vein wall IFNα was significantly reduced with anti-DLL4 treatment compared with IgG control \( (P=0.01, n=5) \) (Figure 5), but neither IL-1α nor IL-2 was significantly altered (data not shown).

To characterize the vein wall genetic expression of certain ligands related to TLR and NOTCH signaling, there was neither significant induction of TLR9 or DLL4 in the vein wall after VT nor a difference in the WT and the TLR9−/− mice in either Jagged 1 to 3 or Notch 1 to 3 (data not shown). There was also no induction of vein wall TLR3 or TLR4 gene expression (data not shown). However, IL-1α gene expression was reduced by \( \approx 3\)-fold \( (P=0.03, n=6 \text{ to } 8) \) in TLR9−/− compared with WT at 2 days.

TLR9 Agonist Accelerates VT Resolution

To determine whether we could accelerate early thrombus resolution via stimulation of TLR9 signaling, we treated the WT mice with immunostimulatory ODN-1826, using a scrambled ODN as control. The ODN-1826-treated mice had 29% smaller thrombi \( (P=0.001, n=12 \text{ to } 13) \) (Figure 6) compared with the ODN control. The thrombus PMN and TLR9 counts were not significantly altered by ODN-1826 administration compared with ODN controls at 2 days (data not shown).

Evaluating the thrombus parameters, TAT complex was reduced by \( \approx 10\% \) in the ODN-1826-treated mice compared

Figure 2. A and B, By trichrome thrombus analysis, thrombus maturation was found to be impaired in TLR9−/− mice compared with WTs. Significantly less collagen was found in the TLR9−/− thrombus sections as shown at ×100 and ×400. C and D, Less thrombus neovascularization was found in the TLR9−/− mice at 8 days, denoted by von Willebrand factor–positive channels (×400) (arrows). \( \ast P<0.05 \). vWF indicates von Willebrand factor; hpf, high-power fields; T, thrombus.
with the ODN controls ($P<0.01$, $n=6$ to 7). Thrombus FX clot levels were also reduced with ODN-1826 treatment ($P<0.01$, $n=7$). Thrombus free fibrinogen and plasmin activity were not significantly different between groups.

Comparing the cytokine milieu, we found no significant difference in vein wall IFNγ or IL-1α levels. However, IL-2 was elevated 2.5-fold with ODN-1826 compared with the ODN control ($P=0.02$, $n=7$).

**Discussion**

Recent models have begun to illuminate the processes mediating sterile inflammation with the TLR9 pathway directly involved. The data herein suggest a similarly important role, supported by these major findings: (1) thrombus resolution is impaired with genetic deletion and exogenous inhibition of TLR9, as well as DLL4, but not MYD88 pathways; (2) in vivo thrombus clotting mechanisms are altered and Th1 cytokines are reduced in TLR9−/− mice; and (3) CpG ODN stimulation of TLR9 accelerates early VT resolution.

Deletion of TLR9 was associated with significantly increased thrombus size, with less neovascularization and fibrosis, despite increased thrombus PMN and monocyte influx. The increase in thrombus cellularity with deletion of TLR9 is consistent with other experimental models. Prior work has shown directly and indirectly that thrombus leukocyte influx is essential for VT resolution, but only with normal leukocyte functioning. As TLR9 gene expression was not induced, nor were TLR9 protein or cells detected in the vein wall after the VT insult, this suggests that influxing cells with intact TLR9 signaling are important for VT resolution rather than the resident vein wall response. These data also suggest that TLR9 signaling affects downstream leukocyte functions for thrombus clearance, rather than being a function of the total number of leukocytes. For example, PMNs are known to release plasminogen activators and other proteases, but it is not known whether the TLRs affect these processes. However, TLR9 stimulation may directly activate PMNs, including IL-8 release. Related work has suggested the importance of CXCR2-PMN role in experimental VT resolution, with a similar time frame of impaired resolution (eg, increased VT size at 2 and 8 days). Lastly, although the TLR9−/− was less fibrotic, our model does not allow us to determine
whether this is more lysable or less likely to embolize to the pulmonary circulation, which would be a critical outcome parameter in humans to evaluate.

Although our model does not allow us to determine thrombotic propensity, it is likely that thrombolysis was not affected, as neither plasmin nor the activating protease urokinase plasminogen activator and its inhibitor, plasminogen activator inhibitor-1, was affected in the TLR9/H11002/H11002 mice. Because our model reflects the relative balance of thrombosis and thrombolysis at any given time point, the data suggest that in vivo but not ex vivo thrombogenesis was affected by TLR9 deletion, as decreased free fibrinogen and increased TAT complexes in the TLR9/−/− mouse thrombi were observed without a change in thromboelastographic parameters. Consistently, exogenous CpG stimulation of TLR9 was associated with less thrombus TAT. This is the first observation to suggest that TLRs play a role in the hemostatic processes and may modulate thrombogenesis. Alternatively, TLR9 deficiency may promote ongoing thrombogenesis by impairing the clearance of extracellular procoagulant RNA22 or by preventing the release of normal antiinflammatory mediators.23

It was surprising that MyD88 did not confer the same phenotype as the TLR9/H11002/H11002 mouse, given the central position

Figure 5. DLL4 antibody inhibition experiments. Compared with WT given IgG control, thrombus size was increased with DLL4 blockade at 2 days (A), thrombus fibrinogen was reduced (B), and vein wall IFNα was reduced (C). D, Gene expression of IL-1α was reduced in TLR9/−/− mice. d indicates days;Ctl, control. *P<0.05.

Figure 6. Stimulation with ODN-1826 (CpG) TLR9 agonist at 2 days. Compared with control, thrombus size was reduced (A), TAT was reduced (B), FX was reduced (C), and vein wall IL-2 was elevated (D). d indicates days. *P<0.05.
of MyD88 in TLR9 signaling.11,24 However, MyD88 is converged on by multiple TLRs and may have canceled out their effects in VT resolution. For example, TLR9−/− and MyD88−/− have differing phenotypes in experimental lupus models.25 Consistently, no increase in proinflammatory cytokines was observed after VT (eg, tumor necrosis factor-α or IL-12), a known function of the MyD88 signaling pathway.24 In contrast, DLL4 inhibition produced a phenotype similar to that of TLR9−/−, with larger thrombi and less IFNα, but less alteration in PMN influx and thrombogenic parameters. Of note, NOTCH–DLL4 signaling has been shown to be important in arterial vascular homeostasis, although this study is the first to assess its role in VT resolution.26 Taken together, these results suggest that TLR9 may signal via other pathways besides MyD88.16

Other pathways are also important for sterile inflammation resolution, including IL-1α,8 and were significantly reduced in the TLR9−/− mice. IL-1α may promote several processes to allow clearance of apoptotic cells, including induction of the caspase enzymes. Consistently, we observed decreased caspase-1 levels at 8 days in TLR9−/− mice. Caspases are important enzymes for activation of apoptotic pathways and modulating resolution of inflammation.8 Interestingly, although much of the cellular breakdown products are assumed to be from leukocytes, platelets also have functional mRNA27 and make up a large portion of a VT. Exactly how the necrotic and apoptotic cellular DNA and RNA is processed in a VT has not been defined. Although extracellular DNA may be primary, other cell peptides, including high-mobility group Box-1 and heat shock protein-1, as well as uric acid, may also be important.8,10

The immune adjuvant TLR9 agonist ODN-1826 has been tested in various disease models with strong Th1 effects.13,14 The host recognizes self DNA for normal homeostasis and is both a TLR9-dependent and TLR9-independent process.28 The use of ODN-1826 was associated with a significant decrease in VT weight. This is striking, given that our model is full venous stasis, and LMWH has only delayed thrombus resolution effects.29 The Th1 cytokine IL-2 was elevated with ODN-1826 treatment but not IL-1α or IFNα. These data suggest that a pro-Th1 thrombus milieu seems important for VT resolution, as previously observed.1,6 Determining whether IL-2 is directly responsible for accelerating VT resolution or is simply a surrogate will require further work. Unlike the TLR9−/− mice, which had less IFNα and IL-α expression, these factors were not increased in the ODN-1826-treated mice. These data suggest that both Th1 modulation and in vivo thrombogenesis may be conferred in part through TLR9 signaling. Our experiments do not show which of these factors is most important mechanistically, but future work will define the stimulatory factors for TLR9, as well as further VT modulation mechanisms associated with TLR9 signaling.

Although new therapies are on the horizon for DVT treatment, these all primarily target the coagulation cascade, and the bleeding risks remain.3 Indeed, a concern of those new agents is the lack of antidotes.30 The next generation of DVT therapy should have rapid onset, require little monitoring, and have little bleeding risk. Stimulation of the sterile inflammatory processing pathway in the DVT may be ideal in this regard.

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Disclosures
None.

References


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