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In situ assays demonstrate that interferon-gamma suppresses infection-stimulated hepatic fibrin deposition by promoting fibrinolysis

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Summary

Inflammatory cytokines potently impact hemostatic pathways during infection, but the tissue-specific regulation of coagulation and fibrinolysis complicates studies of the underlying mechanisms. Here, we describe assays that quantitatively measuring prothrombinase (PTase), protein C-ase (PCase) and plasminogen activator (PA) activities in situ, thereby facilitating studies of tissue-specific hemostasis. Using these assays, we investigate the mechanisms regulating hepatic fibrin deposition during murine toxoplasmosis and the means by which interferon-gamma (IFN γ) suppresses infection-stimulated fibrin deposition. We demonstrate that *Toxoplasma* infection upregulates hepatic PTase, PCase, and PA activity. Wild type and gene-targeted IFN γ -deficient mice exhibit similar levels of infection-stimulated PTase activity. By contrast, IFN γ -deficiency is associated with increased PCase activity and reduced PA activity during infection. Parallel analyses of hepatic gene expression reveal that IFN γ -deficiency is associated with increased expression of thrombomodulin (TM), a key component of the PCase, increased expression of thrombin-activatable fibrinolysis inhibitor (TAFI), a PC substrate, and reduced expression of urokinase PA (uPA). These findings suggest that IFN γ suppresses infection-stimulated hepatic fibrin deposition by suppressing TM-mediated activation of TAFI, thereby destabilizing fibrin deposits, and concomitantly increasing hepatic uPA activity, thereby promoting fibrinolysis. We anticipate that further application of these in situ assays will improve our understanding of tissue-specific hemostasis, its regulation by cytokines, and its dysregulation during coagulopathy.

Keywords

Cytokines; Fibrinolysis; Infection

Introduction

In response to vascular trauma, hemostatic pathways promote the deposition of fibrin, an insoluble extracellular matrix that helps to restrain blood loss. The mechanisms prompting trauma-associated fibrin deposition have been studied extensively. Exposure of plasma to

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tissue factor (TF)-bearing extravascular cells activates coagulant pathways that culminate in the formation of a prothrombinase (PTase) enzyme that converts prothrombin to thrombin. Thrombin then cleaves fibrinogen, prompting its polymerization and deposition as fibrin [1–3]. Fibrin deposition is antagonized by anticoagulant pathways that inhibit thrombin's activity and/or suppress its generation, and also by fibrinolytic pathways that degrade fibrin. A principal component of the anticoagulant response is thrombomodulin (TM), a protein that binds thrombin and modulates its proteolytic specificity [4,5]. The TM/thrombin complex converts protein C to activated protein C, a powerful suppressor of thrombin production. Notably, this TM-dependent protein C-ase (PCase) also activates thrombin-activatable fibrinolysis inhibitor (TAFI), an enzyme that cleaves terminal lysines from fibrin, thereby suppressing its degradation by fibrinolytic enzymes [6]. Consequently, PCase activity simultaneously suppresses further fibrin formation, via activation of protein C, and stabilizes fibrin deposits, via activation of TAFI. The primary mediator of fibrinolysis is plasmin, which is formed upon partial proteolysis of plasminogen by plasminogen activators (PA) [7]. Mammals produce two major PA, tissue-type PA (tPA) and urokinase-type PA (uPA), and their activities are antagonized by PA inhibitor 1 (PAI1) [8]. Thus, levels of fibrin deposition reflect a balance of highly regulated hemostasis-related pathways in which PTase, PCase and PA activities play major roles.

There are extensive interactions between hemostatic and inflammatory pathways, with elements of each stimulating aspects of the other [9,10]. With regard to the clinically significant setting of sepsis-associated disseminated intravascular coagulation (DIC), the cytokines tumor necrosis factor- α (TNF α), interleukin (IL)-1, and IL-6 appear to play prominent roles in disturbing the hemostatic balance. Studies of the underlying mechanisms have concluded that TNF α , IL-1 and IL-6 primarily upregulate expression of procoagulant TF, and that TNF α and IL-1 also downregulate expression of anticoagulant TM while upregulating expression of antifibrinolytic PAI1 [9–13]. These conclusions derive primarily from experiments that measured plasma-based hemostatic biomarkers *in vivo* and cytokine-induced changes in hemostatic protein expression *in vitro*. However, recent data clearly indicate that hemostasis is regulated in a tissue-specific manner [14,15]. Whether inflammatory cytokines similarly impact all tissues remains to be determined.

A number of groups, including our own, have demonstrated that infection-stimulated fibrin deposition can perform critical protective roles during infection [16–23]. One striking example is infection with *Toxoplasma gondii*, where mice acutely succumb to infection-stimulated hemorrhagic pathology if they cannot produce fibrin [21]. Given that cytokines contribute to coagulopathy during sepsis, we investigated their impact on the protective fibrin deposition that accompanies toxoplasmosis. We reasoned that studies in this protective setting may reveal natural counter-regulatory pathways that function to limit and/or resolve infection-stimulated fibrin deposition. Indeed, we found that interferon- γ (IFN γ) potently suppresses *T. gondii*-stimulated hepatic fibrin deposition [24]. Here, we dissect the underlying mechanisms by developing novel assays that quantitatively measure PTase, PCase, and PA activities *in situ*. We demonstrate that infection upregulates each of these activities within hepatic tissue and that IFN γ suppresses fibrin deposition by promoting fibrinolysis.

Materials and methods

Mice

C57BL/6 mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) or were bred at Trudeau Institute. C57BL/6-backcrossed mice deficient in IFN γ , uPA, tPA or PAI1 were purchased from The Jackson Laboratory. C57BL/6-backcrossed mice with very low levels of TF activity (low-TF mice) were bred at Trudeau Institute. These animals lack

expression of mouse TF due to its inactivation by gene targeting, and instead express a human TF transgene, which imparts low-level TF activity [25]. All animals were housed in a specific pathogen-free facility, and all animal experiments were reviewed and approved by the Trudeau Institute Animal Care and Use Committee.

Infections and assays for D-dimer and fibrin

Oral infections with 10 strain ME49 *T. gondii* cysts were performed as previously described [21]. All mice were between 6- and 8-weeks old at the time of infection. At day 8 postinfection, mice were treated with heparin (500 units, intravenously) just prior to euthanasia by carbon dioxide narcosis. Plasma was collected by cardiac puncture and assayed for D-dimer by ELISA following instructions provided by the assay manufacturer (Diagnostica Stago). Hepatic fibrin was measured by quantitative Western blotting, as described previously [21].

In situ assays

For PTase assays, hepatic tissue was removed from heparinized mice, embedded in Tissue-Tek OCT compound (Krackler Scientific), and flash frozen in liquid nitrogen. Tissue sections (7 μ m) were then prepared in duplicate with a cryostat, mounted onto Superfrost Plus slides (Fisher Scientific), and air-dried for 15 min at room temperature before storing up to two weeks at -20°C . Immediately prior to assay, slides were thawed for 10 min at room temperature. The tissue sections were encircled with a PAP isolator pen (Zymed Laboratories) and then overlaid with 65 μ l of 36 $\mu\text{g/ml}$ human prothrombin (Enzyme Research Laboratories) in PTase assay buffer (25 mM HEPES pH 7.4, 135 mM NaCl, 4 mM KCl, 15 mM glucose, 3 mM CaCl_2 , 0.3% BSA). After 12 min in a 37°C humidified incubator, the reaction buffers were removed from each section, transferred to a polypropylene 96-well microtiter plate, and mixed by pipetting. A 30 μ l aliquot of each sample was then added to 120 μ l of 50 mM Tris pH 7.4, 135 mM NaCl, 400 mM EDTA, and 50 μ l of this diluted sample was transferred, in duplicate, to a 96-well MaxiSorp plate (Nunc), along with 50 μ l of similarly prepared standards and buffer blanks. Purified human alpha-thrombin (Enzyme Research Laboratories) was used as standard. Thrombin levels were then quantified by adding 150 μ l of 270 μM Spectrozyme TH (American Diagnostica), incubating at room temperature for 75 min, measuring absorbance at 450 nm, and interpolating against the standard curve. Finally, thrombin production was normalized to the area of the tissue sections by staining with hematoxylin, capturing a digital image on a flatbed scanner, and calculating pixel areas using PhotoShop 5.5 (Adobe). Where indicated, 100 μM TENSTOP (American Diagnostica) was included in the PTase assay buffer.

PCase assays were performed similarly to PTase assays, with the following modifications: (i) prothrombin was replaced by human protein C (3.1 $\mu\text{g/ml}$; Enzyme Research Laboratories), (ii) the initial assay buffer consisted of 50 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl_2 , 0.3% BSA, (iii) the initial incubation was extended to 50 min, and (iv) the reaction buffer was mixed with 50 mM Tris pH 7.4, 100 mM NaCl after its removal from the tissue sections. The standard curve was prepared using activated human protein C (Enzyme Research Laboratories), and activated protein C levels were measured using 533 μM Spectrozyme PCA (American Diagnostica) and incubating at room temperature for 20 hr. Where indicated, tissues were pretreated with 25 μM Phe-Pro-Arg-chloromethylketone (PPACK; Haematological Technologies) in PCase assay buffer for 6 minutes, washed with assay buffer, and then assayed as above. Pretreatment with PPACK under these conditions did not affect the Spectrozyme PCA assay, as the activity of exogenous activated protein C was unaffected by its co-incubation with PPACK-treated/washed tissue.

PA assays were performed similarly to PTase and PCase assays, with the following modifications: (i) prothrombin was replaced by human Glu-plasminogen (150 µg/ml; Enzyme Research Laboratories), (ii) the initial assay buffer consisted of 50 mM Tris pH 7.4, 100 mM NaCl, 3 mM CaCl₂, 0.3% BSA, (iii) the initial incubation was extended to 60 min, and (iv) the reaction buffer was mixed with 50 mM Tris pH 7.4, 100 mM NaCl after its removal from the tissue sections. The standard curve was prepared using purified human plasmin (Enzyme Research Laboratories), and plasmin levels were measured using 200 µM Spectrozyme PL (American Diagnostica) and incubating at room temperature for 20 hr. Where indicated, 100 ng/ml murine PAI1 (American Diagnostica) was included in the PA assay buffer.

Real-time PCR

Tissue levels of TM, TAFI, uPA, tPA and PAI1 mRNA were measured by real-time PCR and normalized using the housekeeping gene GAPDH as described [26], but using the following primers: TM forward TGTATGTGGTCCTGCCTCAA, TM reverse CGAAGCACAACTCATGCT, TM probe TCAGAGCCTGACAGATGGAAGCACC, TAFI forward TTCGAGAAGTACCCACTTTATGTT, TAFI reverse CCATTCTCTGGCATGGATT, TAFI probe AATGCCATCTGGATCGACTGTGG, uPA forward CTGCAGGAACCTGACAACC, uPA reverse GCCACACTGGAAGCCTTGTT, uPA probe TGCTCTCTTAGCAAAAAGCCTTCTTCGTCTGTAGA, tPA forward TTGTGGACTGGCTTTCCCAT, tPA reverse TCTGCGTTGGCTCATCTCTG, tPA probe TGACCAGGGAATACATGGGAGGTTTCAGA, PAI1 forward GAGCCAATCACAAGGCACCT, PAI1 reverse TCCCAGAGACCAGAACCAGG, PAI1 probe TCAGGATGCAGATGTCTTCAGCCCTTG. These primers were designed to span an intron within the target gene. Preliminary studies confirmed that they amplify a fragment from liver cDNA that encodes the message of interest and that they do not amplify genomic DNA.

Statistics

Statistical significance was determined by Student's t test using the program Prism 4.0 (GraphPad Software, Inc.).

Results

IFN γ -deficiency upregulates infection-stimulated fibrin deposition in liver tissue and levels of D-dimer in plasma

Mice rendered deficient in IFN γ , either by administration of neutralizing antibody [24], or by gene-targeting (Figure 1A), deposit nearly 100-fold increased levels of hepatic fibrin at day 8 after *Toxoplasma* infection. Studies employing plasma-based assays previously demonstrated evidence of increased fibrinolysis in humans treated with recombinant IFN γ [27,28]. Consistent with those studies, we found that *Toxoplasma* infection increases plasma levels of D-dimer, a fibrin degradation product (Figure 1B; $p < 0.05$), and that IFN γ -deficiency further upregulates D-dimer levels (Figure 1B; $p < 0.05$). While elevated D-dimer levels suggest increased fibrinolysis, increased fibrinolysis cannot explain the increased hepatic fibrin deposition in IFN γ -deficient mice.

IFN γ -deficiency does not impact infection-stimulated hepatic PTase activity

Given recent evidence that hemostasis is regulated in a tissue-specific manner [14,15], we next developed *in situ* assays to specifically measure hemostasis-related activities within liver tissue. In the coagulation pathway, TF activates proteolytic cascades that culminate in the formation of a PTase comprised of activated factor V and activated factor X (factor Xa),

which then converts prothrombin to thrombin. To measure levels of hepatic PTase activity in situ, we prepared fresh-frozen liver sections, overlaid buffer containing prothrombin, and measured its conversion to thrombin. We found that *Toxoplasma* infection significantly increased hepatic PTase activity (Figure 2A; $p < 0.0005$). Both the basal and infection-stimulated PTase activity were fully suppressed by TENSTOP, a factor Xa antagonist, thereby confirming that the assay measures a factor Xa-dependent PTase activity (Figure 2A). Likewise, transgenic low-TF mice displayed significantly reduced infection-stimulated hepatic PTase activity, as compared with littermate controls, thereby confirming that the in-situ assay measures a TF-dependent PTase activity (Figure 2B; $p < 0.0001$).

Using this in situ assay, we next investigated whether IFN γ suppresses hepatic fibrin deposition during infection by suppressing PTase activity. We found similar basal levels of PTase activity in both wild type and IFN γ -deficient mice (Figure 2C). Moreover, infection upregulated PTase activity to a similar extent in both wild type and IFN γ -deficient mice (Figure 2C). Consistent with that finding, IFN γ -deficiency did not significantly alter hepatic levels of mRNA encoding TF (data not shown). Together, these observations suggest that IFN γ does not suppress infection-stimulated hepatic fibrin deposition via impacts on PTase activity.

IFN γ -deficiency increases infection-stimulated hepatic PCase activity, TM expression, and TAFI expression

We next investigated whether IFN γ regulates hepatic fibrin deposition via impacts on the protein C pathway. We employed an assay design analogous to that described above for PTase activity to measure levels of PCase activity, which results from a complex of thrombin bound to TM. In the PCase assay, we replaced prothrombin with protein C and measured its conversion to activated protein C. We found that infection stimulated a 3.5-fold increase in hepatic PCase activity (Figure 3A). Pretreating the tissue with the thrombin antagonist PPACK fully suppressed this activity, thereby demonstrating that this assay measures a thrombin-dependent PCase (Figure 3A).

Having established a PCase assay, we assessed whether altered PCase activity could account for the capacity of IFN γ to suppress infection-stimulated hepatic fibrin deposition. We found similar levels of basal hepatic PCase activity in both wild type and IFN γ -deficient mice (Figure 3B). Interestingly, IFN γ -deficiency significantly increased the infection-stimulated upregulation of hepatic PCase activity (Figure 3B; $p < 0.0005$). Real-time PCR revealed that levels of PCase activity correlated with levels of TM mRNA within hepatic tissue, suggesting that IFN γ regulates PCase activity, at least in part, by impacting levels of TM expression (Figure 3C).

In addition to converting protein C to activated protein C, PCase activity also converts TAFI to activated TAFI. Thus, PCase activity both suppresses fibrin formation, via the anticoagulant activity of activated protein C, and stabilizes fibrin deposits, via the antifibrinolytic activity of activated TAFI. Increased activation of protein C cannot readily explain the increased fibrin deposition in IFN γ -deficient mice, but increased activation of TAFI could potentially explain the increased fibrin. Consistent with that possibility, we found that infection significantly upregulates hepatic levels of TAFI mRNA (Figure 3D; $p < 0.0001$), and that IFN γ -deficiency further increases TAFI levels (Figure 3D; $p < 0.0001$). Taken together, these data suggest that IFN γ suppresses infection-stimulated hepatic fibrin deposition, at least in part, by decreasing levels of TM and TAFI, thereby reducing PCase activity and TAFI-mediated fibrin stabilization.

IFN γ -deficiency suppresses infection-stimulated hepatic PA activity and uPA expression

Fibrinolysis is primarily accomplished by plasmin, a proteolytic enzyme derived from plasminogen upon its partial proteolysis by PA activity. To assess whether altered PA activity contributes to the increased infection-stimulated fibrin deposition in IFN γ -deficient mice, we developed an in situ PA assay. The assay design is analogous to that described above for the measurements of PTase and PCase activity, except that we incubate tissue sections with plasminogen and measure its conversion to plasmin. We observed that infection stimulated a 9-fold increase in hepatic PA activity (Figure 4A). Adding the physiologic PA antagonist PAI1 to the assay buffer significantly suppressed this infection-stimulated increase in PA activity (Figure 4A; $p < 0.0001$). To investigate specific roles for PAI1, tPA and uPA in the regulation of hepatic PA activity during infection, we analyzed PAI1-deficient, tPA-deficient and uPA-deficient mice. Neither PAI1- nor tPA-deficiency significantly impacted the infection-stimulated increase in hepatic PA activity (Figure 4B and 4C). However, uPA-deficiency significantly decreased both basal hepatic PA activity (Figure 4D; $p < 0.02$) and the infection-stimulated increase in hepatic PA activity (Figure 4D; $p < 0.005$). These observations suggest that uPA is a primary regulator of hepatic PA activity.

We next assessed whether altered PA activity contributes to the increased fibrin deposition in IFN γ -deficient mice. We found that IFN γ -deficiency significantly suppresses the infection-stimulated increase in PA activity (Figure 4E; $p < 0.0001$). To investigate the mechanism by which IFN γ -deficiency suppresses PA activity, we measured levels of hepatic mRNA encoding PAI1, tPA, and uPA. Infection upregulated hepatic PAI1 mRNA levels more than 100-fold in wild type mice, and IFN γ -deficiency further increased PAI1 expression (Figure 4F; $p < 0.02$). Similarly, infection upregulated hepatic tPA mRNA levels 4-fold in wild type mice, and IFN γ -deficiency further increased tPA expression (Figure 4G; $p < 0.02$). Infection also upregulated hepatic uPA mRNA levels 10-fold in wild type mice (Figure 4H). However, unlike tPA, IFN γ -deficiency significantly decreased expression of uPA (Figure 4H; $p < 0.0001$). Together, these data indicate that infection increases hepatic PA activity, and suggest that altered expression of uPA contributes to the suppressed hepatic PA activity in IFN γ -deficient mice.

Discussion

Regardless of the causative microorganism, severe sepsis often disturbs hemostasis and promotes coagulopathy [29]. Despite this pathological capacity of infection-stimulated coagulation, mice must deposit fibrin to survive *Toxoplasma* infection [21]. As such, murine toxoplasmosis provides an opportunity to study mechanisms regulating balanced, protective, infection-stimulated coagulation. We recently reported that IFN γ functions as an endogenous antagonist of fibrin deposition during *Toxoplasma* infection [24]. Others previously reported that IFN γ also suppresses fibrin deposition during bacterial peritonitis [30,31] and organ transplantation [32,33]. In each of these settings, the mechanisms by which IFN γ regulates fibrin deposition have yet to be established.

Human patients treated with IFN γ exhibit changes in plasma biomarkers that suggest decreased activation of fibrinolytic pathways [27,28]. Likewise, a plasma-based D-dimer assay provided us with data consistent with IFN γ acting to suppress fibrinolysis. However, IFN γ -suppressed fibrinolysis was difficult to reconcile with our observation that IFN γ suppresses hepatic fibrin deposition. Consequently, we developed in situ assays to specifically investigate hemostasis-related activities in the liver.

The in situ PTase assay revealed that infection by *Toxoplasma* upregulates hepatic coagulant activity and that this upregulation is TF-dependent. Others have reported that IFN γ regulates

TF expression *in vitro* [34–36]. However, IFN γ -deficiency did not alter hepatic PTase activity during *Toxoplasma* infection. While it remains possible that IFN γ regulates TF-dependent PTase activity in other settings, our data suggest that IFN γ does not suppress infection-stimulated hepatic fibrin deposition by downregulating PTase activity.

The *in situ* assays also demonstrated that *Toxoplasma* infection upregulates hepatic PCase activity, and moreover, that IFN γ suppresses this upregulation. Subsequent analyses revealed that IFN γ reduces infection-stimulated expression of hepatic TM and TAFI. Together, these observations suggest that IFN γ suppresses fibrin deposition by decreasing TM-mediated activation of TAFI, thereby preventing TAFI-mediated stabilization of fibrin deposits. Consistent with these findings, a prior study found that IFN γ destabilizes TM mRNA, thereby decreasing expression of TM by primary human monocytes [37]. To our knowledge, our study is the first to demonstrate that IFN γ regulates TM *in vivo*. This is also the first report that IFN γ regulates expression of TAFI. Further studies will be required to definitively establish that IFN γ -regulated expression of TM and TAFI impact TAFI activation *in vivo*.

The *in situ* assays also indicated that IFN γ promotes hepatic PA activity during infection. Further analysis revealed that IFN γ increases hepatic uPA expression and modestly decreases tPA and PAI1 expression. The physiologic significance of altered tPA and PAI1 expression is uncertain, as neither tPA-deficiency nor PAI1-deficiency significantly impact hepatic PA levels (Figure 4B and 4C). The unaltered levels of PA activity in PAI1-deficient mice were particularly unexpected given the robust induction of PAI1 during infection (Figure 4F). It may be that other PAI play dominant roles in this model or that regulation of uPA expression suffices to control PA levels. Alternatively, the *in situ* assay, which does not entail tissue homogenization, may be revealing levels of regulation related to the localization and compartmentalization of fibrinolytic proteins within tissue [38]. Yet another possibility is that uPA may be present in its single chain form, which is not bound by PAI1 but could be converted to active two-chain uPA during the course of our assay [7]. Regardless, it is clear that uPA impacts hepatic PA levels in this model (Figure 4D) and, thus, our data suggest that IFN γ increases PA activity by increasing expression of uPA (Figure 4H). Notably, others previously reported that IFN γ upregulates expression of uPA by macrophages [39] but antagonizes expression of uPA by activated endothelial cells [40–42]. In combination with our findings, these reports suggest that IFN γ primarily regulates hepatic PA activity through effects on leukocytes. Presently, we are addressing this possibility via the generation and analysis of mice expressing uPA in specific cell types.

As noted above, our finding that IFN γ promotes fibrinolysis contrasts with prior reports suggesting that IFN γ suppresses fibrinolysis [27,28]. However, those prior conclusions derived from plasma-based assays of hemostatic biomarkers, which often cannot discern whether increased analyte (ex. D-dimer) reflects increased substrate (i.e. fibrin) or increased enzyme activity (i.e. PA). Moreover, plasma-based assays may fail to accurately depict the hemostatic state of all tissues, particularly when one considers accumulating evidence that hemostasis is regulated by tissue-specific mechanisms [14,15]. During septic coagulopathy, plasma-based assays reveal net activation of all hemostatic pathways, and provide the clinician with little information regarding the specific tissues and hemostatic activities that require therapeutic modulation. We anticipate that further application of the *in situ* assays described herein will provide greater insight into the mechanisms underlying tissue-specific hemostasis, their regulation by cytokines, and their dysregulation during coagulopathy.

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Figure 1. Fibrin and D-dimer levels during toxoplasmosis in wild type and IFN γ -deficient mice
 Plasma and hepatic tissue were harvested from sham-infected mice (open symbols) and mice infected 8 days prior with *Toxoplasma* (closed symbols). Each symbol represents an individual animal. (A) *Toxoplasma* infection significantly increased hepatic fibrin deposition in wild type mice (WT; $p < 0.0001$), and that increase was further upregulated in mice homozygous for IFN γ -deficiency (IFN γ -KO; $p < 0.0001$). (B) *Toxoplasma* infection significantly increased plasma D-dimer in WT mice ($p < 0.05$), and that increase was further upregulated in mice homozygous for IFN γ -deficiency ($p < 0.05$).

Figure 2. Levels of tissue factor-dependent hepatic PTase activity during toxoplasmosis in wild type and IFN γ -deficient mice

Hepatic tissue was harvested from sham-infected mice (open symbols) and mice infected 8 days prior with *Toxoplasma* (closed symbols). Each symbol represents an individual animal. Hepatic PTase activity was measured as described in the *Materials and Methods*. (A) *Toxoplasma* infection significantly increased PTase activity in wild type (WT) mice ($p < 0.0005$), and the factor Xa antagonist TENSTOP significantly suppressed that increase ($p < 0.0001$). (B) *Toxoplasma* infection significantly increased hepatic PTase activity in mice heterozygous for murine TF-deficiency and expressing a human TF transgene (mTF^{+/-}/hTF⁺; $p < 0.005$), and this infection-stimulated increase in PTase activity was significantly suppressed in mice homozygous for murine TF-deficiency and expressing a human TF transgene (mTF^{-/-}/hTF⁺; $p < 0.0001$; N.D., not determined). (C) *Toxoplasma* infection significantly increased hepatic PTase activity in WT mice ($p < 0.0001$), and that activity was not significantly altered in mice homozygous for IFN γ -deficiency (IFN γ -KO; $p = 0.5$).

Figure 3. Levels of hepatic PCa activity and mRNA encoding TM and TAFI during toxoplasmosis in wild type and IFN γ -deficient mice

Hepatic tissue was harvested from sham-infected mice (open symbols) and mice infected 8 days prior with *Toxoplasma* (closed symbols). Each symbol represents an individual animal. Hepatic PCa activity was measured as described in the *Materials and Methods*. (A) *Toxoplasma* infection significantly increased PCa activity in wild type (WT) mice ($p < 0.005$), and the thrombin antagonist PPACK significantly suppressed that increase ($p < 0.0005$). (B) *Toxoplasma* infection significantly increased hepatic PCa activity in WT mice ($p < 0.0001$), and that activity was significantly increased in mice homozygous for IFN γ -deficiency (IFN γ -KO; $p < 0.0005$). (C–D) Levels of hepatic mRNA encoding TM and TAFI were measured by real-time PCR and expressed as log₁₀ fold change relative to sham-infected WT mice. *Toxoplasma* infection significantly increased levels of hepatic TM and TAFI mRNA in WT mice (both $p < 0.0001$). Levels of TM and TAFI mRNA were further increased in mice homozygous for IFN γ -deficiency (IFN γ -KO; $p < 0.001$ and $p < 0.0001$ for TM and TAFI, respectively). Notably, IFN γ -deficiency did not significantly impact basal levels of PCa activity or mRNA encoding TM or TAFI.

Figure 4. Levels of hepatic PA activity and mRNA encoding PAI1, tPA and uPA during toxoplasmosis

Hepatic tissue was harvested from sham-infected mice (open symbols) and mice infected 8 days prior with *Toxoplasma* (closed symbols). Each symbol represents an individual animal. Hepatic PA activity was measured as described in the *Materials and Methods*. (A) *Toxoplasma* infection significantly increased PA activity in wild type (WT) mice ($p < 0.005$), and the PA antagonist PAI1 significantly suppressed that increase ($p < 0.005$). (B–D) The *Toxoplasma* infection-stimulated increase in hepatic PA activity was not significantly altered in mice homozygous for PAI1-deficiency (PAI1-KO; $p = 0.7$) or tPA-deficiency (tPA-KO; $p = 0.6$), but was significantly decreased in mice homozygous for uPA-deficiency (uPA-KO; $p < 0.005$). (E) The *Toxoplasma* infection-stimulated increase in hepatic PA activity was also significantly decreased in mice homozygous for IFN γ -deficiency (IFN γ -KO; $p < 0.0001$). (F–H) Levels of hepatic mRNA encoding PAI1, tPA, and uPA were measured by real-time PCR and expressed as log10 fold change relative to sham-infected WT mice. *Toxoplasma* infection significantly increased levels of hepatic PAI1, tPA, and uPA mRNA in WT mice (all $p < 0.0001$). Levels of PAI1 and tPA were further increased in mice homozygous for IFN γ -deficiency (both $p < 0.02$), whereas levels of uPA mRNA were significantly decreased in mice homozygous for IFN γ -deficiency ($p < 0.0001$).