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MAJOR ARTICLE

Interferons Induce STAT1–Dependent Expression of Tissue Plasminogen Activator, a Pathogenicity Factor in Puumala Hantavirus Disease

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Hantaviruses are zoonotic viruses that show various degrees of vasculopathy in humans. In this study, we analyzed the regulation of 2 fibrinolytic parameters, tissue plasminogen activator (tPA) and its physiological inhibitor, plasminogen activator inhibitor 1 (PAI-1), in Puumala hantavirus (PUUV)–infected patients and in human microvascular endothelial cells. We detected strong upregulation of tPA in the acute phase of illness and in PUUV-infected macaques and found the tPA level to positively correlate with disease severity. The median levels of PAI-1 during the acute stage did not differ from those during the recovery phase. In concordance, hantaviruses induced tPA but not PAI-1 in microvascular endothelial cells, and the induction was demonstrated to be dependent on type I interferon. Importantly, type I and II interferons directly upregulated tPA through signal transducer and activator of transcription 1 (STAT1), which regulated tPA gene expression via a STAT1-responsive enhancer element. These results suggest that tPA may be a general factor in the immunological response to viruses.

Keywords. tissue plasminogen activator; interferon; STAT1; hantavirus; hemorrhagic fever; innate immunity.

Hantaviruses (genus *Hantavirus*) are rodent- and insectivore-borne enveloped viruses that belong to the family *Bunyaviridae* [1, 2]. Pathogenic hantaviruses are carried exclusively by rodents [3], and they are transmitted to humans through aerosolized rodent excreta [4]. When transmitted to humans, hantaviruses may cause 2 severe diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS).

In both diseases, the vasculature is severely affected, leading to bleeding in HFRS and to lung edema in HCPS. Hantaviruses infect the endothelial cells of the blood vessels both in vitro and in vivo but do not cause direct cytopathic effects that would explain the increased vascular permeability. Hantavirus infection in humans does, however, lead to an acute inflammatory reaction characterized by the induction of proinflammatory cytokines, thrombocytopenia, leukocytosis, endothelial cell activation, complement activation, and hemostatic abnormalities [5–7]. Excessive activation of the fibrinolytic system, as reflected by the high level of fibrin degradation products (FDPs) in comparison with thrombin formation is seen in Puumala hantavirus (PUUV)–caused HFRS [8].

Tissue-type plasminogen activator (tPA) is mostly responsible for stimulating fibrinolysis in the vasculature. The main inhibitor of tPA is plasminogen activator inhibitor 1 (PAI-1), the active form of which irreversibly inactivates PA through a covalent 1:1 interaction [9]. In this study, we found that tPA is upregulated in the acute phase of PUUV infection and that high tPA levels are associated with the severity of infection in patients and hantavirus-infected macaques. In addition, we demonstrate that hantavirus infection of cultured primary microvascular endothelial cells induces tPA production that is dependent on type I interferons (IFNs), which are known to be efficient antiviral substances against hantavirus infection [10]. Importantly, we further demonstrate that IFNs induce tPA through signal transducer and activator of transcription 1 (STAT1), which regulated the *tPA* gene via a STAT1-responsive enhancer element. Our results suggest that tPA plays a central role in hantavirus pathogenesis and might be a general factor in the immunological response to viruses.

MATERIALS AND METHODS

PUUV Strain

PUUV Kazan strain was propagated in Vero E6 cells (green monkey kidney epithelial cell line; ATCC no. CRL-1586). Virus titers were measured as previously described [11, 12].

Primary Endothelial Cell Cultures

The primary blood microvascular endothelial cells (BECs) were used during passages 3–6 and grown in reduced growth medium

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for experiments. Subconfluent BECs underwent PUUV infection or mock infection in Vero E6 growth medium at a multiplicity of infection of 10. In indicated experiments, the BEC reduced growth medium was supplemented with sheep neutralizing antibodies against type I IFNs [13].

Messenger RNA (mRNA) Quantification

Extracted RNA was subjected to reverse transcription with hexamers and relative quantitative polymerase chain reaction analysis. Primers used to detect tPA, uPA, and PAI-1 are described by Muth et al [14], those for myxovirus resistance gene A (MxA) are described by Verma et al [15], those for retinoic acid inducible gene I (RIG-I) are described by Prescott et al [16], those for STAT1 are described by Schmeisser et al [17], and those for the PUUV S segment are described by Koivula et al [18]. RNA polymerase 2 was used as the housekeeping gene [19]. Relative quantification of individual mRNAs was performed by the comparative cycle threshold method [20].

Immunofluorescence

Infected BECs grown on coverslips were fixed and stained with rabbit polyclonal antibody to detect hantavirus N protein [21].

Patient Samples

The study cohort consisted of 38 patients treated for serologically confirmed acute PUUV infection at the Tampere University Hospital, Finland, during September 2000–January 2004. All 38 patients were also participants in our larger study to determine complement activation during acute PUUV infection [22, 23].

tPA Activity Assays

The tPA activity in plasma samples was measured as previously described [24].

Knock Down of STAT1 by Short Interfering RNA (siRNA)

BECs were either nontransfected or transfected with scrambled negative control or STAT1-specific siRNA. One day after transfection, the cells were treated with 10 ng/mL of IFN-α, IFN-β, or IFN-γ for 2 days and analyzed for the expression of tPA, PAI-1, and STAT1 mRNAs in cells and of tPA and PAI-1 protein levels in the supernatants as previously described.

Chromatin Immunoprecipitation

Chromatin from BECs treated with IFN-α, IFN-β, or IFN-γ for 1 hour was cross-linked to its bound proteins and sheared using an ultrasonicator (Bandelin Sonopuls). Immunoprecipitation was performed using 2 μg of p65 or STAT1 antibodies, and total DNA was isolated. Real-time polymerase chain reaction analysis was performed to evaluate the amount of DNA precipitated by STAT1 as compared to that precipitation by p65 antibodies, using primers specific for the enhancer, intermediate, and promoter regions of the regulatory elements of *tPA*. As positive and negative controls, primers specific for IFN regulatory factor 1 (IRF1) promoter and integrin β3 subunit exon, respectively, were used (primers sequences are specified in Supplementary Table 3).

RESULTS

Regulation of Fibrinolytic Factors tPA and PAI-1 in the Acute Phase of Human PUUV Infection

PUUV-infected patients have excessive fibrinolysis [8]. Since the major activator and inhibitor of fibrinolysis are tPA and PAI-1, respectively [9], we decided to measure their levels during the acute and recovery phases of PUUV infection by enzyme-linked immunosorbent assays. We observed that the plasma level of tPA in the acute phase (median, 23.2 ng/mL) was significantly higher than in the convalescent phase (10.9 ng/mL; $P < .01$), whereas similar PAI-1 levels were measured in both groups (median, 31.7 ng/mL vs 31.7 ng/mL; $P = .4$; Figure 1*A*). The tPA level in the acute phase correlated with the following parameters: length of hospitalization, gain of weight (reflecting fluid retention during the oliguric phase), minimum platelet count, leukocytosis, and high levels of the terminal complement complex (TCC), interleukin 6 (IL-6), and hematocrit (reflecting plasma leakage; Supplementary Table 1). High acute-phase PAI-1 level correlated strongly with the length of hospital stay, and a close to statistically significant correlation was observed with leukocytosis ($P = .05$) and high hematocrit ($P = .051$). The increased tPA plasma level prompted us to study tPA activity, since plasma contains inhibitors of tPA (such as PAI-1). A statistically significant increase in tPA activity was observed in the plasma samples collected during the acute phase (median, 0.23 IU/mL) versus those collected in the convalescent phase (median, 0.10 IU/mL; $P < .01$; Figure 1*A*). Furthermore, we found the activity of tPA to correlate with the amount of tPA (protein level) and also with the length of hospital stay, the amount of weight loss, and the TCC, C-reactive protein (CRP), and IL-6 levels (Supplementary Table 1). Taken together, our results indicate that acute PUUV infection induces tPA production and that, since the levels of its inhibitor, PAI-1, were not generally altered, the overall tPA activity is increased.

PUUV causes a mild form of HFRS, and hemorrhagic manifestations were present in 10% of the patients included in this study. We found higher levels (median, 61.2 ng/mL vs 22.0 ng/mL) and increased activity (median, 0.55 IU/mL vs 0.20 IU/mL) of tPA in acute-phase plasma samples from patients with hemorrhagic manifestations as compared to specimens from patients without hemorrhagic manifestations, while the levels of PAI-1 in both groups were similar (median, 30.4 ng/mL vs 31.6 ng/mL; Figure 1*B*). Taken together, these results suggest that tPA plays a major role in PUUV pathogenesis.

Kinetics of tPA Upregulation in PUUV Infection

An experimental infection model of wild-type PUUV infection that mimicked the disease in humans to a high extent was established earlier in a monkey model (cynomolgus macaques) [25]. To get an insight on the kinetics of tPA induction during PUUV

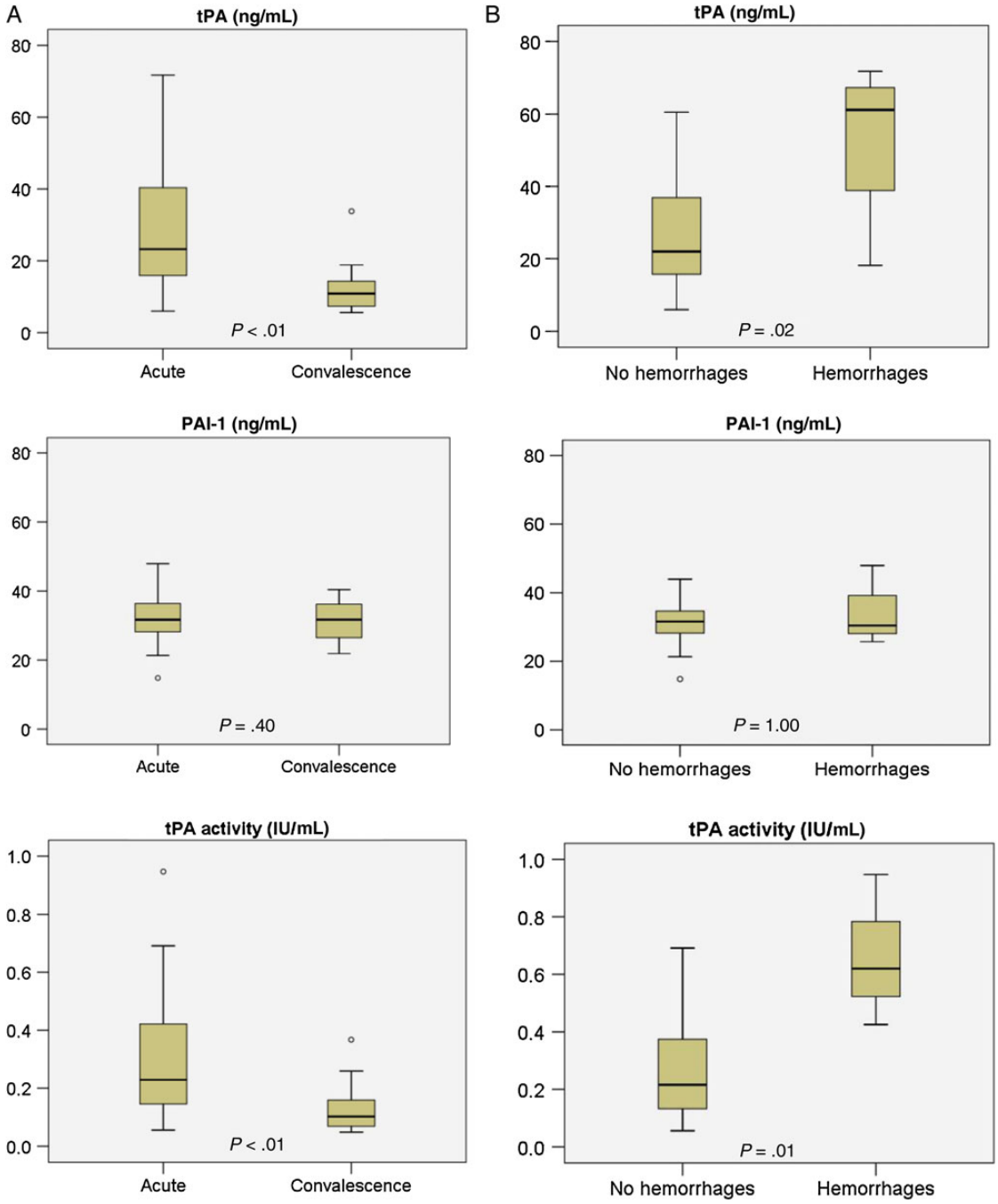

Figure 1. Levels of tissue plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-1), and tPA activity in acute human Puumala hantavirus (PUUV) infection. Box plots of the levels of tPA (n = 38), PAI-1 (n = 32), and tPA activity (n = 35) in patient plasma samples of acute versus convalescent phases of PUUV infection (*A*) and in acute-phase samples of reported hemorrhagic versus nonhemorrhagic PUUV infection (*B*). Box plot illustrates the median value (center horizontal line), interquartile range (the lower and upper quartiles), and the highest and lowest values (whiskers) that are not outliers. The outliers are presented as circles. The normal values of tPA and PAI-1 in plasma of healthy individuals were 10.5 ± 3.1 and 39.6 ± 7.5 ng/mL, respectively, showing that elevated tPA levels during the acute phase return to baseline levels at the convalescent phase. The statistical significance was analyzed using the Wilcoxon signed rank test (*A*) and the Mann–Whitney *U* test (*B*).

infection, we analyzed the tPA levels in archival serum samples from 3 monkeys that showed disease with varying severity, as judged initially by the loss of appetite and general affectedness (the degree of severity was greatest in animal 59, followed by animal 23 and animal 25). Of the 2 more severely affected animals, one had an elevated CRP level (Figure 2*B*) and the other had an increased creatinine level (Figure 2*C*) during infection, indicating inflammation and kidney dysfunction, respectively. All animals produced neutralizing antibodies with similar kinetics (Figure 2*D*), showing successful virus challenge. We found

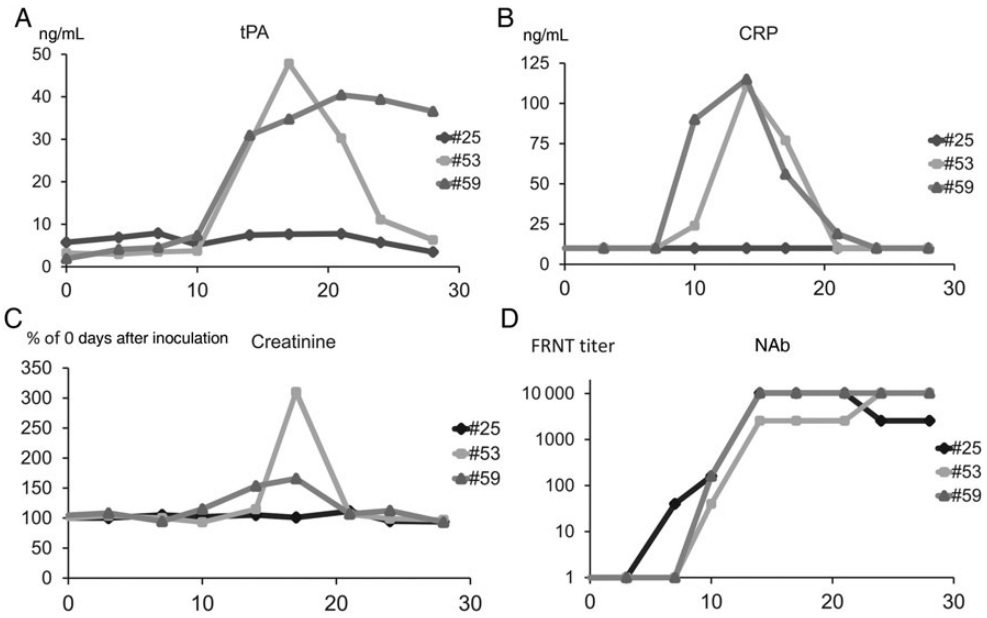

Figure 2. Levels of tissue plasminogen activator (tPA) in experimental monkey model of Puumala hantavirus (PUUV) infection. Three cynomolgus macaques were infected with wild-type PUUV and levels of tPA (*A*) were measured from serum samples collected at 0, 3, 7, 10, 14, 17, 21, 24, and 28 days after inoculation. These samples have been previously used to measure the levels of C-reactive protein (CRP; *B*), creatinine (*C*; measured originally as micromoles and shown here as percentage of initial value at 0 days after inoculation), and neutralizing antibody (NAb) titers (*D*; measured by focus reduction neutralization test [FRNT]) to mark inflammation, kidney dysfunction, and successful virus challenge, respectively. As judged by general affectedness, the monkeys manifested a hemorrhagic fever with renal syndrome–like disease with disease severity greatest in animal 59, followed by animals 53 and 25.

tPA to be upregulated (level, 30–50 ng/mL) in the 2 more severely affected animals (ie, animals 59 and 53; Figure 2*A*) 14 days after inoculation. We then compared the tPA values to other parameters previously measured from these samples [25], and we found that tPA upregulation significantly correlated with the induction of the proinflammatory cytokines tumor necrosis factor α (TNF-α), IL-6, and IFN-γ (Supplementary Table 2). We were unable to reliably measure PAI-1 levels in these samples, likely because PAI-1 is released by platelet activation during serum processing [26].

PUUV Infection Induces tPA in Primary Blood Microvascular Endothelial Cells

Because endothelial cells lining the vasculature are important for tPA production and hantaviruses infect these cells both in vitro and in vivo [5], hantavirus infection could directly induce tPA production in vascular endothelial cells. To study the regulation of genes involved in fibrinolysis (ie, those encoding tPA, PAI-1, and uPA) by hantavirus infection in vitro, we infected primary human BECs with hantaviruses. Growth factors and hydrocortisone, present in the fully supplemented growth medium of BECs, regulate tPA production in vascular endothelial cells [27–29]. Therefore, we grew BECs in nonsupplemented and reduced (from 5% to 1% fetal bovine serum) growth medium and infected the cells with ultraviolet light (UV)–inactivated PUUV (PUUV-UV) and live PUUV. We monitored the kinetics of hantavirus infection by immunofluorescence staining of viral nucleocapsid (N) protein 1, 3, and 5 days after inoculation We observed that the amount of N protein increased from 1 to 3 days after inoculation but declined 5 days after inoculation (Figure 3*A*). We further verified the downregulation of viral infection 5 days after inoculation by quantifying the viral RNA levels (relative amounts of viral S segments; Figure 3*B*).

Hantaviruses are susceptible to the antiviral action of IFNs [10]. Thus, we decided to study whether the observed downregulation of viral infection in BECs could have been due to the innate immune response, and we measured the levels of IFN-β in the supernatants of mock-, PUUV- and PUUV-UV–infected BECs. We found that IFN-β was induced by PUUV infection 3 days after inoculation (Supplementary Figure 1*A*). We also observed strong induction of IFN-inducible genes, MxA, RIG-I, and STAT1 concomitant with the upregulation of IFN-β (Supplementary Figure 1*B*). The activation of an antiviral state in infected BECs likely explains the rapid decline in viral RNA and protein amount at the later stages of infection (Figure 3*A* and 3*B*).

Next, we measured the expression levels of genes involved in fibrinolysis. We observed upregulation of tPA in response to PUUV 3 and 5 days after inoculation, whereas uPA and PAI-1 remained at levels comparable to those in mock-infected and PUUV-UV–infected cells (Figure 3*C*). We detected an approximately 10-fold induction of tPA mRNA by PUUV and

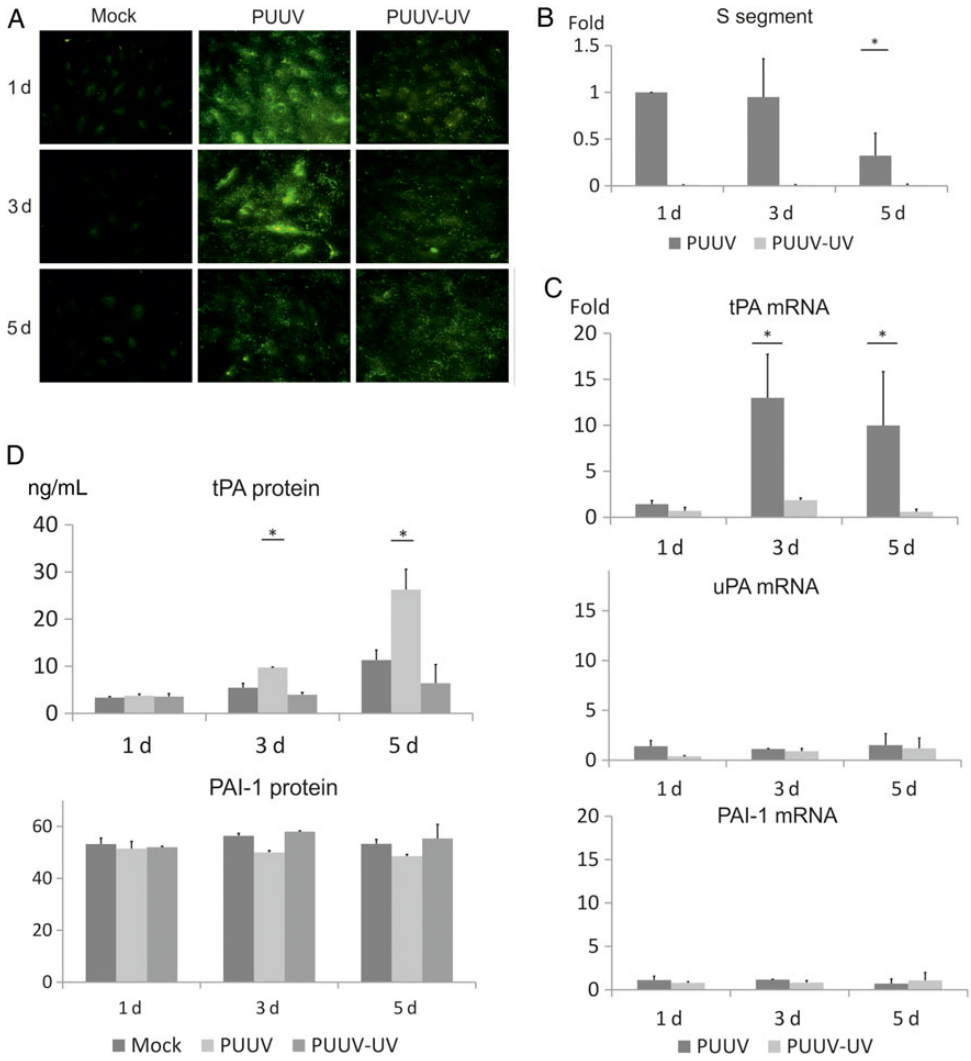

Figure 3. Characterization of Puumala hantavirus (PUUV) infection in blood microvascular endothelial cells (BECs). BECs were either mock infected or infected with PUUV or ultraviolet light–inactivated PUUV (PUUV-UV) and collected 1, 3, or 5 days after inoculation. *A*, Immunofluorescence staining of hantavirus N protein by a polyclonal antibody. *B*, Real-time quantitative polymerase chain reaction (qPCR) analysis of PUUV S segment RNA in infected BECs. Fold change on *y*-axis is relative to infected cells 1 day after inoculation. $^*P < .05$, by the Student *t* test, compared with infected cells 1 day after inoculation. *C*, Real-time qPCR analysis of tissue plasminogen activator (tPA), uPA, and plasminogen activator inhibitor 1 (PAI-1) messenger RNA (mRNA) in BECs. Fold change on *y*-axis is relative to mock-infected cells at indicated days after inoculation. *D*, Findings of enzyme-linked immunosorbent assays of tPA and PAI-1 in supernatants of BECs. Data are presented as means±SD (n = 3). $^*P < .05$, by the Student *t* test, compared with the mock-infected sample. The experiments were independently performed at least 3 times, with similar results.

confirmed tPA induction in PUUV-infected BECs versus mock-infected or PUUV-UV–infected BECs at the protein level from the cell culture supernatants (Figure 3*D*).

Interestingly, we did not record increased tPA activity in supernatants of mock-infected or PUUV-infected BECs (data not shown). Hence, we studied the ability of BECs to inactivate tPA by incubating the supernatants of mock-infected and PUUV-infected BECs with active exogenous tPA and measuring the remaining tPA activity in the samples. The supernatants of PUUV-infected cells retained >80% of tPA activity, while only about 50% activity was left in mock-infected and PUUV-UV–infected cell supernatants (Supplementary Figure 1C). This indicates that PUUV-infected BECs produce active tPA, which reduces the ability of BEC supernatants to inactivate exogenous tPA. The inactivation of tPA is likely due to an excess of PAI-1, relative to tPA, in BEC supernatants (Figure 3*D*).

PUUV-Induced Expression of tPA Is Dependent on Type I IFN

We found upregulation of tPA (3 and 5 days after inoculation; Figure 3*C* and 3*D*) to occur concurrently with downregulation of viral RNA and N protein expression (Figure 3*A* and 3*B*). This suggests that tPA upregulation in virus infection would, instead of viral replication, be due to induction of cytokines or growth factors. To study the role of innate immunity (ie, type I IFNs) in the regulation of tPA expression, we infected BECs with PUUV in the presence and absence of IFN-α and

IFN-β function-neutralizing antibodies (anti–IFN-I). As a result of anti-IFN-I treatment we detected significantly elevated amounts of N protein (Figure 4*A*) and viral RNA (Figure 4*B*) in cells, as well as a higher number of infectious virus (Figure 4*C*), in the supernatant. This indicates that type I IFNs possess marked antiviral effects toward PUUV in BECs.

Next, we analyzed mRNA levels of tPA and PAI-1 in BECs infected with PUUV, with or without treatment with anti-IFN-I, and those in uninfected BECs treated with anti–IFN-I and compared the mRNA levels to those in mock-infected cells 1 and 3 days after inoculation (Figure 4*D*). We found that the level of PUUV-induced tPA mRNA was clearly downregulated by anti–IFN-I treatment 3 days after inoculation. Anti-IFN treatment downregulated the level of PUUV-induced tPA close to basal levels, also on protein level (Figure 4*E*). Contrary to tPA, the levels of PAI-1 mRNA and protein remained constant between the samples (Figure 4*D* and 4*E*). These results suggest that type I IFNs contribute to tPA induction in PUUV-infected BECs.

The ability of hantaviruses to cause IFN-dependent upregulation of tPA in BECs is not restricted to PUUV, since we obtained similar results by using another HFRS-causing hantavirus (Hantaan virus [HTNV], which causes a severe form of HFRS in Asia; Supplementary Figure 2).

Type I and II IFNs Induce tPA in BECs

To study whether type I IFNs could induce tPA independent of virus infection, we investigated whether exogenously added

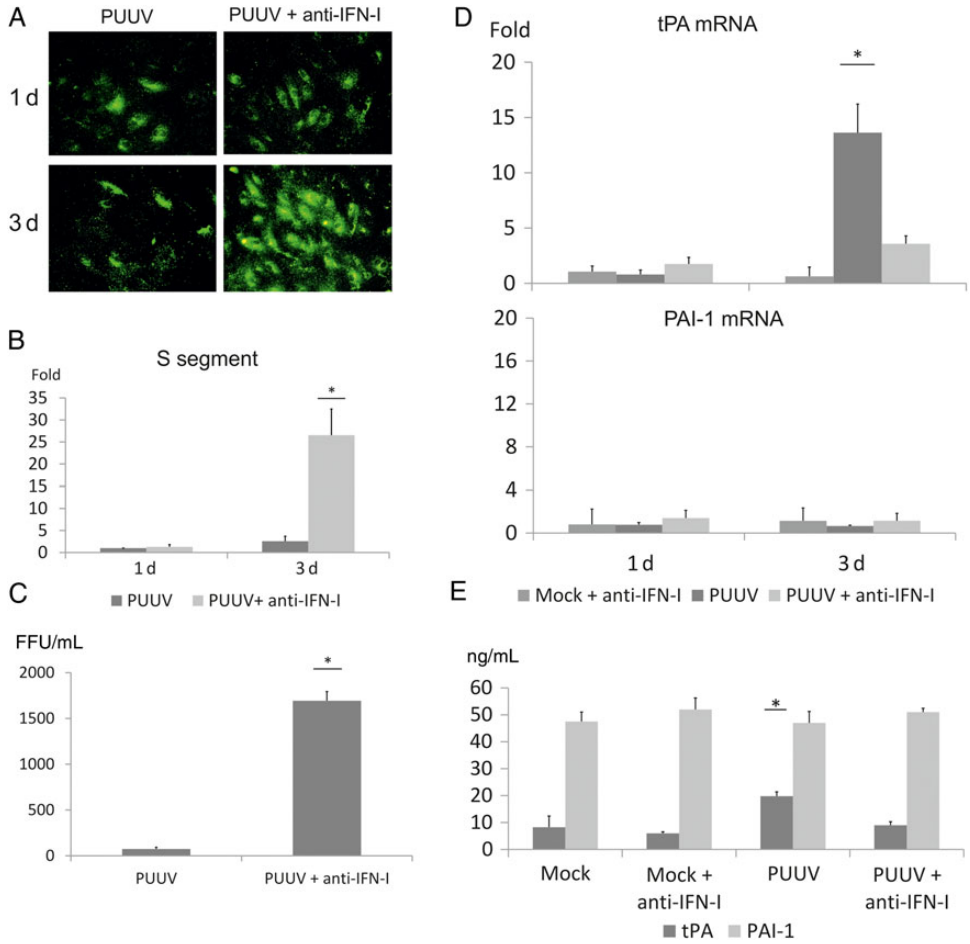

Figure 4. Effect of type I interferon (IFN)–neutralizing antibodies on Puumala hantavirus (PUUV) replication and PUUV-induced tissue plasminogen activator (tPA) in blood microvascular endothelial cells (BECs). BECs were mock or PUUV infected, polyclonal sheep antibodies against IFN-α and IFN-β (1:100 dilutions; anti-IFN-I) were added where indicated, and cells were collected 1 or 3 days after inoculation. *A*, Immunofluorescence staining of PUUV N protein by a polyclonal antibody. *B*, Real-time quantitative polymerase chain reaction (qPCR) analysis of PUUV S segment RNA. Fold change on the *y*-axis is relative to infected, nontreated cells 1 day after inoculation. **P* < .05, by the Student *t* test, compared with infected, nontreated cells 1 day after inoculation. *C*, Quantification of the release of infectious virus in supernatants of BECs 3 days after inoculation, using by a focus-forming unit assay. *D*, Real-time qPCR analysis of tPA and plasminogen activator inhibitor 1 (PAI-1) messenger RNA (mRNA). Fold change on the *y*-axis is relative to mock-infected cells at indicated days after inoculation. *E*, Findings of enzyme-linked immunosorbent assays of tPA and PAI-1 in supernatants of BECs 3 days after inoculation. Data are presented as means±SD (n = 3). **P* < .05, by the Student *t* test, compared with the mock-infected sample. The experiments were independently performed at least 3 times, with similar results.

IFN-α or IFN-β would induce tPA in BECs. We also studied whether other proinflammatory cytokines (TNF-α, IL-6, and IFN-γ) that have been previously suggested to play a role in hantavirus disease pathogenesis [5] would induce tPA production. We observed a 10-fold increase in tPA mRNA in response to stimulation of the cells with IFN-α or IFN-β (Figure 5*A*). Unexpectedly, we also found that IFN-γ (type II IFN) induced tPA, whereas TNF-α and IL-6 did not alter its expression. The mRNA levels of PAI-1 remained unaltered in the presence of all cytokines. These findings were corroborated at the protein level (Figure 5*B*). These data suggest that type I IFNs are responsible for the upregulation of tPA in hantavirus-infected BECs but also indicate that tPA might play a general role in the innate immune response.

STAT1 Mediates the Induction of tPA by Type I and II IFNs

Type I and II IFNs induce different signaling cascades in target cells. However, the activation of STAT1 transcription factor is common to both pathways. To analyze the activation of tPA by type I and II IFNs further, we decided to knock down STAT1 induction in BECs by siRNA prior to treatment by the cytokines. To begin with, we confirmed that the STAT1-specific siRNA downregulates the STAT1 protein in BECs as compared to the negative control, scrambled siRNA (Figure 6*A*). As expected, we found that both type I and II IFNs induced STAT1 mRNA, which was downregulated by STAT1-specific siRNA (Figure 6*B*). Interestingly, we observed the IFN-induced levels of tPA to be downregulated by knock down of STAT1, while the levels PAI-1 mRNA remained unaltered (Figure 6*B*). We also confirmed the regulation of tPA, but not PAI-1, by STAT1 at the protein level (Figure 6*C*).

STAT1 Directly Regulates the Enhancer Region of *tPA*

Next, we asked whether activated STAT1 could directly enhance the transcription of *tPA*. First, we analyzed whether the regulatory regions of *tPA* would contain one of the two canonical STAT1 binding sites, IFN-stimulated response elements (activated by type I IFNs) or IFN-γ activation sites (activated by type II IFN). We were, however, unable to find by computer search any putative STAT1 binding sites on the regulatory elements of *tPA*. The regulation of *tPA* is achieved through cooperation with the gene promoter and enhancer regions (Figure 7*A*). Both contain elements that are regulated by specificity protein 1 (Sp1), and the enhancer region also contains a binding site for retinoic acid receptor transcription factors [30, 31]. We thus wanted to study whether STAT1 directly binds either the enhancer or promoter regions of *tPA*. We used the chromatin immunoprecipitation assay and compared the ability of STAT1 and p65 (another commonly expressed transcription factor) to precipitate chosen DNA sequences in mock-treated, type I IFN–treated, or type II IFN–treated BECs. We observed a 5-fold enhancement in the level of enhancer region DNA in cells treated with type I or II IFN. The amount of intermediate and promoter region DNA elements remained at a similar level as compared to those in mock-treated cells (Figure 7*B*). The IRF-1 promoter and an exon to integrin β_3 were used as positive and negative controls, respectively, for the activity of IFNs, and both locate to a different chromosome than that on which *tPA* is located. The results show that STAT1 induced by type I or II IFN binds the enhancer region either directly or via another protein to induce the transcription of *tPA*.

DISCUSSION

Hemostasis is a well-regulated mechanism that stops the loss of blood in the case of vascular injury. It is increasingly evident that hemostasis, together with innate immunity (recently referred to as "immunothrombosis" [32]), plays a role in counteracting invading microbes. In this report, we demonstrated that tPA is upregulated at the protein level in the acute phase of a hantavirus infection, whereas PAI-1 is not, which results in

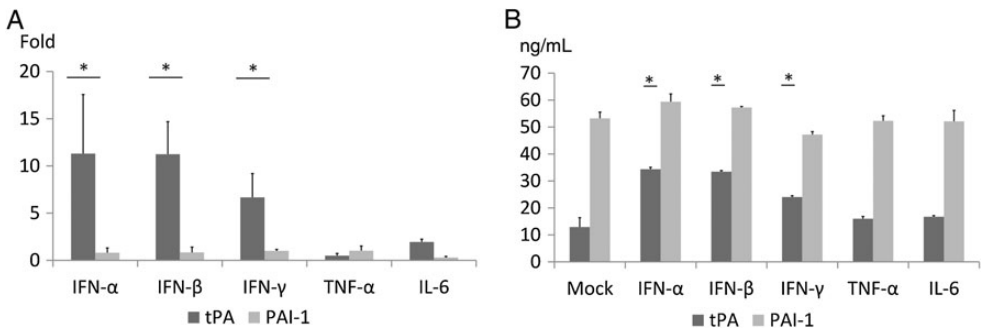

Figure 5. Type I and II interferons (IFNs) induce tissue plasminogen activator (tPA) in blood microvascular endothelial cells (BECs). BECs were nontreated or treated with IFN-α (0.3 ng/mL), IFN-β (0.3 ng/mL), IFN-γ (10 ng/mL), tumor necrosis factor α (TNF-α; 10 ng/mL), or interleukin 6 (IL-6; 10 ng/mL) and collected after 3 days. *A*, Real-time quantitative polymerase chain reaction analysis of tPA or plasminogen activator inhibitor 1 (PAI-1) messenger RNA (mRNA). Fold change on the *y*-axis is relative to mock-infected cells. *B*, Findings of enzyme-linked immunosorbent assays of tPA and PAI-1 in supernatants of BECs. Data are presented as means±SD (n = 3). **P* < .05, by the Student *t* test, compared with the nontreated sample. The experiments were independently performed at least 3 times, with similar results.

significantly enhanced tPA activity. The elevated tPA activity most likely results in activation of fibrinolysis, which has been reported in the acute phase of PUUV infection [8]. Impaired hemostasis and bleeding complications are common findings in hantaviral diseases. In HTNV infection, low plasminogen levels and increased levels of fibrin degradation products have been observed [33]. In the present study, we observed tPA upregulation in HTNV-infected BECs. Since HTNV infection is typically associated with more-severe bleeding complications than PUUV, it would be of interest to analyze the tPA levels in patients with acute HTNV infection. Recently, it was reported that PAI-1 levels are induced by SNV in acute HCPS and strongly correlate with a fatal outcome [34]. This implies that fibrinolysis is significantly reduced in severe HCPS. In concordance, no increased FDPs (a marker of fibrinolysis) were observed in Sin Nombre virus–infected rhesus macaques that had a disease mimicking HCPS [35]. This suggests that tPA activity is not increased by HCPS-causing viruses and might explain the lack of hemorrhages in HCPS. Given the relationship between IFNs and tPA presented in this report, it is likely that tPA is also upregulated in HCPS but that concomitant induction of PAI-1 counteract its effects. Taken together, these findings indicate that vascular hemostasis is differentially regulated by hantavirus infections of varying disease severity and implies that excessive fibrinolysis might even protect from the most severe forms of the disease.

tPA is mainly produced by endothelial cells, wherein hantavirus replication also primarily occurs [5]. Recently, PUUV infection of human umbilical vein endothelial cells (HUVECs) was shown to result in upregulation of the PAI-1 level [36]. HUVECs are derived from veins, whereas BECs are capillary endothelial cells that are mainly responsible for the interchange of liquids between blood and tissues and are more likely to interact with viruses and other components of plasma. The results with

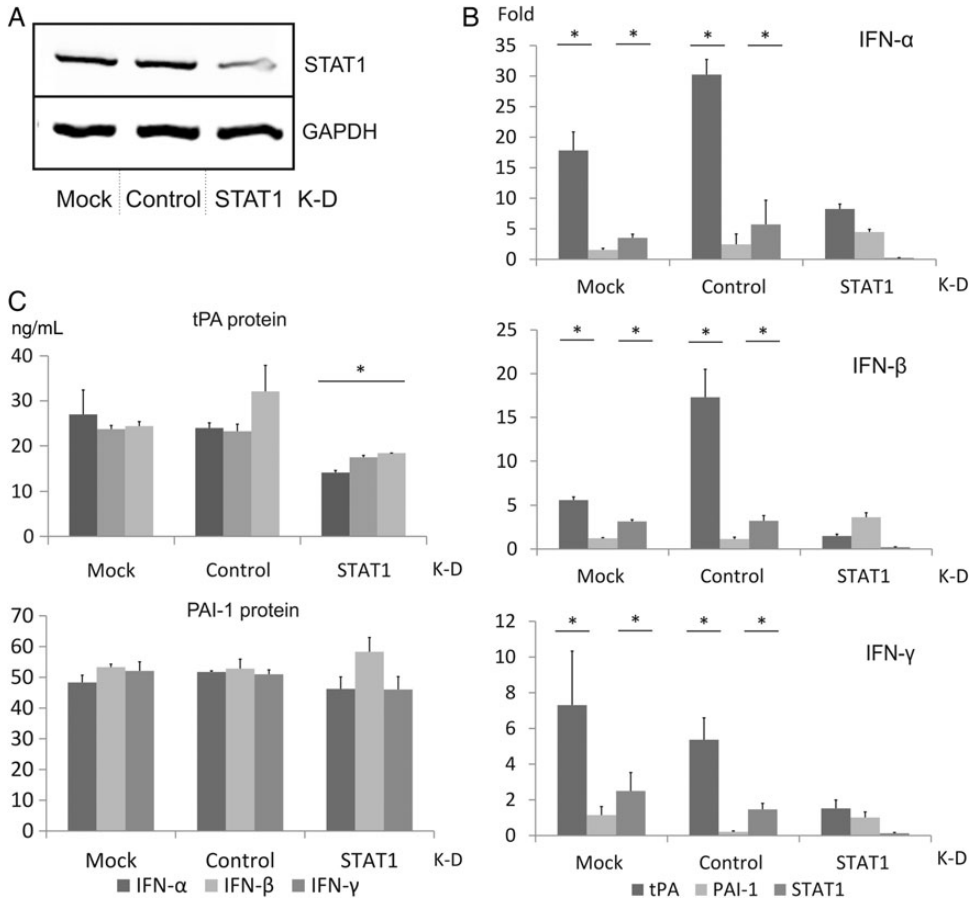

Figure 6. Knock down of STAT1 abolishes the induction of tissue plasminogen activator (tPA) by interferons (IFNs). Blood microvascular endothelial cells (BECs) were nontransfected (mock) or transfected with control or STAT1-specific short interfering RNA (siRNA) and treated with 10 ng/mL of IFN-α, -β, or –γ on day 1 and collected on day 3 after transfection. *A*, Western blot analysis of STAT1 and GAPDH in non–IFN-treated cells 3 days after transfection. *B*, Real-time quantitative polymerase chain reaction (qPCR) analysis of tPA, plasminogen activator inhibitor 1 (PAI-1), or STAT1 messenger RNA (mRNA). Fold change on the *y*-axis is relative to mock-transfected, nontreated cells. **P* < .05, by the Student *t* test, for the difference between tPA or STAT1 mRNA levels and the STAT1-specific siRNA-treated sample. *C*, Findings of enzyme-linked immunosorbent assays of tPA and PAI-1 in supernatants of IFN-treated and mock-transfected, control, or STAT1-siRNA–transfected BECs. Data are presented as means±SD (n = 3). **P* < .05, by the Student *t* test, compared with the nontreated sample. The experiments were independently performed at least twice, with similar results.

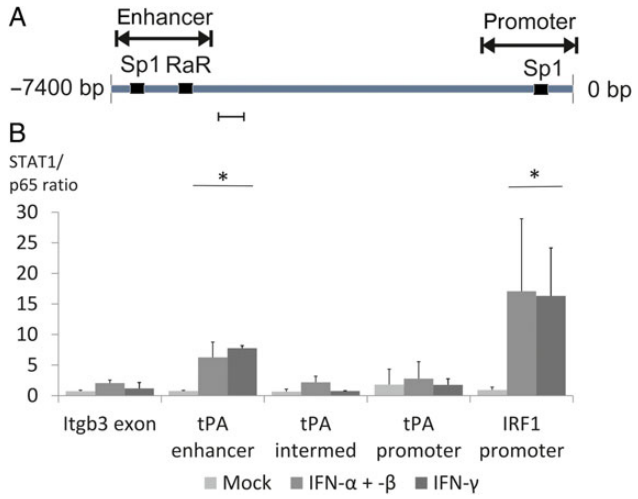

Figure 7. Chromatin immunoprecipitation (CHIP) analysis of STAT1 reveals its direct involvement in regulating the gene encoding tissue plasminogen activator (tPA). *A*, Schematic diagram of the regulatory elements of *tPA*, showing enhancer and promoter regions at 7400 base pairs upstream and proximal, respectively, to the tPA transcription start site. Bolded areas indicate known transcription factor binding sites. Polymerase chain reaction amplicon sites, which were chosen for our CHIP analysis, are also shown. *B*, Blood microvascular endothelial cells (BECs) were nontreated (mock) or treated with type I interferons (IFNs; 10 ng/mL of IFN-α and -β) or type II IFNs (10 ng/mL of IFN-γ) and collected 1 h after treatment. CHIP analysis, using STAT1 or p65 polyclonal antibodies, was performed on the previously defined sequences located in the tPA regulatory elements. We chose p65 as our negative control for STAT1 binding since it did not precipitate any of the analyzed DNA elements as compared to beads without an antibody. The ITGβ3 exon or IRF1 promoter were used as negative and positive controls, respectively. The ratio between STAT1- and p65-precipitated DNA, relative to input, is indicated. $*P < .05$, by the Student *t* test, compared with mock-treated cells. Data are presented as means±SD (n = 3). The experiments were independently performed at least twice, with similar results.

HUVECs indicate that, in addition to tPA, hantaviruses have the capability to also induce PAI-1 in endothelial cells, and this might be highly important in HCPS, in which PAI-1 levels are upregulated.

A major finding of this study is that IFNs induce tPA in microvascular endothelial cells. To our knowledge, this is the first time that type I (or II) IFNs have been shown to induce tPA in endothelial cells. However, IFN-α has been recognized to induce tPA in macrophages [37, 38]. Elevated expression of tPA and fibrinolysis have also been observed due to IFN-α therapy in humans [39, 40]. tPA is known to cause enhanced leakage of capillaries [41], which might facilitate diapedesis of leukocytes to underlying tissue during inflammation. In concordance with this, type I IFNs have been implicated in lethal vascular leakage [42]. On the other hand, tPA could also play a role in the resolution of infection by clearing thrombi that have been produced as part of the innate immune response to the intruding microbe.

We found that STAT1 plays a crucial role in the induction of tPA by type I and II IFNs. *tPA* is regulated by, in addition to its promoter, an enhancer element located approximately 7400 base pairs upstream of the transcription initiation site, which we found to be the target of STAT1. Interestingly, the chromatin immunoprecipitation assay revealed that this region was associated with IFN-γ–activated STAT1 in HeLa cells [43]. However, this region does not contain canonical STAT1 binding sites that would directly explain the mechanism of STAT1 association with this DNA region. Thus, whether STAT1 binds a nontypical DNA sequence present in the *tPA* enhancer or indirectly associates with this region by binding another transcription factor remains to be determined. The *tPA* enhancer is known to be regulated by Sp1 and retinoic acid receptor transcription factors [30, 31], and STAT1 is known to cooperate with Sp1 [44], favoring the indirect association to *tPA* enhancer.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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