Thrombin-activatable fibrinolysis inhibitor and bacterial infections
Valls Serón, M.

Citation for published version (APA):

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Download date: 28 Dec 2018
Chapter 6

Murine TAFI improves survival against Streptococcus pyogenes

Mercedes Valls Serón, Stefan R. Havik, Joris J.T.H. Roelofs, Charlotte Spaendonk, Heiko Herwald, Philip G. de Groot and Joost C.M. Meijers
Abstract
Thrombin-activatable fibrinolysis inhibitor (TAFI) is synthesized in the liver and secreted into the bloodstream in a zymogen form. TAFI is activated by thrombin, either free or in complex with thrombomodulin. Active TAFI (TAFIa) is involved in down regulation of plasmin formation and plays a role in inflammatory processes by inactivation of inflammatory mediators. We investigated the role of TAFI in the haemostatic and immune response during infection of mice with *Streptococcus pyogenes*. Wild-type and TAFI-knockout mice were infected using a subcutaneous air-pouch model with *S. pyogenes*. Mice were terminated after 24 or 48 hours or observed in a 5-day survival study. Our results showed that absence of TAFI did not have a consistent effect on bacterial dissemination into systemic organs in the first 48 hours. In addition, wild-type and TAFI-knockout presented no differences in activation of coagulation or fibrinolysis, as reflected by comparable plasma levels of thrombin-antithrombin complexes and D-dimers. TAFI-deficiency had no effect on lung, liver, spleen or kidney histopathology, or cytokine levels in plasma. Remarkably, TAFI-knockout mice had increased susceptibility to infection, with a 35% survival of TAFI-knockout compared to 87% of wild-type mice. Immunohistochemistry revealed that both wild-type and TAFI-knockout mice accumulated megakaryocytes in spleen during the 5-day infection. Megakaryocyte levels were significantly lower in TAFI-knockout compared to wild-type mice, which was associated with mortality. These data suggest that murine TAFI is necessary for survival after *S. pyogenes* infection and that TAFI plays a role in megakaryopoiesis in spleen. In addition, megakaryocytes in spleen may contribute to host defense towards bacteria.
Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a 56-kDa metallo-carboxypeptidase that is predominantly synthesized in the liver and circulates in plasma as a zymogen. Upon activation during coagulation by thrombin or the complex of thrombin with thrombomodulin, TAFI becomes active (TAFIa) and modulates fibrinolysis by cleaving off the C-terminal lysine residues from partially degraded fibrin. Plasminogen and tissue tissue-type plasminogen activator (t-PA) bind to those lysine residues [1] and therefore removal of the lysines attenuates the fibrin cofactor function of t-PA-mediated plasminogen activation, resulting in less plasmin formation and reduced fibrinolysis [2]. TAFI can also be activated by plasmin, trypsin, neutrophil elastase and meizothrombin [3-6].

TAFI activation occurs after proteolytic cleavage of the Arg92-Ala93 bond. TAFIa is characterized by a temperature-dependent instability, showing a spontaneous decay with a half-life less than 10 minutes at 37°C or several hours at 22°C [7]. TAFIa’s self-destruction mechanism was elucidated by the crystal structure [8,9], in which it was shown that a highly dynamic structure is responsible for its own regulation.

Besides fibrin, a number of other TAFIa substrates have been described. TAFIa inactivates the pro-inflammatory mediators bradykinin (BK), complement factors C3a and C5a, thrombin-cleaved osteopontin and fibrinopeptide B [10-13].

TAFIa can therefore have two different effects during infection: regulating plasmin generation, and inactivating inflammatory mediators. This dual function makes TAFI an interesting protein to study in relation to infection.

Streptococcus pyogenes is a common bacterial human pathogen that causes a variety of diseases affecting the skin or the upper respiratory tract [14,14]. These pathological conditions are relatively mild such as impetigo and pharyngitis [14,15]. However, S. pyogenes can cause invasive diseases such as toxic shock syndrome and necrotizing fasciitis which over the last twenty years have been reported to increase world-wide [16]. Serious sequeale following S. pyogenes infections are rheumatic fever and glomerulonephritis.

Although several studies have investigated the role of TAFI after LPS challenge [17,18] or during sepsis induced by Gram-negative intact bacteria [19], the in vivo role of TAFI after Gram-positive bacterial infection has not been investigated. In the present study, we analyzed the role of TAFI during infection caused by S. pyogenes in TAFI-deficient mice.

Materials and Methods

Animals

C57BL/6 mice (wild-type) were supplied by Harlan (Venray, The Netherlands). TAFI-KO mice (backcrossed at least 10 times to a C57BL/6 background) [20-22] were bred in the animal facility at the Academic Medical Center, The Netherlands. Animals were kept on a controlled 12 h light/dark cycle and food and water were provided ad libitum. Experiments were carried
with 8-12 weeks old male mice and all procedures were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

**Experimental model of S. pyogenes infection**
The *S. pyogenes* strain AP 41 (M41 type) was obtained from the Institute of Hygiene and Epidemiology (Prague, Czech Republic). Bacteria were grown in Todd-Hewitt broth (BD Bioscience, San Jose, CA, USA) at 37°C and an aliquot of these cells were added to fresh media and grown up to the exponential phase of growth (OD$_{620}$=0.5). The cells were washed three times with saline and diluted to 0.5 to 3.7 x 10$^8$/ml in saline.

Wild-type and TAFI-KO mice were subjected to an air-pouch infection model. Briefly, 0.8 ml of air and 0.2 ml of *S. pyogenes* in saline were injected with a 25-gauge needle subcutaneously on the back of the mouse. Mice were sacrificed 24 and 48 hours (16 per group) after infection or were observed in a survival study for 5 days (40 per group). During the 5 days, animal survival was recorded in 40 animals per group; animals were monitored (4-6 times a day) for signs of disease such as hunched posture, ruffed fur, and decreased responsiveness to stimuli. When severe disease signs were observed (moribund), mice were humanely euthanized. Animals (n=20) terminated at day 5 (survivors) or those that were terminated within the 5 day observation (non-survivors) were included for analysis of histopathology. Eight mice injected with saline were used as controls.

**Sample harvesting**
At the time of sacrifice (24 hours, 48 hours or during the 5-days), mice were first anesthetized by inhalation of isoflurane (2L/min O$_2$/2% volume isoflurane, Abbott Laboratories). Blood was drawn after cardiac puncture into a sterile syringe containing citrate and immediately placed on ice. Blood was centrifuged for 20 minutes at 2000 g. Plasma was stored at -80°C until use. Thereafter, spleen, kidney, lung and liver were harvested and processed for measurements of CFU, histology.

**Determination of bacterial outgrowth**
Spleen, kidney, lung and liver were homogenized at 4°C in 4 volumes of sterile isotonic saline with a tissue homogenizer (Biospec Products, Bartlesville, OK, USA) which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates and blood. Thereafter, 50-µl volumes were plated onto sheep-blood agar plates and incubated at 37°C. CFU were counted after 16 h.
Assays
Interferon-γ, TNF-α, IL-6, IL-12, IL-10 and MCP-1 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer’s recommendations. For these cytokine measurements, harvested organs were homogenized as described above. Sample homogenates were lysed in 1.25 volumes lysis buffer (300 mM NaCl, 15 mM Tris [tris(hydroxymethyl) aminomethane], 2 mM MgCl₂, 1% Triton X-100, pepstatin A, leupeptin, and aproatin (each at 20 ng/ml) (pH 7.4)) on ice for 20 min and spun at 3600 g at 4°C for 10 min. The supernatant was frozen at -80°C until assayed. Thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany) and D-dimer (Diagnostica Stago, Asnieres, France) were measured by ELISA.

Histopathology of mouse tissue samples
Directly after sacrifice spleen, kidney, liver and lung were fixed in 4% formalin. Tissue samples were embedded in paraffin, sectioned (5-μm) and stained with haematoxylin and eosin (H-E) using standard methods. Megakaryocytes were detected in spleen by immunohistochemistry staining using an antibody against human von Willebrand factor (anti-vWF; Dakocytomation, Glostrup, Denmark) as primary antibody. Briefly, sections were deparaffined and then immersed in 3% hydrogen peroxide in methanol to block endogenous peroxidase for 20 minutes. Sections were then rinsed with PBS, blocked with 10% normal goat serum in PBS for 30 minutes and incubated overnight with the primary antibody in PBS. After the overnight incubation, the sections were incubated with a secondary antibody (HRP conjugated). Immune complexes were revealed via incubation with 3, 3’-diaminobenzidine (DAB) and sections were counterstained with haematoxylin. Sections were examined using an Olympus BX51 microscope and microscope fields were photographed with an Olympus DP70 camera. To quantify the number of megakaryocytes in spleen from wild-type and TAFI-KO mice, vWF positive megakaryocytes were counted under the microscope in 10 randomly chosen X 4 observation fields. For image analysis the freeware ImageJ v1.45a was used.

Statistical analysis
Differences between groups were calculated using the Mann-Whitney U test. Values were expressed as means ±SE. A p value of < 0.05 was considered statistically significant. Survival curves were analyzed by the Kaplan-Meyer log-rank test. All statistics were performed using GraphPad Prism, version 5.01 (GraphPad Software, San Diego, CA, USA).
Results

**Bacterial outgrowth**

To determine whether TAFI deficiency influences *S. pyogenes* dissemination, we compared the bacterial load in spleen, kidney, lung, liver and blood of TAFI-KO and wild-type mice after 24 and 48 h induction of infection (Fig.1). After 24 and 48 h there were no differences in bacterial loads in organs or blood between mouse strains, suggesting that at early time of infection TAFI deficiency does not influence *S. pyogenes* AP41 spread.

![Figure 1. Bacterial dissemination.](image)

**Figure 1. Bacterial dissemination.** Wild-type (●) and TAFI-KO mice (○) were injected with *S. pyogenes* AP41 (1.4 to 3.7 x10⁶/ml) administrated by subcutaneous injection. After 24 or 48 h mice were sacrificed and the bacterial load in the spleen (A), kidney (B), liver (C), lung (D) and blood (E) was determined. Data are expressed as CFU/ml of homogenated organ or blood from each animal with lines drawn at the median for each group (n=16). The dotted horizontal line represents the detection limit. There were no statistically significant differences between groups (Mann-Whitney test).

**Activation of coagulation and fibrinolysis**

To determine whether TAFI plays a role in the coagulant and fibrinolytic host responses, we determined levels of TATc and D-dimer after 24 and 48 h *S. pyogenes* AP41 infection in plasma. At 24 h TATc levels in TAFI-KO mice tended to be higher, although not significant, compared to wild-type mice. After 48 h, TATc levels were not different between the mouse strains (Fig.2A). D-dimer concentrations were increased after 24 h and 48 h compared to the saline control, but were not different between wild-type and knockout animals (Fig. 2B).
mTAFI improves survival against *S. pyogenes*

**Figure 2. Activation of coagulation and fibrinolysis.** Levels of TATc (A) or D-dimer (B) in plasma at 24 and 48 hours after inoculation of *S. pyogenes* in wild-type mice (black bars) and TAFI-KO mice (white bars). Data are expressed as means ± SE of n= 8 (saline group) or n=16 (infected group). There were no statistically significant differences between groups (Mann-Whitney test).

**Survival**
To determine if TAFI has an impact on mortality during *S. pyogenes* infection, we performed a survival study. The survival of the mice in the two groups over time is illustrated in Figure 3. Despite having similar responses to infection at earlier time points (24, 48 h) as shown by analysis of bacterial loads, coagulation, fibrinolysis and inflammation, TAFI-KO mice exhibited significantly increased mortality towards *S. pyogenes* (*p* < 0.0001). At day 5 post-infection, 35% TAFI-KO animals had survived infection in contrast to 87% of wild-type mice.

**Histopathology and inflammation**
We examined the influence of TAFI deficiency on tissue damage after 24 h, 48 h and during a 5-day survival experiment where survivors and non-survivors were included for analysis. Histopathology examination of organs from infected wild-type and TAFI-KO mice after 24 and 48 h showed no relevant inflammation (data not shown). In addition, cytokines and
Chemokines in plasma were measured. Levels of interferon-γ, TNF-α and IL-12 were below the detection limit in this model, while levels of IL-6, IL-10 and MCP-1 did not differ between wild-type and TAFI-KO mice (data not shown). Histopathology examination of kidney, liver and lung from infected wild-type and TAFI-KO mice during the 5-day infection showed no relevant changes compared to the saline control group (data not shown). In contrast, as shown by haematoxylin and eosin staining of paraffin sections, the spleens from *S. pyogenes* AP 41 infected wild-type and TAFI-KO mice showed evidence of an infiltration with large, multinucleated cells (Fig. 4), that were present in occasional clusters within the red pulpa in both groups and morphologically appeared to be megakaryocytes.

**Figure 4. Histopathology of wild-type and TAFI-KO mice.** Representative H&E stained sections of spleen tissue from *S. pyogenes*-infected wild-type (A, B) and TAFI-KO spleen tissue (C, D). Infected animals were injected with 1 to 3 x10⁸ CFU/ml and followed for 5 days as indicated in methods. Magnifications are indicated in each panel. Data were from two independent experiments, each of which was conducted with 10 mice per group.

Immunohistochemical staining with antibodies against von Willebrand factor confirmed these cells to be megakaryocytes (Fig. 5). The presence of megakaryocytes in spleen was suggestive for extramedullary hematopoiesis. Spleens from TAFI-KO mice showed decreased numbers of megakaryocytes (Fig. 5A,B), in comparison to spleens of wild-type mice (Fig. 5C,D). In spleens from wild-type and TAFI-KO mice injected with saline (controls), megakaryocytes were not observed (data not shown). Quantification of the megakaryocytes in spleen (Fig. 5E) confirmed that TAFI-KO have lower MK numbers in spleen after *S. pyogenes* infection,
suggesting that TAFI could play a role in extramedullary hematopoiesis. Furthermore, non-survivors from both infected groups had lower megakaryocyte numbers in spleen compared to survivors (Fig. 5E), indicating that extramedullary hematopoiesis may play an important role in survival against S. pyogenes.

**Figure 5.** Immunohistochemical staining of spleens of wild-type and TAFI-KO mice. Representative immunohistochemical staining for vWF of sections from S. pyogenes-infected wild-type spleen tissue (A, B) and TAFI-KO mice (C, D). Infected animals were injected with 1 to 3 x10^8 CFU/ml and followed for 5 days. Original Magnifications are indicated in each panel. Arrows indicate megakaryocytes. (E) Quantification of vWF positive megakaryocytes in spleen from wild-type (●) and TAFI-KO (■) mice after S. pyogenes AP41 injection. Horizontal lines in the graphs represent the medians of the groups. Representative images of two independent experiments, each of which was conducted with 10 mice per group. Non survivors (open symbols) are indicated for both groups.

**Discussion**

Upon infection, many complex processes such as inflammation and activation of both the coagulation and the fibrinolytic systems are compromised. TAFI represents a link between coagulation and fibrinolysis and inactivates pro-inflammatory mediators, therefore it can be expected that TAFI is involved in the response to infection. The present study was performed to elucidate the role of endogenous TAFI during infection of the Gram-positive bacteria, S. pyogenes.

Creation of TAFI-KO mice has enabled the possibility of studying the relevance of endogenous TAFI in different experimental settings. Compared to wild type animals, the TAFI-KO mice were normal in many respects, including survival, development, and fertility. In addition, TAFI-KO displayed no bleeding disorders but as expected prolonged clot lysis time [20,23].
Using *S. pyogenes* M41 serotype strain at a dose of 0.5 to 3 x10^8 CFU/ml, we observed 87% survival in wild-type mice after 5 days. TAFI deficiency markedly decreased survival to 35%. Our results suggested that the increased mortality observed in TAFI-KO might be due to impaired regulation of the inflammatory mediators C3a and C5a leading to uncontrolled inflammation. C5a has been suggested to play a role in the increased mortality when TAFI-KO mice were challenged with both LPS and cobra venom factor [17]. However, we were unable to demonstrate an altered local or systemic inflammatory response in TAFI-KO mice, as evidenced by unchanged cytokine levels after 24 and 48 h *S. pyogenes* infection. In addition, bacterial loads were similar after 24 and 48 h *S. pyogenes* infection between wild-type and TAFI-KO mice. It seems likely that acceleration of the disease progression may have occurred later than 48 h after *S. pyogenes* infection.

Upon infection, various hematopoietic factors promote hematopoiesis mainly in liver and spleen (extramedullary hematopoiesis) [24]. It has been suggested that inadequate extramedullary hematopoiesis leads to insufficient production or maturation of blood cells [24]. Extramedullary hematopoiesis can occur as long as there are local production of hematopoietic factors that maintain and induce differentiation of the hematopoietic stem and progenitor cells (HSPCs). In our mouse model, megakaryocytes appearance correlated with reduced mortality in both wild-type and TAFI-KO mice. Surprisingly, in the 20 animals in which immunohistopathology was performed (Fig. 5), the TAFI-KO survivors only represented 25% compared to 80% in the wild-type mice, suggesting that TAFI may be involved in extramedullary hematopoiesis leading to megakaryocyte migration and/or differentiation. To our knowledge, a role for TAFI in these processes has never been described. A potential target for TAFI in this process may be stromal cell-derived factor-1α (SDF-1α). SDF-1α also known as C-X-C type chemokine ligand 12 (Cxcl12) is produced by various cell types including bone marrow stromal cells, inflammatory cells, endothelial cells and osteoblasts [25]. SDF-1α plays a role in regulating the retention of progenitor cells in hematopoietic tissues [26]. It has been suggested that perturbation of the SDF-1α chemoattractant gradient by cleavage of the C-terminal lysine residue of SDF-1α by carboxypeptidase M can lead to mobilization of HSPCs from bone marrow to peripheral blood [27]. This might facilitate entrance of megakaryocyte precursors in peripheral organs such as the liver and spleen leading to megakaryocyte differentiation. Therefore, TAFI activation during *S. pyogenes* infection, additionally to other carboxypeptidases, could allow colonization of the spleen with bone marrow-derived stromal cells by cleaving the C-terminal lysine of SDF-1α. Enhanced TAFI activation could be the result of increased TAFI levels upon infection. Both, TAFI mRNA and TAFI protein have been reported to be upregulated during *Escherichia coli*-induced abdominal sepsis [19]. Moreover, a local increased level of TAFI in the bone marrow can be achieved by a pool of TAFI present in megakaryocytes [28,29].

The spleens of survivors from TAFI-KO and wild-type mice contained higher numbers of megakaryocytes, suggesting that they are important for host defense against *S. pyogenes*. A role for toll-like receptors (TLR) in megakaryocytes has recently been demonstrated. The TLR2
that recognizes the lipoteichoic acid of gram-positive bacteria has been detected in human megakaryocytes and lately, platelets which derive from megakaryocytes were shown to bind and internalize pathogens and release microbial proteins [30,31]. In addition, many studies have identified TLRs on platelets [32-36]. In line with our observation, extramedullary hematopoiesis has been reported before in direct association with infection [37,38]. Recently, we reported the binding of human TAFI to two surface proteins of S. pyogenes, and its subsequent activation at the bacterial surface via plasmin and the thrombin-thrombomodulin complex [39]. The TAFI region involved in binding to the streptococcal collagen-like surface proteins A and B (SclA and SclB) is Gly205 to Asp232 which is 99% conserved in mouse TAFI compared to human TAFI (chapter 4). In addition, mouse TAFI also binds the S. pyogenes serotype used in this study (data not shown). Because both mouse and human TAFI interact with S. pyogenes, it is tempting to speculate that mouse TAFI can also be used by S. pyogenes to its own benefit. However, TAFI-KO mice but not wild-type mice were susceptible to S. pyogenes AP41 infection, suggesting that mouse TAFI is of importance for defense during S. pyogenes infection.

In conclusion, we have shown that TAFI-KO mice are susceptible to S. pyogenes infection. However, the etiology of the disease could not be clarified. It seems likely that TAFI could contribute to protection of infection by facilitating extramedullary hematopoiesis that might be used to counteract the infection.
Reference List


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