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The lectin like domain of thrombomodulin is involved in the defence against pyelonephritis

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ABSTRACT

Pyelonephritis, a common complication of urinary tract infections, is frequently associated with kidney scarring and may lead to end-stage renal disease. During bacterial infections inflammatory and coagulation pathways and their mutual interaction are playing pivotal roles in the host response. Given that thrombomodulin (TM) is crucially involved in the interplay between coagulation and inflammation, we aimed to investigate the roles of its EGF and lectin-like domains in inflammation during acute pyelonephritis. Indeed, the EGF-like and the lectin-like domains of TM, are especially known to orchestrate inflammation and coagulation in different ways. Acute pyelonephritis was induced by intravesical inoculation of 1×10^8 CFU of uropathogenic *Escherichia coli* in two strains of TM transgenic mice. $TM^{pro/pro}$ mice carry a mutation in the EGF-like domain making them unable to activate protein C, an anticoagulant and anti-inflammatory protein. $TM^{LeD/LeD}$ mice lack the lectin-like domain of TM, which is critical for its anti-inflammatory and cytoprotective properties. Mice were sacrificed 24 and 48 h after inoculation. Bacterial loads, the immune response and the activation of coagulation were evaluated in the kidney and the bladder.

$TM^{LeD/LeD}$ mice showed elevated bacterial load in bladder and kidneys compared to WT mice, whereas $TM^{pro/pro}$ had similar bacterial load as WT mice. $TM^{LeD/LeD}$ mice displayed a reduced local production of pro-inflammatory cytokines and neutrophil renal infiltration. Activation of coagulation was comparable in $TM^{LeD/LeD}$ and WT mice. From these data, we conclude that the lectin-like domain of thrombomodulin is critically involved in host defence against *E. coli* induced acute pyelonephritis.

1. Introduction

Urinary tract infections (UTI) are common bacterial infections, affecting 50% of all women at least once in their lifetimes [1] and 2 to 8% of children under 10 years of age [2]. UTI are most frequently caused by Gram-negative bacteria, by far the most common one being *Escherichia coli* (*E. coli*), followed by *Proteus*, *Klebsiella* and *Enterobacter* [1].

A major and disabling complication of UTI is acute pyelonephritis, which occurs when infection ascends from the bladder through the ureters into the renal pyelum [3]. Prolonged pyelonephritis may lead to kidney scarring which can subsequently lead to end-stage renal disease [4]. The incidence of renal fibrosis following pyelonephritis can be as high as 64% in children [5,6].

Thrombomodulin (TM, CD141) [7,8] is a multi-domain type 1 transmembrane glycoprotein which plays a critical role in both coagulation and inflammation [9,10]. TM is constitutively expressed by endothelial cells [7,11], monocytes [12] and neutrophils [13]. Interestingly, expression of TM by human urothelial cells has also been reported [14].

Structurally, TM consists of 5 domains [9], the N-terminal lectin-like domain, six epidermal growth factor (EGF)-like repeats, a serine/threonine-rich domain, a transmembrane and a cytoplasmic domain.

The domain comprising the 6 EGF-like repeats [9] is critical as a co-factor to thrombin for generation of activated thrombin activatable fibrinolysis inhibitor (TAFIa) and activated protein C (APC) [15–19]. APC has important anticoagulant [20] as well as anti-inflammatory and cytoprotective properties [21–25].

The lectin-like domain (also referred to as TMD1) displays a structural homology with the C-type lectin family and has direct anti-inflammatory effects [26]. Mice lacking the lectin-like domain produced more cytokines such as tumour necrosis factor alpha (TNFα) and interleukin (IL)-1 and exhibited more leukocyte infiltration into the lungs after challenge with

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lipopolysaccharide (LPS) or following inhalation of Gram-negative bacteria [26,27].

The lectin-like domain also binds to the high mobility group box 1 (HMGB1) and LPS. Binding of the lectin-like domain prevents HMGB1 from triggering activation of the downstream pro-inflammatory pathways of its receptor (e.g. receptor for advanced glycation end products or toll-like receptors). In addition, binding of the lectin-like domain to HMGB1 results in degradation of HMGB1, dampening its proinflammatory properties [28]. Binding of soluble recombinant lectin-like domain to the Lewis Y antigen of LPS causes agglutination of the bacteria and facilitates their opsonisation by macrophages [29]. Moreover, soluble recombinant lectin-like domain prevents LPS from binding to CD14, thereby reducing the production of inflammatory mediators [29].

Overall, TM regulates coagulation and plays a pivotal role in inflammation and in host defence against bacteria.

Having established the involvement of clotting [30–33] and inflammation during bacterial infection, we aimed to study the function of TM in regulating these two phenomena during pyelonephritis. More specifically, we have investigated the roles of TM-mediated APC activation and the intrinsic role of TM lectin-like domain in inflammation upon pyelonephritis. In order to do so, we used two TM transgenic mouse strains: $TM^{pro/pro}$ mice, which bear a mutation in the EGF-like domain making them unable to activate protein C [34], and $TM^{LeD/LeD}$ mice which lack the lectin-like domain of thrombomodulin [27]. These mice were subjected to a model of pyelonephritis by intravesical inoculation of uropathogenic *E. coli.*

2. Methods

2.1. Mice

$TM^{LeD/LeD}$ and $TM^{pro/pro}$ mice were generated as described [27,34] and backcrossed at least eight times to a C57BL/6 genetic background. $TM^{LeD/LeD}$ mice express normal antigenic levels of TM and activation of PC is intact [27].

The cell type- and organ-specific distribution of TM was identical in $TM^{pro/pro}$ mice as in WT mice, although antigen levels are significantly reduced [34]. In $TM^{pro/pro}$ mice, protein C activation is reduced 26 fold [34,35]. Age matched pathogen free female wild type (WT) C57BL/6J mice were purchased from Charles River (Maastricht, The Netherlands). Groups of at least 8 mice were used.

Mice were maintained at the animal care facility of the Academic Medical Center (University of Amsterdam), according to national guidelines and had ad libitum access to food and water. The Committee on Use and Care of Animals of the University of Amsterdam approved all experiments.

2.2. Experimental protocol

UTI was induced as previously described [30,33,36]. Uropathogenic *E. coli* 1677 isolated from an uroseptic patient was cultured in sterile Tryptic Soy Broth (TSB) overnight at 37 °C. After a 100 times dilution in 100 mL of TSB, bacteria were further grown for 2 to 3 h at 37 °C to reach an optical density of 1 (600 nm). *E. coli* were washed 3 times in sterile PBS and resuspended in 10 mL of sterile PBS to obtain a suspension containing 10^9 colony forming unit (CFU)/mL.

Acute pyelonephritis was induced under general anaesthesia (10 μL/1 g mouse of FFM mixture containing 1.25 mg/mL midazolam (Roche, Mijdrecht, The Netherlands), 0.08 mg/mL fentanyl citrate and 2.5 mg/mL fluanisone (Janssen Pharmaceutica, Beerse, Belgium)) in 8–12 week old female mice. 100 μL of 10^9 CFU/mL suspension was administered by transurethral injection. Mice were sacrificed 24 and 48 h after the procedure by exsanguination and cervical dislocation performed under general anaesthesia (isoflurane).

2.3. Determination of bacterial outgrowth

The left kidney and the bladder of each mouse were homogenized in, respectively, 4 and 9 volumes of sterile PBS using a tissue homogenizer (Polytron, Luzern, Switzerland) which was cleaned with ethanol after each homogenization.

To quantify bacterial outgrowth, serial 10-fold dilutions of blood, kidney and bladder homogenate were made in sterile PBS and 50 μL of homogenate were plated onto blood agar plates. After overnight incubation at 37 °C, the *E. coli* CFU was counted. Only the mice showing positive bladder and kidney cultures were taken into account in our analysis.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The kidneys and bladders were homogenated in a lysis buffer containing 150 mM NaCl, 15 mM Tris, 1 mM $MgCl.H_2O$, 1 mM $CaCl_2$, 1% Triton and 1% protease inhibitors. Concentrations of macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), interleukin 1ß (IL-1ß), interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) were measured in kidney homogenates by ELISA according to the manufacturer's instructions (R&D System, Abingdon, UK; for KC homogenates were diluted 8.5 times, for others 3 times).

Mouse myeloperoxidase (MPO) concentrations were determined by ELISA in kidney and bladder homogenates (HyCult Biotech, Uden, The Netherlands; kidney homogenates were diluted 40 times, bladder homogenates were diluted 30 times). Concentrations of Thrombin–antithrombin (TAT) complexes in kidney homogenates were measured using the Enzygnost® TAT micro Kit (Siemens Healthcare, Erlangen, Germany).

The concentrations measured by ELISA were normalized to the total amount of protein present in the kidney or bladder homogenates.

Total protein concentration was measured by incubating 1 μL of 10 times diluted homogenates for 30 min at 37 °C in 500 μL of bicinchoninic acid containing 4% of $CuSO_4$, and absorbance was measured at 570 nm.

2.5. Immunohistochemistry

4 μm thick paraffin sections of tissue were prepared. Antigen retrieval with respectively 0.25% pepsin in 0,01 M HCl (15 min at 37 °C) and 0.01 M tri-sodium citrate dehydrate 4.4 mM HCl were carried out for optimal staining. After blocking with 5% normal mouse serum, the sections were immunostained for the lymphocyte antigen 6 (Ly6G) or F4/80 overnight at 4 °C respectively with a rat-anti-mouse-Ly6G-FITC antibody or with a rat-anti-mouse-F4/80 antibody.

Detection of Ly6G and F4/80 staining was achieved with a rabbit-anti-FITC secondary antibody (for Ly6G) or a rabbit-anti-rat antibody (for F4/80), each for 30 min at room temperature, followed by development using the appropriate species specific HRP peroxidase kit (30 min incubation at room temperature, Powervision, Immunologic, Duiven, Netherlands). Staining was developed with UltraDAB (Immunologic, Duiven, Netherlands).

After 30 min blocking of sections with 5% normal goat serum, thrombomodulin immunostaining was performed using 10,000 times diluted monoclonal rat-anti-mouse TM antibody (273-34A, kind gift of Dr. Stephen Kennel). After overnight incubation, the slides were subsequently incubated for 1 h with biotin conjugated rabbit-anti-rat antibody (1/100 dilution, DAKO, Glostrup, Denmark), followed by 30 min incubation with ABC complex (Vectastain, Vector Laboratories, Burlingame, CA, USA) and the staining was developed with UltraDAB (Immunologic, Duiven, Netherlands). The extent of TM immunostaining was evaluated by digital image analysis (Image Pro Plus, MediaCybernetics, Rockville, MD, USA).