The Cytoplasmic Domain of Tissue Factor Contributes to Leukocyte Recruitment and Death in Endotoxemia

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Tissue factor (TF) is an integral membrane protein that binds factor VIIa and initiates coagulation. The extracellular domain of TF is responsible for its hemostatic function and by implication in the dysregulation of coagulation, which contributes to death in endotoxemia. The role of the cytoplasmic domain of tissue factor in endotoxemia was studied in mice, which lack the cytoplasmic domain of TF (TF^{δCT/δCT}). These mice develop normally and have normal coagulant function. Following i.p injection with 0.5 mg of lipopolysaccharide (LPS), TF^{δCT/δCT} mice showed significantly greater survival at 24 hours compared to the wt mice (TF^{+/+}). The serum levels of TNF-α and IL-1β were significantly lower at 1 hour after LPS injection and IL-6 levels were significantly lower at 24 hours in TF^{δCT/δCT} mice compared to TF^{+/+} mice. Neutrophil recruitment into the lung was also significantly reduced in TF^{δCT/δCT} mice. Nuclear extracts from tissues of endotoxemic TF^{δCT/δCT} mice also showed reduced NFκB activation. LPS induced leukocyte rolling, adhesion, and transmigration in post-capillary venules assessed by intravital microscopy was also significantly reduced in TF^{δCT/δCT} mice. These results indicate that deletion of the cytoplasmic domain of TF impairs the recruitment and activation of leukocytes and increases survival following endotoxin challenge. (*Am J Pathol* 2004, 165:331–340)

receptor.¹ TF is the major initiator of coagulation *in vivo*. This function is dependent on the extracellular domain that binds factor VIIa forming a complex that activates both factor IX and factor X.² The TF extracellular domain is also essential for embryonic development,^{3,4} for factor VIIa-induced MAP kinase up-regulation in hamster kidney cells *in vitro*,⁵ and for phosphorylation of ribosomal kinases which stimulates protein synthesis.⁶ The TF cytoplasmic domain consists of 21 amino acids in humans and 20 amino acids in mice. The function of the cytoplasmic domain of TF is less well defined. It has been shown to contribute in cell adhesion and cytoskeleton reorganization via binding with actin binding protein-280 (ABP-280)⁷ and is required for factor VIIa-induced Ca²⁺ fluxes in U937 cells,⁸ suggesting a role in inflammation. TF also contributes to the metastatic potential of human melanoma cells and cell lines. Studies support involvement of both factor VIIa binding to the extracellular domain and phosphorylation of the cytoplasmic domain in this process.^{9,10} Similarly, both the extracellular and cytoplasmic domains of TF have been implicated in increased vascular endothelial growth factor (VEGF) levels.^{11,12}

Endotoxemia is an overwhelming, often fatal, systemic inflammatory condition which results in multi-organ dysfunction syndrome (MODS).¹³ It is initiated by binding of endotoxin to Toll-like receptors in association with CD14.^{14,15} The second messengers involve activation of MyD88, and subsequent NFκB activation and nuclear translocation. This leads to increased transcription and systemic release of pro-inflammatory cytokines including IL-1β, TNF-α, and IL-6.¹⁶ TF expression is also markedly up-regulated on monocytes resulting in systemic dysregulation of coagulation.^{17,18} Tissue factor may be involved in adhesion and trafficking of monocytes through endothelium.¹⁹ The involvement of the extracellular domain of TF in endotoxemia has been demonstrated using functionally inhibitory anti-TF antibody,^{20,21} inactivated factor VIIa,^{22–24} and TFPI.^{25,26} The contribution of the cytoplasmic domain to this process is so far unknown.

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Tissue factor (TF) is a 45-kd integral membrane protein. Its extracellular domain is similar to the type II cytokine

Mice with a deletion in the terminal 18 amino acids of the cytoplasmic domain of TF were generated by a Cre-loxP recombination system and display normal embryonic development, growth, fertility, and coagulation.²⁷ We used these mice to study the contribution of the cytoplasmic domain of TF during endotoxemia. Results revealed that in the absence of the cytoplasmic domain of TF, survival is improved, there is reduced systemic cytokine release, reduced leukocyte trafficking, and reduced NF κ B activation following endotoxin challenge.

Materials and Methods

Animals

Mice with a deletion of 18 carboxyl-terminal amino acids of the cytoplasmic domain of TF (TF ^{δ CT/ δ CT}) mice were generated by the Cre-lox recombination technique on an MF1/129Sv/Swiss strain background and provided by Dr. Peter Carmeleit. These animals display normal fertility, embryonic and postnatal development, and coagulation function.²⁷ Mice were bred and housed under specific pathogen-free (SPF) conditions. For littermate-matched studies, litters from heterozygous TF ^{δ CT/+} parents were genotyped to select homozygous TF ^{δ CT/ δ CT} and TF^{+/+} mice. Genotyping was performed using a standard PCR protocol [forward primer 5'-CATCATTGTGGGAGCAGTGGTGC-3' (Position 865–887 in exon 6) and reverse primers 5'-GCCACCCAGGTTATATGAAAGGC-3' (Position 1287–1310 of the untranslated region)] Gene Accession No. M80785. These primers produce a PCR product of 437 bp for TF^{+/+} mice and 395 bp for TF ^{δ CT/ δ CT} mice.²⁷

LPS-Induced Endotoxemia

Endotoxemia was induced in 8- to 10-week-old TF^{+/+} and TF ^{δ CT/ δ CT} mice (19 to 21 g, of both sexes) by intraperitoneal injection with 0.5 mg of LPS (26 to 24 μ g/g of body weight) from *E. coli* (serotype 0111:B4, Sigma, Melbourne, Australia). A 24-hour survival study was performed in littermate TF^{+/+} ($n = 17$) and TF ^{δ CT/ δ CT} ($n = 12$) mice. Additional studies to assess serum cytokine levels, circulating leukocytes, and plasma thrombin-antithrombin (TAT) complexes levels were performed in TF ^{δ CT/ δ CT} and strain control mice, using groups of 5 to 10 mice, injected with LPS, and killed at 1, 6, 24, or 48 hours. Selected experiments, including studies of 1-hour and 24-hour serum cytokine levels were conducted on littermate-matched TF^{+/+} and TF ^{δ CT/ δ CT} mice. All NF κ B binding studies were performed on tissues collected from littermate-matched mice. All experimental protocols were approved by the Monash University Animal Ethics Committee.

Assessment of Circulating Leukocytes and Subsets

Blood for leukocyte analysis was collected by cardiac puncture (in 3.3% sodium citrate) under methoxyfluorane

anesthesia. Red blood cell (RBC) were lysed using Coulter Q-prep (Coulter Corp., Hialeah, FL) and total white cell numbers were determined by counting using a hemocytometer and light microscopy. After staining with phycoerythrin (PE)-conjugated mAb for CD4⁺ T cells (Pharmingen), apocyanin (APC)-conjugated mAb for CD8⁺ T cells (Pharmingen), fluorescein isothiocyanate (FITC) conjugated mAb for B220⁺ B cells (anti-CD45R) (Pharmingen), FITC-conjugated mAb for M170 (α CD11b) for neutrophils and monocytes, and phycoerythrin (PE)-conjugated mAb for GR-1 (Pharmingen) to separate neutrophils (GR-1^{high} M170⁺) from monocytes (GR-1^{intermediate} M170⁺),²⁸ leukocyte subsets were analyzed by flow cytometry (Mo-flo flow cytometer; Cytomation, Fort Collins, CO). Leukocytes subset numbers were calculated by multiplying the total white cell count (determined by hemocytometer) with the percentage of each individual subset determined by flow.

Measurement of Serum Cytokine Levels

Blood collected for serum cytokine levels was allowed to clot at 4°C for 6 hours and then spun at 1600 $\times g$ for 10 minutes. Serum TNF- α , IL-1 β , and IL-6 concentrations were measured in duplicate samples from each mouse using a cytokine-specific ELISA according to the manufacturer's instructions (Endogen). The sensitivity of these assays was 27.5 pg/ml, 15.6 pg/ml, and 7.6 pg/ml, respectively.

Measurement of Thrombin-Antithrombin Complex (TAT)

Blood was collected by cardiac puncture using 23-G needles into tubes containing 3.3% trisodium citrate. These samples were spun at 1600 $\times g$ for 10 minutes and the plasma was stored at -20°C until analyzed. Levels of TAT complexes in the plasma samples of normal and endotoxemic TF^{+/+} and TF ^{δ CT/ δ CT} mice were analyzed using a commercial ELISA kit (Enzygnost, Dade Behring, Marburg, Germany).

Lung Neutrophil Recruitment

Lung neutrophil recruitment was assessed by myeloperoxidase (MPO) activity²⁹ and confirmed by grid counting in lung sections. Lungs were homogenized in 0.5% hexadecyltrimethylammonium bromide buffer (HTAB buffer) using a polytron homogenizer (PT 1200 CL Selby Biolabs, Vic, Australia). MPO was assayed spectrophotometrically by its ability to form a chromogenic product by cleaving the specific substrate o-dianisidine hydrochloride. Activity was calculated using a kinetic protocol on a BIO-RAD Microplate Manager 5.0 PC (Biorad, CA) designed to measure change in absorbance over 1 minute at 450 nm.

Lungs of the endotoxemic TF^{+/+} and TF ^{δ CT/ δ CT} mice were fixed in Bouin's without prior perfusion and then embedded in paraffin. Three- μ m sections were cut and stained with hematoxylin and eosin (H&E) stain. The num-

bers of polymorphonuclear (PMN) leukocytes (identified by their typical nuclear morphology) were counted in four randomly selected fields at $\times 40$ magnification using a graticule and an average was calculated. Results are expressed as PMN/high power field (hpf) for each mouse. Counts were performed by an observer blinded to the mouse genotype.

Intravital Microscopy

Leukocyte trafficking following LPS challenge was assessed in the microcirculation of the mouse cremaster muscle. Animals were injected intra-scrotally with 10 ng of LPS in 250 μ l of saline. Three hours later, animals were anesthetized by i.p. injection of 10 mg/kg xylazine (Bayer Pharmaceuticals, Pymble, NSW, Australia) and 200 mg/kg ketamine hydrochloride (Caringbah, NSW, Australia) and the cremaster microvasculature was prepared for examination as previously described.³⁰ Recordings of leukocyte trafficking were taken 4.5 hours after LPS challenge.

The cremaster microcirculation was visualized using an intravital microscope (Axioplan 2 Imaging; Carl Zeiss Australia) and a color video camera (Sony SSC-DC50AP). The images were recorded for playback analysis using a videocassette recorder (Panasonic NV-HS950) as previously described.^{30,31} Three to four post-capillary venules (25 to 40 μ m in diameter) were examined in each experiment. Venular diameter and the number of rolling, adherent, and emigrated leukocytes were determined off-line during video playback analysis. Rolling leukocytes were defined as cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocyte rolling velocity was determined by measuring the time required for a leukocyte to roll along a 100- μ m length of venule. This was determined for 20 leukocytes per vessel. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 seconds or longer. The number of emigrated leukocytes was determined by counting the leukocytes present in the extravascular tissue within the field of view (250 $\times$ 200 microns).

Measurement of Tissue Factor Activity

TF functional activity was measured in a one-stage prothrombin assay as previously described.³² Kidney and liver tissues were homogenized in 15 mmol/L β octyl glucopyranoside (Sigma, Australia) in HEPES-buffered saline. Samples were spun at 12,000 $\times g$ for 1 minute and the supernatant was incubated for 15 minutes at 37°C before addition of two volumes of HEPES-buffered saline. Time to clot was determined by adding the tissue samples to citrated mouse plasma with CaCl_2 using a Stago Start 4 automatic coagulation analyzer (Stago, France). TF activity was calculated by reference to a standard curve of dilutions of rabbit thromboplastin (Sigma, Australia), corrected for protein concentration, and expressed as units per mg of total protein.

Measurement of NF κ B Activation by Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from the kidney, heart, and liver of endotoxemic mice 1 hour after LPS administration as previously described.^{33,34} Protein concentrations were measured using the BCA assay (Pierce). Equal amounts of protein (10 μ g) were incubated with 50,000 cpm of ³²P-end-labeled probe (containing the NF κ B consensus binding site: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', sense strand) for 15 minutes at room temperature in 20 μ l of binding buffer (20 mmol/L HEPES pH 8.0, 1 mmol/L EDTA, 10% glycerol, 50 mmol/L KCl, 50 μ g/ml poly (dl:dC), 1 mg/ml bovine serum albumin, 10 mmol/L dithiothreitol) before electrophoresis. Electrophoresis was performed to separate protein-DNA complexes from free DNA using a 5.4% polyacrylamide gel and 0.5X TBE (44.5 mmol/L Tris, 44.5 mmol/L boric acid, 1 mmol/L EDTA, pH 8.0) running buffer for 3 hours at 200V (4°C). Gels were dried and radioactive complexes visualized using a phosphorimager (FLA 200 Fujitsu, Japan) and quantified using the Image Quant 5.1 program (Fuji photo film, Japan). Where antibodies were included in the reaction (directed against p50 or p65, Santa Cruz Biotechnology), protein extract and antibody were pre-incubated on ice for 10 minutes before addition of probe. The specificity of binding of nuclear extracts to NF κ B was demonstrated by incubation with NF κ B (wt or mutant) probes. Wild-type probe being non-radiolabeled NF κ B probe and mutant being non-radiolabeled probe with mutation in NF κ B binding site. NF κ B band intensity was quantitated by densitometry and normalized to the constitutive DNA-binding activity of the proximal region of the PDGF-A promoter using Oligo A (5'-GGG GGG GGC GGG GGC GGG GGC GGG GGA GGG-3') as the probe in EMSA³⁵ with identical amounts of extract used for the NF κ B EMSA.

Statistical Analysis

Difference between 24-hour survival curves of TF^{+/+} and TF ^{δ CT/ δ CT} mice were analyzed using a log rank test. Statistical analysis of other parameters was performed using analysis of variance followed by Tukey's multiple comparison test. Comparison in intravital microscopy measurements and cytokine analysis was performed using unpaired Student's *t*-test. All data are expressed as mean $\pm$ SEM.

Results

Deletion of the Cytoplasmic Domain of TF Leads to Increased Survival Following Endotoxin Challenge

Littermate-matched TF ^{δ CT/ δ CT} showed significantly greater survival rates compared to TF^{+/+} mice over 24 hours following endotoxin challenge. At 24 hours, 9 of 12 (75%) TF ^{δ CT/ δ CT} mice and 6 of 17 (35%) TF^{+/+} mice survived

Figure 1. Survival following endotoxin challenge. $TF^{+/+}$ and $TF^{\delta CT/\delta CT}$ mice were injected i.p. with 0.5 mg of LPS. The mice were monitored for 24 hours following endotoxin challenge. $TF^{\delta CT/\delta CT}$ mice showed a significantly greater survival (9 of 12 mice) as compared to littermate $TF^{+/+}$ mice (6 of 17 mice) *, $P = 0.03$ with respect to $TF^{+/+}$ mice, as determined by log rank test.

($P = 0.03$, by log rank test) (Figure 1). In a separate 48-hour study of strain-matched mice, only 1 of 4 (25%) of $TF^{+/+}$ mice compared to 5 of 7 (71%) of $TF^{\delta CT/\delta CT}$ mice survived to 48 hours following endotoxin challenge.

Decreased Serum Cytokines in $TF^{\delta CT/\delta CT}$ Mice

Serum TNF- α levels peaked at 1 hour in $TF^{+/+}$ and $TF^{\delta CT/\delta CT}$ mice (Figure 2 A) but were significantly lower in $TF^{\delta CT/\delta CT}$ mice ($TF^{+/+}$ 27.0 ± 3.0 ng/ml, $n = 7$, versus $TF^{\delta CT/\delta CT}$ 17.0 ± 3.0 ng/ml, $n = 7$; $P = 0.027$). A similar increase in TNF- α ($TF^{+/+}$ 31.6 ± 6.3 , $n = 3$, versus $TF^{\delta CT/\delta CT}$ 20.5 ± 2.3 , $n = 5$; $P = 0.02$) was also observed 1 hour after endotoxin challenge in littermate-matched mice. Serum TNF- α returned to basal levels at 6 hours. Results in strain control and littermate-matched mice were equivalent. Serum IL-1 β showed a more sustained elevation than TNF- α in $TF^{+/+}$ mice. In $TF^{\delta CT/\delta CT}$ mice, the rise in IL-1 β was slower with significantly lower levels 1 hour following endotoxin challenge compared to $TF^{+/+}$ mice ($TF^{+/+}$ 1.7 ± 0.5 ng/ml, $n = 6$ versus $TF^{\delta CT/\delta CT}$ 0.5 ± 0.3 ng/ml, $n = 7$; $P = 0.02$) (Figure 2B). Serum IL-1 β levels were similar at 6 hours after LPS injection. IL-6 levels were significantly elevated at 1 and 6 hours in both $TF^{+/+}$ and $TF^{\delta CT/\delta CT}$ mice. Levels in surviving mice at 24 hours showed a more rapid decline of IL-6 in $TF^{\delta CT/\delta CT}$ mice (0.3 ± 0.07 ng/ml, $n = 5$) compared to $TF^{+/+}$ mice (30.0 ± 11.3 ng/ml, $n = 4$; $P = 0.01$) (Figure 2C). In littermate-matched mice, IL-6 levels at 1 hour ($TF^{+/+}$ 19.8 ± 6.5 , $n = 3$ versus $TF^{\delta CT/\delta CT}$ 18.0 ± 2.8 , $n = 5$; $P = 0.8$) and 24 hours ($TF^{+/+}$ 57.3 ± 3.2 , $n = 3$ versus $TF^{\delta CT/\delta CT}$ 2.3 ± 0.1 , $n = 2$; $P = 0.0004$) showed a similar pattern to strain control mice.

$TF^{\delta CT/\delta CT}$ Mice Develop Systemic Coagulopathy

Plasma TAT levels in normal mice and following endotoxin challenge were measured to assess the effect of TF cytoplasmic domain deletion on systemic activation of coagulation. Basal TAT levels were equivalent in $TF^{+/+}$ and $TF^{\delta CT/\delta CT}$ mice. TAT levels increased in both $TF^{+/+}$

Figure 2. Cytokine levels following endotoxin challenge. $TF^{\delta CT/\delta CT}$ (broken line) and $TF^{+/+}$ (continuous line) mice were injected i.p. with 0.5 mg of LPS. Serum was collected at each time point ($n = 4$ to 10) and (A) TNF- α , (B) IL-1 β , and (C) IL-6 concentrations were measured by ELISA. Results are expressed as mean $\pm$ SEM. *, $P < 0.05$ of $TF^{\delta CT/\delta CT}$ compared to $TF^{+/+}$ mice.

and $TF^{\delta CT/\delta CT}$ mice 1 hour following endotoxin challenge (Figure 3). Generation of TAT complexes did not appear to be impaired in $TF^{\delta CT/\delta CT}$ mice at any time point compared to $TF^{+/+}$ mice. Six hours after endotoxin challenge, TAT complexes in $TF^{\delta CT/\delta CT}$ mice were significantly higher than in $TF^{+/+}$ mice. The reduced TAT complexes in $TF^{\delta CT/\delta CT}$ mice at 24 hours may be a reflection of the attenuated endotoxin response and improved survival.

Reduced Leukocytosis and Lung Neutrophil Recruitment in $TF^{\delta CT/\delta CT}$ Mice

A significant leukocytosis developed 6 hours following endotoxin challenge, which was maintained at 24 and 48 hours. In $TF^{\delta CT/\delta CT}$ mice, the leukocytosis was significantly attenuated at 6 hours ($TF^{+/+}$ $3.6 \pm 0.3 \times 10^6$ cells/ml, $n = 7$ versus $TF^{\delta CT/\delta CT}$ $2.4 \pm 0.3 \times 10^6$ cells/ml, $n = 6$; $P = 0.006$) and in mice that survived at 24 hours ($TF^{+/+}$ $7.1 \pm 0.4 \times 10^6$ cells/ml, $n = 5$ versus $TF^{\delta CT/\delta CT}$ $5.7 \pm 0.5 \times 10^6$ cells/ml, $n = 5$; $P = 0.019$) (Figure 4 A). The leukocytosis was predominantly due to an increase

Figure 3. Effects of the deletion of the cytoplasmic domain on the basal and LPS-induced levels of TAT complexes. Levels of TAT complexes (mean $\pm$ SEM) in plasma of normal TF^{+/+} (filled bars, $n = 5$) and TF^{δCT/δCT} (open bars, $n = 6$) mice, in mice at 1 hour [TF^{+/+} ($n = 10$), TF^{δCT/δCT} ($n = 13$)], 6 hours [TF^{+/+} ($n = 14$), TF^{δCT/δCT} ($n = 15$)] and 24 hours [TF^{+/+} ($n = 11$), TF^{δCT/δCT} ($n = 11$)] following endotoxin challenge.

in circulating neutrophils. Blood neutrophil counts were significantly lower at 6 and 24 hours after endotoxin challenge in TF^{δCT/δCT} mice (6 hours; TF^{+/+} $1.8 \pm 0.25 \times 10^6$ cells/ml, $n = 5$ versus TF^{δCT/δCT} $1.1 \pm 0.15 \times 10^6$ cells/ml, $n = 5$; $P = 0.045$, 24 hours; TF^{+/+} $2.64 \pm 0.27 \times 10^6$ cells/ml, $n = 5$ versus TF^{δCT/δCT} $1.23 \pm 0.13 \times 10^6$ cells/ml, $n = 5$; $P = 0.002$) (Figure 4B). Blood monocyte numbers were similar in TF^{+/+} and TF^{δCT/δCT} mice throughout the disease with a significant increase above normal at 24 hours in both groups. B cell numbers were significantly lower at 6 hours in TF^{δCT/δCT} mice (TF^{+/+} $5.1 \pm 0.6 \times 10^5$ cells/ml, $n = 6$ versus TF^{δCT/δCT} $1.8 \pm 0.3 \times 10^5$ cells/ml, $n = 6$; $P = 0.0003$), however CD4⁺ and CD8⁺ cells were similar in both groups (Table 1).

Pulmonary leukocyte accumulation following endotoxin challenge was assessed by MPO activity. In normal mice, lung MPO activity was similar in TF^{δCT/δCT} (2.2 ± 0.1 U/g, $n = 4$) and TF^{+/+} mice (2.5 ± 0.5 U/g, $n = 4$). Following endotoxin treatment, MPO activity increased significantly in both groups at 1 and 6 hours. At 1 hour, MPO activity was significantly lower in TF^{δCT/δCT} (15.6 ± 3.6 U/g, $n = 8$) compared to TF^{+/+} mice (30.1 ± 4.5 U/g, $n = 6$; $P = 0.037$) indicating reduced lung neutrophil accumulation. The difference in lung MPO activity was not statistically significant at 6 hours after endotoxin challenge in TF^{+/+} (31.7 ± 5.0 U/g, $n = 7$) and TF^{δCT/δCT} (24.8 ± 2.7 U/g, $n = 5$; $P = 0.19$) (Figure 5). MPO assessment of lung neutrophil recruitment at 1 hour was confirmed by morphological count of PMN in lung sections. TF^{δCT/δCT} mice (72.25 ± 7.3 cells/hpf, $n = 4$) had significantly reduced PMN infiltration as compared to TF^{+/+} mice (124.7 ± 16.2 cells/hpf, $n = 3$; $P = 0.02$).

Reduced Leukocyte Rolling, Adhesion, and Emigration after Local Endotoxin Challenge in TF^{δCT/δCT} Mice

Intravital microscopy in the cremaster muscle showed increased leukocyte rolling, adhesion, and transmigration

Figure 4. Circulating white cell count, neutrophils, and monocytes following endotoxin challenge. Peripheral blood leukocytes (A), neutrophils (B), and monocytes (C) in endotoxin challenged mice were measured by either hemocytometer counts or flow cytometry as described in Materials and Methods. Each time point includes minimum of six animals. *, $P < 0.05$ of TF^{δCT/δCT} (broken line) compared to TF^{+/+} (continuous line) mice. #, $P < 0.05$ as compared to baseline. Plotted values are represented as mean $\pm$ SEM.

in TF^{+/+} mice 4.5 hours following local endotoxin challenge. These responses were similar to previously published data in normal mice of other strains.^{30,36,37} Endotoxin-induced leukocyte rolling (Figure 6 A), adhesion (Figure 6C), and emigration (Figure 6D) were significantly reduced in TF^{δCT/δCT} as compared to TF^{+/+} mice. Furthermore, rolling velocity was also significantly elevated in TF^{δCT/δCT} mice (TF^{+/+} 11.1 ± 1.4 , $n = 7$ versus TF^{δCT/δCT} 29.3 ± 6.9 , $n = 5$; $P = 0.0061$) (Figure 6B).

Kidney and Liver TF Activity during the Development of Endotoxemia Is Equivalent in TF^{+/+} and TF^{δCT/δCT} Mice

Kidney and liver TF activity of normal TF^{+/+} and TF^{δCT/δCT} mice was equivalent. Kidney TF activity was significantly increased above normal in both groups 6 hours after endotoxin challenge. There were no significant differences in kidney or liver TF activity between TF^{+/+} and

Table 1. Circulating CD4⁺, CD8⁺ T Cells and B220⁺ B Cells Following Endotoxin Challenge

		CD4 ⁺ T cells ($\times 10^5$ cells/ml)	CD8 ⁺ T cells ($\times 10^5$ cells/ml)	B 220 ⁺ B cells ($\times 10^5$ cells/ml)
0 hour	TF ^{+/+}	3.40 $\pm$ 0.35	1.82 $\pm$ 0.20	6.00 $\pm$ 0.80
	TF ^{δCT/δCT}	5.40 $\pm$ 1.00	1.70 $\pm$ 0.34	4.30 $\pm$ 0.70
1 hour	TF ^{+/+}	3.24 $\pm$ 1.10	1.54 $\pm$ 0.60	5.10 $\pm$ 1.40
	TF ^{δCT/δCT}	3.70 $\pm$ 1.70	1.02 $\pm$ 0.50	1.10 $\pm$ 0.30*
6 hour	TF ^{+/+}	4.20 $\pm$ 0.50	2.10 $\pm$ 0.50	5.10 $\pm$ 0.60
	TF ^{δCT/δCT}	4.10 $\pm$ 0.80	1.10 $\pm$ 0.30	1.80 $\pm$ 0.30*
24 hour	TF ^{+/+}	6.10 $\pm$ 1.00	2.20 $\pm$ 0.30	4.70 $\pm$ 1.00
	TF ^{δCT/δCT}	5.32 $\pm$ 3.20	1.50 $\pm$ 0.84	3.20 $\pm$ 2.00
48 hour	TF ^{+/+}	2.20 $\pm$ 0.00	0.40 $\pm$ 0.00	1.80 $\pm$ 0.00
	TF ^{δCT/δCT}	3.20 $\pm$ 0.30	1.90 $\pm$ 0.3	4.30 $\pm$ 0.90

Peripheral blood CD4⁺, CD8⁺ T cells, and B220⁺ B cells in endotoxin challenged mice were measured using haemocytometer counts and flow cytometry, respectively. Each time point includes minimum of four animals (excluding 48 hour time point). **p* < 0.05 of TF ^{δ CT/ δ CT} compared to TF^{+/+} mice at equivalent time points.

TF ^{δ CT/ δ CT} at comparable time points following endotoxin challenge (Figure 7, A and B).

NF κ B Binding Activity in the Kidney, Liver, and Heart of TF ^{δ CT/ δ CT} Endotoxemic Mice Is Substantially Reduced as Compared to TF^{+/+} Mice

To investigate the possible molecular mechanisms for reduced levels of IL-1 β , TNF- α , and IL-6 in serum and decreased mortality in TF ^{δ CT/ δ CT} mice, mobilization of NF κ B subunits into cell nuclei was assessed in different tissues (heart, liver, and kidney) using EMSA. The results demonstrated the presence of LPS-responsive NF κ B-containing complexes in each of these tissues following endotoxemia. One hour following endotoxin challenge, NF κ B DNA binding in TF ^{δ CT/ δ CT} mice was reduced compared to TF^{+/+} mice in all tissues (Figure 8a). Competition and super-shift assays demonstrated that both NF κ B-containing complexes in kidney tissues from a TF^{+/+} mouse were abolished in presence of 200-fold molar excess of wild-type probe but not by 200-fold molar excess of mutant probe. When extracts were incubated with antibodies directed against either p50 or p65, super-

Figure 5. Lung neutrophil recruitment following endotoxin challenge. MPO activity in lungs of untreated TF^{+/+} (filled bars) and TF ^{δ CT/ δ CT} (open bars) mice (*n* = 4), and 1 hour (*n* = 6 to 8) and 6 hours (*n* = 5 to 7) after endotoxin challenge. **P* < 0.05 of TF ^{δ CT/ δ CT} compared to TF^{+/+} mice. Plotted values are mean $\pm$ SEM.

Figure 6. Leukocyte recruitment parameters following endotoxin challenge. Leukocyte rolling (A), leukocyte rolling velocity (B), adhesion (C), and emigration (D) in post-capillary venules of cremasteric vasculature in TF^{+/+} (filled bars, *n* = 7) and TF ^{δ CT/ δ CT} mice (open bars, *n* = 5) at 4.5 hours after local LPS administration. **P* < 0.05 compared to TF^{+/+} mice. (Student's *t*-test) (mean $\pm$ SEM).

Figure 7. TF activity in kidney and liver following endotoxin challenge. Kidney (A) and liver (B) extracts were prepared from control ($n = 3$) and LPS-treated $TF^{+/+}$ (continuous line) and $TF^{\delta CT/\delta CT}$ (broken line) mice ($n = 4$ to 9) at different time points. TF procoagulant activity (mean $\pm$ SEM) was measured by a single-stage clotting assay, as described in Materials and Methods. *, $P < 0.05$ compared to basal levels.

shifted complexes were formed indicating the presence of both p50 and p65 subunits (Figure 8b).

Discussion

The aim of this study was to evaluate the role for the cytoplasmic domain of TF in endotoxemia. For this purpose, we used mice in which the terminal 18 amino acids of the cytoplasmic domain of TF were deleted. Previous studies demonstrated that these $TF^{\delta CT}$ mice have normal growth, fertility, embryogenesis, and coagulation.²⁷ These mice have normal circulating levels of coagulation factors, platelets, and normal TF activity in primary embryonic fibroblasts. The current study demonstrates that deletion of the cytoplasmic domain of TF significantly attenuates mortality 24 hours after endotoxin challenge.

Plasma TF antigen levels are elevated in patients with disseminated intravascular coagulation (DIC) associated with endotoxemia^{17,18} and increased TF activity correlates with mortality. Inhibition of TF activity by administration of anti-TF antibody or by infusion of inactivated VIIa or TFPI reduces mortality in endotoxemia.^{20–23,25,26} In the current study, kidney and liver TF activity was similar in $TF^{\delta CT/\delta CT}$ and $TF^{+/+}$ mice both in the normal physiological state and following endotoxin challenge *in vivo*. Systemic activation of coagulation, as indicated by the presence of TAT complexes, was more severe in $TF^{\delta CT/\delta CT}$ mice 6 hours after endotoxin challenge. This suggests that the protection afforded by a lack of the cytoplasmic domain of TF does not appear to be attributable to an impaired TF coagulant activity.

Figure 8. a: NFκB activation in kidney, heart and liver of $TF^{+/+}$ and $TF^{\delta CT/\delta CT}$ mice. Nuclear extracts of kidney, heart and liver were prepared from normal and endotoxemic $TF^{+/+}$ and $TF^{\delta CT/\delta CT}$ mice. NFκB binding activity in the kidney, heart or livers of normal or endotoxemic mice (1 hour after LPS administration) was quantitated by densitometric assessment of nucleoprotein complexes (left panels) expressed as a ratio of constitutive DNA-binding activity using the proximal PDGF-A promoter, which served as a loading control. The y-axis represents NFκB/PDGFR-α promoter binding ratio. The four samples from endotoxemic tissue stimulated with LPS shown in the figure were obtained from four separate animals. The NFκB band quantified densitometrically is indicated (arrow). Histograms (right panels) represent mean $\pm$ SEM of NFκB/PDGFR-α promoter binding ratios. NFκB activation was consistently reduced in $TF^{\delta CT/\delta CT}$ mice compared with $TF^{+/+}$ mice. **b:** Super-shift and competition experiments demonstrating p50 and p65 subunit of NFκB. Super-shift analysis using nuclear extracts of kidney and the NFκB probe incubated in the absence or presence of wild-type or mutant unlabeled probe, or antibodies directed to the p50 or the p65 subunit of NFκB, as indicated in the figure.

Mortality associated with sepsis has been correlated with enhanced pro-inflammatory cytokine levels in serum, activation level of transcription factors such as NFκB, recruitment of the mediators of innate immunity (macrophages and neutrophils), and pronounced coagulopathy leading to DIC. In baboons, treatment with inactivated factor VIIa decreased systemic levels of IL-6, IL-8, and soluble TNF receptor-1,^{22,23} but not TNF-α.²⁴ Systemic release of TNF-α and IL-1β plays a critical role in the inflammatory responses in endotoxic shock and circulating levels of IL-6 have been shown to correlate with mortality.³⁸ Studies using antibodies directed against these cytokines have shown improved survival in animal models.^{39–41} After the induction of endotoxemia, $TF^{\delta CT/\delta CT}$ mice showed reduced levels of TNF-α, IL-1β, and IL-6 in the serum. The early serum spike of TNF-α was attenuated, the IL-1β response was delayed and attenuated, and IL-6 responses were truncated. This may suggest that the cytoplasmic domain of TF promotes the

production of each of these key pro-inflammatory cytokines involved in the endotoxemic response.

Endotoxin binding to the Toll-like receptor 4 (TLR4) requires CD14^{14,15,42} and results in activation of cytoplasmic proteins including MyD88⁴³ and TRAF6 which initiates a cascade of events which then results in the dissociation of I κ B from the I κ B-NF κ B complex.⁴⁴ NF κ B subsequently translocates to the nucleus and up-regulates transcription of a number of pro-inflammatory genes including TNF α , interleukins,¹⁶ and TF.⁴⁵ There are several different subunits of NF κ B, including c-rel, p50, p65, rel-B, and p52, which combine to form hetero- or homodimers of NF κ B with varying functional activities.⁴⁶ The p50/p65 heterodimer plays a major role in induction of cytokine responses following endotoxin challenge.⁴⁶ The p50/p50 homodimer has been reported to induce LPS resistance by inhibiting cytokine production.^{47,48} Endotoxin-induced transcription of the TF gene is largely mediated by the c-Rel/p65 isoform of NF κ B, but other transcription factors such as AP-1 have been demonstrated to drive the transcription of TF.⁴⁵

In the current study, reduced nuclear translocation of NF κ B was demonstrated in TF ^{δ CT/ δ CT} mice in kidney, liver, and heart 1 hour after endotoxin challenge. In the kidney, this impaired nuclear translocation was detected up to 48 hours after challenge (data not shown). It is likely that this reduced activation of NF κ B contributes to the reduced systemic release of TNF α , IL-1 β , and IL-6 following endotoxin challenge in TF ^{δ CT/ δ CT} mice and thus improves the survival in TF ^{δ CT/ δ CT} mice. Interestingly, no differences in tissue TF activity in kidney and liver were observed in TF^{+/+} and TF ^{δ CT/ δ CT} mice. It is possible that selective TF cytoplasmic domain-dependent effects on p50/p65, cRel/p65, and p50/p50 NF κ B hetero- or homodimers may contribute to differential effects on cytokine and TF induction following endotoxin challenge.

The role of the cytoplasmic domain of TF in activation of NF κ B has not been reported previously and the molecular mechanisms of this effect remain to be clearly elucidated. They may involve the degradation of I κ B as a result of signaling events following phosphorylation of serine residues in the cytoplasmic domain⁴⁹ or may require signaling via recruitment and interaction with PAR-2 as demonstrated in keratinocytes stimulated with factor VIIa and in cytokine-treated endothelial cells.⁵⁰ However, they are clearly dependent on an intact cytoplasmic domain of TF.

Neutrophils and macrophages are major cellular mediators of the innate immune response to bacterial endotoxin.¹³ Circulating neutrophils were significantly decreased at 6 and 24 hours in TF ^{δ CT/ δ CT} mice, and B cells were lower at 1 and 6 hours following endotoxin challenge. Reduction in these leukocyte subsets appears to be the major contributor to the reduced leukocytosis in the TF ^{δ CT/ δ CT} mice. Pro-inflammatory cytokines such as IL-1 β and TNF- α increase circulating neutrophils by releasing them from marginated pools. Other cytokines such as G-CSF, GM-CSF, and chemokines promote release of neutrophils and B cells from bone marrow. Reduced levels of these cytokines may contribute to the attenuated leukocytosis in TF ^{δ CT/ δ CT} mice.

TF ^{δ CT/ δ CT} mice showed reduced lung accumulation of neutrophils. These cells accumulate in tissue by transmigration across post-capillary venules. This process involves increased expression of adhesion molecules including selectins, ICAM, VCAM-1, and PECAM⁵¹ resulting in leukocyte rolling adhesion and emigration.¹³ TF ^{δ CT/ δ CT} mice showed impaired leukocyte recruitment in lungs with histological evidence of reduced lung inflammation and fewer PMN in the alveolar septae. Reduced lung MPO activity provided further evidence of reduced neutrophil recruitment. Impaired leukocyte recruitment was also observed in the cremaster muscle post-capillary venules following local endotoxin challenge. This was associated with increased leukocyte rolling velocity and reduced leukocyte rolling, adhesion, and transmigration in response to endotoxin in TF ^{δ CT/ δ CT} mice as demonstrated by intravital microscopy. Increase in rolling velocity in TF ^{δ CT/ δ CT} mice might be indicative of reduced adhesion molecule interactions between leukocytes and the endothelium. Impaired expression or function of selectins may contribute to the reduction in leukocyte rolling in TF ^{δ CT/ δ CT} mice. These results appear to correlate with the *in vitro* observation that binding of endotoxin-activated monocytes to endothelial cells can be inhibited by an anti-TF antibody.¹⁹

In summary, these studies provide the first *in vivo* evidence for an important role for the cytoplasmic domain of TF in innate inflammatory response. They demonstrate that the cytoplasmic domain of TF contributes to NF κ B activation, pro-inflammatory cytokine production, leukocyte recruitment, and death following endotoxin challenge thus suggesting a direct or indirect role for it in cell signaling events involved in leukocyte activation.

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