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Transglutaminase Type II Is Involved in the Pathogenesis of Endotoxic Shock

Laura Falasca,* Maria Grazia Farrace,† Alessandra Rinaldi,* Loreta Tuosto,‡ Gennaro Melino,§ and Mauro Piacentini2*†

The pathogenesis of sepsis is characterized by the inability of the host to regulate the inflammatory response, and as a consequence, dysregulated inflammatory processes induce organ dysfunctions and death. Altered transglutaminase type II (TG2) expression is associated with the development of many inflammatory diseases. Therefore, in this study, we questioned whether TG2 could also contribute to the pathological inflammatory dysregulation occurring in septic shock in vivo. To this aim, we used as an experimental model the TG2 knockout mice, in which the process of septic shock was elicited by treatment with LPS. Interestingly, our results demonstrated that TG2 ablation leads to partial resistance to experimental sepsis. The increased survival of TG2−/− mice was reflected in a drastic reduction of organ injury, highlighted by a limited infiltration of neutrophils in kidney and peritoneum and by a better homeostasis of the proinflammatory mediators as well as mitochondrial function. We also showed that in wild-type mice, the TG2 expression is increased during endotoxemia and, being directly involved in the mechanisms of NF-κB activation, it may cause a continuous activation cycle in the inflammatory process, thus contributing to development of sepsis pathogenesis. We propose that the inhibition of TG2 could represent a novel approach in the treatment of inflammatory processes associated with sepsis.


Inflammation is usually a healing response, but in some instances, if targeted destruction and stop signals are not properly phased, acute local inflammation leads to a body-wide response, with tissue damage and consequent multiple organ failure (1, 2). Sepsis is characterized by the inability to regulate the inflammatory response, involving both cellular and humoral defense mechanisms. There is a general agreement that it is the host response, rather than the infection itself, that determines the outcome of sepsis (3), and of particular relevance, the development of acute renal failure and heart failure (4, 5).

Two cell types play a key role during the onset of sepsis, as follows: macrophages, which produce a large amount of powerful proinflammatory mediators, and polymorphonuclear neutrophils (PMN), which have been implicated as key factors in the development of systemic inflammation and crucial tissue injury (1, 6).

The cytotoxic substances released by these cells include cytokines (i.e., IL-6, IFN-γ), chemokines, and reactive oxygen species (7). Among them, especially IL-6 appears to have an important prognostic value in sepsis. In fact, persistently elevated IL-6 levels are associated with both multiple organ failure and death (8–10). A central event, in the induction of cytokines and inflammatory mediators network leading to the pathophysiology of septic shock, is the activation of NF-κB (11). It has been demonstrated that NF-κB activity is markedly increased in every organ, both in animal models of septic shock and in human subjects with sepsis. In addition, increased levels of NF-κB activity are associated with a higher rate of mortality and worse clinical outcome (11). In agreement with these observations, the inhibition of NF-κB activation prevents septic multiple organ dysfunction and improves survival in rodent models of septic shock (12).

Despite recent advances in the comprehension of the sepsis’ pathophysiology, therapeutic approaches are still not effective. The explanation for the failure of the anti-inflammatory approaches is that the progress of the disease to septic shock and death depends on a complex interaction of inflammatory cascades, with the contribution of several intracellular signal pathways (13, 14). Recent findings have also shown that outcome of septic shock (and particularly heart failure) is also associated with acute mitochondrial dysfunction due to respiratory chain impairment (15).

A number of studies suggest that type II transglutaminase (TG2) could be a common central factor in the septic disorders. TG2 is a multifunctional enzyme involved in a variety of biological functions, including cell death, signaling, cytoskeleton rearrangements, and extracellular matrix stabilization (16). TG2 catalyzes posttranslational modifications of proteins, such as protein-protein cross-linking, incorporation of amines and glutamine deamidation. In addition, TG2 is a multifunctional enzyme involved in a variety of biological functions, including cell death, signaling, cytoskeleton rearrangements, and extracellular matrix stabilization (16). TG2 catalyzes posttranslational modifications of proteins, such as protein-protein cross-linking, incorporation of amines and glutamine deamidation. In addition, TG2 can act as a G protein and as protein disulfide isomerase (16–19). Aberrant activation of TG2 or deregulation of its function(s) is involved in a variety of human inflammatory diseases, such as celiac disease, diabetes, neurodegenerative diseases, multiple sclerosis, and rheumatoid arthritis (reviewed in Ref. 20). As far as septic shock is concerned, TG2 expression has been reported to be induced by LPS.
in several tissues and organs (21–23). Use of in vitro cell lines has shown that an increase in TG2 activity is associated with NF-κB activation (24).

Interestingly, TG2 knockout mice are prone to develop inflammatory pathologies (25, 26). Furthermore, we recently showed that these mice have an impaired capacity to clear apoptotic cells and this defect is reflected in a deregulated inflammatory cytokine production by macrophages (27). These data indicate a possible role of TG2 in the control of inflammatory processes. We recently demonstrated that TG2 also affects mitochondrial function and regulates the cell’s energy balance (28, 29); in fact, TG2 contains a BH3 domain and colocalizes with mitochondria, and overexpression of TG2 leads to mitochondrial hyperpolarization resulting from an enhanced proton pumping from the respiratory chain (28–30).

To get further insights into the possible role exerted by TG2 in the molecular and cellular mechanisms leading to the improper regulation of the inflammatory response involved in sepsis, we performed an in vivo study during experimental LPS-induced septic shock in mice.

**Materials and Methods**

**Animals**

TG2 knockout (TG2−−) mice (C57BL/6) were generated, as described (31). Wild-type (WT) and TG2−− female mice, 8–10 wk of age, were used. Animals were housed in a germfree environment and given access to standard laboratory chow and water. All care and procedures were performed according to approved protocols and in accordance with institutional guidelines.

**Experimental protocol**

Endotoxic shock was induced by a single i.p. injection of LPS (from *Escherichia coli*, serotype 055:B5; Sigma-Aldrich). To determine the best dose of LPS for the induction of lethal effect in C57BL/6 mice strain, WT animals were preliminarily injected with LPS at three different concentrations: 20, 40, or 80 mg/kg. Because 100% of mice died soon upon injection with 80 mg/kg, and 90% of animals injected with 20 mg/kg survived, the dose of 40 mg/kg was chosen to compare the survival of WT and TG2−− mice. For the treatment, LPS was dissolved in PBS; control animals received an equivalent volume of vehicle (200 µl).

**Sample collection**

At the indicated times (1.5, 4, and 24 h after LPS injection), mice were sacrificed. Heart and kidneys were rapidly dissected. Fragments of the organs were immediately formalin fixed for histology and immunohistochemistry analysis, or stored at −70°C until used. For the cytokeratin analysis, blood sera were collected and stored at −70°C.

**Histological and immunohistochemical analysis**

For histopathological analysis, paraffin-embedded kidney and heart were cut in sections of 3 µm and stained with H&E. Scoring of the histopathological changes was performed under light microscopy in blind conditions. The evaluation of heart changes by light-microscopic examination included study of alignment and orientation of muscle cells, orientation of myofibrils, nuclear changes, myocard hyper trophy, and degenerative changes.

PMN cell infiltration into the kidney was measured after myeloperoxidase (MPO) staining. Immunohistochemical staining was performed on paraffin-embedded kidney sections (5 µm) using an anti-MPO rabbit polyclonal Ab (DakoCytomation). The slides were counterstained with hematoxylin, PMN cells were counted (>40 magnification), and the average number of cells was recorded.

**Electron microscopy**

Transmission electron microscopy analysis was undertaken of myocardium tissue from WT and TG2−− mice at 24 h after LPS treatment. Heart fragments were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4°C. Postfixation was performed with 1% OsO4. Samples were then dehydrated in a graded ethanol series and embedded in Spurr resin. Ultrathin sections were stained with 2% uranyl acetate and observed under a Zeiss EM900 transmission electron microscope. Images were captured digitally with a Mega View II digital camera (SiS).

**Measurement of cytokine release using a protein array system**

For the cytokine analysis, blood sera were collected 1.5, 4, and 24 h after LPS injection.

Cytokine levels were measured using a Mouse Cytokine Array I kit (Panomics) consisting of 22 different cytokine and chemokine Abs spotted on nitrocellulose onto a membrane (Cytomeg Lab). The membranes were processed according to the instructions of the manufacturer. Briefly, membranes were incubated with 4 ml of 1× blocking buffer at room temperature for 1 h, washed twice with 1× wash buffer II, and incubated with 2 ml of sample (mouse serum or macrophage culture medium) for 2 h at room temperature. After decanting the samples, membranes were washed three times with 4 ml of 1× wash buffer I, 5 min per wash, followed by two washes with 1× wash buffer II. 5 min per wash. The membranes were then incubated with 1.5 ml of diluted biotin-conjugated Abs at room temperature for 1 h, and washing steps were repeated as before. Membranes were incubated for 1 h at room temperature with a dilution of streptavidin-conjugated peroxidase. Following a thorough wash, the membranes were exposed to the mixed detection buffers, according to the instructions of the manufacturer, for 5 min in the dark before imaging. Spots were visualized using ECL (Amersham Biosciences). Membranes exposed to Kodak X-OMAT radiographic film were then processed. Each film was scanned, and spot densities were measured with Scion Image for Windows (Scion; National Institutes of Health). The densities were exported into Excel, and the background intensity was subtracted before analysis.

**Peritoneal cell analysis**

Cells from peritoneal lavage, collected from untreated animals or 24 h after septic challenge by LPS, were stained with anti-Ly-6G FITC-conjugated mAb (BD Pharmingen). Negative controls included cells incubated with or without fluorochrome-conjugated control Abs. Erythrocytes were lysed using lysis buffer (BD Pharmingen). Analyses were performed on a FACSCalibur using CellQuest software (BD Biosciences). Neutrophils were identified based on side scatter (SSC) and Ly-6G expression.

Peritoneal cells were also spun in a cytocentrifuge onto microscopic slides and stained with DiffQuick. For differential counts, 500 cells per slide were scored.

**Preparation of heart mitochondria**

Mitochondrial fractions were prepared from heart of WT and TG2−− mice, as previously described (29). Mitochondria, freshly prepared, were briefly sonicated, and protein concentrations were determined using a DC-protein assay kit from Bio-Rad and BSA as a standard. The purity of mitochondria preparation was assessed by Western blot checking subunit I of cytochrome c oxidase.

**Assays of respiratory chain activities**

Spectrophotometric analysis of the respiratory complex I and II activities was performed on mitochondria isolated from heart, as previously described. All assays were performed at 30°C in a final volume of 1.0 ml using an Ultron 940 Spectrophotometer (Kontron Instruments). The NADH-ubiquinone oxidoreductase activity of complex I was measured quantifying the decrease in UV absorbance accompanying the oxidation of NADH. The reaction mixture consisted of 20 mM potassium phosphate (pH 7.2), 10 mM MgCl2, 1 mM KCN, and 100 µM durosquine. The reaction was initiated by adding 0.15 mM NADH to 20 or 40 µg of mitochondrial proteins and monitored through the linear absorbance decrease in 3 min at 340 nm. Rotenone (10 µM) was added, and any rotenone-insensitive activity was measured for an additional 3 min. Succinate-ubiquinone oxidoreductase activity was determined according to Taylor et al. (32), by following the reduction of 2,6-dichlorophenolindophenol when coupled to complex II-catalyzed reduction of decyldubiquinone, monitoring the absorbance at 600 nm. Mitochondrial proteins (20 or 40 µg) were preincubated in 25 mM potassium phosphate (pH 7.2), 5 mM MgCl2, 3 mM EDTA, and 20 mM succinate for 10 min at 30°C. Rotenone (2 µg/ml), antimycin A (2 µg/ml), and 2,6-dichlorophenolindophenol (50 µM) were then added, and blank rate was recorded for 3 min. The reaction was started by the addition of 50 µM decyldubiquinone and monitored through the change in absorbance for 3 min at 600 nm. Sp. act. was validated by inhibition with 4 mM 3-nitropropanic acid. Values are expressed in nmol of substrate consumed/min/mg protein.

**Western blot analysis**

Heart tissues from WT and TG2−− animals were removed immediately after sacrifice and homogenized in 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and protease inhibitor (Roche), and mitochondria were...
isolated (33). Approximately 20 μg of proteins was separated on a 5–12% precast SDS-polyacrylamide gel (Invitrogen Life Technologies) and transferred to nitrocellulose membrane. Membranes were probed using mouse primary mAbs against TG2 (NeoMarkers), IκBα (Cell Signaling Technology), NF-xB p65 (Santa Cruz Biotechnology), the 17-kDa subunit of complex I (Molecular Probes), and the 70-kDa subunit of complex II (Molecular Probes). Detection was achieved using HRP-conjugated secondary Ab (The Jackson Laboratory). The signals were detected with Supersignal (Pierce).

Chromatin immunoprecipitation (ChIP) assay

To analyze the role of TG2 on LPS-induced NF-xB transcriptional activity, we did ChIP assays, as previously described (34). Briefly, peritoneal macrophages, isolated by peritoneal lavage from WT and TG2−/− mice treated or not with LPS for 18 h, after fixing in 1% formaldehyde, were lysed for 5 min in 50 mM Tris (pH 8.0), 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol supplemented with protease inhibitors. Nuclei were resuspended in 50 mM Tris (pH 8.0), 1% SDS, and 5 mM EDTA. Chromatin was sheared by sonication, centrifuged, and diluted 10 times in 50 mM Tris (pH 8.0), 0.5% Nonidet P-40, 0.2 M NaCl, and 0.5 mM EDTA. After preclearing with a 50% suspension sperm-saturated protein A, lysates were incubated at 4°C overnight with the indicated Abs. Immune complexes were collected with sperm-saturated protein A and washed three times with high salt buffer (20 mM Tris (pH 8.0), 0.1% SDS, 1% Nonidet P-40, 2 mM EDTA, and 500 mM NaCl) and five times with 1× Tris/EDTA. Immune complexes were extracted in 1× Tris/EDTA containing 1% SDS, and protein-DNA cross-links were reverted by heating at 65°C overnight. DNA was extracted by phenol-chloroform, and ∼1/20 of the immunoprecipitated DNA was used in each PCR. The IL-6 promoter-specific primers used were as follows: 5′-GACATGCTCAAGTGCTGAGTCAC-3′ and 5′-AGATT GCACAATGTGACGTCG-3′. Anti-RelA/p65 (C-20) was purchased from Upstate Biotechnology. Anti-NF-xB/p50 (06-886) was purchased from Upstate Biotechnology.

Statistical analysis

Values were expressed as means ± SD. Analysis was performed by two-tailed Student’s t test for comparison between two groups. Statistical significance was set at p < 0.05. GraphPad Prism version 4.00 for Windows (GraphPad) was used to perform the analysis. Survival analysis was performed by Kaplan-Meier analysis.

Results

TG2 ablation causes an increased survival after LPS-induced sepsis

To verify whether TG2 could be involved in septic shock, we first investigated, by means of Western blot analysis, the expression of TG2 in WT and TG2−/− mice treated or not with LPS. Notably, we found that the LPS treatment induced a significant increase of TG2 protein levels (2-fold increase) in the heart, when compared with controls (Fig. 1A). Prompted by this result on sepsis, we examined the survival of WT and TG2−/− mice during acute endotoxic shock. Animals were treated with LPS (40 mg/kg), and survival was assessed every 6 h. After LPS injection, both WT and TG2−/− mice displayed the typical symptoms of murine endotoxic shock, such as decreased motor activities and ruffled fur. However, the difference in mortality between the two animal groups was highly significant (p < 0.0001) (Fig. 1B). In fact, survival at 24 h was 37% in WT and 90% in the knockout animals. One hundred percent of the WT mice succumbed within 36 h, whereas no further death was observed in the TG2−/− mice (Fig. 1B).

Renal and myocardium tissue damage

To determine whether the difference in the mortality between WT and TG2−/− mice was associated with a different inflammatory response and different degree of organ injury, we analyzed kidney and heart histology.

A crucial aspect during sepsis is acute renal failure (35), highlighted by massive neutrophil infiltration. Kidneys from WT and TG2−/− mice were examined 24 h after LPS injection. Renal histology from WT LPS-treated mice showed a moderate sign of tubular injury (Fig. 2c), whereas in TG2−/− renal tissue histological features appeared quite normal (Fig. 2d). Abundant neutrophil infiltration particularly in venules, but sometimes even in glomeruli, was detected by MPO immunostaining in WT mice after LPS (Fig. 2e). Quantitation of neutrophil infiltration (Fig. 2g) showed a 4-fold increase in WT LPS-treated mice compared with baseline (10.1 ± 2.12 vs 1.35 ± 0.62 neutrophils per high power field; p < 0.005). Interestingly, the absence of tissue injury observed in TG2−/− mice after LPS treatment was accompanied by an insignificant increase in neutrophil numbers (1.17 ± 0.75 vs 2.97 ± 0.96 neutrophils per high power field) (Fig. 2, f and g).

During sepsis, a key aspect in the development of the disease is the corresponding evolution of myocardial dysfunction. H&E-stained sections and transmission electron microscopy were used
for evaluation of myocyte degenerative changes and to study alignment and orientation of muscle cells, cross-striations, and organelles. Analysis of H&E-stained longitudinal sections of mice heart, 24 h after LPS, revealed strong muscular degradation in WT (Fig. 2a), in which the principal histological changes observed were myocytolysis (necrosis of myocardial cells). On the contrary, these alterations were not observed in TG2−/− mice (Fig. 2b). The ultrastructural analysis of myocardium from LPS-treated WT mice showed myofibrillar disarray, consisting in nonparallel arrangement and sometimes in focal lysis, Z band abnormalities, and intracytoplasmic vacuolations (Fig. 3c). However, the most evident changes found in these samples concern the mitochondria (Fig. 3e). They were irregular in shape, dilated, and associated with the presence of small mitochondria. Degeneration of cristae was often visible, and sometimes mitochondria appeared ruptured (Fig. 3e).

Interestingly, these changes were not displayed by myocytes from TG2−/− LPS-treated mice. In these animals, the ultrastructure of myocardium appeared preserved, or with very mild signs of alterations, both concerning disorganization of myofibrils and damage to mitochondria (Fig. 3, d and f).

**Effect of LPS on mitochondrial respiratory chain activity in WT and TG2−/− mice**

We have recently reported that TG2 plays an important regulatory role on mitochondria under stressful cellular condition (30). Thus, considering the above described lack of ultrastructural changes of mitochondria induced by septic shock in TG2−/− mice, we analyzed the effect of TG2 ablation on mitochondrial respiratory chain activity compared with WT. The activity of respiratory chain complexes I and II was measured in mitochondria isolated from hearts of both WT and TG2−/− animals treated or not with LPS. As previously reported, under basal conditions complex I activity was significantly lower (45% reduction) in mitochondria isolated from TG2−/− mice compared with WT (Fig. 4A). By contrast, mitochondria obtained from TG2−/− mice displayed a significantly higher activity of complex II (205% increase; p < 0.001) (Fig. 4A). As expected, in WT mice, the activity of complex I and II was substantially reduced after LPS-induced septic shock, and this reduction was more pronounced for complex II (−45%). In TG2−/−
mice, the activity of both complexes was not affected by LPS treatment (Fig. 4A). Of note, the functional differences of the respiratory complexes were not due to changes in complex I and II protein levels, as demonstrated by Western blot analysis in extracts from myocardial mitochondrial fractions of WT and TG2 mice (Fig. 4B).

**Neutrophil recruitment into the peritoneal cavity following LPS**

During acute peritonitis, neutrophils are recruited from the circulation to the peritoneal cavity. The process is temporally regulated to prevent inflammation-induced tissue injury; thus, after the initial phase, PMN undergo apoptotic cell death and are removed (36).

We analyzed by flow cytometry the presence of neutrophils into the peritoneal cavity after 24 h of LPS treatment. As shown in Fig. 4B, in absence of inflammatory stimulation PMN (LY-6G^high^) constituted a very small percentage of the peritoneal different cell types infiltrates: together with neutrophils, an important recruitment of mast cells and lymphocytes, compared with TG2^−/−^ animals, was found (Fig. 5b).

**Inflammatory cytokine production in LPS-treated mice**

To examine the inflammatory response following LPS administration, we measured serum levels of a number of inflammatory mediators.

The evaluation of cytokine expression profile was performed using a highly sensitive cytokine Ab array method, enabling the simultaneous detection of low concentration of multiple cytokines in one assay (pg/ml range). To evaluate whether the improved survival observed in TG2^−/−^ mice was reflected in a difference in circulating cytokine, we analyzed the sera obtained from control and LPS-injected mice at different times of treatment (Fig. 6). The analysis was performed under basal untreated conditions (controls) and at 1.5, 4, and 24 h following LPS treatment; among all cytokines and chemokines analyzed, the following five showed significant differences: IL-6, IFN-γ, G-CSF, soluble TNFRI (sTNFRI), and MCP-1. As shown in Fig. 6, basal level of circulating IL-6, IFN-γ, G-CSF, and MCP-1 did not vary in the sera of WT and TG2^−/−^ mice. By contrast, their expression changes significantly after LPS challenge. In particular, the levels of IL-6, IFN-γ, and
FIGURE 6. Circulating inflammatory cytokine in mice blood serum, under control untreated conditions, and after LPS challenge. a, Representative membrane protein arrays incubated with the sera obtained from WT and TG2−/− mice, untreated (Ctr.) or treated with LPS for 1.5 (LPS 1.5h) and 24 (LPS 24h) h. Differences in cytokine expression can be observed for each sample in the absence or in the presence of treatment, and between samples from WT and TG2−/− mice after 24 h from LPS injection. b, Quantitative analysis of IL-6, IFN-γ, G-CSF, sTNFRI, and MCP-1. Similar profiles were detected in untreated conditions comparing WT vs TG2−/−, apart from sTNFRI, whose expression was significantly different (knockout showed higher levels, p < 0.05). The release of all proinflammatory cytokines is significantly up-regulated by LPS treatment, reaching a peak after 1.5–6 h in both groups of animals (p < 0.005). The levels of IL-6, IFN-γ, and G-CSF were still elevated (p < 0.05) in WT mice; on the contrary, their expression was found back to normal values in TG2−/− mice. Values represent spot densities as measured by Scion Image. Data represent the mean ± SEM of four independent experiments.
demonstrated that TG2 catalyzes the polymerization of I-κB, and no difference was observed in the absence of TG2. It has been revealed that LPS induced a significant increase of p65 subunit in the nucleus of cells from WT animals, whereas the levels of p65 subunit in the cytoplasm, were induced by LPS in macrophages from WT animals; no changes were induced in the absence of TG2. Tubulin was used as loading control. a, ChIP analysis was performed using anti-p65, anti-p50, or anti-p52 Abs. Immunoprecipitated DNA was analyzed by PCR with IL-6 promoter-specific primers. Data are representative of three independent experiments.

G-CSF at 24 h appear to discriminate between survivors and nonsurvivors. Serum concentration of these cytokines dramatically increased, reaching a peak at 1.5–6 h after LPS injection in both groups of animals (p < 0.005); at 24 h, the cytokine levels remained heightened in WT (p < 0.05), whereas in TG2−/− mice they returned to the steady-state values.

**NF-κB activation**

Activation of the nuclear transcription factor NF-κB plays a key role in the inflammatory process by inducing the transcription of proinflammatory mediators (11). We examined the activation of NF-κB in peritoneal macrophages isolated from control and LPS-treated WT and TG2−/− mice (Fig. 7).

Western blot analysis, performed on cytoplasmic and nuclear extracts (Fig. 7A), revealed that LPS induced a significant increase of p65 subunit in the nucleus of cells from WT animals, whereas no difference was observed in the absence of TG2. It has been demonstrated that TG2 catalyzes the polymerization of I-κBα, leading to NF-κB activation (24). In keeping with this notion, we analyzed by Western blot the presence of polymerized I-κBα in the cytoplasmic extracts. Data obtained (Fig. 7A) clearly showed the presence of dimeric form of I-κBα in LPS-treated WT mice, which is undetectable in TG2−/− mice.

Considering that IL-6 is one of the most important proinflammatory cytokines involved in the pathogenesis of sepsis, and that it is differently modulated in WT vs TG2−/− mice, we performed ChIP assays on the promoter region of IL-6, using Abs specific to NF-κB p65, p50, and p52 (Fig. 7B). Results obtained on extracts from peritoneal macrophages of unstimulated WT mice showed that the promoter was mainly occupied by the p52 subunit (Fig. 7B). The transcriptionally inactive p50 subunit was also constitutively bound to the promoter, but following treatment with LPS, this binding complex is displaced by the recruitment to the IL-6 gene promoter of p65 NF-κB subunit, indicating the induction of gene transcription (Fig. 7B). Interestingly, in unstimulated macrophages from TG2−/− mice, the p56 subunit appeared constitutively present at the binding site of the promoter, together with p50, whereas it did not contain the p52. After LPS stimulation, p65 remained bound, whereas the p50 repressive subunit was displaced and substituted by p52, as observed in WT (Fig. 7B).

**Discussion**

The precise mechanism leading to sepsis and the systemic inflammatory response syndrome remains largely unknown, but it is clear that the development of the disease is multifactorial. In this study, we provide evidence that TG2 could be an important factor in the mechanism through which sepsis develops, acting at multiple levels. Previous studies have shown that TG2 inhibitors can be useful in reversing some inflammatory processes (37). In the present study, we demonstrated that the ablation of TG2 confers resistance to LPS-induced septic shock in mice. TG2 knockout mice display enhanced survival (90%) to LPS challenge. The absence of TG2 is associated with a profound reduction of the inflammatory response and attenuated organ damage. Production of proinflammatory mediators is elicited in TG2−/− mice by LPS treatment, indicating that the capacity to respond to endotoxic stimulus is still present in these animals, but as opposed to the WT, TG2−/− mice have the capacity to restore the initial equilibrium. IL-6 and IFN-γ were released at significantly high amounts in the early phase after LPS injection both in WT and TG2−/− mice. These molecules are responsible for the development of fever and cachexia (38), the symptoms of endotoxic shock observed initially also in TG2 animals. However, the levels of IL-6, IFN-γ, and G-CSF found at 24 h appear to discriminate between survivors and nonsurvivors, as follows: WT mice had significantly higher, and long lasting, proinflammatory cytokine levels when compared with TG2−/−.

The data reported in this study demonstrate that no major differences in the basal levels of cytokines exist between WT and TG2−/− mice, with exception of the sTNFRI. TNF, through the interaction with TNFRI, is a major mediator of apoptosis as well as inflammation and immunity, and it has been implicated in the pathogenesis of a wide spectrum of human diseases, including sepsis (39). The absence of detectable levels of TNF in serum of both WT and TG2−/− mice could be related to other factors present during endotoxemia, such as metabolism and degradation, which may influence the earlier disappearance of TNF, as observed in septic patients (40). Although we found significant differences in the levels of sTNFRI between WT and TG2−/− control animals (knockout showed higher levels, p < 0.05), the level of TNFRI was higher in WT at 24 h, but this difference did not reach statistical significance.

The interaction of TNF with TNFRI triggers a series of intracellular events that ultimately result in the activation of two major transcription factors, NF-κB and c-Jun (39). NF-κB plays a critical role in numerous cellular processes. As concerns sepsis, NF-κB is activated not only via the TNFRI, but also by LPS, leading to induction of cytokines and inflammatory mediators (11). This activation is the result of phosphorylation and subsequent degradation of the inhibitory factor I-κBα induced by I-κB kinase complex (41). In this study, we confirmed recent data demonstrating that even the overexpression of TG2 can lead to activation of NF-κB, through an I-κB kinase-independent pathway, which is mediated by the polymerization of I-κBα (24). Our findings showed that, under unstimulated conditions, the TG2−/− mice display a different pattern of distribution of the various NF-κB subunits, p65/p50/p52 on the target gene promoter. In particular, in nuclear extracts of TG2−/− macrophages, the p65 subunit appeared constitutively

**FIGURE 7.** NF-κB activation. a, Cytoplasm (Cyt) and nuclear (Nuc) extracts from peritoneal macrophages isolated from control and LPS-treated WT and TG2−/− mice were assayed for NF-κB, and I-κBα (Cyt and Nuc) and IκBα (cytoplasm) contents by Western blotting. An increase of p65 subunit in the nucleus, and the formation of polymerized I-κBα in the cytoplasm, were induced by LPS in macrophages from WT animals; no changes were induced in the absence of TG2. Tubulin was used as loading control. b, ChIP analysis was performed using anti-p65, anti-p50, or anti-p52 Abs. Immunoprecipitated DNA was analyzed by PCR with IL-6 promoter-specific primers. Data are representative of three independent experiments.
present at the binding site of the IL-6 promoter, although it does not contain the p52, whereas the transcriptionally inactive p50 subunit was also bound to the IL-6 promoter. In some systems, p50 has been described to exert repressive activity and to prevent NF-κB-dependent gene expression (42). This is consistent with the fact that despite the presence of p65 on the promoter of IL-6, we did not find a constitutive expression of this cytokine in TG2−/− mice. LPS stimulation induced the displacement of the p50 repressive subunit from the promoter, and the recruitment of p65/p52 subunits in both WT and in knockout, in agreement with the observed expression of IL-6 induced by LPS. Taking into account that TG2 expression can be induced directly by NF-κB activation, because the TG2 promoter contains a NF-κB-binding motif (43), our results would suggest that the absence of TG2 could be an advantage during endotoxic shock, because this deficiency appears associated with an activation of NF-κB that is transient, thus allowing the restoration of the immunological equilibrium. On the contrary, in WT, the increased expression of TG2 induced by LPS might cause a continuous activation cycle in the inflammation process. Similarly, it has been reported that overexpression of TG2 in cancer cells induces constitutive activation of NF-κB (44).

The present study provides evidence that TG2 deficiency also results in other benefits that contribute to determine survival during septic shock. TG2−/− mice displayed a lesser extent of renal and myocardial tissue damage. Kidneys from TG2−/− animals after LPS treatment showed an unchanged histology associated with an insignificant neutrophil infiltration. In these mice, endotoxemia did not affect the PMN population into the peritoneal cavity, as opposed to WT, in which we observed a strong increase of neutrophil number. Neutrophils are crucial elements for the destruction of infective agents, but they can also damage cells and tissue of the host (1, 6). Thus, during septic shock, the lower number of infiltrating neutrophils in TG2−/− could improve survival, as a consequence of less organ injury. Our data are in agreement with results by others showing that in the absence of TG2 neutrophils exhibited attenuated chemotaxis and diminished extravasation capacity, but displayed enhanced phagocytic activity (45). After LPS treatment, TG2−/− mice also have well-preserved cardiac tissue. A growing body of evidence indicates that functional rather than structural changes seem to be responsible for myocardial damage, and that mitochondrial dysfunction contributes significantly to cardiac failure (46, 47). The mitochondrial respiratory chain includes four enzyme complexes, whose activity can be inhibited by reactive oxygen and nitrogen species. These reactive species are produced in excess during sepsis, causing mitochondrial impairment (48, 49). Therefore, the reduced activity of complexes I and II found in WT mice after LPS treatment was expected. In contrast, in TG2−/− mice, the activity of both complexes was not affected during endotoxemia. As recently shown by our group (30), the ablation of TG2 leads to defective function of mitochondrial respiratory complex I in knockout mice, which is associated with an increased activity of the complex II. It could be hypothesized that in TG2−/− mice, the compensatory increase in complex II activity found under basal conditions could account for the resistance to mitochondria failure induced by LPS; thus, it could even explain the reduced myocardial tissue damage.

In conclusion, data reported in this study suggest for the first time that TG2 overexpression contributes to the pathogenesis of sepsis. Therapeutic approaches for treatment of septic shock are focused on interrupting the inflammatory cascade, and are thus based on antagonists to microbial products, cytokine and inflammatory mediators (13). Moreover, due to the different interacting pathways activated in course of the disease, most therapies are still not effective and the identification of novel therapeutic strategies is needed. In this study, we showed that inhibition of TG2 improves survival of mice to septic shock, and due to the different substrates and functions of the enzyme, the benefit appears related to multiple involvement of TG2 at different sites of action (i.e., NF-κB activation, cytokine homeostasis, mitochondrial function). Based on these results, TG2 inhibitors may be taken into account for developing future therapies for sepsis.

Disclosures
The authors have no financial conflict of interest.

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