Increased Mortality and Inflammation in Tumor Necrosis Factor-Stimulated Gene-14 Transgenic Mice after Ischemia and Reperfusion Injury

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TSG-14/PTX3 is a gene inducible by tumor necrosis factor (TNF)- α , interleukin-1 β , and lipopolysaccharide in fibroblasts, macrophages, and endothelial cells. It encodes a 42-kd secreted glycoprotein that belongs to the pentraxin family of acute-phase proteins. Recently, we demonstrated that TSG-14 transgenic mice (TSG-14tg) overexpressing the murine TSG-14 gene under control of its own promoter are more resistant to lipopolysaccharide-induced shock and to polymicrobial sepsis caused by cecal ligation and puncture. Here we show that after ischemia and reperfusion (I/R) injury, TSG-14tg mice have an impaired survival rate, which appeared secondary to a markedly increased inflammatory response, as assessed by the local (duodenum and ileum) and remote (lung) enhancement in vascular permeability, hemorrhage, and neutrophil accumulation. Moreover, tissue concentrations of TNF- α , interleukin-1 β , KC, and MCP-1 were higher in TSG-14tg as compared to wild-type mice after I/R injury. Of note, elevated TNF- α concentrations in serum were only observed in TSG-14tg mice and blockage of TNF- α action prevented lethality of TSG-14tg mice. These results demonstrate that transgenic expression of TSG-14 induces an enhanced local and systemic injury and TNF- α -dependent lethality after I/R. Taken together, our data point to a critical role of TSG-14 in controlling acute inflammatory response in part via the modulation of TNF- α expression. (*Am J Pathol* 2002, 160:1755–1765)

Tumor necrosis factor (TNF) is a pleiotropic, pro-inflammatory cytokine with a multitude of biological effects in a variety of cells within and outside the immune system. TNF- α seems to be a key cytokine in regulating inflammation and its expression can be protective as well as deleterious for the host.¹ As a way of unraveling the

molecular mode of action of TNF, various groups have succeeded in isolating new TNF- α -stimulated genes and, with less success, characterized the functions of these TNF-stimulated genes (TSGs).

TSG-14/PTX3 was originally described as a gene inducible by TNF- α in human fibroblasts² and, soon after, it was also identified as being induced by interleukin (IL)-1 β in endothelial cells.³ TSG-14 gene encodes a 42-kd secreted glycoprotein that belongs to the long pentraxin family of proteins. At the c-terminal half, it shares homology with the short pentraxin family of acute-phase proteins such as C-reactive protein (CRP) and serum amyloid P component (SAP) but, at the n-terminal half, no homology with known proteins can be found.^{4,5} The expression of the TSG-14 gene can be detected in fibroblasts,² endothelial cells,⁴ chondrocytes, and synovocytes,⁶ and cells of the monocyte/macrophage lineage⁷ stimulated with TNF- α ,² IL-1 β ,⁴ lipopolysaccharide (LPS),⁸ or components of the mycobacterial cell wall.⁹ At a discrepancy with other pentraxins and acute-phase response proteins, TSG-14 gene is not expressed in liver and its induction occurs mainly in endothelial cells of skeletal and myocardic muscle and in macrophages exposed to components of bacterial cell wall, LPS, and lipoarabinomannan (LAM).^{10,11}

Previous studies have suggested a role for TSG-14 in the inflammatory process: TSG-14 binds to the C1q component of the complement cascade;¹² high levels of TSG-14 are detected in the serum of LPS-injected humans and mice,^{10,13} and in the joint fluid of rheumatoid arthritis patients;⁶ and the expression of TSG-14 is regulated by the inflammatory cytokines TNF- α and IL-1 β .^{10,13}

To characterize the functions of TSG-14 *in vivo*, we generated transgenic mice having two or four extra copies of the TSG-14 gene under the control of its own promoter.¹⁴ In these mice, the expression of the transgene parallels that of the resident gene and is detected

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only in heart and muscle of LPS-treated mice. Interestingly, TSG-14 transgenic (tg) mice are more resistant to the lethal effects of LPS injection and have increased survival rate when submitted to cecal ligation and puncture (CLP), an experimental model of peritonitis. Moreover, on LPS injection, transgenic mice produce higher levels of circulating TNF- α . Finally, we also demonstrated that peritoneal macrophages from transgenic mice produce higher amounts of nitric oxide (NO) when stimulated with interferon- γ (IFN- γ) alone or in combination with LPS or TNF- α .

If an enhanced inflammatory response is beneficial after microbial infection, excessive tissue and systemic inflammation may also lead to enhanced mortality in several inflammatory diseases and even after microbial infection.¹⁵ Events that lead to acute ischemia, such as after trauma or acute vascular events are associated with hypoxemic damage in organs and endothelium. Reperfusion of ischemic tissue is the treatment of choice in these acute events but is known to worsen the damage of ischemia. Reperfusion-induced injury involves the recruitment of activated leukocytes, especially neutrophils, and the release of soluble mediators of inflammation, such as pro-inflammatory cytokines, notably TNF- α , and reactive oxygen and nitrogen species.^{16–21} Because TSG-14tg mice were shown to produce higher levels of TNF- α and NO¹⁴ and because the TSG-14 gene was shown to be expressed by endothelial cells,^{3,13} we used TSG-14tg mice to evaluate the role played by this molecule in the cascade of events leading to inflammatory injury and lethality after ischemia and reperfusion of the superior mesenteric artery (SMA).

Here, we show that mice overexpressing the TSG-14 gene have a reduced survival rate as compared to wild-type (WT) mice, with an augmented production of pro-inflammatory cytokines, a higher degree of tissue damage, and hemorrhage both locally and in the lungs. As demonstrated in other models of ischemia and reperfusion (I/R) injury, TNF- α also seemed to be the key mediator of the enhanced cellular damage observed in TSG-14tg mice, because injection of a soluble form of TNF receptor rescued mice from death. Our data demonstrate a role for TSG-14 in controlling TNF- α production in a relevant pathophysiological model and further support the notion that TSG-14 is an important player during acute inflammation. Interference with TSG-14 action may be a potential target for the development of strategies aimed to minimize tissue damage when reperfusion is required.

Materials and Methods

Mice

Generation and the initial characterization of TSG-14tg mice (CD1 background) were described earlier.¹⁴ Two lineages of transgenic mice having two (Tg2 line) or four (Tg4 line) extra copies of the mouse TSG-14 gene under control of its own promoter, always in heterozygosity, were used in this study. TNF receptor 1 knockout mice

(p55^{-/-}, mixed 129SvEv/C57B/L6 background)²² were a kind gift of Dr J. S. Silva (USP, São Paulo, Brazil). Mice were housed in a clean, temperature-controlled room and received sterile food and acidic water *ad libitum*. For all experiments, mice were used at the age of 6 to 8 weeks and WT controls were always of the same background as the mutant line (CD1 for TSG-14tg and 129SvEv/C57B/L6 for p55^{-/-}).

Ischemia and Reperfusion

Mice were anesthetized with urethane (140 mg/kg, i.p.) and laparotomy was performed. The SMA was isolated and ischemia was induced by totally occluding the SMA for 60 minutes. For measuring percentage of surviving mice, reperfusion was re-established, and mice were monitored for indicated time periods. When indicated 5 minutes before reperfusion of the SMA, mice were injected intravenously with 3 μ g/mouse of soluble TNF receptor 1-IgG chimera (sTNFR),²³ calibrated against a standard preparation from the National Institute of Biological Standards and Control (England). For the other parameters, reperfusion was allowed to occur for 30 minutes (I60R30) or indicated time points (as in the case of neutrophil counts), when mice were sacrificed. Sham-operated animals were used as controls.

Evaluation of Changes in Vascular Permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability. Evans blue (20 mg/kg) was administered intravenously (1 ml/kg) via a femoral vein 2 minutes before reperfusion of the ischemic artery. Thirty minutes after reperfusion, segments of the duodenum and ileum (~10 cm) were cut open and allowed to dry in a Petri dish for 24 hours at 37°C. The dry weight of the tissue was calculated and Evans blue extracted using 3 ml of formamide (24 hours at room temperature). The amount of Evans blue in the tissue was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in an enzyme-linked immunosorbent assay plate reader. Results are presented as the amount of Evans blue (in μ g) per 100 mg of tissue. The right ventricle was flushed with 20 ml of phosphate-buffered saline (PBS) to wash the intravascular Evans blue in the lungs. The left lung was then excised and used for Evans blue extraction. The right lung was used for the determination of myeloperoxidase activity as described below.

Myeloperoxidase Concentrations

The extent of neutrophil accumulation in the intestine and right lung tissue was measured by assaying myeloperoxidase activity (MPO) as previously described.²⁴ Briefly, a portion of duodenum, ileum, and the flushed right lungs of animals that had undergone I/R injury were removed and snap-frozen in liquid nitrogen. On thawing, the tissue (1 g of tissue per 19 ml of buffer) was homogenized in pH 4.7 buffer (0.1 mol/L NaCl, 0.02 mol/L NaPO₄, 0.015 mol/L

Na-ethylenediaminetetraacetic acid), centrifuged at $260 \times g$ for 10 minutes and the pellet subjected to hypotonic lyses (15 ml of 0.2% NaCl solution followed 30 seconds later by the addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After a further centrifugation, the pellet was resuspended in 0.05 mol/L NaPO_4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and re-homogenized. One-ml aliquots of the suspension were transferred into 1.5-ml Eppendorf tubes followed by three freeze-thaw cycles using liquid nitrogen. The aliquots were then centrifuged for 15 minutes at $10,000 \times g$, the pellet was resuspended to 1 ml and samples of intestine, mesentery, and lungs were diluted before assay. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density (OD) at 450 nm using tetramethylbenzidine (1.6 mmol/L) and H_2O_2 (0.5 mmol/L). Results were expressed as total number of neutrophils by comparing the OD of tissue supernatant with the OD of murine peritoneal neutrophils processed in the same way. Neutrophils were isolated from the peritoneum cavity of mice injected with 3 ml of casein 5%. A standard curve of neutrophil (>95% purity) numbers *versus* OD was obtained by processing purified neutrophils as above and assaying for MPO activity.

Measurement of Hemoglobin Concentrations

The determination of hemoglobin concentrations in tissue was used as an index of tissue hemorrhage. After washing and perfusing the intestines to remove excess blood in the intravascular space, a sample of ~100 mg of duodenum was removed and homogenized in Drabkin's color reagent according to the instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). The suspension was centrifuged for 15 minutes at $3000 \times g$ and filtered using 0.2- μm filters. The resulting solution was read using an enzyme-linked immunosorbent assay plate reader at 520 nm and compared against a standard curve of hemoglobin.

Measurement of Cytokine and Chemokine Concentrations in Serum, Intestine, and Lungs

One hundred mg of tissue of WT and TSG-14tg mice were homogenized in 1 ml of PBS (0.4 mol/L NaCl and 10 mmol/L NaPO_4) containing anti-proteases (0.1 mmol/L phenylmethyl sulfonyl fluoride, 0.1 mmol/L benzethonium chloride, 10 mmol/L ethylenediaminetetraacetic acid, and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 minutes at $3000 \times g$ and the supernatant immediately used for enzyme-linked immunosorbent assay at a 1:2 dilution in assay dilution buffer. For determination of circulating TNF- α , serum was obtained from coagulated blood, stored at -20°C and assayed at a 1:3 dilution. The concentration of TNF- α , IL-1 β , KC, and MCP-1 in samples was measured using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN).

RNA Extraction and Determination of TSG-14 mRNA Levels

Organs were removed as indicated and total RNA was isolated by using the Trizol Reagent in accordance with the instructions provided by the manufacturer (Gibco-BRL). For reverse transcriptase-polymerase chain reaction (PCR), 1 μg of total RNA was reverse-transcribed with the aid of oligo-dT and superscript (Amersham Biosciences), following the manufacturer's instructions. For PCR, 2 μl of first-strand synthesis reaction was used as template and a fragment of TSG-14 cDNA was amplified with primers 5' cct.gct.ttg.tgc.tct.ctg.gt 3' and 5'tct.cca.gca.tga.tga.aca.gc 3' corresponding to positions 117 to 292 of murine TSG-14 mRNA (accession no. X83601). Amplification was performed for 25 cycles that corresponds to nonsaturable conditions. Amplified products were fractionated through an agarose gel, blotted onto nylon membranes, and hybridized with ^{32}P - αdCTP -labeled TSG-14 cDNA probe.¹⁴ The same samples were also amplified, blotted, and hybridized for the detection of HPRT transcripts to ensure equal amounts of template.

Histopathology

Organs (duodenum, ileum, and lungs) were removed from anesthetized mice at indicated time points and immediately fixed in formalin for 24 hours and tissue fragments were embedded in paraffin. Tissue sections (4- μm thick) were stained with hematoxylin and eosin.

Statistical Analysis

Where indicated, results are shown as the means $\pm$ SEM. Differences were compared by analysis of variance followed by Student-Newman-Keuls post hoc analysis. Data were considered significant when $P < 0.05$. For survival curves, differences between groups at different time points were compared using Fisher's exact test and considered significant when $P < 0.05$.

Results

Kinetics of the Inflammatory Changes after I/R Injury in WT CD1 Mice

Initial experiments were designed to investigate the kinetics of the reperfusion-induced inflammatory injuries, which occur after ischemia of the SMA in CD1 mice. As shown in Figure 1, there was a time-dependent increase in duodenal concentrations of Evans blue, an index of vascular permeability (Figure 1A); myeloperoxidase, an index of neutrophil influx (Figure 1B); and hemoglobin, an index of tissue hemorrhage (Figure 1C). Similarly to the inflammatory lesions observed in the duodenum, we also observed time-dependent inflammatory lesions in the ilea and lungs of reperfused CD1 mice (data not shown). Overall, our kinetic studies demonstrate that inflammatory changes were marginally elevated from control back-

Figure 1. Kinetics of tissue injury after I/R of the SMA. CD1 WT mice were submitted to ischemia (60 minutes) of the SMA. Reperfusion was allowed for 15, 30, and 60 minutes and tissue injury (duodenum) was determined. **A:** Changes in vascular permeability were evaluated by measuring the extravasation of Evans blue ($\mu\text{g}/100$ mg of tissue). **B:** Neutrophil infiltration was determined by measurement of tissue myeloperoxidase activity, as described in Material and Methods. **C:** Hemorrhage was evaluated by measuring the concentration of hemoglobin in 100 mg of tissue. Data represent the average value for five mice at each time point and SEM is indicated. $\#$, $P < 0.05$ and $P < 0.001$ for comparison of values observed after 30 minutes and 60 minutes, respectively, with the respective sham-operated group.

grounds after 15 minutes but were significantly elevated by 30 minutes ($P < 0.05$ for Evans blue and hemorrhage) and even higher at 60 minutes of reperfusion ($P < 0.001$ for all parameters) (Figure 1). Likewise, the concentra-

tions of pro-inflammatory cytokines TNF- α and IL-1 β and of the chemokine MCP-1 also increased in a time-dependent manner in all tested organs (see Table 1 for changes in the duodenum and lungs). Increases in the concentration of KC were only detectable in the lungs (Table 1). Of note, changes in cytokine/chemokine concentrations already occurred at the end of the ischemic period (Table 1), whereas edema formation, hemorrhage, and neutrophil influx were only detected at significant levels after reperfusion (Figure 1). As inflammatory changes were marked after 30 minutes of reperfusion (Figure 1, Table 1) and most TSG-14tg animals died after 30 minutes (see Figure 3), this time point was used in all further experiments.

Expression of TSG-14 Gene in Response to I/R

Next, we compared the levels of TSG-14 mRNA in WT and TSG-14tg mice. Target organs of mice submitted to sham operation or I/R were collected and total RNA was extracted. TSG-14 mRNA was measured by reverse transcriptase-PCR under nonsaturated conditions. As shown in Figure 2, TSG-14 mRNA was elevated in both WT and TSG-14tg mice in response to I/R injury when compared to sham controls. Higher levels of TSG-14 mRNA were detected in the duodenum of both transgenic lines than in WT mice (Figure 2). We also observed higher levels of TSG-14 mRNA in the lungs of transgenic mice in comparison to those from WT mice when both groups were submitted to I/R injury, but the overall expression levels were lower than those observed in duodenum (data not shown).

Increased Lethality in TSG-14 Transgenic Mice in Response to I/R

Having shown that TSG-14 mRNA is expressed in response to I/R, we next compared the survival rate in WT and in TSG-14tg mice harboring either two or four copies of the transgene (Tg2 and Tg4). As shown in Figure 3, death in the WT group was first noticed ~60 minutes after reperfusion and 100% of mice were dead by 120 minutes. In contrast, death was already observed after 20 minutes of reperfusion in both groups of transgenic mice and virtually all transgenic mice were dead by 60 minutes after reperfusion (Figure 3). There were no deaths among the sham-operated WT or transgenic mice (Figure 3). We also did not observe statistically significant differences when the two transgenic lines were compared.

Increased Tissue Injury in TSG-14 Transgenic Mice Submitted to I/R

We then assessed, comparatively, the inflammatory injuries induced by 30 minutes of reperfusion in WT and TSG-14tg animals. Overall, there was a twofold to threefold increase in vascular permeability (Figure 4, A and B) and neutrophil influx (Figure 4, C and D) in the duodenum and lungs of transgenic mice than in WT mice. There was

Table 1. Kinetics of Reperfusion-Induced Changes in the Expression of TNF- α , IL-1 β , KC, and MCP-1 in the Duodenum and Lungs after Ischemia (60 Minutes) of SMA in CD1 Mice (pg of Cytokine or Chemokine/100 mg of Tissue)

	Sham	0	15	30	60
Duodenum					
TNF- α	<X	8.4 (0.7)	7.3 (1.5)	122.5 (22.4)	125.8 (13.6)
IL-1	38.0 (5.4)	68.54 (5.7)	91.8 (9.0)	88.3 (7.0)	170.9 (25.9)
MCP-1	21.3 (4.7)	671.2 (65.8)	1933.6 (272.4)	1978.3 (199.0)	3488.6 (376.5)
KC	<X	<X	<X	<X	<X
Lungs					
TNF- α	<X	<X	16.7 (0.9)	48.6 (5.6)	194.4 (22.4)
IL-1 β	121.9 (15.2)	224.2 (20.3)	267.0 (26.8)	425.9 (49.1)	633.6 (69.6)
MCP-1	1014.0 (99.7)	1426.0 (211.1)	1006.0 (114.0)	3918.0 (387.2)	3769.2 (358.4)
KC	<X	16.3 (1.6)	50.2 (4.9)	113.6 (5.7)	212.7 (9.0)

Results are shown as the mean (SEM) of five animals in each time point. Results below the detection limit of the assay are shown as <X.

also a similar fold increase in hemorrhage in the duodenum after I/R in TSG-14tg over WT mice (Figure 4E). Moreover, there was no significant difference when we compared mice harboring two or four copies of the transgene (Figure 4). In the ileum, there was a significant increase in the recruitment of neutrophils when we compared sham-operated WT mice and mice submitted to I/R ($P < 0.001$) and such an increase was comparable in both WT and transgenic mice (number of neutrophils in the ileum after I/R injury: WT, $6.2 \pm 0.5 \times 10^6$; Tg2, 6.8 ± 0.6 ; Tg4, 9.7 ± 1.3 ; $n = 6$; $P > 0.05$).

The histopathological changes observed in the intestine and lungs of reperused animals are shown in Figure 5. In the intestine of reperused WT mice, the villi were widened with edema and leukocyte infiltration, especially neutrophils (Figure 5B). In TSG-14tg mice, there was much greater tissue damage, with increased cellular infiltration and a more pronounced edema with enlargement and erosions on the tips of the villi (Figure 5D). Not only could we observe injury to the intestines, the organ irrigated by the SMA, but there also was a significant remote injury to the lungs (Figure 5; E to H). In reperused WT animals, there was a marked hyperemia; interstitial infiltration of leukocytes, es-

pecially neutrophils; and interstitial edema (Figure 5F). In transgenic animals, the number of neutrophils in the interstitium and hyperemia greatly increased and, in addition to the interstitial edema, alveolar flooding was frequently observed (Figure 5H). It was also possible to observe that interstitial edema was present in both WT and TSG-14tg mice submitted to I/R but it was greatly enhanced in the latter group (not shown).

TSG-14 Transgenic Mice Have Increased Levels of Pro-Inflammatory Cytokines and Chemokines in Response to I/R

The acute inflammatory response that occurs after reperfusion of an ischemic vascular bed is accompanied by significant local and systemic production of pro-inflammatory cytokines, such as TNF- α .^{19,25-28} In WT mice, TNF- α concentrations in duodenum, ileum, and lungs 30 minutes after reperfusion were markedly increased when compared to sham-operated mice (Table 1 and Figure 6). However, I/R led to a much greater increase in TNF- α concentration in duodenum and lungs of transgenic mice than that observed in WT mice (Figure 6, B and D). Moreover, after I/R, TNF- α was not measurable in serum of WT mice whereas, in transgenic mice, it was greatly

Figure 2. Induced expression of the *TSG-14* gene on I/R of the SMA. CD1 WT or TSG-14tg mice were sham-operated or submitted to I/R of the SMA (ischemia for 60 minutes and reperfusion for 30 minutes). Total RNA (1 μ g) was isolated from the duodenum, reverse-transcribed, and used as a template for PCR as indicated in Material and Methods. PCR products were fractionated through a 1% agarose gel and detected by Southern blot using a ³²P α -dCTP-labeled *TSG-14* cDNA probe. Using the same methodology, HGPRT mRNA was measured to control for RNA loading.

Figure 3. Survival curve of CD1 and TSG-14tg mice submitted to ischemia of the SMA. Mice ($n = 10$ in each group) were anesthetized and submitted to ischemia of the superior mesenteric for 60 minutes. Tissue perfusion was then re-established and survival was monitored. The differences observed between WT and the two transgenic lines submitted to I/R are statistically significant, as determined by Fischer's exact test ($P < 0.05$).

Figure 4. Evaluation of tissue injury in WT and in TSG-14tg mice submitted to I/R of the SMA. WT or TSG-14tg mice having two (Tg2) or four (Tg4) copies of the transgene were sham-operated or submitted to 60 minutes of ischemia of the SMA and reperfusion was allowed for 30 minutes. **A** and **B**: Changes in vascular permeability were evaluated by measuring the extravasation of Evans blue ($\mu\text{g}/100\text{ mg}$ of tissue). **C** and **D**: Neutrophil infiltration was determined by measurement of tissue myeloperoxidase activity, as described in Material and Methods. **E**: Hemorrhage was evaluated by measuring the concentration of hemoglobin in 100 mg of tissue. Tested organs were the duodenum (**A**, **C**, and **E**) and lungs (**B** and **D**). Data represent the average value for five to six mice at each time point and SEM is indicated. $\#$, $P < 0.01$ for comparison with individual groups with their respective sham-operated group. $*$, $P < 0.01$ for comparisons between WT and transgenic mice submitted to I/R.

increased (Figure 6A). Interestingly, in the ileum there were similar increases in concentrations of $\text{TNF-}\alpha$ after I/R injury in WT and TSG-14tg animals (Figure 6C).

Overall, the concentrations of $\text{IL-1}\beta$, MCP-1, and KC in reperused tissues followed the same pattern of $\text{TNF-}\alpha$ expression, ie, their concentrations were higher in mice that underwent I/R than in sham-operated mice (Table 2). When we compared the levels of these cytokines in transgenic and WT mice, we observed that, in the duodenum and ileum, they were higher in TSG-14tg than in WT mice.

In the lungs, transgenic and WT mice had comparable levels of $\text{IL-1}\beta$, MCP-1, and KC (Table 2).

Soluble TNFR1 Can Rescue Transgenic Mice from I/R-Induced Death

It has been well documented that $\text{TNF-}\alpha$ plays a central role in tissue injury and in lethality after severe I/R.^{19,29} To confirm a role for $\text{TNF-}\alpha$ in our model of I/R, we compared

Figure 5. Tissue damage in lungs and duodenum of WT or TSG-14tg mice submitted to I/R of the SMA. WT or TSG-14tg mice having two copies of the transgene (Tg2) were sham-operated (**A** and **E** and **C** and **G**, respectively) or submitted to 60 minutes of ischemia of the SMA and reperfusion was allowed for 30 minutes (**B** and **D** and **F** and **H**, respectively). Mice were anesthetized, sacrificed, and the duodenum (**A–D**) and lungs (**E–H**) were processed for histological analysis after H&E staining. Original magnification, $\times 100$.

the reperfusion-induced lethality in $TNFR1^{-/-}$ and WT mice. As expected, lethality was greatly reduced in mice lacking $TNFR1$ (Figure 7A). As TSG-14tg mice expressed higher levels of $TNF-\alpha$ and died faster than WT animals, it

was of interest to examine whether lethality in these mice was also $TNF-\alpha$ -dependent. To answer this question, transgenic and WT mice were treated with a chimeric form of soluble $TNFR1^{23}$ just before reperfusion and le-

thality was examined. Whereas most TSG-14tg animals were dead by 60 minutes of reperfusion, there were no deaths in transgenic mice treated with sTNFR1-IgG up to 120 minutes after reperfusion (Figure 7B).

Discussion

The restoration of blood flow of an ischemic vascular bed, ie, reperfusion, is a major therapeutic objective after ischemia of an organ or tissue.¹⁶ However, reperfusion may be accompanied by significant local and systemic inflammatory injury, limiting the potential benefits of blood flow restoration. Thus, understanding the pathophysiology of the inflammation that occurs after reperfusion may be useful in the development of novel therapeutic strategies that limit the injury caused by the reperfusion process.¹⁶ Several studies have now shown a central role for TNF-α in mediating I/R injury in several vascular beds, including that irrigated by the SMA.^{19,25,30-32} However, much less is known about the mediators of the inflammatory process, which regulate the local and systemic production of TNF-α.

TSG-14/PTX3 is a TNF-α-inducible protein that shares homology with the short pentraxin family of acute-phase proteins, such as CRP and SAP but, at the n-terminal half, no homology of known proteins can be found.^{4,5} Interestingly, expression of the *TSG-14* gene is controlled by TNF-α^{2,5} and, in a positive feedback, TSG-14 appears to enhance the production of TNF-α. For example, we have recently shown that mice that overexpress the *TSG-14* gene under the control of its own promoter have enhanced production of TNF-α when injected with LPS.¹⁴

Considering that TNF plays a central role in the inflammatory response as well as in lethality induced by I/R in different animal models,^{19,25,30-32} and considering that TSG-14tg mice produced higher levels of TNF when exposed to LPS, we decided to evaluate the survival of TSG-14tg mice when submitted to I/R injury. In agreement with our previous observation, TSG-14tg mice produced higher levels of TNF-α (Figure 6) and had impaired survival when compared to their WT counterparts (Figure 3). Interestingly, TSG-14tg mice also showed higher levels of TSG-14 mRNA, both locally (duodenum, Figure 2) and at a distance (lungs, data not shown) suggesting that the TNF-α/TSG-14 loop is functioning *in vivo* and appears to amplify the inflammatory response. Whereas our data does not allow us to suggest that TSG-14 modulates TNF-α expression in a direct or in an indirect manner, it was reported earlier that *TSG-14* is a primary responsive gene in TNF-α-treated cells.² Moreover, the molecular events related to the enhanced recruitment of leukocytes

Figure 6. Levels of TNF protein in serum and tissue of mice submitted to I/R of the SMA. WT or TSG-14tg mice having two (Tg2) or four (Tg4) copies of the transgene were sham-operated or submitted to 60 minutes of ischemia of the SMA and reperfusion was allowed for 30 minutes. Mice were anesthetized, sacrificed, and the concentration of TNF-α in serum (A), duodenum (B), ileum (C), and lungs (D) were measured by enzyme-linked immunosorbent assay. Data represent the average value for six mice at each time point and SEM is indicated. #, $P < 0.01$ for comparison with individual groups with their respective sham-operated group. *, $P < 0.01$ for comparisons between WT and transgenic mice submitted to I/R.

Table 2. Concentration of IL-1 β , KC, and MCP-1 in Duodenum, Ileum, and Lungs of Reperfused WT and TSG-14tg Mice after Ischemia and Reperfusion of SMA (pg of Cytokine or Chemokine/100 mg of Tissue)

	Sham			I60R30		
	WT	Tg2	Tg4	WT	Tg2	Tg4
Duodenum						
IL-1 β	45.6 (3.2)	35.9 (4.2)	52.9 (4.5)	94.5 (9.1)*	221.4 (29.5)*†	295.3 (36.5)*†
MCP-1	26.5 (3.4)	35.6 (32.4)	56.3 (10.5)	1658.0 (202.3)*	2596.5 (193.5)*†	2394.9 (195.7)*†
KC	<X	<X	<X	<X	29.8 (3.6)*†	32.6 (5.9)*†
Ileum						
IL-1 β	19.6 (2.4)	25.3 (3.1)	31.4 (4.2)	75.8 (4.7)*	125.6 (9.8)*†	111.4 (18.2)*†
MCP-1	194.6 (27.8)	296.2 (32.4)	212.4 (25.4)	1128.4 (201.3)*	2456.2 (181.8)*†	2295.2 (453.2)*†
KC	<X	<X	<X	<X	19.5 (2.4)*†	25.6 (7.3)*†
Lungs						
IL-1 β	95.8 (11.4)	88.7 (9.3)	78.9 (9.1)	458.4 (51.4)*	478.9 (3.6)*	544.6 (6.5)*†
MCP-1	956.3 (99.8)	856.6 (98.4)	912.6 (94.0)	3753.4 (402.2)*	3946.5 (425.0)*	4012.3 (56.9)*
KC	<X	<X	<X	103.6 (5.7)*	212.6 (21.0)*†	118.5 (24.4)*

Results are shown as the mean (SEM) of five to six animals in each time point.

Results below the detection limit of the assay are shown as <X.

*, $P < 0.01$ for sham $\times$ I/R.†, $P < 0.01$ for tg $\times$ WT. **Figure 7.** Survival curve of mice submitted to I/R of the SMA. Mice were anesthetized and submitted to ischemia of the SMA for 60 minutes. Reperfusion was then re-established and survival was monitored. **A:** Comparison of survival of WT and p55^{-/-} mice ($n = 10$ in each group, both groups in 129Sv/C57/BL6 background). **B:** Tg2 mice (CD1 background) were left untreated or injected with 3 μ g of soluble TNFR1 receptor immediately before reperfusion ($n = 8$ in each group). Survival was monitored as indicated and survivors were sacrificed after 120 minutes. Differences between groups in **A** and **B** were compared using Fisher's exact test and considered significant ($P < 0.05$).

and/or the release of other cytokines and chemokines observed in TSG-14tg mice remains to be elucidated.

It is possible that, during I/R-induced injury, TSG-14 is functioning as a pro-inflammatory signal, as it has been suggested in experimental models of cecal ligation and puncture and LPS injection¹⁴ or after infection by *C. albicans* or *C. neoformans*.³³ As demonstrated here, TSG-14tg mice produced higher levels of IL-1 β than WT mice. Because blockage of IL-1 receptors reduces TNF- α production during I/R,³⁰ it is conceivable that augmented IL-1 β also contributes to the elevated TNF- α production and increased lethality observed in these mice.

Consistent with this pro-inflammatory activity, we observed an enhanced local expression of various mediators of inflammation, including the chemokines KC and MCP-1 and the cytokines IL-1 β and TNF- α . KC and MCP-1 are well-characterized mediators of leukocyte migration and are found in elevated concentrations in serum and tissue after I/R injury.^{34,35} In agreement with these observations, we found that in the lung and in the duodenum of mice submitted to I/R both KC and MCP-1 are induced and tissue levels are higher in TSG-14tg mice as compared to WT mice. The enhanced production of chemokines and cytokines was mirrored by an enhanced tissue injury. As shown in Figure 5, there was a parallel between tissue levels of KC and MCP-1 and tissue infiltration by neutrophils, as revealed by MPO activity in duodenum and lungs (Figure 4), as well as by the histopathological analysis. In the case of transgenic mice, we observed extensive and more severe damage in the lungs as well as in duodenum, with enhanced congestion, vascular permeability, and inflammation.

In addition, and probably more relevant than the enhancement of tissue pathology and mediator release, TSG-14tg mice died much faster than WT animals after I/R injury. Indeed, virtually all animals had deceased by 60 minutes, when the first deaths were observed in reper-fused WT animals. The enhanced lethality correlated with the much greater and much earlier (Figure 6 and data not shown) concentrations of TNF- α in serum of reper-fused TSG-14tg than in WT mice. Moreover, blockage of TNF- α

in vivo with a soluble chimeric form of the TNF- α receptor (TNFR1-IgG) prevented death in reperfused TSG-14tg mice (Figure 7) suggesting that TNF- α is necessary for the lethality observed in our experimental system. Finally, we also demonstrate that, together with TNF- α , augmented production of other pro-inflammatory signals such as IL-1 β , MCP-1, and KC contribute to the aggravated response in TSG-14tg mice that showed an enhanced systemic inflammation and tissue damage (Figures 4 and 5). These results argue for a critical role of TSG-14 in the control of acute inflammation, at least in part, via the modulation of TNF- α expression.

A proper and controlled inflammatory response is essential for the ability of a host to deal with tissue injury, either mediated by an infectious agent or by other inflammatory stimuli, such as I/R. Our studies clearly demonstrate a role for TSG-14 in the control of the inflammatory process. It has been suggested that TSG-14 could modulate complement activity,¹² clearance of apoptotic cells,³⁶ and cytokine expression.¹⁴ During peritoneal bacterial infection in the cecal ligation and puncture model, an excessive inflammatory response was associated with a more effective control of infection and greater survival in TSG-14tg mice.¹⁴ On the other hand, an exacerbated inflammatory response, which followed the reperfusion of the ischemic SMA in TSG-14tg mice, was undoubtedly deleterious and associated with enhanced lethality (the present study). At first sight, the outcome of these two experimental models might seem to be contradictory but, in our opinion, it clearly shows that kinetics and intensity of cytokine production as well as their final balance determine different outcomes depending on the nature of the insult. In the cecal ligation and puncture model, augmentation of TNF- α in association with the protective effects of NO against the release of LPS³⁷ resulted in improved survival. During I/R injury, augmentation of pro-inflammatory cytokines was faster and not directed against an invading microorganism, resulting in increased tissue infiltration of inflammatory cells, exacerbation of tissue damage, and enhanced lethality. A further understanding of the molecular mechanisms by which TSG-14 enhances the production of TNF- α and of other mediators of inflammation may help us to better understand the molecular events during acute inflammation. Moreover, our results suggest that TSG-14 may be a relevant therapeutic target for the pharmacological treatment of the inflammatory lesions and lethality that follow the reperfusion of an ischemic vascular bed.

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