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Chronic Inflammation and Protection from Acute Hepatitis in Transgenic Mice Expressing TNF in Endothelial Cells[1]

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Endothelial activation is an important feature of many inflammatory diseases and has been implicated as the cause of vascular complications in disorders such as diabetes, atherosclerosis, and transplant rejection. One of the most potent activators of the endothelium is TNF, which can also be expressed by endothelial cells, causing a permanent, autocrine stimulatory signal. To establish a model of continuous endothelial activation and to elucidate the role of endothelial derived TNF in vivo, we generated transgenic mice expressing a noncleavable transmembrane form of TNF under the control of the endothelial-specific tie2 promoter. Adult tie2-transmembrane TNF-transgenic mice developed chronic inflammatory pathology in kidney and liver, characterized by perivascular infiltration of mononuclear cells into these organs. Along with the infiltrate, an up-regulation of the adhesion molecules ICAM-1 and VCAM-1, but not E-selectin, in the endothelium was observed. Despite predisposition to chronic inflammation these mice were protected from immune-mediated liver injury in a model of Con A-induced acute hepatitis. Although the blood levels of soluble TNF and IFN-γ were increased in transgenic animals after challenge with Con A, no damage of hepatocytes could be detected, as assessed by the lack of increase in plasma transaminase activities and the absence of TUNEL staining in the liver. We conclude that expression of transmembrane TNF in the endothelium causes continuous endothelial activation, leading to both proinflammatory and protective events. ***The Journal of Immunology,*** **2001, 167: 3944–3952.**

Tumor necrosis factor is a pleiotropic, proinflammatory cytokine, mainly produced by activated macrophages and T cells. It is involved in tissue remodeling, defense against infection, and inflammation and can induce necrosis in certain experimental tumors. Furthermore, it has been directly implicated in severe pathological situations, such as septic shock, cachexia, graft-vs-host diseases, and the autoimmune disorders multiple sclerosis, Crohn's disease, diabetes, and rheumatoid arthritis (1). TNF appears in two biologically active forms, namely the transmembrane-spanning precursor (tmTNF)[3] and the soluble cytokine (sTNF) that is released from the surface by proteolytic cleavage (2). TNF signaling is mediated by two high affinity receptors, TNFRI and TNFRII. Whereas TNFRI is widely expressed and constitutively active, TNFRII is found mainly on cells of the hemopoietic lineage upon appropriate stimulation (1). In principle, both receptors are capable of binding sTNF as well as tmTNF, but the binding affinities as well as the kinetics are different. Under physiological conditions, sTNF preferentially binds to TNFRI (3), whereas tmTNF is the prime activating ligand of TNFRII (4).

The involvement of sTNF, tmTNF, and both TNF receptors has been intensively studied in a model of Con A-induced hepatitis in vivo (5). Application of the T cell mitogen Con A to mice causes the release of several proinflammatory cytokines, leading to hepatocyte damage and severe liver injury (6, 7). Immune-mediated liver cell death in this model is likely to occur without activation of caspases and seems to depend on an early destruction of sinusoidal endothelial cells (6, 8–10). The intrahepatic formation of tmTNF as well as the release of sTNF and IFN-γ are likely to be directly involved in the induction of hepatocyte death (7, 11). TNF-deficient animals are protected against Con A-induced liver failure, whereas transgenic animals expressing the noncleavable tmTNF mutant (mtmTNF) bred into TNF-deficient mice ("tmTNF-knockin mice") were even more susceptible than wild-type animals (12). Both TNF receptors are involved in this model of immune-mediated hepatotoxicity, because animals deficient in either one of the receptors do not respond to Con A (12). Finally, mice overexpressing the human TNFRII are even more susceptible to Con A-induced liver injury (12).

Endothelial cells express both TNF receptors and represent one of the major and primary targets for TNF (1). TNF induces the up-regulation of ICAM-1, VCAM-1, and E- and P-selectin in endothelial cells, which mediate the attachment and transmigration of leukocytes to and through the endothelium. In addition, TNF causes the release of chemokines such as monocyte chemoattractant protein-1, IL-6, and IL-8 from endothelial cells and likewise contributes to the extravasation of leukocytes to the sites of inflammation. Recently, several reports demonstrated that upon appropriate stimulation endothelial cells are, in principle, able to express endogenous TNF, leading to autocrine activation. The TNF mRNA or protein could be detected in HUVEC (13–16), porcine pulmonary artery endothelial cells (17), and rat brain endothelial cells (18) after stimulation with bacterial cell wall components or proinflammatory cytokines. Evidence for endothelial expression of TNF in vivo is provided by our recent observation that the tumor

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[3] Abbreviations used in this paper: tmTNF, transmembrane TNF; ALT, alanine aminotransferase; AST, aspartate aminotransferase; mtmTNF, mutant form of mTNF; PECAM-1, platelet endothelial cell adhesion molecule-1; sTNF, soluble TNF.

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endothelium of the Meth A fibrosarcoma produces endogenous TNF (16). The hypothesis that endothelial cells may express TNF under pathophysiological conditions is also supported by the detection of TNF-positive endothelial cells in multiple sclerosis lesions (19, 20) and in human atheromas (21), although these studies do not distinguish between endothelial cells binding or producing TNF. We have shown recently that expression of tmTNF in endothelial cells leads to the continuous activation of NF-κB and the p38/SAPK2, resulting in a constitutive expression of IL-6 and tissue factor in endothelial cells. We could further demonstrate that such activation is permissive for an unrelated cytokine, vascular endothelial growth factor, to act as a permeability-inducing factor (16).

To elucidate the role of endothelial tmTNF in vivo and to establish a model of continuous endothelial activation, we generated transgenic mice expressing a noncleavable mtmTNF under control of the endothelial-specific tie2 promoter. These mice develop a chronic inflammatory pathology in liver and kidney that is accompanied by up-regulation of ICAM-1 and VCAM-1 in the endothelium of these organs. Conversely, in a model of Con A-induced acute hepatitis tie2-tmTNF-transgenic mice are protected from immune-mediated hepatic injury.

Materials and Methods

Construction of the transgene and generation of tie2-tmTNF-transgenic animals

The cDNA for the murine tmTNF mutant (mTNFΔ1–9,K(11)E) (22) was constructed using a PCR cloning strategy based on the cDNA for mTNF (provided by W. Fiers, Department of Molecular Biology, Ghent University, Ghent, Belgium). Two PCR fragments of mTNF, introducing the mutation, were amplified using the primers MTNF1A (5′-AAAAACGCT GCAGCCACCATGAGCACAGAAAGCATGATCCGC-3′), MTNF2A (5′-GCCGGGGTACCGGTTCGTCTGTGAGGGTCTGGGCCATA GAACT-3′), MTNF3 (5′-GCCGGGGGAACCGGTAGCCCACGTCG TAGCAAACCAC-3′), and MTNF4 (5′-GGCTCCAGTGAATTCG GAAAGCCC-3′), ligated via the introduced restriction site *Age*I, and cloned into pBluescript (Stratagene, Amsterdam, The Netherlands). mtmTNF cDNA was than excised and cloned between the tie2 promoter and the tie2 first intron of the plasmid pHHNS (23) replacing the *lacZ* gene. In the resulting construct a 570-bp fragment of *lacZ* remained downstream of the mtmTNF cDNA 3′ of the SV40 polyadenylation signal sequence. Transgenic mice were generated by microinjection of the linear 14.3-kb tie2-tmTNF construct into fertilized C57BL6×C3H/He oocytes as previously described (24). Tie2-tmTNF-transgenic mice were identified by Southern blot analysis of tail biopsy DNA as previously described (25). An *Acc*I-*Bam*HI fragment (570 bp) of the transgenic construct served as a probe containing parts of the *lacZ* and SV40 polyadenylation sequence. Transgenic lineages were maintained heterozygous by backcrossing them to C57BL/6 wild-type mice (Harlan, Borchen, Germany). Litters were routinely screened by PCR analysis of tail biopsy or yolk-sac DNA as described using the primer pair tie2-T5 (25) and muTNF (5′-GCACCACTAGTTGGTTGTCTTTG-3′). Nontransgenic littermates and, after backcrossing of more than four generations, C57BL/6 animals served as wild-type controls.

In situ hybridization

In situ hybridization of mouse embryos was performed as previously described (26). Either the complete cDNA of mtmTNF (TNF probe, recognizing also wild-type TNF) or a *Mlu*I-*Xba*I fragment (570 bp) of *lacZ* (LacZ probe, recognizing specifically the transgene) were used as templates for generation of sense and antisense riboprobes to detect the mRNA of the transgene. A riboprobe for tie2 (27) was used to verify endothelial specific staining. Template-containing plasmids were linearized and ^{35}S-labeled riboprobes were made by in vitro transcription with T3 and T7 RNA polymerases (Stratagene). Hybridization was performed overnight at 48°C and washing at 37°C. Slides were then coated with photographic emulsion (NTB-2, Eastman Kodak, Rochester, NY), exposed for 2–3 wk, and counterstained with toluidine blue before photography.

RT-PCR

Total RNA was extracted from embryos or the indicated organs of transgenic or wild-type mice using the PeqGold RNA Extraction Kit (PeqLab, Erlangen, Germany). Contaminating DNA was removed by subsequent DNase digestion with RQ1 RNase-free DNase (Promega, Mannheim, Germany) according to the manufacturer's protocol. First-strand cDNA was synthesized by RT of 2 μg total RNA with 200 U SuperScript II reverse transcriptase (Life Technologies, Rockville, MD). For subsequent PCR analysis, 1 μl cDNA was amplified with 1U *Taq* polymerase (Promega). Primers used to specifically amplify the tmTNF mutant were 5′-ACCCT CACAGACGAACCGGTA-3′ and 5′-AGATAGCAAATCGGCTGACG G-3′. In a parallel reaction, primers for mouse GAPDH were used to ensure comparable cDNA content of samples: 5′-ACCACAGTCCATGCCAT CAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. To exclude any DNA contamination in the RNA samples, PCR analysis was performed with RNA before reverse transcription. To determine the IL-6 and IL-10 mRNA contents of the liver, the following primers were used: IL-6, 5′-GCCTAT TGAAAATTTCCTCTG-3′ and 5′-GTTTGCCGAGTAGATCTC-3′; and IL-10, 5′-GTTACTTGGGTTGCCAAG-3′ and 5′-GATCATCATGTAT GCTTC-3′. Semiquantitative evaluation was performed using the Gel Doc 2000 System (Bio-Rad, Munich, Germany).

Immunofluorescence, immunohistochemical staining, and TUNEL analysis

Organs of transgenic or wild-type mice were dissected, rinsed in ice-cold PBS, and embedded in Tissue-Tek OCT (Sakura, Zoeterwoude, The Netherlands). Cryosections of 6 μm were fixed for 10 min in acetone at −20°C and rehydrated in TBS (20 mM Tris-HCl (pH 7.4) and 50 mM NaCl). Sections were blocked with 10% rabbit serum and were incubated overnight with either hybridoma supernatant or 10 μg/ml purified monoclonal rat anti-mouse Ab at 4°C: 9B5 (isotype control), Mec13.3 (PECAM-1), 25ZC7 (ICAM-1), 9DB3 (VCAM-1), UZ4 (E-selectin), M1/9 (CD45), M1/70 (CD11b), F4/80 (Dianova, Hamburg, Germany), Ly-6G (GR1, BD PharMingen, Hamburg, Germany), and anti-CD3e (Dunn Labortechnik, Asbach, Germany). Bound Ab was detected using either the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) and the AEC Chromogen kit (Sigma, Deisenhofen, Germany) according to the manufacturer's instructions or a biotin-conjugated goat anti-rat IgG secondary Ab (Dianova, 1/100) and streptavidin-coupled PE (Dianova, 1/1000). Sections were counterstained with Gill's hematoxylin I and mounted with Aquatex (Merck Eurolab, Frankfurt, Germany) or a solution of Mowiol 488 (Calbiochem, Bad Soden, Germany). Staining of liver and lung sections for TNF was performed as previously described (28) using the Ab IP-400 (Genzyme, Ruesselsheim, Germany) or a human sTNFRI/Fc fusion protein (R&D Systems, Wiesbaden, Germany) (29). Isotype-matched controls were rabbit normal serum and human IgG, respectively. DNA fragmentation was detected using the In Situ Cell Death Detection kit, Fluorescein (Roche, Mannheim, Germany), according to the manufacturer's instructions. Quantification of double-positive cells was performed by counting three to five pictures of 900 μm^2 for each group by six independent observers.

Dosage and application route

Con A was purchased from Sigma. Con A (20 mg/kg) was administered i.v. in 200 μl pyrogen-free saline. Two hours after challenge with Con A blood was taken from the tail vein for determination of cytokine levels, and after 8 h, animals were sacrificed to collect livers and blood by puncture of the heart.

Analysis of liver enzymes and cytokine determination

Hepatocyte damage was assessed by measuring the plasma enzyme activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (30) using an automated procedure. Cytokine contents of plasma samples were determined by a sandwich ELISA using flat-bottom, high binding, polystyrene microtiter plates (Greiner, Nurtingen, Germany). Abs were purchased from BD PharMingen. Streptavidin-peroxidase (Jackson ImmunoResearch, West Grove, PA) and the peroxidase chromogen tetramethylbenzidine (Roche) were used according to the manufacturer's instructions.

Statistical analysis

The results were analyzed using Student's *t* test if two groups were compared and Dunnett's test if more groups were tested against a control group. If variances were inhomogeneous, the results were analyzed using the Welsh test. All data in this study are expressed as the mean ± SEM. Values of $p < 0.05$ were considered significant.

Results

Generation and analysis of tie2-tmTNF-transgenic mice

To direct TNF expression to endothelial cells in transgenic mice we chose the tie2 promoter in conjunction with the intronic enhancer element, which has been demonstrated previously to mediate specific endothelial expression (23, 31). Based on our observation that endothelial expressed TNF displays biological activity in its pro form at the cellular surface in vitro and in vivo (16), we inserted the cDNA for the uncleavable mtmTNF (22) into the transgene construct (Fig. 1*A*). Microinjection of the DNA into fertilized oocytes revealed 10 transgenic founder mice. Of nine lineages, three lineages showed low, four lineages showed intermediate, and two lineages showed high expression compared with each other (data not shown).

To test endothelial-specific expression of the transgene, we performed in situ hybridization of E12.5 mouse embryos of transgenic animals and nontransgenic littermates. Using a probe recognizing specifically a part of the transgene mRNA (LacZ probe) we could detect hybridization signals on vascular structures of transgenic embryos such as the heart, the dorsal aorta, and the capillaries. Hybridization with a probe recognizing the TNF mRNA (wild-type TNF as well as mtmTNF) revealed similar signals on transgenic embryos, but no specific signal on nontransgenic littermates (Fig. 1). Sense controls of both probes gave no hybridization signal (data not shown). Endothelial specificity of the transgene was demonstrated by hybridization studies of adjacent sections with a tie2 probe, which gave a similar signal pattern as the LacZ and TNF probes (data not shown).

Expression of tmTNF in organs of adult transgenic mice

Animals originating from the two highest expressing lineages (Tg 4328 and Tg 5382) were further analyzed for transgene mRNA levels in adult organs. RT-PCR using tmTNF mutant-specific primers was performed with total RNA extracted from the lungs, kidneys, and livers of transgenic mice and wild-type controls (Fig. 2). The transgene was amplified as a 230-bp fragment from all tested organs of transgenic mice, whereas the mRNA for endogenous TNF was not detected, as the primers used are specific for the tmTNF mutant (Fig. 2). PCR of RNA samples before RT revealed no amplification product, excluding the possibility of contaminating DNA in the RNA preparation (data not shown). Production of the TNF protein by endothelial cells in organs of adult tie2-tmTNF-transgenic animals (Tg 4328) was checked by immunofluorescent double staining against PECAM-1 and TNF. TNF-positive endothelial cells could be detected in the liver and lung of transgenic animals, whereas the respective organ of wild-type mice showed only background staining (Fig. 3). Isotype-matched control reagents revealed no staining (data not shown). In all other organs tested (heart, kidney, pancreas, brain, and intestine), several vessels in wild-type animals exhibited endogenously a basal TNF expression that made the additional overexpression of transgenic TNF difficult to assess (data not shown).

Chronic inflammatory lesions in organs of transgenic mice

In principle, the tie2-tmTNF-transgenic animals of all lineages tested develop and grow normally, but some animals showed striking abnormalities. Three founders of the low expressing lines died at the age of 6–10 mo, and pathological analysis revealed a diffuse inflammation of the bowel. However, this phenotype was never observed in any of the littermates of these lines. In addition, two animals (of all transgenic mice born) of two lines with an intermediate to high expression developed an inflammatory skin phenotype with epidermal hyperproliferation and fibrosis. A more detailed histological analysis of the organs of tie2-tmTNF-transgenic mice revealed a chronic infiltration of inflammatory cells into the kidney, liver, and lung of animals originating from the two highest expressing lines (Tg 4328 and Tg 5382), whereas mice from the lower expressing lines were not affected. All other organs tested (muscle, brain, heart, gut, and pancreas) showed no pathological changes. The chronic inflammatory regions could be observed in transgenic mice from 4–19 mo and appeared in the highest expressing line (Tg 4328) in the kidneys and livers of all animals tested ($n = 5$), whereas the lung showed significant changes in only one of three mice. In mice originating from the line with the second most expression (Tg 5382), all kidneys, two of three livers, and only one lung (of three) contained infiltrates. However, the extent of inflammation in this line seemed to be less severe and covered a smaller area of the organs compared with that in the

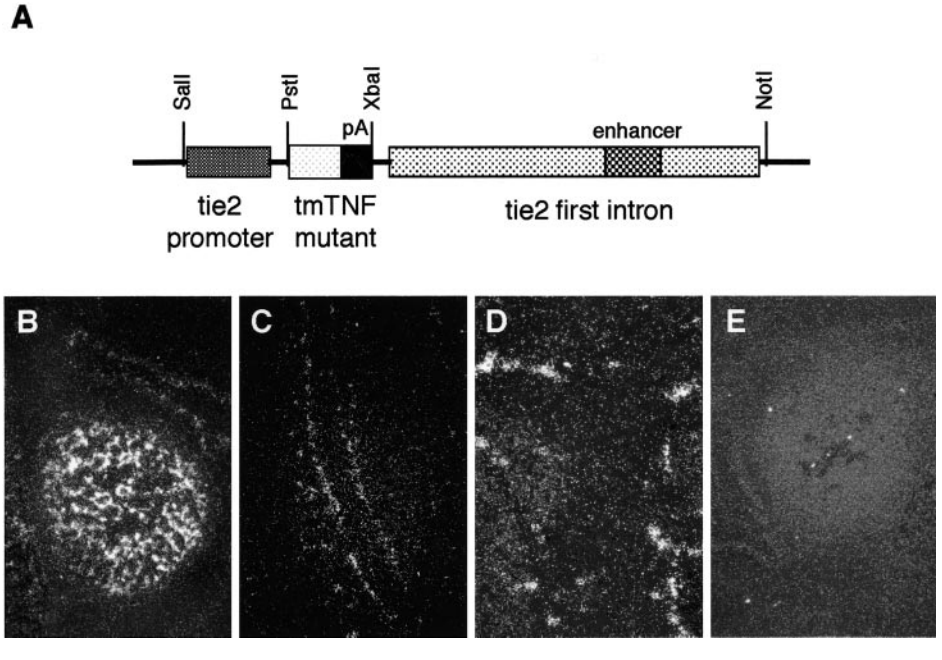

FIGURE 1. Endothelial-specific expression of mtmTNF in transgenic mice. *A*, Transcription of the uncleavable mutant of murine TNF is controlled by the tie2 promoter and its intronic enhancer element in the transgenic construct. Endothelial-specific expression was checked by in situ hybridization analysis of transgenic embryos (embryonic day 12.5) with a probe recognizing either TNF-mRNA (TNF probe, *B*, *C*, and *E*) or specifically a part of the transgene-mRNA (LacZ probe, *D*), giving similar results. Specific hybridization signals were observed in the heart (*B*), dorsal aorta (*C*), and capillaries of the brain (*D*) of transgenic embryos, but not of nontransgenic littermates (heart; *E*).

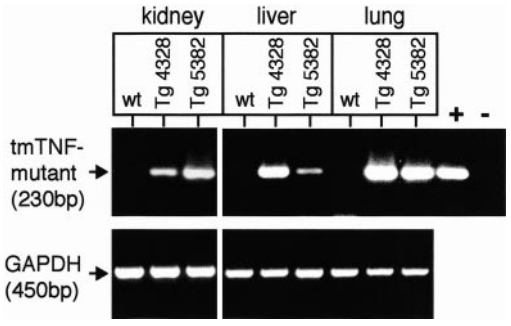

FIGURE 2. Expression of tmTNF in adult organs of tie2-tmTNF-transgenic mice. RT-PCR with total RNA extracts of the indicated organs of transgenic mice from two different lines (Tg 4328 and Tg 5382) or wild-type mice (wt) was performed. Primers specifically recognizing the tmTNF mutant amplify a fragment of 230 bp in adult organs of transgenic mice, which represents the mRNA of the transgene. Wild-type TNF, possibly present in the organs of wild-type animals, is not recognized with the primers used. Amplification of the mRNA for GAPDH served as the loading control. +, Positive control (plasmid containing murine mtmTNF); −, negative control (plasmid containing murine wild-type TNF).

highest expressing line. All animals analyzed to date have been heterozygous for the tie2-tmTNF transgene, with the exception of one animal from a line with intermediate expression. Heterozygous mice of this line developed no inflammatory phenotype. However, when one animal of this line was crossed to homozygocity, it showed the same infiltration in the kidney, but not in the liver or lung, as the animals from the higher expressing lines. This argues for a dose dependency of the transgene as far as manifestation of the pathology is concerned.

To further analyze the origin of the infiltrating cells, we subjected sections of the liver, kidney, and lung to immunohistochemical staining using cell-specific markers. The infiltrated cells formed big clusters within the tissue and consisted exclusively of leukocytes, as assessed by staining against the leukocyte marker CD45 (Fig. 4). In the liver, we detected these clusters in periportal areas and also around big collecting veins as well as smaller collections of inflammatory cells within the liver parenchyma. These clusters are composed of either T cells, because they stained positively for CD3, or macrophages, as assessed by staining against the macrophage-marker F4/80 (Fig. 4). The remaining cells are in part positive for CD11b, which is present in activated monocytes, neutrophils, and NK cells, or the granulocyte-marker GR1 (Fig. 4). Besides the clustered leukocyte infiltration we observed a strong increase in F4/80-positive cells scattered over the liver of transgenic animals compared with age-matched wild-type controls. These cells most likely comprise infiltrating macrophages and not Kupffer cells, because we also detected CD11b-positive cells all over the liver (Fig. 4). Moreover, some granulocytes, but no T cells, can be found scattered in the liver parenchyma. The chronic

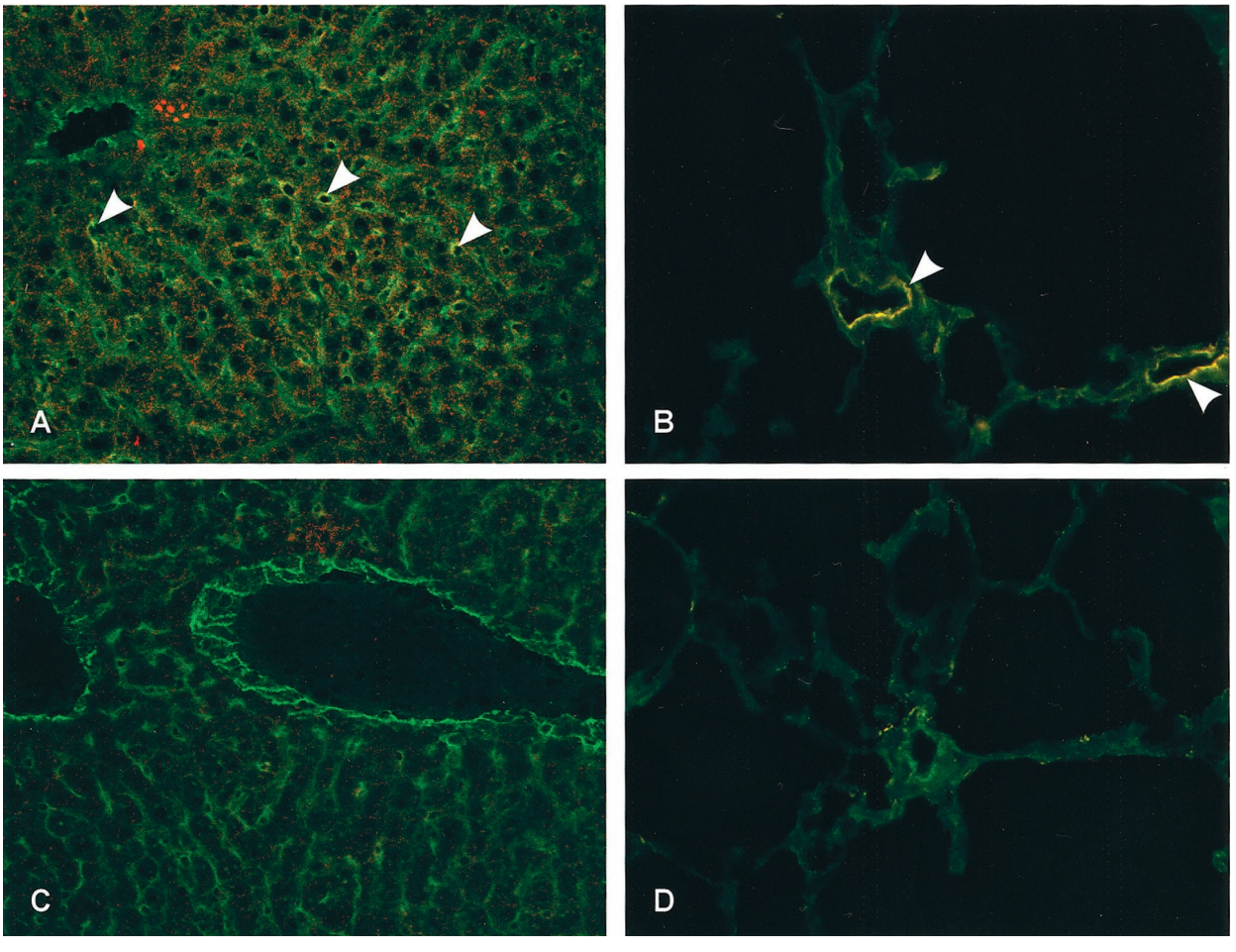

FIGURE 3. Expression of TNF in endothelial cells in the liver and lung of adult tie2-tmTNF-transgenic mice. Confocal laser imaging of double-immunofluorescent-stained liver and lung sections of transgenic (*A* and *B*) and wild-type mice (*C* and *D*). Endothelial cells are stained with an Ab against PECAM-1 (green). TNF (red) was detected using a specific Ab or a TNFRI/Fc fusion protein that recognizes cellular tmTNF (29). Arrows point to TNF-positive endothelial cells (yellow).

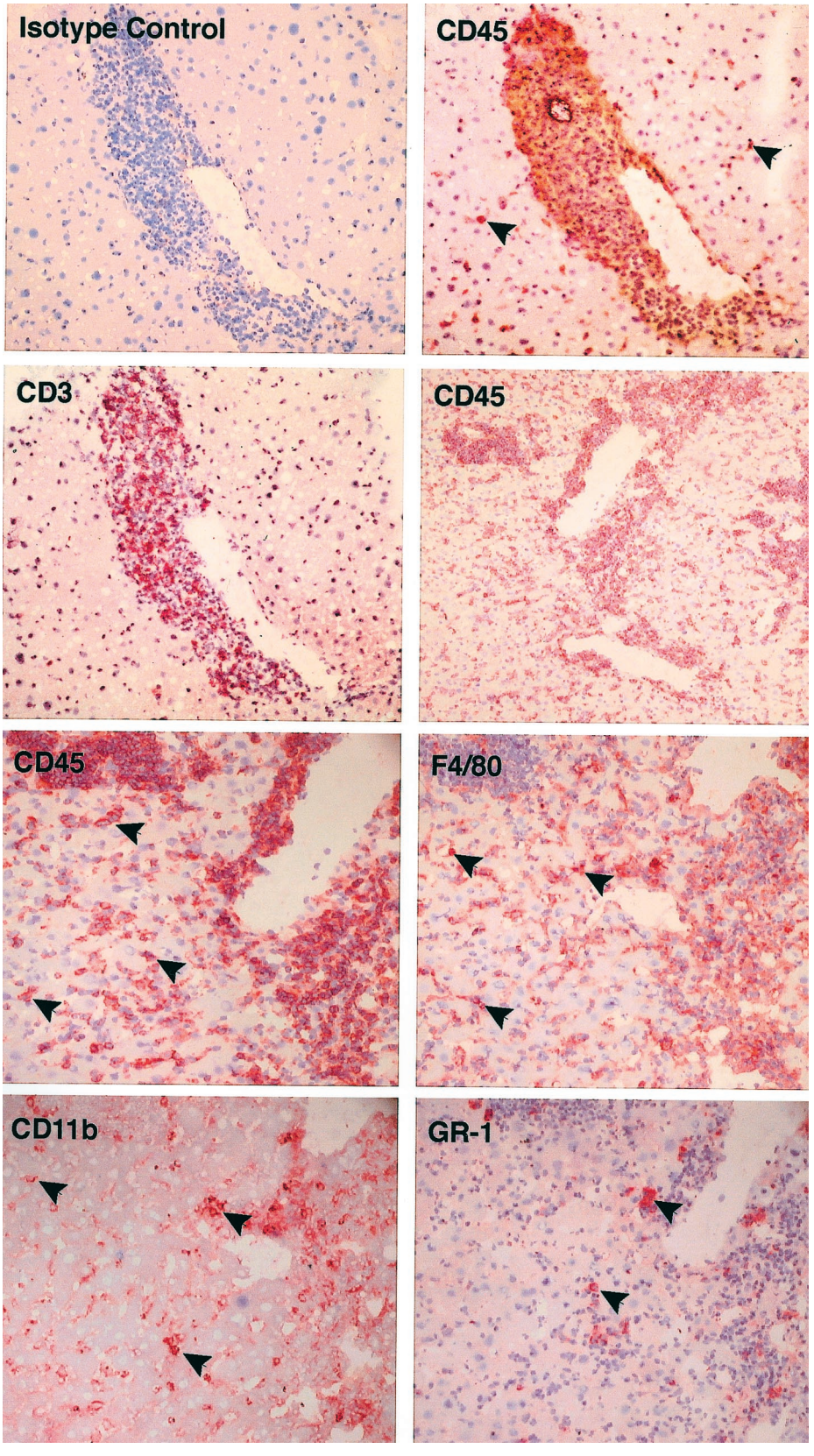

FIGURE 4. Infiltrated leukocytes in the liver of tie2-tmTNF-transgenic mice. Immunohistologic analysis of liver sections with Abs against CD45, CD11b, CD3, the macrophage marker F4/80, the granulocyte marker GR-1, or an isotype-matched control Ab. Clusters of infiltrated cells are composed exclusively of leukocytes and contain mainly macrophages or T cells. Arrowheads point to scattered leukocytes within the liver parenchyma.

inflammation observed in the kidneys of transgenic animals resembles that in the liver. Big clusters of leukocytes accumulate mainly around big veins in the cortex and medulla, but not at renal arteries or glomeruli of the kidney (data not shown). In the lung, the leukocytes cluster at vessels of intermediate size and around the bronchi (data not shown).

Up-regulation of cell adhesion molecules in the endothelium of transgenic liver and kidney

A prerequisite for the extravasation of leukocytes from the bloodstream into the inflamed tissue is the expression of cell adhesion molecules such as ICAM-1, VCAM-1, and E- and P-selectin in the

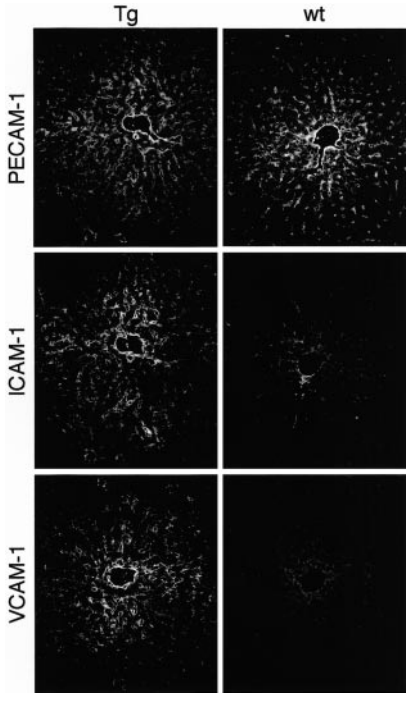

FIGURE 5. Up-regulation of cell adhesion molecules in the endothelium of transgenic liver. Immunofluorescent staining of liver sections of transgenic (Tg) vs wild-type (wt) mice with Abs against PECAM-1, ICAM-1, and VCAM-1.

endothelium (32). Because each of these molecules is known to be up-regulated upon stimulation with sTNF (33), we investigated the expression patterns in the livers and kidneys of tie2-tmTNF-transgenic mice vs wild-type animals. In the livers and kidneys of wild-type control mice, neither E- selectin (data not shown) nor VCAM-1 and only rare expression of ICAM-1 could be seen (Fig. 5). In contrast, transgenic livers showed a strong up-regulation of ICAM-1 and VCAM-1, but not of E-selectin (data not shown), in endothelial cells, as verified by staining of adjacent sections for the endothelial marker PECAM-1 (Fig. 5). The use of an isotype-matched control Ab revealed no staining (data not shown). Portal and central veins as well as arteries and sinusoidal endothelial cells showed increased expression of ICAM-1 and VCAM-1. This up-regulation was observed at sites of clustered leukocyte infiltrates as well as in many areas all over the liver. Whereas VCAM-1 expression appeared to be locally increased, forming patches of activated areas within the liver, the ICAM-1 up-regulation covered nearly all liver endothelial cells. Also in the kidneys of transgenic animals, an up-regulation of ICAM-1-positive endothelial cells and less prominent VCAM-1-positive endothelial cells could be observed (data not shown).

Tie2-tmTNF-transgenic mice are protected from hepatocellular damage upon Con A treatment

The induction of a proinflammatory state by constitutive tmTNF expression in tie2-tmTNF-transgenic mice prompted us to investigate the sensitivity of these animals toward Con A-induced hepatitis, i.e., in an experimental mouse model of immune-mediated liver injury that is mediated by tmTNF (12). In this model, hepatocellular damage and liver injury can be assessed by the formation of TUNEL-positive hepatocytes (34) as well as by an increase in plasma transaminase activities (6). As demonstrated by confocal laser imaging and double-immunofluorescent staining (Fig. 6), i.v. injection of 20 mg/kg Con A to wild-type mice induced massive formation of TUNEL-positive hepatocytes as well as the appearance of TUNEL- and PECAM-1-positive endothelial cells. TUNEL staining was largely absent in Con A-treated tie2-tmTNF-transgenic mice (Fig. 6), indicating protection from DNA-damage in hepatocytes and endothelial cells in these animals.

Tie2-tmTNF-transgenic mice are protected from Con A-induced hepatitis

According to the prevention of DNA damage in hepatocytes of Con A-challenged tie2-tmTNF-transgenic mice, these animals released significantly less ALT and AST into the circulation compared with the wild-type animals (Fig. 7). The protective effect was not due to an inhibition of the production of hepatotoxic mediators in response to Con A because the tie2-tmTNF-transgenic mice released even more TNF and IFN-γ upon Con A treatment compared with the wild-type animals (Fig. 7). A similar effect of TNF-dependent protection against Con A-induced hepatitis was observed by direct injection of recombinant murine TNF to BALB/c mice 12 h before Con A challenge (Con A treatment: ALT, 1860 ±

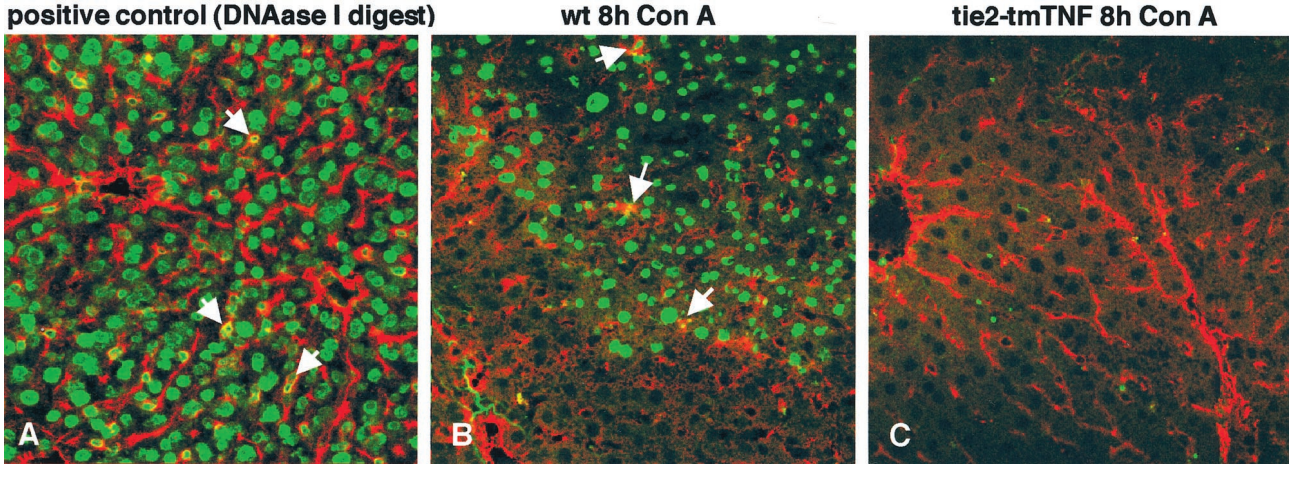

FIGURE 6. Tie2-tmTNF-transgenic mice are protected from Con A-induced DNA damage in the liver. Confocal laser imaging of double-immunofluorescent TUNEL (green)- and PECAM-1 (red)-stained liver sections of Con A-treated (20 mg/kg) wild-type mice (*B*) and tie2-tmTNF-transgenic mice (*C*). *A*, DNase I treatment (positive control). Arrows point to TUNEL-positive endothelial cells (yellow). Quantification of TUNEL-positive endothelial cells revealed (± SD): wild-type, untreated, 1.8 ± 1.2 cells/mm^2; wild-type, 8 h Con A, 17.1 ± 8.2 cells/mm^2; tie2-tmTNF, untreated, 1.0 ± 1.0 cells/mm^2; and tie2-tmTNF, 8 h Con A, 8.2 ± 4.1 cells/mm^2.

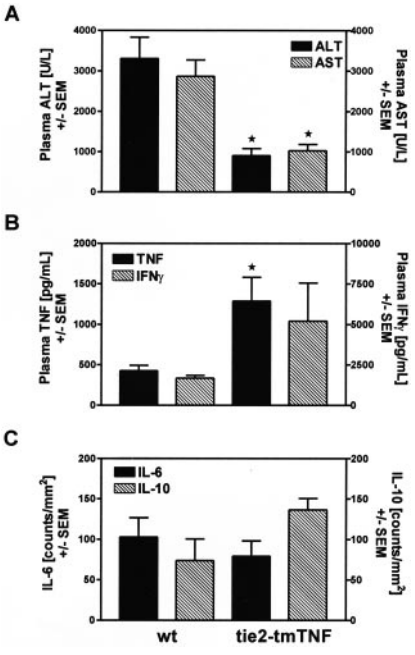

FIGURE 7. Release of aminotransferases and cytokines in tie2-tmTNF-transgenic and wild-type (wt) mice. *A*, Plasma aminotransferase (ALT and AST) levels 8 h after challenge with Con A. *B*, Plasma levels of TNF and IFN-γ 2 h after Con A treatment were determined by ELISA. *C*, Production of IL-6 and IL-10 in the liver of untreated animals as determined by RT-PCR. ∗, $p < 0.05$.

506 U/l; recombinant murine TNF pretreatment 12 h before Con A treatment: ALT, 450 ± 120 U/l ($p \leq 0.03$)).

Prophylactic injection of either IL-6 or IL-10 has been shown to protect mice from Con A-induced liver damage (35, 36). Because the protective effect of these cytokines can only be achieved when administered before injection of Con A, we investigated whether IL-6 or IL-10 is already produced in untreated tie2-tmTNF-transgenic mice. In the livers of transgenic animals no significant changes in IL-6 and IL-10 expression could be observed compared with wild-type controls (Fig. 7*C*), and analysis of the plasma levels of untreated animals revealed no detectable cytokine (data not shown). After treatment with Con A, the concentration of IL-6 in the plasma increased dramatically in both transgenic and wild-type mice, but without significant differences between the two groups (data not shown). These data argue for an IL-6- and IL-10-independent mechanism for hepatic protection in tie2-tmTNF-transgenic mice.

Discussion

To analyze the function of endothelial-derived TNF in vivo, we have generated transgenic mice that overexpress tmTNF under control of the endothelial cell-specific tie2 promoter. Furthermore, to restrict the TNF activity to the endothelium we have chosen a mutant nonsecretable form of murine TNF for the transgenic construct. This mutant has been shown to stay unprocessed at the cellular surface and exhibit full activity compared with wild-type TNF (22). Similar mutants of murine or human TNF have been successfully used under the control of various promoters in transgenic animals (37–39). We investigated the transgenic expression and could demonstrate that tmTNF is expressed exclusively in endothelial cells of tie2-tmTNF-transgenic embryos and also in organs of adult animals. Tie2-tmTNF-transgenic mice developed, dependent on the expression level of the transgene, a chronic inflammatory phenotype in the adult liver and kidney that was characterized by infiltration of mainly T cells and macrophages. In addition, we observed a strong up-regulation of ICAM-1 and VCAM-1 in the endothelium of these organs that occurred independently of leukocyte clusters.

Induction of ICAM-1 and VCAM-1 as a prerequisite for the attachment and subsequent transmigration of leukocytes to and through the endothelium has first been demonstrated by sTNF (1). As overexpression of tmTNF in endothelial cells can also induce the expression of ICAM-1 and VCAM-1, causing increased transendothelial migration of monocytes in an in vitro transmigration assay (A. Willuweit, S. Hippenstiel, N. Suttorp, and M. Clauss, manuscript in preparation), we propose that the expression of tmTNF in endothelial cells of tie2-tmTNF-transgenic mice activates the endothelium and causes up-regulation of ICAM-1 and VCAM-1. This enhanced adhesion molecule expression is the most likely explanation for the observed infiltration of leukocytes in the transgenic animals.

It has been reported earlier that tissue-specific expression of TNF in transgenic mice leads to inflammatory infiltrates in the respective organ, e.g., heart, pancreas, brain, and lung (40–43). However, systemic release of soluble TNF by transgenic overexpression can also be the cause of endothelial activation and inflammation in some susceptible organs (44). In addition, a multiorgan inflammation has been observed in mice overexpressing the human TNFRII, leading to a phenotype very similar to that described here (45). By localizing TNF to the endothelium in tie2-tmTNF-transgenic animals, we could show that the presence of TNF at the vascular wall is sufficient to induce such a systemic inflammatory phenotype. This observation further stresses the central role of an activated endothelium in inflammatory disorders.

Given the fact that untreated tie2-tmTNF-transgenic mice already display elevated amounts of macrophages and T cells in the liver, both of which mediate Con A-induced hepatitis and produce TNF upon Con A stimulation in vivo (6, 28), we investigated the contribution of endothelial tmTNF in the model of Con A-induced hepatitis. This animal model shares typical features with acute stages of chronic active hepatitis B and C in humans as well as human autoimmune liver disease. These disorders are characterized by infiltration of T cells in the liver, the release of Th1-like cytokines, and elevated transaminase levels (46, 47). Production of IFN-γ and TNF by infiltrating T cells and Kupffer cells has been directly implicated in the induction of hepatocellular damage leading to severe liver injury (7, 11, 48), and especially tmTNF has been shown to be sufficient to induce hepatotoxicity (12). Unexpectedly, mice expressing tmTNF in the endothelium are protected from hepatic injury rather than being more sensitive to Con A treatment. Neither a dramatic release of aminotransferases nor widespread staining of hepatocytes by TUNEL could be observed in Con A-treated transgenic mice, which demonstrates that hepatocytes were indeed not damaged. Moreover, tmTNF signaling in endothelial cells also seemed to prevent DNA damage in these cells, because the amount of TUNEL-positive endothelial cells was apparently reduced in Con A-treated transgenic animals. Destruction of sinusoidal endothelial cells is an early event in Con A-induced liver injury, and inhibition of endothelial detachment has been discussed to contribute to the protection from immune-mediated hepatic damage (9, 10). Moreover, the unresponsiveness of the tie2-tmTNF-transgenic mice to Con A is not the cause of an overall insensitivity of the immune system, because plasma levels of IFN-γ and TNF are even further increased compared with those in Con A-treated wild-type mice.

Although TNF has been postulated to mediate mainly cytotoxic effects in inflammatory and autoimmune disorders, there are increasing numbers of publications showing that TNF can be

involved in cytoprotection as well. In models of autoimmune-mediated demyelination (49), autoimmune lupus nephritis (50), and diabetes (51), TNF was found to protect mice from severe injury. One possible mechanism for the cytoprotective effect of TNF is *trans*-activation of NF-κB, leading to the induction of anti-apoptotic genes, such as the members of the inhibitor of apoptosis family of proteins (52). Therefore, our previous observation that expression of tmTNF in endothelial cells leads to the constitutive activation of NF-κB (16) provides a possible explanation for the protection from endothelial and subsequent hepatocellular damage in Con A-treated tie2-tmTNF-transgenic animals. However, we have not been able to detect an increased activation of NF-κB in the liver of transgenic compared with wild-type mice using either immunofluorescence or mobility shift analysis (data not shown). Although these results argue against the involvement of NF-κB in hepatic protection in tie2-tmTNF-transgenic animals, it cannot be excluded that the techniques used are not sensitive enough to detect a low and constitutive activation of NF-κB.

The induction of protective cytokines such as IL-6 and IL-10 is another possible mechanism for the observed protection from Con A-induced liver damage, because prophylactic administration of either IL-6 or IL-10 has been shown to prevent hepatocellular damage in this model (35, 36). However, none of these cytokines was up-regulated in untreated tie2-tmTNF-transgenic animals compared with wild-type mice, which argues for an IL-6- and IL-10-independent mechanism of hepatic protection in our transgenic animals. Up-regulation of other putative protective cytokines in untreated transgenic animals or after administration of Con A could also play a role in hepatic protection and remains to be elucidated. Such a cytokine stimulatory network has been reported for the induction of desensitization after repeated administration of endotoxin or TNF to rodents (53–56). The exact mechanism of cytokine tolerance is not fully understood, but it has been proposed that in desensitized cells alteration of the signaling cascades, rather than down-regulation of the corresponding receptors, plays a role (57, 58). In our tie2-tmTNF-transgenic mice, desensitization of T cells and macrophages is unlikely, because we could show increased production of IFN-γ and sTNF after challenge with Con A. Alternatively, desensitization of endothelial cells by TNF via down-regulation of signaling steps such as the Jun NH_2-terminal kinase pathway (59) could comprise a further protective mechanism in Con A-treated tie2-tmTNF-transgenic animals.

Our observation that tie2-tmTNF-transgenic animals are, on the one hand, predisposed to chronic inflammation in the liver and, on the other hand, protected from immune-mediated acute hepatitis provides an example for the dual role of TNF. The ability of TNF to induce the transcription of both proinflammatory and cytoprotective/anti-apoptotic genes may explain why this cytokine can cause opposing effects, i.e., toxicity and protection. In conclusion, our results suggest an active involvement of TNF-expressing endothelial cells in limiting immune-mediated inflammatory processes. However, whether this "beneficial role" of an activated endothelium is also of importance in diseases such as diabetes, transplant rejection, and atherosclerosis has not been addressed. The tie2-tmTNF-transgenic animals provide a new model for an activated endothelium and may be useful to investigate the participation of activated endothelial cells in these and other vascular disorders.

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