Abnormal TNF activity in *Timp3*^{-/-} mice leads to chronic hepatic inflammation and failure of liver regeneration

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Tumor-necrosis factor (TNF), a pleiotropic cytokine, triggers physiological and pathological responses in several organs. Here we show that deletion of the mouse gene *Timp3* resulted in an increase in TNF- α converting enzyme activity, constitutive release of TNF and activation of TNF signaling in the liver. The increase in TNF in *Timp3*^{-/-} mice culminated in hepatic lymphocyte infiltration and necrosis, features that are also seen in chronic active hepatitis in humans. This pathology was prevented when deletion of *Timp3* was combined with *Tnfrsf1a* deficiency. In a liver regeneration model that requires TNF signaling, *Timp3*^{-/-} mice succumbed to liver failure. Hepatocytes from *Timp3*^{-/-} mice completed the cell cycle but then underwent cell death owing to sustained activation of TNF. This hepatocyte cell death was completely rescued by a neutralizing antibody to TNF. Dysregulation of TNF occurred specifically in *Timp3*^{-/-}, and not *Timp1*^{-/-} mice. These data indicate that TIMP3 is a crucial innate negative regulator of TNF in both tissue homeostasis and tissue response to injury.

Mutation of *TIMP3*, which encodes a member of the tissue inhibitor of metalloproteinase (TIMP) family, results in the human retinal degenerative disease Sorsby's fundus dystrophy (SFD)¹. Deletion of *Timp3* in mice results in spontaneous pulmonary air space enlargement and enhanced mammary epithelial apoptosis^{2,3}. Biochemical studies suggest that TIMP3 is unique in its ability to inhibit TNF- α converting enzyme (TACE; also known as disintegrin and metalloproteinase domain 17 or ADAM17; refs. 4,5). Deletion of the gene encoding TACE, *Adam17*, results in the defective release of membrane-bound TNF^{6,7}, and TACE is important for shedding several ligands and receptors⁸⁻¹². TNF has a central role in physiological processes such as immunity and organ regeneration, as well as in diseases including rheumatoid arthritis, diabetes, chronic inflammation and autoimmune disorders^{13,14}.

We investigated whether deletion of *Timp3* alters TNF bioactivity and signaling *in vivo*. Our results show that TIMP3 controls TNF and, as a consequence, affects inflammation and organ regeneration.

RESULTS

Constitutive TNF shedding in *Timp3*^{-/-} mice

We first examined several organs to determine whether basal TNF levels were altered in *Timp3*^{-/-} mice. TNF is normally undetectable in the liver, spleen, lung, brain, serum (Fig. 1a) and kidney (data not shown) of wild-type mice. In *Timp3*^{-/-} mice, TNF was measurable only in the liver (Fig. 1a). We detected an increase in TNF by enzyme-

linked immunosorbent assay (ELISA) in liver protein homogenates of *Timp3*^{-/-} mice, which was confirmed by an increase in hepatic TNF detected immunohistochemically. Immunohistochemical analysis also localized TNF to the hepatic sinusoids (Fig. 1b,c). In the sinusoids, TNF immunostaining colocalized with Kupffer cells, the resident macrophages of the liver, as shown by immunodetection with antibodies to F4/80 in adjacent sections (Fig. 1d,e).

Histomorphometric quantification showed that the number of F4/80-positive cells was not altered in *Timp3*^{-/-} mice (data not shown), suggesting that the increase in TNF might be due to an increase in the production or release of this cytokine by Kupffer cells. Membrane-bound TNF is a 26-kDa protein that is proteolytically processed into a 17-kDa soluble form^{15,16}. Western blotting showed that there was more of both forms of TNF, but particularly soluble TNF, in the livers of *Timp3*^{-/-} mice compared with those of wild-type mice (Fig. 1f). By real-time TaqMan RT-PCR, we found no difference in the expression of *Tnf* mRNA in livers of wild-type and *Timp3*^{-/-} mice (data not shown).

We sought to confirm the presence and localization of *Timp3* expression in livers of wild-type mice. *Timp3* mRNA is abundant in mouse liver¹⁷ and is primarily expressed in hepatocytes¹⁸. *In situ* hybridization on wild-type liver tissue showed that *Timp3* mRNA localized to hepatocytes (Fig. 1g-j). Because hepatocytes constitute ~95% of the liver volume, it is difficult to attribute *Timp3* expression to smaller liver cell populations, such as Kupffer cells. We used bone

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Figure 1 Increased TNF in *Timp3*^{-/-} liver. **(a)** Quantification of TNF by ELISA in organs of 12-week-old wild-type (+/+) and *Timp3*^{-/-} (-/-) mice. **P* < 0.05. **(a,b)** Immunohistochemical staining for TNF in livers of wild-type **(b)** and *Timp3*^{-/-} **(c)** mice. **(e,f)** Adjacent sections immunostained for the macrophage-specific marker F4/80 in livers of wild-type **(d)** and *Timp3*^{-/-} **(e)** mice. **(f)** Appearance of soluble TNF (17 kDa) in livers of *Timp3*^{-/-} mice (100 μg of protein loaded per lane). Soluble recombinant TNF (rTNF) was used as a positive control. Membrane-bound TNF is visualized as a 26-kDa band. **(g-j)** *In situ* hybridization on livers of wild-type mice with *Timp3* sense and antisense riboprobes. **(k)** Quantitative real-time TaqMan RT-PCR of *Timp3* mRNA in livers of wild-type mice (*n* = 3) and *Timp3*^{-/-} mice with (*n* = 3) and without (*n* = 2) bone marrow transplantation (BMT) of GFP-tagged wild-type bone marrow. Data are the mean ± s.e.m. Scale bars: **b-e**, 40 μm; insets in **d,e**, 0.1 mm; **g,h**, 50 μm.

marrow transplantation to test for the production of *Timp3* mRNA by the intrahepatic bone marrow-derived compartment that includes Kupffer cells¹⁹. *Timp3*^{-/-} mice that received transplants of green fluorescent protein (GFP)-labeled wild-type bone marrow showed detectable expression of *Timp3* in the liver by real-time TaqMan RT-PCR, indicating that intrahepatic bone marrow-derived cells could express *Timp3* (Fig. 1k). As expected, this level of *Timp3* expression was 100 times lower than that found in livers of wild-type mice, where hepatocytes also contributed to the production of *Timp3* (Fig. 1k).

Increased TACE activity in livers of *Timp3*^{-/-} mice

Next, we used a fluorogenic TACE peptide substrate to determine TACE activity and found that cleavage was 1.8 times higher in liver extracts from *Timp3*^{-/-} mice than in those from their control littermates (Fig. 2a). Adding recombinant TIMP3, but not recombinant TIMP1, to liver extracts reduced this enzymatic activity (Fig. 2a). This differential response to TIMP3 is the signature of TACE activity⁴. Adding the serine protease inhibitor aprotinin or the matrix metalloproteinase (MMP)-specific inhibitor BMS-275291 (ref. 20) did not

reduce the increase in enzymatic activity in liver extracts from *Timp3*^{-/-} mice (Fig. 2a).

The membrane-type MMPs MMP14 and MMP17 have TNF convertase activity *in vitro*^{21,22}. We found that only recombinant TACE, and not MMP14 or MMP17, efficiently cleaved the fluorogenic TACE peptide substrate, verifying the specificity of this cleavage assay for TACE (Fig. 2b). As above, recombinant TACE activity was inhibited only by recombinant TIMP3 and not by recombinant TIMP1 (Fig. 2b). MMP7 can also shed TNF *in vivo*²³. Using an MMP7 peptide substrate (GLARE), we ruled out any increase in MMP7 activity in the liver as a result of *Timp3* deficiency (Fig. 2c). In fact, MMP7 was undetectable by immunoblotting in livers of both wild-type and *Timp3*^{-/-} mice (data not shown). In addition, we detected no increase in general MMP activity in two independent assays that used either gelatin or fibronectin as substrates (Fig. 2d,e).

To confirm the ability of these MMP activity assays to detect MMP14 and MMP17, we added the recombinant proteins to GLARE, gelatin and fibronectin substrates. Both GLARE and gelatin were cleaved by MMP14 and, to a lesser extent, by MMP17

Figure 2 Livers of *Timp3*^{-/-} mice show an increase in activity of TACE but not MMP. **(a)** Fluorometric measurements of TACE peptide cleavage in livers of 12-week-old wild-type and *Timp3*^{-/-} mice in the absence of any inhibitors or in the presence of recombinant TIMP1 (rTIMP1; 100 nM), recombinant TIMP3 (rTIMP3; 100 nM), aprotinin (5 μg ml⁻¹) or BMS-275291 (1 μM). Data are the mean ± s.e.m. (*n* = 3 mice for each genotype). **P* < 0.05. **(b)** Cleavage of TACE peptide substrate by 50 nM recombinant TACE (rTACE), recombinant MMP14 (rMMP14) or recombinant MMP17 (rMMP17), in the absence or presence of 100 nM recombinant TIMP1 (rTIMP1) or TIMP3 (rTIMP3). **(c-e)** Fluorometric measurements indicating cleavage of GLARE **(c)**, gelatin **(d)** or fibronectin **(e)** in livers of wild-type and *Timp3*^{-/-} mice. Data are the mean ± s.e.m. (*n* = 3 mice per genotype). As a positive control, 100 nM recombinant MMP7 (rMMP7), MMP14 (rMMP14) or MMP17 (rMMP17) was added in these assays.

(Fig. 2c–e), verifying the sensitivity of the assays to these membrane-type MMPs. Real-time TaqMan RT-PCR analyses also showed that there was no difference in the expression of *Adam17* mRNA in livers of wild-type and *Timp3*^{−/−} mice. Taken together, these results indicate that livers of *Timp3*^{−/−} mice have increased TACE activity but no difference in MMP activity.

Activation of TNF signaling in livers of *Timp3*^{−/−} mice

Activation of the transcription factor NF-κB, a heterodimer composed of p65 (also called RelA), RelB or cRel and p50 or p52 subunits²⁴, is central to TNF signaling. In the liver, NF-κB is normally present as the p65–p50 heterodimer. Notably, loss of p65 in mice blocks NF-κB activation, resulting in embryonic lethality owing to massive TNF-induced apoptosis in the liver²⁵. These mice can be rescued by deleting the gene *Tnfrsf1a*, which encodes TNF receptor 1 (ref. 26). When NF-κB is activated, p65 is translocated from the cytoplasm to the nucleus.

To investigate whether an excess of TNF resulted in increased activation of NF-κB in livers of *Timp3*^{−/−} mice, we examined the localization of p65 by immunohistochemistry. In the absence of TIMP3, p65 showed a preferentially nuclear localization, in contrast to its cytoplasmic granular appearance in age-matched wild-type controls (Fig. 3a–d). We quantified the cells showing nuclear localization of p65 by counting five random fields in pericentral regions of the liver at ×40 magnification. The number of cells with nuclear NF-κB localization was more than twice as high in livers of *Timp3*^{−/−} mice (45.2 ± 6.6%; mean ± s.e.m.) versus wild-type mice (18.6 ± 4.9%; Fig. 3e).

We confirmed activation of the TNF signaling pathway by assessing the expression of interleukin-6 (IL-6), which is a transcriptional target of NF-κB²⁷. Hepatic quantities of IL-6 measured by ELISA were 2.6 times higher in livers of *Timp3*^{−/−} mice (Fig. 3f). Expression of IL-6 in *Timp3*^{−/−} mice was localized to the Kupffer cells and to the cytoplasm of pericentral hepatocytes (Fig. 3g–j). Thus, in the absence of TIMP3, a low concentration of TNF activated the TNF signaling pathway in the liver. Notably, immunostaining of both p65 and IL-6 was most prominent in the pericentral regions.

Hepatic inflammation with aging in *Timp3*^{−/−} mice

Given the aberrant activation of TNF signaling in livers of *Timp3*^{−/−} mice, we determined whether the mutant mice had associated liver pathology. At 18–22 months of age, periportal lymphocytic infiltrates progressively appeared in the livers of *Timp3*^{−/−} mice, with substantial alteration of the periportal lobular architecture (Fig. 4a,b). This was not observed in age-matched *Timp3*^{+/−} mice (Fig. 4c). Hepatic necrosis was also evident in some areas (Fig. 4b). We also looked for evidence of hepatic fibrosis by trichrome staining in the livers of *Timp3*^{−/−} mice but found no excessive deposition of collagen (Fig. 4d).

Characterization of the lymphocytic infiltration showed that it comprised cytologically mature lymphocytes of a non-neoplastic nature. These hepatic infiltrates predominantly contained CD3⁺ T cells with occasional foci of B220⁺ B cells (Fig. 4e,f). Further characterization indicated that the T-cell population comprised mostly CD4⁺ cells and few CD8⁺ cells (Fig. 4g,h). A mouse neutrophil-specific antibody showed that neutrophils were occasionally present in the infiltrates (Fig. 4i). There was no evidence of cell turnover in these infiltrates, as assessed by measuring apoptosis by TUNEL and cell proliferation by immunohistochemistry of phosphorylated histone H3 (data not shown). The lack of cell turnover and presence of immunohistochemical heterogeneity confirmed that these mononuclear infiltrates are not clonogenic or tumoral.

Lymphocyte infiltration and hepatic necrosis are associated with chronic active hepatitis in humans²⁸. They have been also reported in transgenic mice overexpressing interferon-γ (IFN-γ)²⁹. We therefore determined whether IFN-γ was induced in the livers of aged *Timp3*^{−/−} mice. We detected lymphoid cells immunopositive for IFN-γ in the periphery of these infiltrates (Fig. 4j). To confirm that TNF signaling

Figure 3 Nuclear localization of NF-κB and activation of IL-6 in 12-week-old *Timp3*^{−/−} mice. (a–d) Representative images at low and high magnification of immunohistochemical staining for p65 in livers of wild-type (a,b) and *Timp3*^{−/−} (c,d) mice. Arrowheads in d indicate positive nuclei. (e) Histomorphometric quantification (original magnification, ×40) of positive nuclei in pericentral hepatocytes of wild-type and *Timp3*^{−/−} mice. (f) Quantification of IL-6 by ELISA in livers of wild-type and *Timp3*^{−/−} mice. (g–j) Immunohistochemical staining for IL-6 in liver sections from wild-type (g,h) and *Timp3*^{−/−} (i,j) mice. Arrowheads indicate positive staining. Data are the mean ± s.e.m. (*n* = 3 mice per group). **P* < 0.05. Scale bars: a,c,h,j, 0.1 mm; b,d,g,i, 40 μm; h,j, insets, 50 μm.

Figure 4 Lymphocytic infiltration of aged livers in *Timp3*^{-/-} mice. (a–c) Hematoxylin and eosin staining of liver sections from *Timp3*^{-/-} (a,b) and *Timp3*^{+/+} (c) mice. Arrowheads mark the borders of lymphocyte-infiltrated areas and the asterisk indicates hepatocyte necrosis. (d) Trichrome staining of liver from *Timp3*^{-/-} mice. (e–j) Immunohistochemical detection of CD3 (e), B220 (f), CD4 (g), CD8 (h), neutrophils (i) and IFN-γ (j) in livers of *Timp3*^{-/-} mice. Arrowheads indicate areas of positive stain. (k,l) Hematoxylin and eosin staining of liver sections from *Tnfrsf1a*^{-/-} *Timp3*^{-/-} (k) and *Tnfrsf1a*^{+/+} *Timp3*^{-/-} (l) mice. Arrowheads mark the borders of lymphocyte-infiltrated areas. Images are representative of six 22-month-old mice of each genotype. Scale bars, 0.1 mm.

was responsible for hepatic inflammation in *Timp3*^{-/-} mice, we generated mice deficient in both *Timp3* and *Tnfrsf1a*. We found no evidence of lymphocytic infiltration or hepatic necrosis in the livers of *Tnfrsf1a*^{-/-} *Timp3*^{-/-} mice at 18–22 months of age (Fig. 4k) compared with age-matched *Tnfrsf1a*^{+/+} *Timp3*^{-/-} mice (Fig. 4l). Thus, blocking TNF signaling through TNFRSF1A prevented hepatic inflammation in *Timp3*^{-/-} mice.

Mortality of *Timp3*^{-/-} mice after hepatectomy

Given the liver pathology observed in unchallenged aged *Timp3*^{-/-} mice, we reasoned that an acute challenge might further indicate the importance of TIMP3 in controlling TNF bioavailability. We examined the regenerative response after partial hepatectomy in mice aged 9–12 weeks, because partial hepatectomy induces a rapid release of TNF³⁰. In addition, liver regeneration is dependent on signaling through TNFRSF1A and activation of NF-κB and

IL-6 (ref. 27). Regeneration of the liver can be monitored by measuring liver mass, which is restored 8–10 d after partial hepatectomy in mice³¹.

Liver to body mass ratios after partial hepatectomy increased similarly in wild-type and *Timp3*^{-/-} mice until 72 h after partial hepatectomy (Fig. 5a). Beyond this time point, we observed a marked decline in liver mass of all *Timp3*^{-/-} mice (Fig. 5a). By 144 h after partial hepatectomy, four of six *Timp3*^{-/-} mice were moribund, and the remaining two, though not moribund, had decreased liver to body mass ratios. By contrast, all six wild-type mice appeared normal and had healthy liver to body mass ratios.

Accelerated cell cycle in regenerating livers of *Timp3*^{-/-} mice

In the resting liver, hepatocytes are in the G0 state³¹. After partial hepatectomy, a surge of TNF at 1 h triggers hepatocyte entry into the cell cycle³⁰. We followed hepatocyte cell-cycle progression in *Timp3*^{-/-}

Figure 5 Failure of liver regeneration despite accelerated progression of the cell cycle in hepatocytes in *Timp3*^{-/-} mice. (a) Liver to body mass ratio over time after partial hepatectomy (PHx) in wild-type (dashed line) and *Timp3*^{-/-} (solid line) mice. (b) Representative western blot of cyclin D1 and PCNA in regenerating livers of wild-type and *Timp3*^{-/-} mice (50 μg of protein loaded per lane). (c–f) Immunohistochemical detection of phosphorylated histone H3 in regenerating livers of wild-type (c,e) and *Timp3*^{-/-} (d,f) mice. Arrowheads indicate positive nuclei. (g) Histomorphometric quantification (original magnification, ×40) of nuclei positive for phosphorylated histone H3 in regenerating livers of wild-type and *Timp3*^{-/-} mice. PHx, partial hepatectomy. Data are the mean ± s.e.m. (*n* = 3 and *n* = 6 mice of each genotype, aged 9–12 weeks, at time points 0–96 h and 144 h, respectively). **P* < 0.05. Scale bars: c–f, 0.1 mm; insets in c–f, 20 μm.

mice after partial hepatectomy by measuring cyclin D1 to mark the G1-S transition, proliferating cell nuclear antigen (PCNA) to indicate S phase, and phosphorylated histone H3 to quantify mitosis. Immunoblotting showed that cyclin D1 and PCNA were expressed earlier in the absence of TIMP3 than in wild-type mice (Fig. 5b). Hepatocyte cell division also occurred earlier in livers of *Timp3*^{-/-} mice, as indicated by a substantial increase in the number of nuclei positive for phosphorylated histone H3 ($21.1 \pm 5.9\%$ in *Timp3*^{-/-} mice versus $1.8 \pm 2.1\%$ in wild-type mice) at 48 h (Fig. 5c–g). This level of mitosis was observed only at 72 h in wild-type mice (Fig. 5g). Taken together, these findings suggest that *Timp3*^{-/-} hepatocytes rapidly complete the cell cycle.

Extensive necrosis of regenerating livers of *Timp3*^{-/-} mice

We investigated the cause of mortality in *Timp3*^{-/-} mice undergoing liver regeneration. During the window of hepatocyte cell-cycle progression (0–48 h), livers of *Timp3*^{-/-} mice appeared normal and showed characteristics of regeneration, such as hepatocyte hypertrophy and perturbed architecture, similar to those of wild-type mice (Fig. 6a–c,g–i). By contrast, by 72 h *Timp3*^{-/-} mice had developed multiple foci of liver necrosis (Fig. 6d,j), which became more marked with time (Fig. 6e,k). Histologically, necrosis was liquefactive in nature, and by 144 h no normal lobules could be seen, owing to widespread hepatocyte vacuolization (Fig. 6f,l).

Sustained activation of TNF leads to hepatocyte apoptosis

Because TNF can stimulate cell proliferation and induce programmed cell death^{13,32}, we examined whether apoptosis accompanied hepatic necrosis in *Timp3*^{-/-} mice. Histomorphometry after TUNEL staining detected numerous apoptotic nuclei by 48 h in regenerating livers of *Timp3*^{-/-} but not wild-type mice (Fig. 7a–c). Molecular analyses indicated that there was a pronounced cleavage of BID at 24 h (Fig. 7d) and activation of caspase 3 at 48 h (Fig. 7e) after partial hepatectomy in *Timp3*^{-/-} mice. α -tubulin was used as a loading control (Fig. 7f). These observations suggest that TNF-mediated hepatocyte apoptosis may initiate hepatocyte death, although other more complex mechanisms may contribute to the eventual collapse of liver regeneration in *Timp3*^{-/-} mice.

One such mechanism may involve the upregulation of MMPs that are induced by TNF¹⁸. By real-time TaqMan RT-PCR, we found a substantial increase in the mRNA expression of *Mmp9* at 72 h, *Mmp13* at 1 h, *Mmp14* between 24 and 96 h, and *Mmp15* at 96 h after partial hepatectomy in livers of *Timp3*^{-/-} mice relative to those of wild-type controls (data not shown). Gelatin zymography on liver extracts from regenerating livers of wild-type and *Timp3*^{-/-} mice showed that there was an increase in MMP9 activity (data not shown). Notably, none of these MMPs had increased expression in livers of *Timp3*^{-/-} mice before partial hepatectomy.

We investigated hepatic TNF levels over the time course of liver regeneration. As expected in wild-type mice, the level of TNF peaked sharply at 1 h after partial hepatectomy and then declined to baseline levels (Fig. 7g). Loss of TIMP3 function caused substantial perturbations in TNF throughout the course of liver regeneration: TNF was upregulated throughout the regeneration and did not decline as it did in wild-type mice (Fig. 7g). This effect was specific to *Timp3*^{-/-} mice and was not observed in regenerating livers of *Timp1*^{-/-} mice, which maintained a TNF expression profile similar to that of wild-type controls (Fig. 7h).

IL-6 is a key target of TNF signaling during liver regeneration²⁷. We confirmed the perturbation of TNF signaling by assessing IL-6 levels by ELISA during the regenerative response. Consistent with higher

Figure 6 Widespread hepatic necrosis in regenerating livers of *Timp3*^{-/-} mice. Representative images of sections stained with hematoxylin and eosin from regenerating livers of wild-type (a–f) and *Timp3*^{-/-} (g–l) mice aged 9–12 weeks. Arrowheads indicate necrotic areas. Scale bars, 0.1 mm.

levels of TNF, IL-6 levels were substantially higher and dysregulated in regenerating livers of *Timp3*^{-/-} mice (Fig. 7i). Notably, both TNF and IL-6 were highest at 144 h after partial hepatectomy, coincident with the mortality of *Timp3*^{-/-} mice (Fig. 7g,i). Peroxisome proliferator activated receptor- γ (PPAR- γ), a nuclear hormone receptor with an anti-inflammatory function^{33,34}, is a marker of chronic cytokine exposure. We found a large increase in PPAR- γ at 48 h after partial hepatectomy in livers of *Timp3*^{-/-} mice (Fig. 7j). These data suggest that livers of *Timp3*^{-/-} mice have sustained TNF activity and inappropriate hepatocyte cell death.

Figure 7 Increased TNF and apoptosis in regenerating livers of *Timp3*^{-/-} mice. **(a)** Quantification of TUNEL-positive nuclei in livers of wild-type and *Timp3*^{-/-} mice after partial hepatectomy (PHx). **(b,c)** Representative TUNEL-stained liver sections from wild-type **(b)** and *Timp3*^{-/-} **(c)** mice at 48 h after partial hepatectomy. Representative western blots of BID **(d)**, caspase 3 **(e)** and α -tubulin **(f)** in regenerating livers of wild-type and *Timp3*^{-/-} mice (50 μ g of protein loaded per lane). Wild-type mouse liver treated with Fas agonist was used as a positive control for apoptosis markers (C), and α -tubulin was used as a protein loading control. **(g,h)** Quantification of TNF by ELISA in regenerating livers of *Timp3*^{-/-} **(g)** and *Timp1*^{-/-} **(h)** mice and their respective wild-type controls. **(i)** Quantification of IL-6 by ELISA in regenerating livers of wild-type and *Timp3*^{-/-} mice. **(j)** Temporal expression of PPAR- γ (50 μ g protein loaded per lane) in regenerating livers of wild-type and *Timp3*^{-/-} mice. Data are the mean $\pm$ s.e.m. ($n = 3$ mice, aged 9–12 weeks, at each time point per group). PHx, partial hepatectomy. * $P < 0.05$. Scale bars, 40 μ m.

TNF neutralizing antibody rescues *Timp3*^{-/-} mice

We considered that TIMP3 is essential for controlling hepatic TNF activity and that excessive TNF is responsible for the untimely activation of hepatocyte cell death in regenerating livers of *Timp3*^{-/-} mice. We therefore tested whether a neutralizing antibody to TNF could rescue these mice. Because TNF signaling in the first 6 h is required for hepatocyte DNA synthesis, we administered the neutralizing antibody or vehicle to *Timp3*^{-/-} mice at 12 h after partial hepatectomy and examined livers at 96 h for organ pathology. Substantial necrosis was apparent in mice treated with vehicle alone (**Fig. 8a–c**). By contrast, all three mice treated with the TNF blocking antibody showed no signs of hepatic necrosis (**Fig. 8d–f**).

Consistent with the rescued pathology, *Timp3*^{-/-} mice had considerably higher liver to body mass ratios (**Fig. 8g**). We first deter-

mined hepatic TNF in these mice and found that TNF was reduced in mice treated with the neutralizing antibody (data not shown); however, we found that the antibody interfered with measurements of TNF by ELISA. Thus, to confirm the effective blocking of TNF activity, we measured IL-6 in liver homogenates by ELISA and found that hepatic IL-6 levels were 6.6 times lower in mice treated with the antibody (**Fig. 8h**). These results show that TNF dysregulation is responsible for the phenotype of *Timp3*^{-/-} mice.

DISCUSSION

We showed here that TIMP3 is a crucial innate negative regulator of TNF both in tissue homeostasis and in tissue response to injury. *Timp3* deficiency leads to an increase in TACE activity and a failure to control the release of TNF, leading to inappropriate induction of inflammation and TNF-mediated cell death.

Figure 8 Rescue of livers of *Timp3*^{-/-} mice from cell death by neutralizing antibody to TNF. **(a–f)** Hematoxylin and eosin staining of regenerating livers of *Timp3*^{-/-} mice treated with vehicle (phosphate-buffered saline) alone **(a–c)** or neutralizing antibody to TNF **(d–f)**. A single dose was administered 12 h after partial hepatectomy and livers were collected at 96 h after partial hepatectomy. Arrowheads indicate necrotic areas. **(g)** Quantification of liver to body mass ratio at 96 h after partial hepatectomy in *Timp3*^{-/-} mice treated with vehicle (gray bar) or neutralizing antibody (black bar). **(h)** Quantification of IL-6 by ELISA in regenerating livers of the *Timp3*^{-/-} mice treated as above. Data are the mean $\pm$ s.e.m. ($n = 3$ mice, aged 9–12 weeks, per group). * $P < 0.05$, ** $P = 0.001$. Scale bars, 0.1 mm.

Previous studies have reported that, of the four members of the TIMP family, only TIMP3 inhibits TACE activity⁴. Shedding mediated by TACE extends the effects of TNF from juxtacrine to paracrine and provides an additional level of pericellular control of TNF activity. We found that deletion of *Timp3* increased TACE activity in the liver, as shown by an increase in the cleavage of a TACE peptide substrate, which was inhibited only by recombinant TIMP3 and not by recombinant TIMP1. Notably, we also observed the constitutive appearance of the soluble 17-kDa TNF species, as well as an increase in levels of the 26-kDa membrane-bound form of TNF. Expression of *Adam17* mRNA was not increased in livers of *Timp3*^{-/-} mice before or during regeneration (data not shown), although there was a trend towards an increase in mature TACE protein in livers of *Timp3*^{-/-} mice before partial hepatectomy (data not shown).

The activities of MMP7, the only other metalloproteinase shown to affect TNF release *in vivo*²³, or of MMP14 and MMP17, which have been reported to cleave TNF *in vitro*^{21,22}, were not elevated in livers of *Timp3*^{-/-} mice before partial hepatectomy. These findings are consistent with the interpretation that TIMP3 is a physiological regulator of TACE activity and TNF shedding. TNF can induce its own expression in a positive-feedback manner, as well as the expression of several molecules including MMPs and TACE³⁵. Whether changes in TNF or TACE expression in the absence of TIMP3 also contribute to an increase in TNF activity needs to be tested further.

Suppression of both basal and inducible TNF was compromised in *Timp3*^{-/-} mice. Continuous, low-level exposure to TNF over the lifespan of *Timp3*^{-/-} mice led to abnormal lymphocyte infiltration. This infiltration was prevented when *Tnfrsf1a* was deleted, indicating that this TNF signaling pathway is required for the observed pathology. Notably, lymphocytic infiltration is also observed in human livers afflicted with chronic active hepatitis^{28,29}. We propose that TIMP3 constitutes a previously unknown point of control in TNF-mediated inflammation.

In addition to its ability to inhibit TACE, TIMP3 is also unique in that it binds to the extracellular matrix on secretion³⁶. Notably, both TACE and TIMP3 are abundantly expressed in heart, kidney, lung, liver and brain¹⁷. In the liver, hepatocytes have been reported to be the primary source of TIMP3 (ref. 18). We confirmed this localization by *in situ* hybridization and found that livers of *Timp3*^{-/-} mice containing wild-type bone marrow-derived cells expressed TIMP3 at low levels. Thus, TIMP3 is abundant in the liver, and its secretion by hepatocytes and Kupffer cells into the pericellular microenvironment positions it well for regulating TNF processing. The precise mechanism by which TIMP3 inhibits TACE and TNF release, however, requires further investigation. Similarly, the various biological effects of TNF shedding may be dependent on the context³⁷⁻³⁹ and need to be fully understood. Combining noncleavable transmembrane TNF transgenic expression with *Timp3* deficiency will allow further investigation of the pathological consequences that arise from altered TNF shedding.

Macrophages are a principal source of TNF. In *Timp3*^{-/-} mice, TNF was produced by Kupffer cells, the resident macrophage population of the liver. These cells are positioned to respond rapidly to infection or injury by initiating cytokine cascades. Maintenance of the normal noninflammatory state results not only from the absence of stimuli, but also from the active suppression of inappropriate inflammatory responses⁴⁰. Tissue-specific inflammatory conditions spontaneously occur in several genetically altered mice⁴⁰. The liver is one of the sites most frequently affected, probably owing to its large size and unique function⁴⁰. Kupffer cells constitute the largest population of resident macrophages in the body⁴¹; are respon-

sible for clearing gut-derived bacteria and lipopolysaccharide, a potent inducer of TNF⁴²; and are a chief source of proinflammatory cytokines⁴¹. Kupffer cells therefore have an important role in liver tissue homeostasis and in the hepatic inflammatory response. It may be imperative to actively suppress an aberrant inflammatory reaction while Kupffer cells carry out their function. Thus, the presence of TIMP3 may be more crucial for liver tissue homeostasis in this context than in others.

The essential role of TIMP3 was more obvious after the induction of TNF signaling by partial hepatectomy, which resulted in massive hepatic necrosis, apoptosis and morbidity in *Timp3*^{-/-} mice, owing to their inability to downregulate hepatic TNF levels. TNF is normally tightly regulated and is transiently induced after hepatectomy in wild-type mice. Levels of PPAR- γ were also increased in regenerating livers of *Timp3*^{-/-} mice, which may be a reactive response to prolonged cytokine exposure and an attempt to negatively regulate cytokine production and macrophage activation^{33,34}.

The dual role of TNF in cell survival and cell death is well recognized^{13,14} and was highlighted in our organ regeneration studies. At first, the increase in TNF accelerated hepatocyte cell-cycle progression in *Timp3*^{-/-} mice, but then the sustained upregulation of TNF triggered programmed cell death and necrosis. Neutralizing antibody to TNF completely rescued hepatocyte cell death in *Timp3*^{-/-} mice after partial hepatectomy. Consistent with the function of TIMP3 in controlling TNF, endogenous *Timp3* was transcriptionally induced after the peak of TNF induction during liver regeneration in wild-type mice (data not shown). Our findings show that TIMP3 functions upstream of TNF. This metalloproteinase inhibitor alters both the amplitude and the duration of TNF activation, switching the effect of TNF from prosurvival to proapoptotic. Overall, TIMP3 regulation of TNF is essential for successful liver regeneration.

Inappropriate induction or sustained activation of TNF signaling has been implicated in the pathogenesis of numerous common human diseases¹⁴ and is recapitulated in TNF transgenic mouse models^{43,44}. We provide evidence that TIMP3 is a crucial negative regulator of TNF release and signaling *in vivo*. We also show that TIMP3 has an essential role in hepatic inflammation and regeneration. The pathogenetic mechanisms leading to SFD, including the role of mutant TIMP3 proteins^{45,46}, are currently not well understood. Macrophages and inflammation are reported to have a role in the development of age-related macular degeneration, which has a pathology closely resembling that of SFD⁴⁷. It is plausible that TIMP3 is involved in the inflammation underlying age-related macular degeneration and possibly SFD. It will be important to explore the role of TIMP3 and inflammation in clinical conditions including age-related macular degeneration, cardiovascular disease, sepsis and rheumatoid arthritis.

METHODS

Experimental mice. We generated 129J/C57BL6 *Timp3*^{-/-} mice as described² and backcrossed them eight times into the C57BL/6 background. Unlike the original mixed strain, C57BL/6 *Timp3*^{-/-} mice do not succumb to morbidity through lung dysfunction. We obtained C57BL/6 mice deficient in *Tnfrsf1a* (p55), originally generated by the laboratory of T.W. Mak (Ontario Cancer Institute), from Jackson Laboratory. We did PCR genotyping of *Timp3* as described². Primer sequences are available on request. We fed mice 4% fat chow *ad libitum* and cared for them in accordance with protocols approved by the Ontario Cancer Institute Animal Care Committee and with Canadian Council on Animal Care guidelines.

Liver regeneration studies. We used male *Timp3*^{-/-} mice, 9–12 weeks old, and their age- and sex-matched wild-type littermates as controls. We anesthetized mice with isoflurane and carried out 70% partial hepatectomy as described⁴⁸. We did the surgeries consecutively on knockout and control pairs. We killed

mice by cervical dislocation and collected liver tissue. We flash-froze livers in liquid nitrogen for biochemical analyses, fixed them in 4% formaldehyde and embedded them in paraffin for histology.

ELISA assays. We extracted total liver protein by homogenization of frozen tissue in extraction buffer containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM $\text{Na}_2\text{P}_2\text{O}_7$, 50 mM NaF, 200 μM Na_3VO_4 , 2 mM PMSF, 5 $\mu\text{g ml}^{-1}$ of aprotinin, 1 $\mu\text{g ml}^{-1}$ of pepstatin A and 2 μg of leupeptin. We centrifuged the suspension (at 14,000 r.p.m. for 20 min at 4 °C) and stored the supernatant at -70 °C. We determined protein concentration using a Dc BioRad assay. For each sample, we analyzed 4 μg of protein in triplicate by TNF and IL-6 ELISA kits used in accordance with the manufacturer's instructions (PharMingen).

Western-blot analyses. We extracted liver proteins as described above for all western-blot analyses, except those for TNF and TACE, in which 1% SDS replaced 1% Triton X-100 in the buffer. We separated proteins by SDS-PAGE and transferred them to nitrocellulose membrane. We blocked membranes with 5% milk in TBST (20 mM Tris (pH 7.5), 500 mM NaCl and 0.05% Tween-20) and probed them with primary antibodies to TNF (Endogen), TACE (Chemicon), cyclin D1 (Oncogene), PCNA (Novacastra), BID (R&D Systems) or caspase 3 (Signal Transduction), followed by secondary antibodies to rabbit or to mouse (Cell Signaling) conjugated to horseradish peroxidase. We used enhanced chemiluminescence developing reagents from Pierce.

In situ hybridization. We generated ^{35}S -labeled riboprobes in both sense and antisense orientations from 300 bp of the *Timp3* cDNA using T3 and T7 polymerases, respectively. We removed paraffin from serial 5- μm sections of liver and rehydrated them through a series of graded ethanol solutions, permeabilized them with proteinase K and postfixed them with 4% paraformaldehyde. We hybridized each section overnight at 50 °C with 2×10^6 c.p.m. of sense or antisense probe diluted in buffer for *in situ* hybridization (Dako). The next day, we washed the sections repeatedly at high stringency ($0.1 \times \text{SSC}$, 55 °C), dehydrated them and dipped them in NBT-2 emulsion (Kodak). After 8 d of exposure, we developed the slides, counterstained them with Mayer's hematoxylin, mounted them and photographed them with bright-field and dark-field filters at $\times 40$ magnification.

Bone marrow transplantation. We isolated bone marrow from the tibia and femur of FVB mice universally expressing GFP driven by the chicken β -actin promoter (Jackson Laboratory). We removed red blood cells using Lympholyte-M (Cedarlane). We lethally irradiated 12-week-old FVB *Timp3*^{-/-} mice with 900 rad and then injected them intravenously with 10^6 bone marrow-derived cells. To increase the number of bone marrow-derived Kupffer cells, we injected transplanted mice intraperitoneally with gadolinium chloride (20 mg per kg body weight in phosphate-buffered saline; Sigma). We killed mice 14 d after this treatment. Successful transplantation was confirmed by directly imaging freshly isolated spleen and liver by using fluorescence microscopy, followed by immunostaining with an antibody to GFP (Molecular Probes).

RNA preparation and real-time RT-PCR. We prepared RNA from frozen tissues using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. We designed oligonucleotide primers and fluorescently labeled TaqMan probes to recognize only full-length *Timp3* RNA species by binding a region that is deleted in *Timp3*^{-/-} RNA. We quantified gene expression as described¹⁷.

TACE activity assay. We extracted liver proteins by homogenization of frozen liver tissue in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P40 and 1% Triton X-100. We incubated 10 μM Abz-LAQAVRSSSR-Dpa (Peptides International) with 100 μg of liver lysates in TACE buffer (50 mM Tris-HCl (pH 7.4), 25 mM NaCl and 4% glycerol) at room temperature for 1 h. We measured fluorescence over 10 min with an excitation wavelength (λ_{ex}) of 320 nm and an emission wavelength (λ_{em}) of 420 nm (ref. 49). We purchased recombinant human TIMP1 and human TIMP3 from Oncogene and R&D, respectively; BMS-275291 was a gift from R.A. Kramer (Bristol-Myers Squibb).

MMP activity assays. We extracted liver proteins in the buffer described for ELISA excluding EDTA. We assayed 25- μl aliquots of liver homogenate at a protein concentration of 1 mg ml^{-1} in Tricine buffer (50mM Tricine (pH 7.4), 0.2 M NaCl, 10 mM CaCl_2 , 50 μM ZnSO_4 and 0.005% Brij 35) containing 12 μl of 'Complete' EDTA-free protease inhibitor (Roche Diagnostics) and 0.8 mg of DQ-gelatin (Molecular Probes), 0.8 mg of DQ-fibronectin (Molecular Probes) or AEDENS/DABCYL-labeled KRPLGLARE peptide³⁰. MMP7 activity was inhibited by the inclusion of 20 μM EDTA or 0.5 μM BMS-275291. We measured fluorescence at 37 °C as a function of time as follows: for DQ-gelatin and DQ-fibronectin, λ_{ex} = 485 nm, λ_{em} = 538 nm; for KRPLGLARE, λ_{ex} = 355 nm, λ_{em} = 538 nm. We purchased recombinant human MMP7 and human MMP17 from Calbiochem and human MMP14 from Chemicon.

Histological and immunohistochemical analyses. We used 5- μm liver sections placed on Superfrost/Plus microscope slides (Fisher Scientific). We removed the paraffin from tissues in toluene, rehydrated them in water and ethanol and stained them with Harris' hematoxylin (Electron Microscopy Sciences) and eosin (Fisher Scientific) or with Masson's trichrome. We used antibodies to phosphorylated histone H3 (Upstate Biotech), TNF (PharMingen), IL-6 (PharMingen), p65 (Santa Cruz), F4/80 (CalTag), B220 (PharMingen), CD3 (Dako), CD4 (PharMingen), CD8 (PharMingen), mouse neutrophil (Serotec) and IFN- γ (Abcam) in accordance with the manufacturer's instructions. We used frozen sections for CD4 and CD8 immunostaining. We carried out TUNEL analysis with an ApoTag kit (Intergen). We quantified positive nuclei by blindly counting five random microscopic fields at $\times 40$ magnification from two random sections for each experimental sample. Livers tested negative by Gram staining for *Helicobacter hepaticus*, which is associated with chronic hepatitis and hepatocellular neoplasia in mice.

TNF neutralizing experiment. We administered 250 μl of monoclonal neutralizing antibody to TNF (1 mg ml^{-1} ; Genzyme) to mice intraperitoneally 12 h after partial hepatectomy. We killed mice 96 h after partial hepatectomy.

Statistical analyses. Data are reported as the mean $\pm$ s.e.m. We carried out statistical analysis with Excel software (Microsoft). Comparisons were made by Student's *t*-test and analysis of variance.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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