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Stromal TIMP3 Regulates Liver Lymphocyte Populations and Provides Protection against Th1 T Cell-Driven Autoimmune Hepatitis

Aditya Murthy,¹ Yang Washington Shao,¹ Virginie Defamie, Christopher Wedeles, David Smookler, and Rama Khokha

Lymphocyte infiltration into epithelial tissues and proinflammatory cytokine release are key steps in autoimmune disease. Although cell-autonomous roles of lymphocytes are well studied in autoimmunity, much less is understood about the stromal factors that dictate immune cell function. Tissue inhibitor of metalloproteinases 3 (TIMP3) controls systemic cytokine bioavailability and signaling by inhibiting the ectodomain shedding of cytokines and their receptors. The role of TIMP3 in cytokine biology is emerging; however, its contribution to cellular immunity remains unknown. In this study, we show that TIMP3 produced by the hepatic stroma regulates the basal lymphocyte populations in the liver and prevents autoimmune hepatitis. TIMP3 deficiency in mice led to spontaneous accumulation and activation of hepatic CD4⁺, CD8⁺, and NKT cells. Treatment with Con A in a model of polyclonal T lymphocyte activation resulted in a greatly enhanced Th1 cytokine response and acute liver failure, which mechanistically depended on TNF signaling. Bone marrow chimeras demonstrated that TIMP3 derived from the stromal rather than hematopoietic compartment provided protection against autoimmunity. Finally, we identified hepatocytes as the major source of Timp3 in a resting liver, whereas significant Timp3 gene transcription was induced by hepatic stellate cells in the inflamed liver. These results uncover metalloproteinase inhibitors as critical stromal factors in regulating cellular immunity during autoimmune hepatitis. The Journal of Immunology, 2012, 188: 000–000.

As a primary site of pathogen encounter, the liver microenvironment has a unique makeup of local immune cells. The epithelial and stromal cells of the liver such as hepatocytes, endothelial cells, and stellate cells can additionally act as nonclassical APCs and generate exquisite sensitivity to entering pathogens without arming liver dendritic cells (1). Autoimmunity involves infiltration of lymphocytes into the liver and T cell activation, which accelerates disease progression during viral, autoimmune, or toxin-induced hepatitis (1, 2). This process typically relies on a proinflammatory cytokine milieu that culminates in hepatoxicity (3, 4). The lectin Con A causes polyclonal T cell activation and is used as an experimental means of inducing immune cell function and wound healing during liver injury (10). We and others have previously demonstrated that TIMP3 functions in a context-dependent manner to direct cytokine signaling during acute and chronic hepatic stress (11–14). However, the physiologic role of TIMPs in cellular immunity remains completely unknown. In this study, we show that TIMP3 deficiency results in spontaneous lymphocyte infiltration into the liver. Con A administration to TIMP3 deficient mice results in enhanced Th1 cytokine production and TNF-dependent liver damage. Using bone marrow chimeras and sorting of liver parenchymal and non-parenchymal cells, we identify that TIMP3 derived from the stromal sources, specifically hepatocytes, and hepatic stellate cells provides hepatoprotection against Con A-induced autoimmunity.

Materials and Methods

Mice

All mice used in this study were backcrossed at least 10 generations into the C57BL/6 background. Timp3⁻/⁻ mice have been described previously (12). Tnf⁻/⁻ mice were obtained from the Jackson Laboratory and were crossed with Timp3⁻/⁻ mice to generate Timp3⁻/⁻;Tnf⁻/⁻ mice. C57BL/6-Ly5.1 (CD45.1) mice were provided by Dr. Norman Iscove (Professor, University of Toronto, Department of Medical Biophysics, MaRS center, Toronto, Canada). Male mice aged 12–15 wk were used for all the experimental procedures. Mice were housed and cared for in accordance with the guidelines approved by the Canadian Council for Animal Care and the Animal Care Committee of the Ontario Cancer Institute.
Induction of hepatitis, generation of bone marrow chimeras, Kupffer cell depletion

Con A (10 μg/g; Sigma) was injected into the tail vein of 12-wk-old male mice dissolved in 200 μl sterile PBS to induce hepatitis. Congenic bone marrow transplants were performed by exposing 12-wk-old recipient mice to a single dose of 9 Gy (900 rad) ionizing radiation, followed by reconstitution with 4 × 10^6 donor bone marrow cells by injection into the tail vein. Bone marrow and peripheral reconstitution was allowed for 8 wk prior to induction of hepatitis. Depletion of liver macrophages (Kupffer cells) was accomplished with i.p. injection of 60 μg/g GdCl₃ in 400 μl sterile PBS at 48 and 24 h prior to administration of Con A. PBS alone was used as a control.

Isolation of liver cell subsets

Parenchymal, nonparenchymal, and immune cell subsets were isolated from the liver as described previously (15). Livers were obtained from mice 18 h after injection of Con A or Con A + PBS. Con A was used for retrograde perfusion (13) and single-cell suspensions generated by passing through a 70-μm nylon mesh. Hepatocytes were isolated by low-speed centrifugation at 50 × g for 10 min. The supernatant containing nonparenchymal and immune cells was pelleted at 400 × g for 10 min and then subjected to a two-step OptiPrep gradient of 8.2% and 17.6% by centrifugation at 1400 g for 10 min. Hepatic stellate cells were obtained from the 8.2% interface. Next, the 17.6% interface containing Kupffer cells and liver sinusoidal epithelial cells (LSECs) was obtained by centrifugation at 400 × g for 10 min. These cells were incubated with anti-LEC magnetic microbeads (Miltenyi Biotec) and passed through a magnetic selection column. The flow-through containing Kupffer cells was incubated with anti-CD11b magnetic microbeads (Miltenyi Biotec) and passed through a second magnetic selection column. Both columns were washed, and positively selected cells were obtained by analysis. Purity was quantified by quantitative PCR analysis of the following lineage-specific markers: hepatocytes—Asglr1; hepatic stellate cells—Glp1r1; LSECs—Cd31; Kupffer cells—Cd68. Timp3 mRNA expression was measured in all cell types. Primer sequences are provided in Supplemental Table I.

CD4⁺ T cell culture

CD4⁺ T cells were isolated from spleens of wild type (WT) and Timp3⁻/⁻ mice by negative selection using MACS (Miltenyi Biotec). Splenocytes were obtained from mice and passed through a 70-μm nylon mesh to generate single-cell suspensions. Red blood cells were removed and remaining cells were resuspended in PBS supplemented with 0.5% BSA and 2 mM EDTA. Negative selection was performed using an Ab mixture containing anti-CD8α (Ly-2), anti-CD11b (Mac-1), anti-CD45R (B220), anti-DX5 and anti-TER-119 Abs conjugated to biotin. Greater than 95% purity of CD4⁺ T cells was confirmed by flow cytometry. Cells were cultured in RPMI 1640 medium containing 10% FBS and 50 μM β-mercaptoethanol. For T cell activation, 1 × 10⁶ CD4⁺ T cells were stimulated with 1.0 μg/ml Con A for 24, 48 and 72 h. Proliferation was measured using a standard MTS assay, with light absorption at 450 nm (OD450) as a measurement of cell quantification. Cell culture supernatant was obtained at indicated time points to measure cytokine levels of IL-2 and TNF by ELISA following the manufacturer’s instructions (R&D Systems).

Serum analysis

Serum was obtained before and after Con A injection at 2 h intervals by bleeding the tail vein. Hepatotoxicity was assessed by measuring serum levels of alanine transaminase (ALT) and aspartate aminotransferase. Concentrations of cytokines were measured using a multiplexed cytokine bead array on a BD FACSArray Bioanalyzer system (BD Biosciences). Concentrations of cytokines were measured using an anti-CD45R Ab and brefeldin A. Intracellular cytokine staining was performed using anti-CD3, anti-CD4, and anti-CD8 Abs followed by intracellular Fixation/Permeabilization Kit (BD Biosciences). T cell isolation was verified by flow cytometry. Cells were cultured in RPMI 1640 medium containing 10% FBS and 50 μM β-mercaptoethanol. For T cell activation, 1 × 10⁶ CD4⁺ T cells were stimulated with 1.0 μg/ml Con A for 24, 48 and 72 h. Proliferation was measured using a standard MTS assay, with light absorption at 450 nm (OD450) as a measurement of cell quantification. Cell culture supernatant was obtained at indicated time points to measure cytokine levels of IL-2 and TNF by ELISA following the manufacturer’s instructions (R&D Systems).

Immunoblotting

Total liver protein was extracted by mortar and pestle homogenization of frozen tissue. RIPA extraction buffer containing 25 mM Tris-HCl pH 7.6, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 5 mM EDTA, 50 mM NaCl, 200 μM Na₂VO₄, 2 mM PMSF, and appropriate dilution of Complete Mini, EDTA-free protease inhibitor mixture tablets (Roche) was used to lyse all tissue and cells. Lysate was stored at −70°C; 30 μg protein was loaded on SDS-PAGE gels for Western blotting. The following anti-mouse Abs were used: anti-phosphorylated JNK (p-JNK, Thr183/Tyr185) anti-JNK (Cell Signaling).

Histology and immunohistochemistry

Liver lobes were fixed in 4% paraformaldehyde for 24 h, transferred to 70% ethanol, and processed for embedding in paraffin. The following stains were applied: Harris’ hematoxylin (Electron Microscopy Sciences), eosin (Fisher Scientific), TUNEL staining to measure hepatocyte apoptosis, anti-F4/80 Ab to visualize macrophages/Kupffer cells. All staining was performed by institutional core facilities.

Flow cytometry

For splenocyte analysis, RBCs were lysed prior to further processing. Liver lymphocytes were obtained by density centrifugation using Percoll. Livers were disaggregated in 20 ml of 40% Percoll dissolved in William’s E medium and passed through a 100-μm nylon mesh to create single cell suspensions. The cell suspension was underlaid with 70% Percoll dissolved in sterile HBSS and centrifuged to generate an interface containing liver lymphocytes. The interface was removed and processed for flow cytometry. Cell numbers were counted using a Beckman Coulter Vi-Cell counter, and viability measured using trypan blue. The following Abs were used for flow cytometry (eBiosciences): anti-CD3 (clone 145-2C11), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-B220 (clone RA3-6B2), anti-NK1.1 (clone PK136), anti-Gr-1 (clone RB6-8C5), anti-CD11b (clone M1/70), anti-F4/80 (clone BM8), anti-CD62L (clone MEL-14), anti-CD44 (clone IM7), and anti-CD69 (clone 1H1.2F3). Propidium iodide uptake was used to label dead cells. Data was obtained on a BD FACSCalibur system and analyzed using FlowJo software.

RNA preparation and quantitative RT-PCR

RNA was prepared from total liver, sorted liver cell subsets, or splenic CD4⁺ T cells using TRIzol reagent (Invitrogen), and cDNA was obtained using a qScript cDNA supermix protocol (Quanta Biosciences). Gene expression was measured using SYBR Green reagent (Quanta Biosciences). All gene expression was measured using SYBR Green reagent (Quanta Biosciences). Gene expression levels were normalized to Actb (β-actin) or Hprt as indicated. Relative amount of each product was calculated using the 2⁻ΔΔCT method. Timp3 mRNA levels in liver parenchymal and nonparenchymal cells were measured by absolute quantification followed by normalization to absolute amounts of Hprt. Primer sequences are provided in Supplemental Table I.

Statistical analyses

Data are reported as mean ± SD or SEM. Comparisons were made by two-tailed Student t test and ANOVA; comparisons between Kaplan-Meier survival curves were made by log-rank test.

Results

Basal increase in CD4⁺ T cell and NKT cell populations in Timp3⁻/⁻ mice

We investigated the effect of Timp3 deficiency on steady-state composition of immune cells in the periphery by characterizing the lymphocyte subsets of liver and spleen from WT and Timp3⁻/⁻ mice. Flow cytometry analysis of liver lymphocytes showed a relative increase in CD4⁺ T cells and NKT cells (characterized as CD3⁺NK1.1⁺ cells; Fig. 1A). Specifically, absolute cell counts of basal CD4⁺ T cells and NKT cells were more than 2-fold higher, and this was reflected in an overall increase in the number of mononuclear cells (MNCs; Fig. 1B). However, splenic numbers of the same subsets, along with total MNCs, were comparable between WT and Timp3⁻/⁻ mice in basal conditions (Fig. 1C, 1D).

Spontaneous activation of liver CD4⁺ T cells and NKT cells in Timp3⁻/⁻ mice

Given that lymphocyte activation precedes infiltration into the periphery (5, 8, 16), we next analyzed the activation marker CD69 on splenocytes and liver lymphocytes by flow cytometry. A higher number of CD69 expressing CD4⁺ and CD8⁺ T cells as well as NKT cells were observed only in resting livers of Timp3⁻/⁻ mice (PBS-treated group; Fig. 2A), whereas comparable numbers existed in the spleen (PBS-treated group; Fig. 2B). After treatment with Con A, we also observed the induction of splenomegaly accompanied by an increase in cell surface CD69 expression on CD4⁺, CD8⁺, NKT, and NK cells in Timp3⁻/⁻ (Con A-treated
groups; Fig. 2A, 2B; representative histograms depict the gating strategy used to quantify CD69+ cells). ADAM17 is known to perform ectodomain shedding of L-selectin, an important early step in T cell activation. In vitro studies have suggested that TIMP3 inhibits this process in human lymphocytes (17, 18).

Consistent with these observations, we noted spontaneous shedding of L-selectin from CD4+ splenocytes in Timp3−/− mice (Supplemental Fig. 1). Thus, loss of TIMP3 results in a spontaneous basal increase of total liver lymphocytes that are composed of higher numbers of activated CD4+, CD8+, and NKT cells.

**FIGURE 1.** Timp3−/− mice exhibit spontaneous accumulation of hepatic CD4+ T cells and NKT cells. (A) Flow cytometry analysis of cell surface CD4/CD8 expression gated on CD3+ lymphocytes, and CD3/ NK1.1 resting lymphocytes from livers of 12-wk-old WT and Timp3−/− mice. (B) Counts of total MNCs and indicated immune cell subsets of liver lymphocytes calculated from (A). (C) Flow cytometry analysis of splenocyte populations in WT and Timp3−/− spleens. CD4/8 plots are gated on CD3+ cells. (D) Counts of total splenocyte numbers and indicated splenocyte subsets in untreated WT and Timp3−/− mice. Data are representative of at least four mice per condition. *p < 0.05, mean ± SD (n = 4).

**FIGURE 2.** TIMP3 deficiency increases basal liver lymphocyte activation, which is enhanced following Con A treatment. Total cell counts and representative flow cytometry histograms of activated liver lymphocyte (A) and splenocyte (B) subsets gated on CD69+ cells. WT and Timp3−/− mice were treated intravenously with PBS or 10 μg/g Con A, and lymphocyte activation was measured by cell surface CD69 expression using flow cytometry. Bars in the representative histograms depict the gating for CD69+ cells of the indicated immune cell subset. *p < 0.05, mean ± SD (n = 4).
TIMP3 deficiency sensitizes mice to T cell-mediated hepatitis induced by Con A

We have found previously that Timp3−/− mice have enhanced sensitivity to LPS-induced hepatitis caused by increased TNF bioactivity, but exhibit resistance to Fas-mediated fulminant hepatitis resulting from compound alterations in TNFR1 and EGFR signaling (13, 14). These models rely on resident macrophage (Kupffer cell) activity and hepatocyte-intrinsic responses to apoptotic stimuli, but do not address lymphocyte function in modulating hepatitis. Intravenous administration of Con A offers a model of autoimmune hepatitis induced by lymphocytes (19, 20); we therefore examined the susceptibility of Timp3−/− mice to Con A-induced hepatitis. Significantly more Timp3−/− mice succumbed to a low dose (10 μg/g) of Con A compared with WT controls (Fig. 3A). A greater increase in serum transaminase (ALT) levels at 6 and 18 h indicated enhanced hepatotoxicity (Fig. 3B). Histologic examination showed severe liver damage with extensive necrosis in Timp3−/− mice 18 h after treatment with Con A (Fig. 3C, arrows depict necrotic regions). Thus, TIMP3 deficiency exacerbates autoimmune hepatitis in mice.

Liver damage in Timp3−/− mice is dependent on TNF
TIMP3 is a negative regulator of TNF shedding in vivo, and this cytokine is a key contributor to Con A-induced hepatitis (3, 7, 12, 14, 19, 21, 22). Immunoblotting showed phosphorylation of the TNF signaling effector JNK/SAPK at early (6 h) and late (18 h) time points after Con A administration in Timp3−/− mice and found that loss of Tnf in WT and Timp3−/− backgrounds protected against hepatotoxicity as indicated by lower serum ALT (Fig. 4A). We tested whether increased TNF bioactivity underlies accelerated induction of autoimmune hepatitis in Timp3−/− mice and found that loss of Tnf in WT and Timp3−/− backgrounds protected against hepatotoxicity as indicated by lower serum ALT (Fig. 4B). Histologic analyses confirmed negligible liver damage and hemorrhaging (Fig. 4C, arrows), and TUNEL staining revealed an absence of hepatocyte apoptosis in Tnf−/− or Timp3−/−, Tnf−/− mice versus Timp3−/− mice (Fig. 4D).

Amplified Th1 cytokine response in Timp3−/− mice following Con A administration
Analysis of early serum cytokine release over 6 h following Con A administration showed accelerated kinetics of cytokine release in Timp3−/− mice compared with all control groups. Tnf−/− and Timp3−/−, Tnf−/− mice exhibited significant abrogation of cytokine release (Fig. 5A). Of the Th1 cytokines, TNF peaked at 2 h and remained high until 6 h, IFN-γ exhibited a transient 5-fold greater level at 4 h, IL-6 continued to rise dramatically over 6 h, and the Th2 cytokine IL-4 peaked at 2 h. IL-6 plays paradoxical roles in acute hepatic inflammation depending on the duration of its bioavailability (23, 24), and IL-4-dependent signaling through STAT6 is required for promoting T cell-mediated hepatitis (20, 25). These data demonstrate that TIMP3 deficiency enhances the Th1 cytokine response during autoimmune hepatitis. Indeed, Timp3−/− mice displayed sustained increase in Th1 cytokines 18 h after Con A treatment. We also identified increases in factors that promote the Th1 response, namely IL-1β, IL-12, and the chemotractant MCP-1 (Fig. 5B). Of note, IL-12 release by activated macrophages promotes T cell activation (8, 26), and consistent with this we observed that immunohistochemical analysis showed greater association of F4/80+ macrophages to areas of necrosis in Timp3−/− livers (Fig. 5C). Finally, measurement of local cytokine production revealed dramatically higher gene expression of hepatic Il1b, Tnfα, Ifng, Il6 and Il12p35 in Timp3−/− mice following Con A administration (Fig. 5D). These data indicate that TIMP3 suppresses Th1-driven inflammation in the liver.

Loss of Timp3 and Tnf elevates serum IL-10 and IL-17A levels and depletes liver CD4+ T cells
We observed increased serum IL-17A and IL-10 in Timp3−/−; Tnf−/− mice, which is particularly intriguing because loss of Timp3 alone only modestly affected these cytokines (Supplemental Fig. 2A). The role of Th17 cells is still emerging in autoimmune hepatitis and is primarily associated with hepato-protective IL-22 production (27). IL-10 is also suggested to be protective through its suppression of the Th1 response and its involvement in immunologic tolerance (6). In addition, we measured decreases in splenic and hepatic CD4+ T cell numbers, consistent with abrogated liver damage in Timp3−/−, Tnf−/− mice (Supplemental Fig. 2B). Further study is needed to elucidate how metalloproteinase inhibitors affect these immunoregulatory cytokines.

Cell-intrinsic TIMP3 is not required for CD4+ T cell activation
Together the four TIMPs inhibit metalloproteinase activity of all known 24 MMPs, nine active ADAMs and four active ADAM-TS enzymes, but the regulation of lymphocyte function by TIMPs remains uninvestigated (9). We examined the cell-autonomous role of TIMP3 in lymphocyte activation in vitro. First, gene expression analysis of Timp and specific Adam and Mmp genes in resting splenic CD4+ T cells showed low levels of Timp1, Timp3, and Timp4, but significantly higher expression of Adam10 and Adam17 (Fig. 6A). Stimulation of cultured WT and Timp3−/− CD4+ T Cells (1.0 μg/ml Con A over 72 h) showed comparable expansion of CD4+ T cells in both genotypes when measured by MTS (Fig. 6B). IL-2 is required for expansion and growth of Ag-specific T cells subsequent to TCR ligation, and its concentration was comparable between WT and Timp3−/− CD4+ T cell culture media (Fig. 6C). TNF levels also remained comparable across all time points (Fig. 6D). These data demonstrate that TIMP3 is dispensable for cell-autonomous activation, expansion, and effector function of CD4+ T cells.

TIMP3 derived from nonhematopoietic tissue regulates liver lymphocyte infiltration
To evaluate the physiologic relevance of hematopoietic and non-hematopoietic TIMP3 in vivo, we generated congenic bone-
marrow chimeras as depicted in Fig. 7A. WT (CD45.1\textsuperscript{+}) recipients were reconstituted with Timp3\textsuperscript{2/2} (CD45.2\textsuperscript{+}) bone marrow cells and Timp3\textsuperscript{2/2} recipients with WT bone marrow, achieving >90% reconstitution at 8 wk after transplant (Fig. 7A, bar graphs). We observed that only stromal TIMP3 deficiency reproduced the spontaneous basal increase in CD3\textsuperscript{+} liver lymphocytes (Fig. 7B) as described in nonirradiated Timp3\textsuperscript{2/2} mice (Fig. 1). Thus, non-hematopoietic TIMP3 negatively regulates the spontaneous infiltration of circulating lymphocytes into the hepatic microenvironment.

FIGURE 4. TNF bioactivity is causal to enhanced hepatotoxicity in Timp3\textsuperscript{2/2} mice. (A) Immunoblots of JNK phosphorylation in liver lysates from WT and Timp3\textsuperscript{2/2} mice treated with PBS or Con A. (B) Serum transaminase (ALT) levels in mice of indicated genotypes 6 h after treatment with PBS or Con A. (C) H&E stain of livers treated with PBS or Con A. Arrowheads depict necrosis. (D) TUNEL staining of livers 6 h after PBS or Con A treatment. Brown nuclei depict apoptotic cells. Histology is representative of at least four mice per condition. Scale bars, 25 μm. *p ≤ 0.04, mean ± SEM (n = 4).

FIGURE 5. TIMP3 deficiency enhances Th1 cytokine response and macrophage activation following Con A administration. (A) Measurement of serum cytokine levels over 6 h following Con A administration using a cytokine bead array. (B) Serum levels of indicated cytokines and chemokines and growth factors measured 18 h after PBS or Con A administration. (C) Immunohistochemical staining for F4/80 (brown cells) depicting increased macrophage association with areas of necrosis in Timp3\textsuperscript{2/2} livers 18 h following Con A administration. (D) Quantitative PCR measurement of mRNA levels of indicated cytokines in total liver obtained from mice treated with PBS or 10 μg/g Con A for 6 h. Gene expression is normalized to Hprt. Images are representative of at least three mice per condition. Scale bars, 100 μm. *p ≤ 0.04, mean ± SEM (n = 4 for A, B; n = 3 for D).
Stromal TIMP3 protects against Con A-induced hepatitis

We treated the chimeric mice generated above with Con A to determine whether TIMP3 derived from nonhematopoietic tissue would provide hepatoprotection against autoimmune hepatitis. Treatment with Con A induced serum toxicity and liver damage at 6 h in Timp3<sup>−/−</sup> recipients harboring WT bone marrow, whereas WT recipients with Timp3 null hematopoietic cells did not exhibit hepatotoxicity (Fig. 8A, 8B). TUNEL staining showed extensive hepatocyte death only in Timp3<sup>−/−</sup> recipients (Fig. 8C). Time course analysis of serum cytokines showed significant elevations in Th1 cytokines IFNγ, IL-2, and IL-6 over 6 h after Con A administration in these mice (Fig. 8D). It is important to consider that Kupffer cells may exhibit radioresistance, and thus existing recipient liver macrophage populations may contribute to the enhanced liver damage in Timp3<sup>−/−</sup> mice (28). To address this, WT and Timp3<sup>−/−</sup> mice were pretreated with gadolinium chloride (GdCl<sub>3</sub>) to deplete liver macrophages prior to Con A administration (Supplemental Fig. 3A). WT mice exhibited a significant decrease in serum toxicity and liver damage following Kupffer cell depletion; however, Timp3<sup>−/−</sup> mice remained highly sensitized to Con A-induced hepatitis regardless of the presence or absence of Kupffer cells (Supplemental Fig. 3B, 3C). This finding indicates that the increased liver lymphocyte populations rather than liver macrophage activation may be the effectors of hepatic cytotoxicity. Finally, to directly identify relevant cellular sources of TIMP3 in the resting and inflamed hepatic environment, individual cell types were sorted from WT livers treated with PBS or Con A for 18 h. Hepatocytes (parenchymal cells), hepatic stellate cells, liver sinusoidal endothelial cells (nonparenchymal cells) and Kupffer cells (hematopoietic cells) were isolated and Timp3 mRNA levels were compared (Fig. 8E, 8F). Intriguingly, hepatocytes were observed to be the dominant TIMP3 source in resting livers; however, upon induction of hepatitis, hepatocytes dramatically downregulated Timp3 transcription and hepatic stellate cells showed significant induction of Timp3 gene expression (Fig. 8F). By comparison, sinusoidal endothelial cells and Kupffer cells were not significant sources of hepatic TIMP3. These data show that hematopoietic TIMP3 does not affect cytokine release and activation of T lymphocytes, whereas TIMP3 in nonhematopoietic tissues provides protection against Con A-induced hepatitis.

Discussion

In this study, we demonstrate that the metalloproteinase inhibitor TIMP3 regulates liver lymphocyte infiltration and proinflammatory function during acute hepatitis. Loss of Timp3 led to spontaneous expansion of liver CD4<sup>+</sup> T and NKT cells, and this resulted in increased hepatotoxicity following Con A treatment. Examination of TNF signaling revealed that elevated serum TNF levels and signaling were causal to enhanced morbidity in Timp3<sup>−/−</sup> mice. We noted that CD4<sup>+</sup> T cells isolated from spleen expressed comparably low levels of Timp genes in WT mice; furthermore, WT and Timp3<sup>−/−</sup> CD4<sup>+</sup> T cells exhibited comparable expansion and cytokine production following in vitro stimulation with Con A. We thus hypothesized that TIMP3 generated by nonhematopoietic tissue, such as the liver parenchyma, inhibited lymphocyte infiltration, and we tested this by the generation of bone marrow chimeras. Indeed, Timp3<sup>−/−</sup> recipients of WT bone marrow exhibited spontaneous increase of CD3<sup>+</sup> cells and total liver mononuclear cells. In these chimeric mice, induction of Con A-mediated hepatitis reproduced the enhanced cytokine response and hepatotoxicity. Fig. 8G models the role of Timp genes in autoimmune hepatitis, illustrating the requirement of nonhematopoietic TIMP3 in maintaining hepatic lymphocyte homeostasis and controlling a Th1 cytokine response.

The liver has a unique role in maintaining tolerance to food Ags and gut flora that drain in, while generating protection against exogenous biological insults such as hepatitis B and C virus, bacterial Ags, and nonbiologic agents. Kupffer cells, sinusoidal endothelial cells, and even hepatocytes participate in local Ag

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**FIGURE 6.** TIMP3 is dispensable for cell-autonomous activation and proliferation of CD4<sup>+</sup> T cells. (A) Gene expression of indicated Timps, Adams, and Mmps in resting CD4<sup>+</sup> T cells isolated from spleens of WT mice. Data are one of three independent analyses, normalized to β-actin (Actb). (B) Proliferation of WT and Timp3<sup>−/−</sup> CD4<sup>+</sup> T cells following stimulation with Con A over 72 h. (C and D) Cytokine release of (C) IL-2 and (D) TNF into culture media of CD4<sup>+</sup> T cells stimulated with Con A. Data are mean ± SD (n = 3) and representative of two independent experiments.

**FIGURE 7.** Depletion of nonhematopoietic TIMP3 results in spontaneous liver lymphocyte accumulation. (A) Schematic depicting generation of radiation chimeras. Stacked graphs indicate comparable bone marrow reconstitution in WT and Timp3<sup>−/−</sup> recipients as measured by flow cytometry (anti-CD45.1 versus anti-CD45.2 staining of hematopoietic cells). (B) Counts of total MNCs and subsets of liver lymphocytes isolated from radiation chimeras 8 wk after reconstitution. Liver lymphocytes were isolated and counted by flow cytometry using indicated Abs: T cells, CD3<sup>+</sup>NK1.1<sup>+</sup>; NKT cells, CD3<sup>+</sup>NK1.1<sup>+</sup>; NK cells, CD3<sup>−</sup>NK1.1<sup>+</sup>; macrophages (MΦ), F4/80<sup>+</sup>. *p < 0.01, mean ± SD (n = 3).
**FIGURE 8.** Stromal TIMP3 protects against a Th1 proinflammatory cytokine response and autoimmune hepatitis. (A) Serum transaminase (ALT) levels in WT and Timp3−/− recipients 6 h after Con A treatment. (B) Histologic analysis depicting liver damage (H&E) 6 h after Con A treatment. (C) Immunohistochemical TUNEL staining (brown nuclei) indicating hepatocyte death following Con A treatment. (D) Serum levels of indicated cytokines over 6 h following Con A treatment as measured by cytokine bead array. (E) Semi-quantitative RT-PCR assays confirming purity of liver cell sorting protocol. Electrophoresis is representative of three mice. (F) Quantitative PCR measurement of Timp3 mRNA levels in sorted liver cell populations obtained from WT mice treated with PBS or 10 µg/g Con A for 18 h. Gene expression is normalized to Hprt. (G) Schematic illustrating the regulation of lymphocyte infiltration and cytokine release by nonhematopoietic TIMP3. Activation and accumulation of liver lymphocytes generates a positive-feedback loop and predisposes to autoimmune hepatitis. Histology represents at least three mice per condition. Scale bars, 25 µm. *p < 0.05, mean ± SEM (n = 3). Hep, hepatocytes; HSC, hepatic stellate cells; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells.

Presentation and expression of innate immune receptors such as TLR4; this process is postulated to generate tolerance to frequently encountered Ag (29). Alternatively, circulating dendritic cells can contribute to protective proinflammatory responses during infection by recruiting lymphocytes to the liver microenvironment following an encounter with a pathogen (30, 31). Our findings introduce the role of metalloproteinase inhibitors in tolerance versus hepatoprotection. The use of more sophisticated systems (e.g., hepatitis B virus, hepatitis C virus, and CMV models of viral infection; Listeria monocytogenes and Mycobacterium tuberculosis models of bacterial infection) will prove useful in delineating the contribution of Timp genes to acute CD8+ CTL or CD4+ T cell responses against virus (32, 33), and T helper subset function against bacterial granulomas (34). Given the overabundance of CD4+ T cells and NKT cells observed in Timp3 null mice, we may find enhanced pathogen clearance and protection in an Ag-specific manner. Potentially, TIMP3 can regulate the sensitization threshold required for the cellular immune response in the liver.

Several possible mechanisms may be involved in lymphocyte infiltration upon loss of TIMP3. Disruption of chemokine gradients is a prominent candidate, as increased hepatic levels of CC- and CXC-chemokines have shown to promote acute liver injury in murine models and patients alike (35, 36). Specifically, increases in liver CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) and CXCR3 ligands (CXCL9-11) have been reported in human liver biopsy specimens of acute liver pathologies such as fulminant hepatitis and acute viral hepatitis (37, 38). Metalloproteinases MMP1, 2, 3, and 14 can cleave CXC-chemokines (CCL2, 7, 8, 13), whereas CXCL11 is shed by numerous MMPs (MMP1, 2, 3, 9, 13, 14) (39). Physiologically, chemokines are immobilized on the extracellular matrix by binding with glycosaminoglycan-associated proteoglycans such as syndecan. Syndecan-1 harbors “sinks” of CXC-chemokines, and its shedding by MMP7 is an example of the involvement of metalloproteinases in generating CXCL1 gradients. l-Selectin shedding from lymphocyte surfaces is an important early step in their egress from lymphoid organs and into the periphery (40). ADAM17/TACE is the only metalloproteinase known to shed l-selectin in vivo, and TIMP3 is the only endogenous inhibitor of ADAM17 (9). In the current study, loss of Timp3 may potentially generate a permissive environment for l-selectin shedding and uncoordinated lymphocyte egress into the systemic microenvironment. Finally, TIMP3 is established as a suppressor of TNF-induced hepatocyte stress signaling during chronic inflammation following partial hepatectomy or in models of hepatosteatosis and type 2 diabetes (11, 12). Elevated production of reactive oxygen species is a feature of TNF-mediated stress signaling and potentially contributes to the immunopathology described in this study (41, 42).

Immune cell and cytokine composition in the periphery determines the progression of autoimmune (43, 44). Our study demonstrates that loss of control over metalloproteinase activity significantly affects multiple cytokines, both systemically and in the liver microenvironment. Indeed, serum and hepatic levels of cytokines involved in Th1-mediated autoimmunity (e.g., IFN-γ, IL-6) were elevated in mice lacking TIMP3. In addition, a dramatic increase was observed in TNF-α, IL-1β, and IL-12p70 levels in TIMP3-deficient mice following induction of hepatitis. These specific cytokines are known to be induced by activated monocytes during an inflammatory response. Although depletion of Kupffer cells did not significantly abrogate the sensitization of Timp3−/− mice to Con A-mediated hepatitis, it is likely that the presence of infiltrated lymphocytes in the steady state liver further enhances Kupffer cell activity in a feedback loop (7, 45). Cumulatively, unchecked metalloproteinase activity results in a hepatic cytokine storm that drives autoimmune disease. Although the identification of precise mechanisms warrants further investigation, our study reveals a regulatory function for endogenous metalloproteinase inhibitors in cellular immunity. Our results show stromal TIMP3 as a noncell-autonomous regulator of lymphocyte entry into the liver microenvironment, implicating its requirement in immunosuppression and prevention of liver injury during autoimmune hepatitis. Stromal TIMP3 suppresses
undesired lymphocyte infiltration and protects against hepatitis opening avenues to better elucidate the role of Timps in autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

References

SUPPLEMENTAL DATA

Supplemental Figure Legends

*Supplemental Figure 1* – Constitutively elevated shedding of L-selectin in CD4⁺ splenocytes from *Timp3⁻/⁻* mice.

Wildtype and *Timp3⁻/⁻* mice were treated with PBS or 10μg/g Con A for 6h. Splenocytes were obtained and flow cytometry analysis of L-selectin and CD44 performed on CD4⁺ T cells. Flow cytometry plots are representative of 3 mice per condition. *P≤0.05 vs. WT, mean ± S.E.M. (n=3).

*Supplemental Figure 2* - *Timp3⁻/⁻;Tnf⁻/⁻* compound deletion elevates serum IL-10, IL-17A and depletes splenic and hepatic CD4⁺ T cells.

(A) Serum levels of IL-10 and IL-17A following con A treatment in mice of indicated genotypes.

(B) Counts of CD3⁺CD4⁺ T cells in spleen and liver of mice 6 hours after treatment with PBS or con A. *P≤0.05 vs. WT, mean ± S.E.M. (n≥3).

*Supplemental Figure 3* – Depletion of Kupffer cells does not completely ameliorate Con A-mediated hepatotoxicity in *Timp3⁻/⁻* mice.

(A) Immunohistochemical staining for F4/80 depicting successful depletion of liver macrophages by gadolinium chloride treatment (GdCl₃), as shown by a lack of brown staining in the GdCl₃ group. Wildtype and *Timp3⁻/⁻* mice were treated with 60μg/g GdCl₃ intraperitoneally 48h and 24h prior to PBS or Con A treatment. (B) Histology (H & E) depicting onset of extensive liver damage and hemorrhaging in *Timp3⁻/⁻* mice 6h after Con A treatment, regardless of Kupffer cell depletion. (C) Serum ALT measurements show sustained liver toxicity in *Timp3⁻/⁻* mice despite
Kupffer cell depletion. All histology is representative of 3 mice per condition. Bars = 100μm.

*P ≤ 0.05, mean ± S.E.M. (n=3).

**Supplemental Tables**

*Supplemental Table 1 – Primer sequences for quantitative RT-PCR analysis and genotyping*

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Murthy et al. Supplemental Figure 1
Murthy et al. Supplemental Figure 2
Supplemental Figure 3

A

WT  Timp3\(^{-/-}\)

PBS

GdCl\(_3\)

B

WT  Timp3\(^{-/-}\)

PBS

GdCl\(_3\) (60\(\mu\)g/g)

Con A (10\(\mu\)g/g)

C

[Serum ALT (U/L x 100)]

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