The Proinflammatory Myeloid Cell Receptor TREM-1 Controls Kupffer Cell Activation and Development of Hepatocellular Carcinoma

Juan Wu1, Jiaqi Li1, Rosalba Salcedo2,3, Nahid F. Mivechi1, Giorgio Trinchieri2, and Anatolij Horuzsko1

Abstract
Chronic inflammation drives liver cancer pathogenesis, invasion, and metastasis. Liver Kupffer cells have crucial roles in mediating the inflammatory processes that promote liver cancer, but the mechanistic basis for their contributions are not fully understood. Here we show that expression of the proinflammatory myeloid cell surface receptor TREM-1 expressed by Kupffer cells is a crucial factor in the development and progression of liver cancer. Deletion of the murine homolog Trem1 in mice attenuated hepatocellular carcinogenesis triggered by diethylnitrosamine (DEN). Trem1 deficiency attenuated Kupffer cell activation by downregulating transcription and protein expression of interleukin (IL)-6, IL-1β, TNF, CCL2, and CXCL10. In addition, Trem1 ablation diminished activation of the p38, extracellular regulated kinase 1/2, JNK, mitogen-activated protein kinase, and NF-κB signaling pathways in Kupffer cells, resulting in diminished liver injury after DEN exposure. Adoptive transfer of wild-type Kupffer cells to Trem1-deficient mice complemented these defects and reversed unresponsiveness to DEN-induced liver injury and malignant development. Together, our findings offer causal evidence that TREM-1 is a pivotal determinant of Kupffer cell activation in liver carcinogenesis, deepening mechanistic insights into how chronic inflammation underpins the development and progression of liver cancer. Cancer Res. 72(16): 3977–3986. © 2012 AACR.
loss of TREM-1–mediated amplification of inflammation on the course of inflammation-associated tumorigenesis.

Materials and Methods

Animals

C57BL/6J mice were purchased from the Jackson Laboratory. The generation of mice deficient in Trem1 (on C57BL/6J genetic background) is described in Supplementary Fig. S1. All mice were housed in a specific pathogen-free environment in the GHSU animal facilities, and all animal procedures were approved by the Institutional Animal Care and Use Committee.

Tumor induction and analysis

Fifteen-day-old male mice were injected intraperitoneally (ip) with 25 mg/kg DEN (Sigma-Aldrich). After 8 or 14 months, mice were sacrificed and their livers removed and separated into individual lobes. Externally visible tumors (≥0.5 mm) were counted and measured. Large lobes were fixed in 4% paraformaldehyde overnight and paraffin embedded. Sections (7 μm) were hematoxylin and eosin stained and tumor-occupied areas were measured. For short-term studies of inflammation and liver injury, 6- to 8-week-old male mice were injected ip with 100 mg/kg DEN. Apoptosis was determined by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (ApopTag Red in Situ Apoptosis Detection Kit; Millipore). The number of TUNEL-positive hepatocytes was determined by manual counting of 5 high-power fields per liver (200 cells per field). The mean of each time point was plotted as a percentage of the number of labeled nuclei. To examine cell proliferation, mice were injected ip with 1 mg/mL bromodeoxyuridine (BrdUrd) (Sigma) 2 hours before sacrifice, and paraffin sections were stained using the BrdU In-Situ Detection Kit (BD Biosciences). Liver injury was examined by measuring circulating alanine aminotransferase (ALT; Pointe Scientific).

Generation of bone marrow–derived macrophages

Bone marrow–derived macrophages (BMDM) were differentiated in vitro from bone marrow cells cultured with macrophage colony-stimulation factor (see Supplementary Methods for details).

Antibodies and flow cytometric analysis

Cells were stained with monoclonal antibodies (mAb) anti-CD11b-APC, -PerCP-Cy5.5 (M1/70, rat IgG2b), anti-F4/80-FITC, or -APC (BM8, rat IgG2a), anti-Ly6C-PE, or –PE/Cy7 (HK1.4, rat IgG2c), anti-Ly6G-FITC, or –PerCP-Cy5.5 (1A8, rat IgG2a) or anti-TREM-1-PE (174031, rat IgG2a). All primary reagents were purchased from BD Biosciences or from e-Bioscience unless specified. Flow cytometric data were collected using a FACSCanto flow cytometer (BD Biosciences) and analyzed with CellQuest software flow cytometer (BD Biosciences).

Detection of cytokine and chemokine production

Interleukin (IL)-1β, IL-6, TNF, CCL2, and CXCL10 in the supernatants of BMDMs or in the serum were measured with ELISA kits (from ebioscience or from BioLegend).

Kupffer cell isolation, ablation, adoptive transfer of Kupffer cells, and preparation of necrotic hepatocytes

Kupffer cells were isolated by collagenase (Worthington) digestion, and density gradient centrifugation followed by MACS magnetic bead separation (Miltenyi Biotec; see Supplementary Methods for details)

Chemical cross-linking for analysis of protein–protein interactions (in vitro)

Cross-linking reactions with dimethyl adipimide (DMA) or bis(sulfosuccinimidyl) suberate (BS3) with spacer arms of 8.6 and 11.4 Å, respectively, were carried out in 20 mmol/L HEPES buffer (pH 7.5; see Supplementary Methods for details).

Binding analysis using surface plasmon resonance

Murine TREM-1, RAGE, and unrelated CsrA protein with a similar isoelectric point were dissolved in HBS-EP buffer (10 mmol/L HEPES, pH 7.4/150 mmol/L NaCl/3.4 mmol/L, EDTA/0.005%, Surfactant P20; GE Healthcare). Surface plasmon resonance (SPR) experiments were carried out by using a BIACore X100 system (GE Healthcare; see Supplementary Methods for details).

RNA isolation and cDNA-based RT Profiler microarray analysis

RNA from total liver cells, hepatocytes, and Kupffer cells were isolated and subjected to analysis using RT Profiler Mouse Toll-Like Receptor Signaling Pathway Microarray (SABiosciences) according to the manufacturer’s protocol. The average fold induction/decrease was obtained by comparison of wild-type (WT) with Trem1–/– mice.

NanoString analysis

NanoString nCounter gene expression assay was carried out using 2 specific probes (capture and reporter) for each gene of interest (see Supplementary Methods for details).

Real-time PCR, histology, immunohistochemistry, immunoprecipitation, and Western blot

These assays were carried out by standard procedures. For more information, see Supplementary Methods.

Statistical analysis

All experiments were carried out with at least 3 to 15 mice. Statistical analyses were carried out using GraphPad Prism software version 5.0 (GraphPad Software Inc.). Differences between 2 groups were evaluated using a Student t test. Differences at P < 0.05 were considered significant.

Results

Deletion of TREM-1 attenuated the development of inflammation-associated HCC in response to the chemical procarcinogen DEN

In this study, we evaluated the role of TREM-1 in DEN-induced hepatocellular carcinogenesis using newly generated Trem1–/– mice. The Trem1–/– mice were generated on a C57BL/6J genetic background as described in Supplementary Fig. S1. HCC is a typical example of inflammation-linked cancer, and both chemically and genetically induced HCCs depend on inflammatory signaling (8–11). A single injection of DEN to
2-week-old WT male mice resulted within 8 months in the induction of α-fetoprotein (AFP)-expressing HCCs, many of which were large with evident neovascularization (Fig. 1A, C, E, G, and H; ref. 28). AFP is transcribed at high levels in the fetal liver and at low levels in the fetal gut. AFP synthesis is rapidly repressed at birth in the liver and gut. Although in adult animals AFP is normally found at extremely low levels in the liver, AFP is reactivated during liver regeneration and in HCC, an important diagnostic marker for liver cancer. Unlike WT mice, Trem1−/− male mice given DEN at the same age were tumor free at 8 months. At 14 months, only 4% of Trem1−/− mice developed small HCCs, whereas all WT mice at that time had developed large numbers of typical HCCs with maximal tumor diameters (Fig. 1B, D, and F–H). These data indicate that efficient HCC induction in response to DEN administration requires TREM-1.

**TREM-1 deletion decreases DEN-induced liver damage and compensatory proliferation during early tumor promotion**

DEN administration to mice causes a number of biochemical changes, including DEN-induced damage, apoptosis, necrosis, and cytokine production, that lead to compensatory proliferation of hepatocytes and create an inflammatory environment, that within several months, results in the development of HCC.
and Trem1 mice but was significant higher in WT mice (Fig. 3A). Both WT and Trem1−/− naive mice had a limited number of neutrophils in their livers. However, DEN administration significantly increased the number of neutrophils in livers from WT mice but not in Trem1−/− mice (Fig. 3A). The number of monocytes in peripheral blood and livers was also significantly lower in DEN-treated Trem1−/− mice than WT mice (Fig. 3B). Thus, the recruitment of inflammatory cells such as neutrophils and monocytes was impaired in Trem1−/− mice after DEN administration, suggesting that TREM-1 controls this important component of inflammatory responses.

Flow cytometric analyses of isolated cells from WT mice revealed that the cells from liver tissues expressing TREM-1 were neutrophils and Kupffer cells, identified as F4/80+ CD11b+ Ly6G− Ly6C+ cells. Immunohistochemistry and flow cytometric analysis of liver tissue revealed that hepatocytes from naïve WT mice did not express TREM-1. As expected, all cell types isolated from the liver of Trem1−/− mice were negative for TREM-1 surface expression (Supplementary Fig. S2C), whereas Kupffer cells and neutrophils in these mice expressed the reporter EGFP (Supplementary Fig. S2D). Injection of DEN into WT mice led to significant upregulation of the Trem1 mRNA accumulation and increased TREM-1 protein surface expression on Kupffer cells (Supplementary Fig. S2A and S2B). DEN treatment did not affect TREM-1 expression on liver neutrophils. Thus, the major population of TREM-1-positive cells in liver is represented by Kupffer cells that are the key cells in induction of the inflammatory responses that drive hepatocarcinogenesis (29).

To understand the impact of DEN-induced inflammation and tissue damage on liver cell types, we analyzed the expression of the gene encoding proinflammatory and signaling molecules in total liver samples, isolated hepatocytes, and purified Kupffer cells using RT-PCR--based TLR signaling pathway microarray. The gene expression patterns of livers...
from untreated WT and Trem1−/− mice were similar. However, 4 hours after DEN exposure, several genes were differentially expressed in the livers from Trem1−/− mice compared with WT mice. The most downregulated genes in Trem1−/− mice compared with WT mice included Ccl2 and Cxcl10 (Fig. 4A). CCL2 and CXCL10 are chemokines important for the regulation of inflammatory and immune cell migration, differentiation, and function. Kupffer cells are the major source of CCL2 and CXCL10 production in liver, and Kupffer cells from Trem1−/− mice had a lower accumulation of the mRNA of these chemokines compared with the levels observed on Kupffer cells from WT mice. When total liver as well as purified hepatocytes and Kupffer cells were analyzed, Kupffer cells were found to be the cell type in which the most striking difference between WT and Trem1−/− mice was with downregulation of Il1b, Il6, and Tnf in the latter mice (Fig. 4C). The hallmarks of DEN-induced liver injury are necrosis, inflammation, and hepatocellular damage. The damage caused by necrosis and inflammation leads generally to proliferation of the remaining hepatocytes, a characteristic of liver regeneration. The RNA-based NanoString analysis revealed that DEN treatment significantly upregulated sets of genes regulating inflammatory responses (e.g., Il1a, Il18, Cxcl1, Sl100a11, and Sl100a16; Fig. 4D), cell-cycle regulation (e.g., Ccnb2, Ccnd1, and Cdk4; Fig. 4E), and apoptosis (e.g., Bcl2l4, Tnfrsf6, and Cdkn1; Fig. 4F), in the liver of WT mice and not in Trem1−/− mice. Moreover, a set of the genes associated with DNA damage (e.g., Msh2, Msh6, and Chek2; Supplementary Fig. S3A), and genes involved in pivotal pathways associated to promotion of liver cancer, including Akt, Met, and β-catenin pathways (Supplementary Fig. S3B) was significantly downregulated in the liver of Trem1−/− mice after DEN treatment.
Adoptive transfer of WT Kupffer cells to Trem1-deficient mice reversed the unresponsiveness to DEN-induced liver injury

To test whether the impaired activation of Kupffer cells in Trem1−/− mice plays a major role in the low response of liver injury to DEN treatment Trem1−/− or WT mice in which Kupffer cells had been depleted by treatment with clodronate-containing liposomes were reconstituted with Kupffer cells isolated from WT mice and analyzed for DEN-induced inflammatory/injurious responses (Fig. 5A). The proportion of i.v. adoptively transferred F4/80-positive cells that migrated into the liver of depleted Trem1−/− and WT recipients at 18 hours was comparable (Fig. 5D). Adoptive transfer of Kupffer cells in both groups of mice slightly increased the levels of serum ALT (Figs. 2F and 5B). In contrast, a robust increase in levels of serum ALT was determined in WT and Trem1−/− mice reconstituted with WT Kupffer cells at 24 and 48 hours after DEN injection (Fig. 5B). The increase in liver-specific enzyme in the serum correlated with an increase in liver cell apoptosis and in the levels of serum IL-6 and IL-1β cytokines and CCL2 chemokine (Fig. 5C and not depicted). The NanoString analysis revealed that the majority of the genes expressed in the livers of Trem1−/− mice reconstituted with WT Kupffer cells responded to DEN treatment in a similar manner to that of WT-reconstituted mice. The most upregulated genes in Trem1−/− mice reconstituted with WT Kupffer cells were those involved in regulating inflammatory response (Il1a, Il18, and Cxcl1), and cell-cycle progression (Ccne1, Ccnd1, and Cdk4) (Fig. 5E and F). These data suggest that Trem1+/+ Kupffer cells are activated and normally transferred in Trem1−/− mice in response to DEN exposure and induce the same magnitude.
of liver injury observed in Kupffer cell–depleted/reconstituted WT mice. These data also show that hepatocytes from Trem1−/− and WT mice responded equally to activated Kupffer cells.

TREM-1 is capable of binding HMGB1 that could represent an activating ligand

In various experimental models, the compensatory hepatocyte proliferation was described to require the production by Kupffer cells of hepatic mitogens such as IL-6 and TNF (8, 30). BMDMs from Trem1−/− mice exposed to necrotic hepatocytes produced significantly lower levels of CCL2, IL-1β, IL-6, and TNF than WT macrophages (Supplementary Fig. S4). The substances released by necrotic cells that are thought to trigger an inflammatory signal transduction cascade and cytokine/chemokine production by Kupffer cells include TREM-1 potential ligands such as High Mobility Group Box 1 (HMGB1) and heat shock protein 70 (HSP70) (31). Immunoblotting of the product of necrotic hepatocytes (PNH) isolated from DEN-treated and nontreated WT mice showed the presence of both HSP70 and HMGB1 proteins (Fig. 6A). However, immunoprecipitation of DEN-treated PNH with TREM-1Fc protein followed by immunoblotting with anti-HMGB1 or HSP70 mAb indicated that HMGB1 and not HSP70 directly interact with TREM-1 in liver cells from WT mice after DEN treatment (Fig. 6B).

The association of TREM-1 with HMGB1 was confirmed by chemical cross-linking assays (Fig. 6C). We observed that TREM-1 can form stable association with HMGB1 by using dimethyl adipimidate (DMA) with spacer arm of 8.6 Å (Fig. 6C, right) and not by bis(sulfosuccinimidyl) suberate (BS3) with spacer arm 11.4 Å, respectively (Fig. 6C, left). The binding between TREM-1 and HMGB1 molecules was further analyzed by SPR. The BIAcore sensograms of the 2 proteins showed a rapid increase of response units (RU) indicating binding of these proteins to the immobilized HMGB1 on the chip followed by a decrease of RU resulting from a loss of the binding molecules upon washing (Fig. 6D). Binding of RAGE and TREM-1 to HMGB1 was concentration dependent (Fig. 6D). The affinity constants, $K_d$, were determined by SPR technique and found to be $K_d = 0.2$ µmol/L for RAGE and HMGB1 and $K_d = 35.4$ µmol/L for TREM-1 and HMGB1, respectively. Thus, a combination of immunoprecipitation, chemical cross-linking of proteins, and SPR indicated that TREM-1 is capable of binding HMGB1 that could represent an activating ligand.

Discussion

The chronic liver injury caused by several factors triggers inflammation, which drives the compensatory proliferation of intact hepatocytes and liver progenitors leading to initiation and progression of HCC development. Exposure to DEN induces hepatocyte death, and increased hepatocyte death results in a more extensive compensatory proliferative response (10). Necrotic hepatocytes are the major source that triggers the activation of Kupffer cells, which plays a critical role in initiation of the inflammatory response. Our microarray, NanoString analysis, flow cytometry, and ELISA data suggest that in DEN-treated WT mice there is an increased...
number of activated Kupffer cells that express upregulated costimulatory molecules CD80/CD86, with a cytokine profile favoring a type I inflammatory response. In contrast, Trem1−/− Kupffer cells show no changes in these activation markers. Thus, initial damage of hepatocytes mediated by DEN may not be able to activate Trem1−/− Kupffer cells. The RNA-based NanoString analysis revealed that the majority of the genes expressed in the livers of Trem1−/− mice reconstituted with WT Kupffer cells respond to DEN treatment in a similar manner as in WT reconstituted mice. The most upregulated set of the genes in Trem1−/− mice reconstituted with WT Kupffer cells was the set of genes involved in regulating inflammatory response (Il1a, Il1b, and Cxcl1) and cell-cycle progression (Ccnd1, Ccne1, Ccnb2, and Cdk4). In the liver, the IL-1 family cytokines, especially IL-1α primarily expressed by hepatocytes and associated with hepaticocyte damage. The hallmarks of DEN-induced liver injury are necrosis, inflammation, and hepaticellular damage. The damage caused by necrosis and inflammation leads generally to proliferation of the remaining hepatocytes, a characteristic of liver regeneration. DEN treatment significantly upregulated sets of genes regulating inflammatory responses, cell-cycle progression, and DNA repair in the liver of WT mice and not in Trem1−/− mice. In contrast, in livers of Trem1−/− mice reconstituted with WT Kupffer cells, these genes become upregulated to the same levels as seen in WT mice reconstituted with WT Kupffer cells. Hepatic mitogens such as IL-6 and TNF, which are produced by Kupffer cells, are required for compensatory hepatocyte proliferation in various experimental models (8, 30). Necrotic hepatocytes trigger production of the proinflammatory cytokines and chemokines IL-1β, IL-6, TNF, CCL2, and CXCL10 in WT BMDMs but only marginally in Trem1−/− BMDMs. Ablation of Trem1 in macrophages also modulated the expression of certain selective genes regulating MAP kinase (MKK3, MEKK1, JNK1, and JNK2), and NF-κB (IkBα, IkBβ) signaling pathways in response to hepatocyte damage. Recent studies suggest that Trem-1 protein synergizes with TLRs and other pattern recognition receptors (PRRs; refs. 20, 32, and 33). It is most likely that TLRs contribute to Trem-1–mediated signaling in macrophages during activation by DEN-induced hepatocyte damage. However, our data show that there was no significant difference in the expression of TLRs such as TLR4 in the Kupffer cells from WT and Trem1−/− mice. In addition, exposure to DEN did not affect substantially the expression of TLRs. To further dissect TREM-1–mediated signaling, it will be necessary to evaluate the effect of the deletion of different TLRs. To further dissect TREM-1–mediated signaling, it will be necessary to evaluate the effect of the deletion of different TLRs, and we are in the process of establishing Th2−/− Trem1−/− and Tlr4−/− Trem1−/− double-knockout mouse lines.

Our data reveal an essential contribution of a DAP12 ITAM signaling receptor such as TREM-1 on Kupffer cells in regulating or coregulating in combination with other PRRs the inflammatory response to inducers of aseptic inflammation in damaged tissues. Thus, we have identified novel induction mechanisms of chronic inflammation leading to carcinogenesis. We showed that HMGB1 is a TREM-1 ligand released by necrotic hepatocytes and likely involved in their ability to
activate Kupffer cells. It is well established that in addition to its nuclear role, HMGB1 also functions as a damage-associated molecular pattern molecule and activates proinflammatory signaling pathways by activating PRRs. Thus, TREM-1/HMGB1 interaction likely plays an important role in promotion of inflammatory response and liver damage. HMGB1 is passively released following necrosis of parenchymal cells, such as hepatocytes and actively secreted by activated macrophages (34, 35). The mechanism of TREM-1 signaling, following HMGB1 ligand binding remains to be identified. More studies are required to understand how HMGB1 binding to TREM-1 triggers or potentiates signal transduction possibly affecting the association of TREM-1 transmembrane domain or cytosolic tail to its interacting proteins, for example, DAP12. Based on the special structure of TREM-1, its potential synergism with TLRs, and its implication in several diseases, we do not exclude the possibility of the existence of other potential ligands for TREM-1. The knowledge gained from this study that determined the mechanisms of protection from HCC by inhibition of the TREM-1 signaling pathway might have translational relevance to improve prevention and possibly management of human HCC. This is supported by the demonstration that TREM-1 is expressed in human Kupffer cells (Supplementary Fig. S5). The restricted expression of TREM-1 in certain inflammatory cells makes TREM-1 a rational target for clinical situations that involve inflammation, resistance to infection, tumors, transplantation, allergy, autoimmune disease, immunodeficiency, and vaccines. The proof-of-concept of the possibility to systemically inhibit TREM-1 has already been provided by the use of soluble receptors or inhibitory peptides. Clinically suitable TREM-1 inhibitors might be developed that would prove especially useful in cancer prevention and therapy as single agents or coadministered for a shorter duration along with other cancer therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Wu, N. Mivechi, G. Trinchieri, A. Horuzsko
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Wu, J. Li, R. Salcedo, G. Trinchieri, A. Horuzsko
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Wu, R. Salcedo, N. Mivechi, G. Trinchieri, A. Horuzsko
Development of methodology: R. Salcedo
Writing, review, and/or revision of the manuscript: G. Trinchieri, A. Horuzsko

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