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

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Abbreviations used: DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; HMGB1, high mobility group box1; HSP70, heat shock protein 70; KC, Kupffer cell; TREM-1, triggering receptor expressed on myeloid cells-1
Abstract

Chronic inflammation drives liver cancer pathogenesis, invasion and metastasis. Liver Kupffer cells (KC) 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 pro-inflammatory myeloid cell surface receptor TREM-1 expressed by KC 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 KC activation by downregulating transcription and protein expression of IL-6, IL-1β, TNF, CCL2 and CXCL10. In addition, Trem1 ablation diminished activation of the p38, ERK1/2, JNK, MAPK and NF-κB signaling pathways in KC, resulting in diminished liver injury after DEN exposure. Adoptive transfer of wild-type KC 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 KC activation in liver carcinogenesis, deepening mechanistic insights into how chronic inflammation underpins the development and progression of liver cancer.
Introduction

A connection between inflammation and cancer has been long suspected (1-6). One clear example of inflammation-related cancer is hepatocellular carcinoma (HCC), a type of tumor that slowly develops on a background of chronic inflammation and is mainly triggered by exposure to infectious agents or toxic compounds. HCC, the most common type of liver cancer, is the leading cause of cancer deaths worldwide (7). In humans, HCC almost inevitably develops in the setting of chronic hepatitis or cirrhosis, conditions in which hepatocytes are killed and resident inflammatory cells (Kupffer cells (KC)), as well as newly recruited inflammatory cells (monocytes, neutrophils), are activated to produce cytokines that drive the compensatory proliferation of surviving hepatocytes (8-11). In animal model of diethylnitrosamine (DEN)-induced HCC, the extensive hepatocyte damage and inflammatory response represent similar pattern of development human HCC (8). Moreover, analysis of gene expression patterns by comparative functional genomics demonstrated that DEN-induced mouse HCC were similar to the group of human HCC with poorer survival (12, 13).

Experimental and clinical evidence suggests that chronic inflammation can promote all phases of carcinogenesis, from favoring the initial genetic alterations that give rise to tumor cells, to acting as a tumor promoter by establishing a tissue microenvironment that allows the tumor to progress and metastasize, and by establishing immunosuppressive mechanisms that prevent an effective immune response against the tumor (14-18).

The triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface receptor and a member of the immunoglobulin superfamily that potently amplifies inflammatory responses by secretion of pro-inflammatory mediators. TREM-1 is known as activating receptor expressed on neutrophils, monocytes, and macrophages (19-21). The intracellular domain of TREM-1 associates with the adaptor DAP12 that is required for surface expression and signaling by TREM-1 (22-24). TREM-1 is upregulated during infection in vivo and following TLR engagement in vitro. Soluble forms of the TREM-1 molecule, a small peptide blocker (LP17), or...
siRNA have been used in animal studies to reduce TREM-1 signaling. All three methods applied to studies of LPS-induced endotoxemia decreased TREM-1 signaling and systemic cytokine production resulting in improved survival of animals (25, 26). Limited data are available on the role of TREM-1 in chronic inflammation and cancer. It has been demonstrated that cancer cells can directly up-regulate TREM-1 expression in patient macrophages, and TREM-1 expression in tumor-associated macrophages was linked with cancer recurrence and poor survival of patients with non-small cell lung cancer (27). The lack of Trem1-deficient mice does not allow the possibility to directly assess the consequences of the 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 i.p. with 25mg/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 H&E 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 i.p. with 100 mg/kg DEN. Apoptosis was determined by the TUNEL assay (ApopTag Red in Situ Apoptosis Detection Kit, Millipore). The number of TUNEL-positive hepatocytes was determined by manual counting of five 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 i.p. with 1 mg/ml BrdU (Sigma) 2 h before to sacrifice, and paraffin sections were stained using the BrdU in situ detection Kit (BD Biosciences). Liver injury was examined by measuring circulating ALT (Pointe Scientific).

**Generation of BMDMs**

BMDMs were differentiated *in vitro* from bone marrow cells cultured with M-CSF. See Supplementary Methods for details.

**Antibodies and flow cytometry analysis**

Cells were stained with mAbs anti-CD11b-APC, or -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 cytometry data were collected using a FACSCanto (BD Biosciences) and analyzed with CellQquest software (BD Biosciences).

**Detection of cytokine and chemokine production**

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).

**KC isolation, ablation, adoptive transfer of KC, and preparation of necrotic hepatocytes**

KC were isolated by collagenase (Worthington) digestion, density gradient centrifugation followed by MACS magnetic beads separation (Miltenyi Biotec). See Supplementary Methods for details.

**Chemical crosslinking for analysis of protein-protein interactions (*in vitro*)**

Crosslinking reactions with dimethyl adipimidate (DMA) or bis(sulfo succinimidyl) suberate (BS3) with spacer arms of 8.6 Å and 11.4 Å respectively, were performed in 20 mM Hepes buffer (pH 7.5). See Supplementary Methods for details.
Binding analysis using Surface Plasmon Resonance (SPR)

Murine TREM-1, RAGE and unrelated CsrA protein with similar isoelectric point were dissolved in HBS-EP buffer (10mM Hepes, pH 7.4/150 mM NaCl/3.4 mM EDTA/0.005% Surfactant P20) (GE Healthcare). SPR experiments were performed 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 KC 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 WT with Trem1/- mice.

NanoString analysis

NanoString nCounter gene expression assay was performed using two specific probes (capture and reporter) for each gene of interest. See Supplementary Methods for details.

Real-Time PCR, Histology and Immunohistochemistry, Immunoprecipitation and Western Blot

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

Statistical Analysis

All experiments were performed with at least 3-15 mice. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc.). Differences between two groups were evaluated using a Student’s t test. Differences at $p < 0.05$ were considered significant.
Results

Deletion of TREM-1 attenuated the development of inflammation-associated hepatocellular carcinoma 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-deficient 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 HCC many of which were large with evident neovascularization (Fig. 1A and C, E, G, H) (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. Whereas in adult animals AFP is normally found at extremely low levels in the liver, it is AFP is reactivated during liver regeneration and in hepatocellular carcinoma, 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 HCC whereas all WT mice at that time had developed a large numbers of typical HCC with maximal tumor diameters (Fig. 1B and D, 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 inflammatory environment, that within several months, results in the development of HCC (11). Forty-eight h post-DEN administration a significant
increase in hepatocyte death was observed in livers of DEN-treated WT mice compared to similarly treated $Trem1^{+/}$ mice (Fig. 2A and B). Measurements of hepatocyte proliferation similarly revealed a significantly increased hepatocyte proliferation in livers of WT mice compared with $Trem1^{+/}$ mice at 48 h following DEN administration (Fig. 2D and E), suggesting that compensatory proliferation, which has a critical role in DEN-induced hepatocarcinogenesis, is higher in WT than in $Trem1^{+/}$ mice. Additionally, the serum level of IL-6, a hepatic mitogen required for compensatory hepatocyte proliferation, was increased following DEN-injected in WT mice but not in $Trem1^{+/}$ mice (Fig. 2C). After DEN administration, $Trem1^{+/}$ mice displayed significantly less hepatic injury than WT mice, as evidenced by reduced liver enzyme alanine transaminase (ALT) release (Fig. 2F). Thus, TREM-1 was essential for the initiation of liver damage by DEN and $Trem1$-deficient mice exhibited less liver damage than WT mice during the early stage of HCC development.

**TREM-1 expressed on Kupffer cells regulating the inflammatory response to inducers of aseptic inflammation in damaged liver tissues leading to carcinogenesis**

To assess the role of TREM-1 in the recruitment of inflammatory cells in DEN-induced hepatocarcinogenesis, bone marrow, blood, and liver were analyzed for the presence of neutrophils and monocytes. DEN administration increased the number of neutrophils in peripheral blood in both mice but was significant higher in WT mice (Fig. 3A). Both WT and $Trem1^{+/}$ naïve 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 significant 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 cytometry analyses of isolated cells from WT mice revealed that the cells from liver tissues expressing TREM-1 were neutrophils and KC, identified as F4/80+ CD11b+ Ly6G− Ly6C− cells. Immunohistochemistry and flow cytometry 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), while KC and neutrophils in these mice expressed the reporter EGFP (Supplementary Fig. S2D). Injection of DEN into WT mice led to significant up-regulation of the Trem1 mRNA accumulation and increased TREM-1 protein surface expression on KC (Supplementary Fig. S2A and B). 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 KC 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 pro-inflammatory and signaling molecules in total liver samples, isolated hepatocytes, and purified KC using RT-PCR-based TLR Signaling Pathway Microarray. The gene expression patterns of livers from untreated WT and Trem1−/− mice were similar. However, 4 h after DEN exposure, several genes were differentially expressed in the livers from Trem1−/− mice compared to WT mice. The most down-regulated genes in Trem1−/− mice compared to 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. KC are the major source of CCL2 and CXCL10 production in liver, and KC from Trem1−/− mice had a lower accumulation of the mRNA of these chemokines compared to the levels observed on KC from WT mice. When total liver, as well as purified hepatocytes and KC were analyzed, KC were found to be the cell type in which the most striking difference between WT and Trem1−/− mice were with down-regulation of Il1b, Il6, and Tnf in the latter mice (Fig. 4A), suggesting that ablation of the Trem1 gene alters KC
activation during the DEN-induced inflammatory response. Among the genes encoding protein involved in signaling pathways, the most affected in Trem1<sup>−/−</sup> mice were NF-κB and MAPK family members including p38 and c-jun-N-terminal kinase (JNK) (Fig. 4B and C). Jun, Jnk1, Jnk2, and Mkk3 genes were markedly down-regulated in on KC, but not on hepatocytes from Trem1<sup>+/+</sup> 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 up-regulated sets of genes regulating inflammatory responses (e.g. Il1α, Il1β, Cxcl1, S100a11, S100a16) (Fig. 4D), cell cycle regulation (e.g. Ccnb2, Ccnd1, Cdk4) (Fig. 4E), and apoptosis (e.g. Bcl2l4, Tnfrsf6, Cdn1) (Fig. 4F), in the liver of WT mice and not in Trem1<sup>−/−</sup> mice. Moreover, set of the genes associated with DNA damage (e.g. Msh2, Msh6, 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 significant downregulated in the liver of Trem1<sup>−/−</sup> mice after DEN treatment.

Adoptive transfer of wild-type KC to Trem1-deficient mice reversed the unresponsiveness to DEN-induced liver injury

To test whether the impaired activation of KC in Trem1<sup>−/−</sup> mice plays a major role in the low response of liver injury to DEN treatment Trem1<sup>−/−</sup> or WT mice in which KC has been depleted by treatment with Clodronate-containing liposomes were reconstituted with KC isolated from WT mice and analyzed for DEN-induced liver inflammatory/injurious responses (Fig. 5A). The proportion of i.v. adoptively transferred F4/80-positive cells that migrated into the liver of depleted Trem1<sup>−/−</sup> and WT recipients at 18 h was comparable (Fig. 5D). Adoptive transfer of KC 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 KC at 24 and 48 h 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-1\(\beta\) cytokines and CCL2 chemokine (Fig. 5C and not depicted). The NanoString analysis revealed that the majority of the gene expressed in the livers of Trem1\(^+/\) mice reconstituted with WT KC responded to DEN treatment similarly manner to WT reconstituted mice. The most up-regulated genes in Trem1\(^+/\) mice reconstituted with WT KC were those involved in regulating inflammatory response (\(Il1a, Il18, Cxcl1\)), and cell cycle progression (\(Ccne1, Ccnd1, Cdk4\)) (Fig. 5E and F). These data suggest that Trem1\(^+/\) KC are activated normally transferred in Trem1\(^+/\) mice in response to DEN exposure and induce the same magnitude of liver injury observed in KC depleted/reconstituted WT mice. These data also demonstrate that hepatocytes from Trem1\(^-/\) and WT mice responded equally to activated KC.

**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 KC of hepatic mitogens such as IL-6, and TNF (8, 30). Bone marrow-derived macrophages from Trem1\(^+/\) mice exposed to necrotic hepatocytes produced significantly lower levels of CCL2, IL-1\(\beta\), 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 KC 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 non-treated WT mice demonstrated 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 crosslinking 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 panel) and not by bis(sulfosuccinimidyl) suberate (BS3) with spacer arm 11.4 Å respectively (Fig. 6C left panel). The binding between TREM-1 and HMGB1 molecules was further analyzed by Surface Plasmon Resonance (SPR). The BIAcore sensograms of the two 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 were concentration-dependent (Fig. 6D). The affinity constants, $K_d$ were determined by SPR technique and found to be $K_d=0.2 \mu M$ for RAGE and HMGB1 and $K_d=35.4 \mu M$ for TREM-1 and HMGB1, respectively. Thus, a combination of immunoprecipitation, chemical crosslinking 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 KC, 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 KC that express up-regulated co-stimulatory molecules CD80/CD86, with a cytokine profile...
favoring a type I inflammatory response. In contrast, \textit{Trem1}^{-/-} KC show no changes in these activation markers. Thus, initial damage of hepatocytes mediated by DEN may not be able to activate \textit{Trem1}^{-/-} KC. The RNA-based NanoString analysis revealed that the majority of the gene expressed in the livers of \textit{Trem1}^{-/-} mice reconstituted with WT KC responds to DEN treatment in a similar manner as in WT reconstituted mice. The most up-regulated set of the genes in \textit{Trem1}^{-/-} mice reconstituted with WT KC was set of the genes involved in regulating inflammatory response (\textit{Il1a, Il18, Cxcl1}), and cell cycle progression (\textit{Ccnb1, Ccne1, Ccnb2, Cdk4}). In the liver, the IL-1 family cytokines, especially IL-1\(\alpha\) primarily expressed by hepatocytes and associated with hepatocyte damage. 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. DEN treatment significantly up-regulated sets of genes regulating inflammatory responses, cell cycle progression, and DNA repair in the liver of WT mice and not in \textit{Trem1}^{-/-} mice. In contrast, in livers of \textit{Trem1}^{-/-} mice reconstituted with WT KC, these genes become up-regulated to the same levels as seen in WT mice reconstituted with WT KC. Hepatic mitogens such as IL-6, and TNF, which are produced by KC, are required for compensatory hepatocyte proliferation in various experimental models (8, 30). Necrotic hepatocytes trigger production of the pro-inflammatory cytokines and chemokines IL-1\(\beta\), IL-6, TNF, CCL2, and CXCL10 in WT BMDMs but only marginally in \textit{Trem1}^{-/-} BMDMs. Ablation of \textit{Trem1} in macrophages also modulated the expression of certain selective genes regulating MAP kinases (M KK3, MEKK1, JNK1, JNK2), and NF-kB (\textit{IkB\&alpha, IkB\&beta}) signaling pathways in response to hepatocyte damage. Recent studies suggest that TREM-1 protein synergizes with TLRs and other pattern recognition receptors (PRRs)(20, 32, 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 KC from WT and Trem1<sup>−/−</sup> 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, and we are in the process of establishing Tlr2<sup>−/−</sup>Trem1<sup>−/−</sup> and Tlr4<sup>−/−</sup>Trem1<sup>−/−</sup> double-knockout mouse lines.

Our data reveal an essential contribution of a DAP12 ITAM signaling receptor such as TREM-1 on KC in regulating or co-regulating 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 KC. It is well established that in addition to its nuclear role, HMGB1 also functions as a damage-associated molecular pattern molecule and activates pro-inflammatory 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 trigger or potentiate signal transduction possibly affecting the association of TREM-1 transmembrane domain or cytosolic tail to its interacting proteins, e.g. 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 learned 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 co-administered for a shorter duration along with other cancer therapies.

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Figure Legends

Figure 1. Deletion of TREM-1 decreases DEN-induced liver tumorigenesis. (A) WT and Trem1<sup>−/−</sup> male mice were given a dose of DEN (25 mg/kg) at 15 days of age. Numbers of tumors (≥ 0.5 mm) and maximal tumor sizes (diameters) in livers of WT (n=9) and Trem1<sup>−/−</sup> (n=7) mice 8 months after DEN injection. ND, not detectable. Bars represent mean ± SD, statistical significance is indicated (**p < 0.001). mo, month. One of two similar experiments is shown. (B) Numbers of tumors and maximal tumor sizes in livers of male WT (n=7) and Trem1<sup>−/−</sup> (n=10) mice 14 months after DEN injection. Bars represent mean ± SD, statistical significance is indicated (**p < 0.01, ***p < 0.001). One of two independent experiments with similar results is shown. Representative microscopic pictures of livers from WT, Trem1<sup>−/−</sup> mice 8 (C) and 14 months (D) after DEN injection. Arrowhead indicates tumor nodules. (E) Histological analysis (H&E) of livers from indicated mice 8 month (E) and 14 month (F) after injection of DEN. N, noncancerous liver tissues; T, tumors. Arrowheads indicate border between noncancerous liver tissues and tumor. Original magnification: x 10. Bars 50 μm. The expression of HCC marker alpha-fetoprotein (AFP) by liver tumor cells determined by immunohistochemistry (G) and by Western blot analysis (H) using an anti-AFP Ab. Bar 20 μm. One of two similar experiments is shown.

Figure 2. TREM-1 deficiency decreases of DEN-induced hepatic injury. WT and Trem1<sup>−/−</sup> male mice were treated for 4 h with DEN (100 mg/kg) or left untreated. (A and B) Livers of WT or Trem1<sup>−/−</sup> mice were assessed for apoptosis by TUNEL staining. Original magnification, x 40. Values are mean ± SD for independent animals (n=5). One of four similar experiments is shown. (D and E) Hepatocyte proliferation in livers of WT or Trem1<sup>−/−</sup> mice was assessed by injecting mice with BrdU (1 mg/ml) 2 hours before the liver was removed. BrdU-positive cells were identified by immunostaining. Original magnification, x 40. Values are mean ± SD for independent animals (n=3). One of three similar experiments is shown. (C) Levels of IL-6 and
ALT (F) in serum of WT and Trem1−/− mice treated with DEN at indicated time. Data are shown as mean ± SD (n=5 mice per group). For all panels statistical significance is indicated (* p < 0.05, ** p < 0.01). h, hour. One of four similar experiments is shown.

**Figure 3. Loss of TREM-1 alters recruitment of neutrophils and monocytes.** Bone marrow, blood, and liver samples of indicated mice were staining with appropriate mAbs and analyzed by flow cytometry. Data are shown as mean ± SD (n=3 mice per group) of (A) neutrophils (CD11b^high, Ly6G+) and (B) monocytes (CD11b^, Ly6C^high) and represent one experiment out of three independent experiments with similar results.

**Figure 4. TREM-1 deletion alters activation of KC in response to DEN and decreases expression of genes involved in inflammatory responses, cell cycle regulation and apoptosis.** (A) WT and Trem1−/− male mice (n=10 mice per group) were treated for 4 h with DEN. Cells from total livers, isolated hepatocytes, or KC were subjected to RNA isolation and microarray analysis. The average fold induction/decrease was obtained by comparison of Trem1−/− mice with WT mice. Fold changes (A) in pro-inflammatory cytokine and chemokine gene expression, (B) in genes related to NF-kB signaling pathway, (C) in genes related to JNK/p38 signaling pathway. Shown is one representative experiment from two independent experiments. Values are mean ± SD. (D, E, F) WT and Trem1−/− mice were treated for 4 h with DEN or left untreated (n=4 mice per group). RNA samples extracted from livers were subjected to NanoString analysis. Decreased expression in genes involved in inflammation (D), cell cycle regulation (E) and apoptosis (F) was observed in Trem1−/− mice post DEN treatment. The results are expressed as mean ± SE. Statistical significant is indicated.

**Figure 5. Adoptively transferred KC from WT mice increased DEN-induced liver injury in Trem1−/− mice.** (A) Immunofluorescence staining of liver sections with FITC-conjugated anti-F4/80 mAb after 48 h injection of Clodronate-containing liposomes. (D) Reconstitution of predepleted livers with KC from WT mice to indicated mice. Adoptively transferred cells were
observed at 18 h by immunofluorescence staining. Bar 20 μm. Mice were given a dose of DEN at 18 h after KC reconstitution and ALT (B) and IL-6 (C) in serum were measured at indicated time. Data shown are means ± SD of four mice per group and representative of two independent experiments. (E, F) 4 h after DEN treatment gene expression profile of reconstituted liver was performed using NanoString Technology. Values indicate mean ± SE of four mice per time point in each group. ns, not significant.

**Figure 6. TREM-1 binds HMGB1.** (A) HMGB1 and HSP70 present in the product of necrotic hepatocytes (PNH). PNH have been isolated from DEN-treated and non-treated WT mice and subjected to Western blot analysis with indicated Abs. Actin was used as a loading control. (B) HMGB1 and not HSP70 associated with TREM-1. NHP isolated from DEN-treated WT mice have been subjected to immunoprecipitation with murine recombinant TREM-1-Fc fusion protein followed by immunoblotting with indicated Abs. (C) Purified murine HMGB1 and TREM-1 proteins were used as cross-linked proteins. Crosslinking reactions with bis(sulfosuccinimidyld) suberate (BS3) (left panel) or dimethyl adipimidate (DMA) (right panel). One of four similar experiments is shown. (D) Surface plasmon resonance (SPR) analysis of TREM-1 and RAGE binding to immobilized HMGB1. The concentrations of RAGE (0 - black, 30 - yellow, 60 - gray, 120 – blue and 240 nM - red (left panel), the identical concentrations of TREM-1 and CsrA were flowed over immobilized HMGB1 on the sensor chip. Data are presented as response units (RU) over time (seconds). Data shown are representative of three experiments.
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The Pro-inflammatory Myeloid Cell Receptor TREM-1 Controls Kupffer Cell Activation and Development of Hepatocellular Carcinoma

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