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Parenchymal, But Not Leukocyte, TNF Receptor 2 Mediates T Cell-Dependent Hepatitis in Mice

Jens Schümann,† Katrin Mühlen,‡ Alexandra K. Kiemer,‡ Angelika M. Vollmar,‡ and Gisa Tiegs*‡

TNF-α is a central mediator of T cell activation-induced hepatitis in mice, e.g., induced by *Pseudomonas* exotoxin A (PEA). In this in vivo mouse model of T cell-dependent hepatitis, liver injury depends on both TNFRs. Whereas TNFR1 can directly mediate hepatocyte death, the in vivo functions of TNFR2 in pathophysiology remained unclear. TNFR2 has been implicated in deleterious leukocyte activation in a transgenic mouse model and in enhancement of TNFR1-mediated cell death in cell lines. In this study, we clarify the role of hepatocyte- vs leukocyte-expressed TNFR2 in T cell-dependent liver injury in vivo, using the PEA-induced hepatitis model. Several types of TNFR2-expressing leukocytes, especially neutrophils and NK cells, accumulated within the liver throughout the pathogenic process. Surprisingly, only parenchymal TNFR2 expression, but not the TNFR2 expression on leukocytes, contributed to PEA-induced hepatitis, as shown by analysis of wild-type → *tnfr2°* and the reciprocal mouse bone marrow chimeras. Furthermore, PEA induced NF-κB activation and cytokine production in the livers of both wild-type and *tnfr2°* mice, whereas only primary mouse hepatocytes from wild-type, but not from *tnfr2°*, mice were susceptible to cell death induced by a combination of agonistic anti-TNFRI and anti-TNFRII Abs. Our results suggest that parenchymal, but not leukocyte, TNFR2 mediates T cell-dependent hepatitis in vivo. The activation of leukocytes does not appear to be disturbed by the absence of TNFR2.


Tumor necrosis factor α was originally identified by its capacity to induce hemorrhagic necrosis in mouse tumors (1). However, its use in vivo as a systemic anticancer chemotherapeutic agent failed because of severe side effects, with liver injury being one of the dose-limiting parameters (2). Under experimental conditions, rTNF-α turned out to be highly hepatotoxic to mice if the animals had been sensitized with an inhibitor of hepatic transcription, i.e., β-galactosamine (3). Furthermore, infections of different kind, fungus poisoning, abuse of drugs, exposure to industrial by-products, or the presence of an autoimmune disease can result in endogenous TNF-α production, and in many of these cases, TNF-α exerts significant hepatotoxicity (4). Although inflammatory liver disease represents an ubiquitous human health problem, it can be managed pharmacologically in only a few cases. Symptomatic treatment is predominant while cure of hepatic dysfunction remains an elusive goal. Therefore, various mouse models of TNF-α-dependent liver injury have been established to facilitate functional studies on the mechanisms of TNF-α-mediated hepatotoxicity (4).

Recently, we introduced a novel model of liver disease in mice induced by the bacterial toxin *Pseudomonas aeruginosa* exotoxin A (PEA),4 that depends on the activation of T cells and Kupffer cells, induction of TNF-α and IL-18, and a perforin-dependent cytotoxic mechanism (5–7). In this model, TNF-α is primarily produced by Kupffer cells, but only if T cells are present (5, 6). Interestingly, in this model, liver injury depends on both TNFRs (5), the 55-kDa TNFR1 (p55), which is the prime receptor for processed soluble TNF-α (8), and the 75-kDa TNFR2 (p75), which is the prime receptor for the biologically active transmembrane, unprocessed TNF-α precursor (9). So far, a similar dependence of liver injury on TNFR1 and TNFR2 has been described for the Con A hepatitis model (10, 11), and other PEA-dependent models, in which subtoxic amounts of PEA were used to sensitize mice against subtoxic amounts of LPS, *Staphylococcus aureus* enterotoxin B, or murine rTNF-α (12). Furthermore, liver pathology induced by infection with adenoviruses is also mediated through TNFR1 and TNFR2 (13).

It is well-established that activation of TNFR1 directly induces apoptosis and necrosis of sensitized hepatocytes (14). Its signaling pathways include the activation of caspases which are implicated in the execution of cell death (15), and consistently, PEA-induced liver injury is characterized by hepatocyte apoptosis and necrosis (5, 6) and can be inhibited by the caspase inhibitor benzoylcarbonyl-Val-Ala-Asp fluoromethylketone (5).

Much less is known about the role of TNFR2. Two scenarios can currently be discussed. The first scenario is that TNFR2 is important on leukocytes, for example on Kupffer cells, by activating NF-κB and by sustaining the production of cytokines such as TNF-α. This mechanism is supported by the finding that overexpression of human TNFR2 in transgenic mice results in a severe inflammatory syndrome, including inflammatory liver disease, that

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4 Abbreviations used in this paper: PEA, *P. aeruginosa* exotoxin A; RT, room temperature; LDH, lactate dehydrogenase; TRAF, TNFR-associated factor.
is accompanied by constitutively increased NF-κB activity in the leukocyte compartment and by dramatically increased levels of endogenous TNF-α (16). In this model, TNFR1 does not appear to be required for TNFR2-mediated effects, because increased levels of human TNFR2 were sufficient to induce the disease even in the absence of TNFR1 (16). The second scenario is that TNFR2 is important on hepatocytes by sustaining TNFR1-induced cell death signaling (“cooperative cell death signaling”). This mechanism is supported by several cell line studies reporting “cooperative cell death signaling” of TNFR2 with TNFR1 (9, 17–22).

During human acute and chronic hepatitis of various etiologies, TNF2 is up-regulated on hepatocytes and mononuclear inflammatory cells, and the plasma level of soluble TNF2 is elevated (23–27). These observations suggest a role of TNF2 in human hepatitis as well and enforce further studies on the role of TNF2 in inflammatory liver disease. Most importantly, it is unknown what role TNF2 plays on which cells in liver pathophysiology in vivo.

This study was designed to clarify whether T cell-dependent liver injury in mice is associated with changes in the intracellular expression of TNF2 and whether parenchymal or leukocyte TNF2 accounts for the TNF2 dependence of T cell-dependent liver injury. Because PEA-induced liver injury is mediated by TNF1 and TNF2 (5), this model is suitable to answer these questions.

Materials and Methods

Mice

Male C57BL/6 mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany). TNF1+/− (28) and infa2+ mouse (29) that have been backcrossed 10 times with C57BL/6 mice were kindly provided by Dr. H. Bluethmann (Hoffmann-La Roche, Basel, Switzerland). Animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. The legal requirements in Germany were met as well. Mice were maintained under controlled conditions (22°C, 55% humidity, 12-h day/night rhythm) and fed a standard laboratory chow (Altromin 1313; Altromin, Lage, Germany) ad libitum.

Animal treatments

PEA (Sigma-Aldrich, Taufkirchen, Germany) was injected in a dose of 85 μg/kg i.v. For depletion of Kuffer cells, C57BL/6 mice were injected with 100 μl of liposome-encapsulated dichloromethylene-bisphosphonate (Cl2MBP liposomes) i.v. 48 h before challenge, as described previously (6, 30). The Cl2MBP liposomes were kindly provided by Dr. N. van Rooden (Vrije Universiteit, Amsterdam, The Netherlands). Cl2MBP was used for preparation of CL2MBP liposomes was a gift of Roche Diagnostics (Mannheim, Germany). In control experiments, C57BL/6 mice were pretreated with saline instead of Cl2MBP liposomes. Saline liposomes were not used because liposomes themselves block macrophage phagocytosis for certain periods of time (31).

Generation of bone marrow chimeras and analysis of successful bone marrow transplantation

Bone marrow chimeras were generated essentially as described previously (32).Recipient C57BL/6 wild-type or infa2−/− mice were irradiated with 9.5 Gy, and 2 × 106 bone marrow cells from infa2−/− or C57BL/6 wild-type mice were injected i.v. into recipients. To analyze the success of bone marrow transplantation, blood leukocytes from each mouse were stimulated with 2 μg/ml Con A (Sigma-Aldrich) for 5 h. This incubation with Con A is a helpful step because it up-regulates TNF2 in blood leukocytes (our unpublished observation). Following incubation and centrifugation onto glass slides, cells were fixed with 1% formalin/PBS at room temperature (RT) for 10 min, postfixed with acetone/methanol (1/1) at 4°C for 10 min, and incubated with 3% BSA/PBS at RT for 30 min. Incubation was continued at 4°C overnight with rabbit-anti-mouse-TNF2 polyclonal IgG (1/50; Sanbio, Beutelsbach, Germany). Binding sites were detected using suitable secondary Abs, diluted in 3% BSA/PBS, with a fluorochrome (Fluorescein isothiocyanate) or peroxidase conjugates (HRP). After rinsing with PBS, binding sites were detected using suitable secondary Abs, diluted in 3% BSA/PBS, with a fluorochrome (Fluorescein isothiocyanate) or peroxidase conjugates (HRP). After rinsing with PBS, sections or cells processed for immunofluorescence were examined by confocal laser scanning microscopy (LS; CompuCyte, Cambridge, MA).

Sampling of material

Mice were lethally anesthetized with 150 mg/kg of i.v. pentobarbital, containing 15 mg/kg of heparin. From anesthetized mice, blood was withdrawn for analysis of plasma transaminases, for plasma cytokine determination, and for quantification of soluble TNF2. Livers were excised and divided into two parts, one part being embedded in tissue embedding medium (Slee, Mainz, Germany) and frozen at −75°C for immunofluorescent staining and confocal laser imaging, the other part being frozen in liquid nitrogen for preparation of RNA and subsequent real-time RT-PCR and for preparation of nuclear extracts followed by EMSA. For flow cytometric analysis of liver leukocytes, complete fresh livers were used.

Analysis of transaminases, cytokines, and soluble TNF2 in plasma samples

Liver injury was quantified by determination of plasma transaminase activities. The activities of alanine aminotransferase and aspartate aminotransferase in plasma were determined using an automated procedure, according to Bergmeyer (33). The plasma concentrations of TNF-α and IL-6 were determined with the help of specific ELISAs purchased from BD PharMingen (Heidelberg, Germany). The plasma concentrations of soluble TNF2 were determined with the help of an ELISA kit purchased from R&D Systems (Wiesbaden, Germany).

Immunofluorescent staining and confocal laser imaging

The expression of different proteins within liver tissue or in primary-cultured hepatocytes (see Results) was analyzed by immunofluorescent staining and confocal laser imaging. Ten-micrometer-thick cryostat sections of livers were thawed onto poly-l-lysine-coated glass slides or primary mouse hepatocytes were allowed to adhere to chamber slides. Liver sections or adherent cells were air-dried and fixed in acetone/methanol (1/1) at 4°C for 10 min before they were incubated with 3% BSA/PBS at RT for 30 min. Incubation was continued with rabbit-anti-mouse-TNF2 Ab (1/50; Sanbio), rat-anti-mouse-macrophage mAb (clone BM 8; 1/100; Dianova), or FITC-labeled mouse-anti-mouse CD45.2 mAb (clone 104; 1/1000; BD PharMingen), or combinations thereof. After rinsing with PBS, binding sites were detected using suitable secondary Abs, diluted in 3% BSA/PBS, at RT for 1 h. These were: Cy3-labeled goat-anti-rabbit IgG (1/400; Dianova) or FITC-labeled goat-anti-rat IgG (1/50; Sigma-Aldrich). After rinsing with PBS, sections or cells were costained with 10% glycerol/PBS, pH 8.6. Sections or cells processed for immunofluorescence were examined by confocal laser scanning microscopy (MRC 1000; Bio-Rad).

Real-time RT-PCR for cytokine mRNAs in liver tissue

RNA was isolated from pieces of ~25 mg liver tissue by use of an RNA purification kit (Qiagen, Hilden, Germany). One microgram of RNA was used for synthesis of cDNA. For real-time RT-PCR, we used the Light Cycler FastStart DNA Master SYBR Green I method, according to the manufacturer’s instructions (Roche Diagnostics). The following oligonucleotide pairs were used: β-actin, 728-751 and 1076-1052 in GenBank X03376; TNF-α, 157-177 and 386-371 in GenBank X02611; IL-6, 374-394 and 680-663 in GenBank X03783. Forty cycles of real-time RT-PCR were run as follows: β-actin: 95°C, 1 s; 56°C, 7 s; 72°C, 15 s; TNF-α: 95°C, 1 s; 57°C, 7 s; 72°C, 10 s; IL-6: 95°C, 1 s; 56°C, 7 s; 72°C, 14 s. mRNA levels were calculated using the comparative cycle threshold (Ct) method (34) and normalization to β-actin. To confirm amplification specificity, RT-PCR products were subjected to a melting-curve analysis. Quantification is reported as the X-fold difference relative to a calibrator cDNA from solvent-treated mice. Because TNF-α and IL-6 mRNA were not detectable within livers of solvent-treated mice, signals that exceeded a ΔCt of 15 were arbitrarily set to 1-fold to enable a calibration. The relation to this arbitrary 1-fold is represented by the data described as “X-fold increase of mRNA.”

Preparation of nuclear extracts and EMSA

Nuclear extracts from frozen livers were prepared and EMSAs were performed, as described elsewhere (35, 36).

Flow cytometric analysis of liver leukocytes

Leukocytes were isolated from livers, as described previously, using the Percoll separation method (37). For flow cytometric analysis, 109 cells were stained using a standard protocol. The following Abs were used: FITC-labeled Armenian hamster-anti-mouse CD45 mAb (clone 145-2C11;
BD PharMingen), PE-labeled mouse-anti-mouse NK1.1 mAb (clone PK136; BD PharMingen), PE-labeled rat-anti-mouse Ly-6G mAb (clone RB6-8C5; BD PharMingen), and biotinylated rat-anti-mouse TNFR2 mAb (clone HM102; Sanbio). Biotin binding sites were detected using streptavidin-CyChrome (0.2 μg/100 μl; BD PharMingen). Flow cytometric analysis was performed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Krefeld, Germany). The numbers of specific cells per liver were calculated by multiplying the percentage of each population with the total number of leukocytes per liver.

Isolation of hepatocytes and determination of cytotoxicity following stimulation with agonistic Abs against TNFR1 and TNFR2

Hepatocytes were isolated by the two-step collagenase perfusion method of Seglen (38) and cultured as described (39). Stimulation was performed with polyclonal agonistic Abs against TNFR1 and TNFR2 (Sanbio) at concentrations of 10 μg/ml each. Experiments were conducted in a 37°C incubator run at 5% CO2 and 40% O2 for 20 h. Cytotoxicity was quantified by measurement of lactate dehydrogenase (LDH) (33) in culture supernatants and in the remaining cell monolayer after lysis with 0.1% Triton X-100, and calculation of the percentage of LDH release from the ratio of supernatant:supernatant plus cell monolayer.

Statistical analysis

The results were analyzed by one-way analysis of variance followed by the Dunnett or the Student-Newman-Keuls multiple comparison tests. All data in this study are expressed as the mean ± SEM. A value of p < 0.05 was considered significant.

Results

PEA-induced changes in the intrahepatic expression of TNFR2

It was analyzed whether PEA causes changes in the intrahepatic expression of TNFR2. To this end, liver sections from PEA-treated mice were stained with an Ab specific for TNFR2 at different stages (0, 3, 6, 9, and 12 h) after injection. The specificity of TNFR2 staining was verified by analysis of liver sections obtained from untreated C57BL/6 wild-type, tnfr1−/−, and tnfr2−/− mice. Basal intrahepatic expression of TNFR2 was observed in C57BL/6 wild-type and tnfr1−/−, but not in tnfr2−/−, mice (Fig. 1A), showing that TNFR2 staining was specific. TNFR2 staining of liver sections from PEA-treated mice was conducted by concomitant use of Abs directed against the leukocyte common Ag CD45 to distinguish between leukocyte and nonleukocyte expression of TNFR2. Four major observations could be made (Fig. 1B): 1) most TNFR2 staining within livers of mice was associated with leukocytes at the time of injection (t = 0 h). Furthermore, expression of TNFR2 by liver cells was faintly, but clearly detectable; 2) the CD45+ leukocytes, which in the naive mouse predominantly expressed TNFR2, lost their TNFR2 and were not any more detectable 9 or 12 h after challenge. The appearance of these cells reminds of Kupffer cells, which are also not any more detectable at the later time points (5) (for specific staining of Kupffer cells, cf Fig. 2); 3) most strikingly, other leukocytes accumulated within the liver of PEA-treated mice which strongly expressed TNFR2; and 4) liver cell TNFR2 disappeared throughout the pathogenic process.

Analysis of TNFR2 expression on Kupffer cells following injection of PEA

Because Kupffer cells are a key cell type in PEA-induced liver injury, producing disease-relevant TNF-α (6), it was important to formally check whether the strongly TNFR2-expressing accumulating leukocytes were Kupffer cells. Double staining, using the
pan macrophage marker BM 8 and anti-TNF2 IgG, revealed that basal leukocyte expression of TNFR2 was associated with Kupffer cells, whereas the accumulating, strongly TNFR2-expressing leukocytes were not macrophages. This is as we expected, because Kupffer cells have a morphological appearance different from that associated with strong TNFR2 expression during the PEA-induced pathogenic process (cf Fig. 1).

Analysis of TNFR2 expression on different intrahepatic leukocyte populations following injection of PEA

To clarify which TNFR2-expressing cells are induced to accumulate within the liver following injection of PEA, we analyzed the following cell types for TNFR2 expression within the population of liver leukocytes by flow cytometry: “conventional” T cells (CD3⁺ NK1.1⁻), NKT cells (CD3⁺ NK1.1⁺), NK cells (CD3⁻ NK1.1⁺), and neutrophils (Ly-6G⁺). A very significant accumulation of the absolute number of TNFR2-expressing neutrophils could be observed within livers 6 h after challenge with PEA (Fig. 3A). This corresponded to a >16-fold increase in TNFR2-positive neutrophils, as compared with basal intrahepatic levels of TNFR2-expressing neutrophils (Fig. 3B). The intrahepatic accumulation of TNFR2-expressing neutrophils can be explained by infiltration of constitutively TNFR2-expressing neutrophils into the liver parenchyma, because the ratio of TNFR2⁺ neutrophils to total neutrophils is unaltered despite of the increase in TNFR2⁺ neutrophils within the liver (Table 1). Furthermore, an increase in the numbers of TNFR2-expressing “conventional” T cells, NKT cells, and NK cells within livers of PEA-injected mice could be observed. This effect was quite weak with respect to T and NKT cells, but very prominent with respect to NK cells (Fig. 3A). Whereas the weak accumulation of TNFR2-expressing T and NKT cells corresponded to a 2- to 3-fold increase in TNFR2-positive T and NKT cells, as compared with basal intrahepatic levels of TNFR2-expressing T and NKT cells, the number of TNFR2-positive NK cells was elevated by a factor of >11 (Fig. 3B). The analysis of TNFR2-expressing T and NKT cells, as compared with total T and NKT cells, revealed that T and NKT cells only weakly express TNFR2, and that TNFR2 expression is not significantly induced in these cells by PEA (Table 1). However, because the ratio of intrahepatic TNFR2-positive NK cells to total NK cells significantly increased after injection of PEA, though not completely by factor 11 (Table 1), the increase in TNFR2-positive NK cells within livers of PEA-treated mice must be explained by two phenomena: 1) infiltration of constitutively TNFR2-expressing NK cells, and 2) induction of TNFR2. Taken together, the accumulating TNFR2-expressing leukocytes that could be observed within livers of PEA-treated mice (cf Figs. 1 and 2) are mainly constitutively TNFR2-expressing infiltrating neutrophils and infiltrating NK cells, in which TNFR2 expression is also induced.

PEA-induced alterations in the plasma levels of soluble TNFR2

Considering that at least liver parenchymal cells and Kupffer cells lost their TNFR2 (cf Fig. 1), we wondered whether this might be

![Image](http://www.jimmunol.org/Downloaded_from/http://www.jimmunol.org/images/1/14/fig2a.png)

**FIGURE 2.** Analysis of TNFR2 expression on Kupffer cells following injection of PEA. Ten micrometer liver sections were subjected to immunofluorescent staining and confocal laser imaging 6 h after injection of solvent or PEA to C57BL/6 mice. Double staining was performed by use of Abs specific for TNFR2 (Cy3, red) together with mAb against macrophages (FITC, green). Costaining is represented by yellow fluorescence.

![Image](http://www.jimmunol.org/Downloaded_from/http://www.jimmunol.org/images/1/14/fig2b.png)

**FIGURE 3.** Analysis of TNFR2 expression on different intrahepatic leukocyte populations following injection of PEA. Intrahepatic leukocytes were subjected to immunofluorescent staining and flow cytometric analysis 6 h after injection of solvent or PEA to C57BL/6 mice. A, Absolute numbers of intrahepatic TNFR2-positive cell types; B, the X-fold increase of intrahepatic TNFR2-positive cell types. Double or triple staining was performed by use of Abs specific for TNFR2 and CD3/NK1.1 or Ly6G. Data are expressed as the mean ± SEM (n = 3). *, p < 0.05 vs solvent.

<table>
<thead>
<tr>
<th>Ratio TNFR2⁺/Total</th>
<th>Solvent (6 h) (%)</th>
<th>PEA (6 h) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺ NK1.1⁻</td>
<td>3.03 ± 0.70</td>
<td>5.10 ± 1.01</td>
</tr>
<tr>
<td>CD3⁺ NK1.1⁺</td>
<td>6.53 ± 0.37</td>
<td>9.37 ± 1.65</td>
</tr>
<tr>
<td>CD3⁻ NK1.1⁺</td>
<td>54.70 ± 1.63</td>
<td>83.6 ± 1.57*</td>
</tr>
<tr>
<td>Ly6G⁺</td>
<td>86.17 ± 0.49</td>
<td>86.57 ± 1.31</td>
</tr>
</tbody>
</table>

*Identification of the ratio of TNFR2-positive to total “conventional” T cells, NKT cells, NK cells, and neutrophils, respectively, showing real induction of surface TNFR2. Intrahepatic leukocytes were subjected to immunofluorescent staining and flow cytometric analysis 6 h after injection of solvent or PEA to C57BL/6 mice. Double or triple staining was performed by use of Abs specific for TNFR2 and CD3/NK1.1 or Ly6G. Data are expressed as the mean ± SEM (n = 3). *, p < 0.05 vs solvent.
a result of increased shedding of TNFR2, as it has also been observed in the Con A model of T cell-dependent hepatitis (11) and in inflammatory liver disease in humans (24–27). Therefore, we determined the plasma levels of soluble TNFR2 following injection of PEA. Determination of soluble TNFR2 in the circulation of healthy mice revealed that TNFR2 is constitutively shed into the circulation (Fig. 4). PEA induced a biphasic increase in the plasma levels of soluble TNFR2, as compared with untreated control mice (Fig. 4). This might point to a rapid shedding of TNFR2, e.g., from hepatocytes and Kupffer cells, which lost their TNFR2 within 2 h, followed by a delayed shedding of TNFR2, e.g., from the accumulating, strongly TNFR2-expressing leukocytes at later time points.

**Relevance of TNFR2-expressing radiation-sensitive, bone marrow transplantable, leukocytes for PEA-induced liver injury**

The remarkable accumulation of strongly TNFR2-expressing non-macrophage leukocytes, such as neutrophils and NK cells (cf Figs. 1–3), prompted us to investigate the importance of this leukocyte TNFR2 expression for PEA-induced liver injury. To this end, irradiated C57BL/6 wild-type mice were transplanted with bone marrow cells from tnfr2°/tnfr2° mice (tnfr2° → wild-type) and vice versa (wild-type → tnfr2°). The success of transplantation was tested by immunofluorescent staining of Con A-stimulated blood leukocytes with an anti-TNFR2 Ab and cytometric analysis using a laser scanning cytometer. Con A-stimulated blood leukocytes from wild-type, wild-type → wild-type, and wild-type → tnfr2° mice were indistinguishable with respect to TNFR2 expression whereas the cells from tnfr2° and tnfr2° → wild-type mice were largely deficient in TNFR2 (Fig. 5A). The small number of TNFR2-positive leukocytes in tnfr2° mice (~5–6%) was also observed by others using flow cytometry (16) and obviously represents unspecific staining. The success of bone marrow transplantation was further confirmed by staining of liver sections from PEA-treated mice with anti-TNFR2 Ab 12 h after challenge, showing that the accumulation of strongly TNFR2-expressing leukocytes was observed within livers of wild-type, wild-type → wild-type, and wild-type → tnfr2° mice, but not within livers of tnfr2° and tnfr2° → wild-type mice (Fig. 5B). After transplantation, the susceptibilities of wild-type → tnfr2° and tnfr2° → wild-type mice to PEA-induced liver injury were tested and compared with the susceptibilities of C57BL/6 wild-type mice, tnfr2° mice, and irradiated and transplanted wild-type mice, which received bone marrow cells from their own strain (wild-type → wild-type). Liver injury in wild-type → tnfr2° mice was significantly attenuated, as compared with PEA-treated wild-type or wild-type → wild-type mice (Fig. 5C). The plasma transaminase activities in PEA-treated wild-type → tnfr2° mice were essentially the same as in nontransplanted tnfr2° mice (Fig. 5C). This result clearly showed that TNFR2 expression by radiosensitive, bone marrow-derived leukocytes is not the cause of TNFR2 dependence of PEA-induced liver injury. This conclusion was confirmed by tnfr2° → wild-type mice which were highly susceptible to PEA-induced liver injury. The plasma transaminase activities in these animals were even higher than in PEA-treated wild-type or wild-type → wild-type mice (Fig. 5C). Because TNFR2 expression on radiosensitive, bone marrow-derived leukocytes was not able to mediate liver injury in PEA-treated wild-type → tnfr2° mice, and because liver injury was not at all attenuated, but even enhanced, in tnfr2° → wild-type mice, it can be concluded that, despite a conspicuous induction of TNFR2 on these cells, the observed dependence of PEA-induced liver injury on TNFR2 is not due to this leukocyte TNFR2 expression.

**Relevance of Kupffer cells and TNFR2 for PEA-induced intrahepatic NF-κB activation, TNF-α, and IL-6 production**

Because macrophages, including Kupffer cells, are known to be largely radioresistant (40, 41), a fact that we could confirm in our experiments (data not shown), the aforementioned results (cf Fig. 5) cannot exclude that TNFR2 might play a role on Kupffer cells in the activation of NF-κB and in sustaining the production of cytokines such as TNF-α, as supported by the constitutively increased NF-κB activity in the leukocyte compartment and by dramatically increased levels of endogenous TNF-α in transgenic mice overexpressing human TNFR2 (16). To clarify whether Kupffer cells and TNFR2 play a role in PEA-induced activation of NF-κB and in PEA-induced synthesis of NF-κB-dependent Kupffer cell-produced cytokine mRNAs, such as TNF-α and also IL-6 mRNA, within the liver, we analyzed 1) whether Kupffer cells and TNFR2 are involved in the activation of NF-κB within livers of PEA-treated mice, and 2) whether Kupffer cells and TNFR2 are involved in the synthesis of TNF-α and IL-6 mRNAs within livers of PEA-treated mice. Intrahepatic activation of NF-κB following injection of PEA was determined by EMSA, performed with nuclear extracts from mouse livers. PEA clearly induced the intrahepatic activation of NF-κB within 2 h (Fig. 6A). PEA-induced intrahepatic activation of NF-κB was inhibited in C32 MBP lipo-some-pretreated, Kupffer cell-deficient mice, i.e., PEA-induced intrahepatic activation of NF-κB clearly depended on Kupffer cells (Fig. 6A). However, tnfr2° mice were fully susceptible to PEA-induced intrahepatic activation of NF-κB, i.e., PEA-induced, Kupffer cell-dependent, intrahepatic activation of NF-κB does not depend on TNFR2 (Fig. 6A). Furthermore, also in tnfr2° mice, PEA-induced intrahepatic activation of NF-κB was inhibited in the absence of Kupffer cells (Fig. 6A), allowing the conclusion that Kupffer cell-expressed TNFR2 does not play a role in intrahepatic activation of NF-κB. Similar results were obtained by the analysis of PEA-induced intrahepatic synthesis of TNF-α and IL-6 mRNAs. Both intrahepatic cytokine mRNAs were clearly induced by PEA within 2 h (Fig. 6, B and C). This intrahepatic induction of TNF-α and IL-6 mRNAs was inhibited in the absence of Kupffer cells, but it was unaffected by the absence of TNFR2 (full TNF-α and IL-6 mRNA responses in tnfr2° mice) (Fig. 6, B and C). As with activation of intrahepatic NF-κB, intrahepatic synthesis of TNF-α and IL-6 mRNAs was completely inhibited in tnfr2° mice that had been depleted of Kupffer cells (Fig. 6, B and C). Taken together, Kupffer cell-expressed TNFR2 does not play a role in intrahepatic activation of NF-κB and synthesis of NF-κB-dependent cytokine mRNAs such as TNF-α and IL-6 mRNAs. This was confirmed by determination of plasma TNF-α and IL-6 levels 12 h after injection of PEA, i.e., at the time point at which plasma

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**FIGURE 4.** PEA-induced alterations in the plasma levels of soluble TNFR2. Plasma levels of soluble TNFR2 were determined at different stages following injection of PEA to C57BL/6 mice by an ELISA. Data are expressed as the mean ± SEM (n = 3). *, p < 0.05 vs t = 0.
cytokine levels can be determined in the PEA model (5). Both plasma cytokine levels were not significantly diminished in PEA-treated \( \text{tnfr}^2 \) mice, as compared with PEA-treated wild-type mice (wild-type: TNF-\( \alpha \), 428 \pm 112 pg/ml; IL-6, 493 \pm 126 pg/ml; \( \text{tnfr}^2 \): TNF-\( \alpha \), 418 \pm 65 pg/ml; IL-6, 383 \pm 84 pg/ml). Hence, we

**FIGURE 5.** Relevance of TNFR2-expressing radiation-sensitive, bone marrow transplantable leukocytes for PEA-induced liver injury. A, Verification of successful bone marrow transplantation. Con A-stimulated blood leukocytes from C57BL/6 wild-type, \( \text{tnfr}^2 \), wild-type \( \rightarrow \) wild-type, wild-type \( \rightarrow \) \( \text{tnfr}^2 \), and \( \text{tnfr}^2 \) \( \rightarrow \) wild-type mice were analyzed for TNFR2 expression using a laser scanning cytometer. The figure shows one representative example of each mouse type of four individuals, respectively. FSC, forward scatter. B, Intrahepatic accumulation of TNFR2-positive leukocytes in bone marrow-transplanted mice. Ten micrometer liver sections from C57BL/6 wild-type, \( \text{tnfr}^2 \), wild-type \( \rightarrow \) wild-type, wild-type \( \rightarrow \) \( \text{tnfr}^2 \), and \( \text{tnfr}^2 \) \( \rightarrow \) wild-type mice were subjected to immunofluorescent staining and confocal laser imaging 12 h after injection of saline or PEA. C, Plasma transaminase activities were determined 12 h after injection of saline or PEA to wild-type or \( \text{tnfr}^2 \) mice, as described in Materials and Methods. Data are expressed as the mean \( \pm \) SEM (\( n = 3 \)). *, \( p < 0.05 \) vs wild-type; #, \( p < 0.05 \) vs PEA.

**FIGURE 6.** Relevance of Kupffer cells and TNFR2 for PEA-induced intrahepatic NF-\( \kappa B \) activation, TNF, and IL-6 production. A, Liver nuclear extracts from wild-type and \( \text{tnfr}^2 \) mice were subjected to NF-\( \kappa B \)-specific EMSA 2 h after injection of saline or PEA. Depletion of Kupffer cells was achieved by pretreatment with Cl\(_2\)MBP liposomes. Specificity of the NF-\( \kappa B \) \( \rightarrow \) DNA binding reaction was confirmed by incubation of nuclear extracts from PEA-injected \( \text{tnfr}^2 \) mice with unlabeled (“cold”) oligonucleotides for NF-\( \kappa B \) or AP-2. B and C, Intrahepatic TNF (B) and IL-6 (C) mRNA were quantified by means of real-time RT-PCR 2 h after injection of saline or PEA to wild-type or \( \text{tnfr}^2 \) mice, as described in Materials and Methods. Depletion of Kupffer cells was achieved by pretreatment with Cl\(_2\)MBP liposomes. Data are expressed as the mean \( \pm \) SEM (\( n = 3 \)). *, \( p < 0.05 \) vs saline; #, \( p < 0.05 \) vs PEA.

\( \text{tnfr}^2 \), and \( \text{tnfr}^2 \) \( \rightarrow \) wild-type mice were subjected to immunofluorescent staining and confocal laser imaging 12 h after injection of PEA. TNFR2 was stained with a polyclonal IgG fraction of rabbit-anti-mouse TNFR2 (Cy3, red). C, Plasma transaminase activities were determined 12 h after injection of PEA to C57BL/6 wild-type, \( \text{tnfr}^2 \), wild-type \( \rightarrow \) wild-type, wild-type \( \rightarrow \) \( \text{tnfr}^2 \), and \( \text{tnfr}^2 \) \( \rightarrow \) wild-type mice. Data are expressed as the mean \( \pm \) SEM (\( n = 4 \)). *, \( p < 0.05 \) vs wild-type and wild-type \( \rightarrow \) wild-type, respectively.
can conclude that TNFR2 is not important on leukocytes for activating NF-κB and sustaining the production of cytokines, such as TNF-α or IL-6.

Role of TNFR2, expressed by cultured primary mouse hepatocytes, in cooperative cell death signaling

Because leukocyte-expressed TNFR2 does not appear to play a role in PEA-induced liver injury, our data are in favor of a role of TNF2 on hepatocytes by sustaining TNFR1-induced cell death of liver parenchymal cells. To investigate whether it is really possible to drive hepatocytes directly to cell death by a TNFR1-dependent, TNFR2-enhanced mechanism, we incubated cultured primary hepatocytes from wild-type and \textit{tnfr2} \textsuperscript{-/-} mice with agonistic Abs against TNFR1 and TNFR2 and determined the cytotoxicity of this treatment using the LDH release assay. First, we checked whether primary hepatocytes from wild-type mice do express TNFR2. To this end, we stained cultured hepatocytes with the very same agonistic anti-TNFR2 Ab that was also used for stimulation. Indeed, primary hepatocytes from wild-type, but not from \textit{tnfr2} \textsuperscript{-/-}, mice clearly expressed TNFR2 (Fig. 7A). Incubation of primary hepatocytes from wild-type mice with either an agonistic Ab against TNFR1 or an agonistic Ab against TNFR2 failed to induce significant LDH release (Fig. 7B). However, combination of these agonistic Abs caused cell death of primary hepatocytes from wild-type mice (Fig. 7B), pointing to the existence of a “cooperative cell death signaling” mechanism in parenchymal cells such as hepatocytes. As a control, we also incubated primary hepatocytes from \textit{tnfr2} \textsuperscript{-/-} mice. In this case, “cooperative cell death signaling” could not be observed (Fig. 7B), proving that the effect was not unspecific, but clearly mediated by activation of TNFR2.

Discussion

In this study, we show that PEA induced changes in the intrahepatic expression of TNFR2. Most obviously, strongly TNFR2-expressing non-Kupffer cell leukocytes accumulated within livers of PEA-treated mice, whereas Kupffer cells and hepatocytes lost their TNFR2, most probably by shedding, as suggested by a rapid increase in soluble TNFR2 in plasma. A similar increase in soluble TNFR2, accompanied by a loss of liver parenchymal TNFR2 expression, has been observed in the Con A mouse model of T cell-dependent hepatitis as well (11), and also inflammatory liver disease in humans is associated with high levels of soluble TNFR2 (24–27). Therefore, this phenomenon appears to be characteristic for inflammatory liver disease. It should be noted that inflammatory liver disease in humans has been associated with increased levels of TNFR2 mRNA and protein within the leukocyte compartment (23, 26, 27), similar to our results. The accumulation of strongly TNFR2-expressing leukocytes might explain the second increase in soluble TNFR2 in plasma at later time points. It has been shown that an increase in the expression of TNFR2 is associated with an increase in soluble TNFR2 in plasma (16). Rapid shedding of high amounts of TNFR2 from Kupffer cells and hepatocytes might provide soluble TNFR2 for binding circulating TNF-α resulting in inactivation of TNF-α (42–44) and enhancement of renal clearance of TNF-α (43), being a protective mechanism. Furthermore, rapid shedding of TNFR2 from hepatocytes might be an attempt for protection from excessive TNF-α-induced parenchymal cell death (see below), because the loss of cell surface TNFR2 should lead to a decreased responsiveness of these cells to TNF-α.

Although we identified several types of strongly TNFR2-expressing leukocyte populations accumulating throughout the pathogenic process, especially neutrophils and NK cells, TNFR2 expression by these cells did not play a role in the development of liver disease. This conclusion could be drawn because \textit{tnfr2} \textsuperscript{-/-} → wild-type bone marrow chimeras were highly susceptible to PEA-induced liver injury with no TNFR2-expressing leukocytes accumulating in the liver. Conversely, PEA-induced liver injury was attenuated in wild-type → \textit{tnfr2} \textsuperscript{-/-} mice, although TNFR2-expressing leukocytes strongly accumulated. Leukocyte TNFR2 even appears to be protective, because \textit{tnfr2} \textsuperscript{-/-} → wild-type mice were significantly more sensitive to PEA-induced liver injury than wild-type or wild-type → wild-type mice. This might be explained by a TNF buffering property of leukocyte TNFR2. It is obviously not the conspicuous PEA-induced leukocyte TNFR2 expression that accounts for the TNFR2 dependence of liver injury. We might suggest that the observed leukocyte TNFR2 expression is just a result of leukocyte activation. Hence, although TNFR2 expression on neutrophils and NK cells does not appear to be important for the manifestation of PEA-induced liver injury, our results might point to a role of these cells in the disease process by other mechanisms, because these cells accumulate and appear to become activated. A role of neutrophils in inflammatory liver disease has been proposed in liver injury induced by combined injection of α-galactosamine and LPS (45) or by ischemia reperfusion (46). A role of NK cells in hepatocyte death has been described for infection models, e.g.,

![FIGURE 7. Role of TNFR2, expressed by cultured primary mouse hepatocytes, in cooperative cell death signaling.](http://www.jimmunol.org/)

A. Primary hepatocytes from wild-type, but not \textit{tnfr2} \textsuperscript{-/-}, mice express TNFR2. Primary naive hepatocytes from untreated wild-type and \textit{tnfr2} \textsuperscript{-/-} mice were subjected to immunofluorescent staining and confocal laser imaging. TNFR2 was stained with a polyclonal IgG fraction of rabbit-anti-mouse TNFR2. Binding sites were detected with a secondary, Cy3-labeled goat-anti-rabbit IgG (red fluorescence). B. Cell death of cultured primary hepatocytes from wild-type and \textit{tnfr2} \textsuperscript{-/-} mice, incubated with medium, agonistic anti-TNFR1 Ab, agonistic anti-TNFR2 Ab, or a combination of both agonistic Abs for 20 h, was determined using the LDH release assay. Data are expressed as the mean ± SEM (\(n = 3\)). * \(p < 0.05\) vs all other incubations.
during infection with adenoviruses (37). Further studies are currently running to clarify the roles of neutrophils and NK cells in the model of PEA-induced T cell-dependent liver injury in mice.

Because macrophages, including Kupffer cells, are known to be largely radioresistant (40, 41), a fact that we could confirm in our experiments (data not shown), and because Kupffer cells are a central cell type being involved in the disease process (6), we further focused on a possible role of Kupffer cell-expressed TNFR2 in PEA-induced intrahepatic activation of NF-κB and in PEA-induced synthesis of typical NF-κB-dependent Kupffer cell-produced cytokines such as TNF-α and IL-6. Our results show that intrahepatic activation of NF-κB and intrahepatic induction of TNF-α and IL-6 mRNA were fully dependent on Kupffer cells, but not on TNFR2. This also supports recent results showing that Con A-induced activation of NF-κB does not require the presence of TNFR2 (47). Taken together, we might conclude that TNFR2 is not important on Kupffer cells for immunostimulatory consequences such as activation of NF-κB and production of Kupffer cell-dependent cytokines. Although this is only indirect evidence, leaving the possibility of relevant TNFR2-mediated events which are independent of NF-κB, we consider a significant role of Kupffer cell-expressed TNFR2 in PEA-induced liver injury unlikely, especially when considering that Kupffer cells play an important role in PEA-induced liver injury by producing TNF-α (6). Hence, leukocyte TNFR2 expression does not appear to play a role in the intrahepatic inflammatory processes induced by PEA. This result supports earlier observations, describing even elevated TNF-α levels in LPS- or Con A-treated mice (10, 29, 48). This is somewhat surprising because of the stereotypy of leukocyte TNFR2 expression in different kinds of inflammatory liver disease (see above) and because of the severe inflammatory syndrome, including inflammatory liver disease, that is accompanied by increased NF-κB activity in the leukocyte compartment and by dramatically increased levels of endogenous TNF-α and of Con A-inducible IFN-γ, caused by overexpression of human TNFR2 in transgenic mice (10, 16). This apparent inconsistency might be explained by the wide distribution of overexpressed TNFR2 in the transgenic mice, in which TNFR2 is strongly expressed on many cell types, including Kupffer cells (16). In our model, Kupffer cells rapidly lost their TNFR2 following injection of PEA, most probably by shedding (see above), and therefore do not continuously express high amounts of TNFR2 on their surface.

It has to be considered that endothelial TNFR2 plays a role in T cell-dependent liver injury, especially by mediating the synthesis of adhesion molecules, which are necessary for the infiltration of leukocytes such as T cells, neutrophils, and NK cells to parenchymal tissues (49, 50). However, we consider this unlikely, because a very detailed study revealed that TNFR2 does not play a role in the induction of endothelial activation and expression of cell adhesion molecules such as ICAM-1, VCAM-1, or E-selectin in another model of T cell-dependent liver injury in mice (11), and because leukocyte infiltration into liver parenchyma was not influenced by the absence of TNFR2 in tnfr2−/− mice, as demonstrated by flow cytometric analysis of intrahepatic CD45+ cells in PEA-treated wild-type as compared with tnfr2−/− mice (data not shown).

Primary hepatocytes from wild-type, but not from tnfr2−/− mice expressed TNFR2 and were susceptible to synergistic cytotoxicity of otherwise non-toxic agonistic Abs against TNFR1 and TNFR2. This strongly supports “cooperative cell death signaling” in hepatocytes being the mechanism by which TNFR2 participates in the disease process, i.e., we propose an important role of TNFR2 on hepatocytes. The term “cooperative cell death signaling” has been created by Grell et al. (9), who showed that activation of TNFR2 by its prime ligand, the transmembrane form of TNF-α, rendered resistant tumor cells sensitive to TNFR1-mediated cytotoxicity. The existence of “cooperative cell death signaling” has been observed in many other cell lines as well (17–22), but it has not been shown before for primary parenchymal cells. The synergistic cytotoxic action of agonistic Abs against TNFR1 and TNFR2 to primary mouse hepatocyte cultures clearly showed the existence of a “cooperative cell death signaling” pathway in hepatocytes. “Cooperative cell death signaling” most probably also occurs in the transgenic mice overexpressing human TNFR2, because transgenic TNFR2 expression was high within the liver (16). Interestingly, overexpression of human TNFR2 potently sensitized transgenic mice to the toxicity of an otherwise sublethal dose of either rTNF-α or LPS (16) or Con A (10).

For a mechanistic explanation of “cooperative cell death signaling”, we refer to the very interesting work performed by Streeetz et al. (27) who showed that primary mouse hepatocytes infected with adenoviruses expressing a dominant negative form of TNF-associated factor (TRAF) 2 or an NF-κB superrepressor were sensitive to otherwise noncytotoxic soluble rTNF-α, which primarily activates TRAF1 (8). Interestingly, the effects of dominant negative TRAF2 and the NF-κB superrepressor were independent from each other, i.e., dominant negative TRAF2 did not block NF-κB activation (27). From cell line experiments it is known that TNFR2 is able to down-regulate TRAF2 (19, 21), i.e., TNFR2 appears to be able to generate a situation similar to the presence of dominant negative TRAF2. Therefore, down-regulation of TRAF2 might also be the mechanism by which TNFR2 mediates “cooperative cell death signaling” in PEA-induced, T cell-dependent liver disease.

In summary, our results strongly suggest a role of parenchymal TNFR2 in T cell-dependent liver injury in vivo. The activation of leukocytes does not appear to be disturbed by the absence of TNFR2. Therefore, antagonization of TNFR2 might be an interesting approach for an at least adjuvant therapy of immune-mediated liver injury and perhaps also of infections with PEA-producing pseudomonads without disturbing host defense.

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