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NK Cells, but Not NKT Cells, Are Involved in *Pseudomonas aeruginosa* Exotoxin A-Induced Hepatotoxicity in Mice[1]

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Pseudomonas aeruginosa **exotoxin A (PEA) causes T cell- and Kupffer cell (KC)-dependent liver injury in mice. TNF-α as well as IL-18 and perforin are important mediators of liver damage following PEA injection. In this study, we focus on the role of NK and NKT cells in PEA-induced liver toxicity. Depletion of both NK and NKT cells by injection of anti-NK1.1 Ab as well as depletion of NK cells alone by anti-asialo GM1 Ab protected mice from PEA-induced hepatotoxicity, whereas mice lacking only NKT cells were susceptible. Additionally, we observed infiltration of NK cells, T cells, and neutrophils into liver parenchyma after injection of PEA. The number of NKT cells, however, remained unchanged. The increase in intrahepatic NK cells depended on KCs and the TNF-α-dependent up-regulation of the adhesion molecule VCAM-1 in the liver, but not on NKT cells. PEA also augmented the cytotoxicity of hepatic NK cells against typical NK target cells (YAC-1 cells). This effect depended on KCs, but not on TNF-α or NKT cells. Furthermore, only weak expression of MHC class I was detected on hepatocytes, which was further down-regulated in PEA-treated mice. This could explain the susceptibility of hepatocytes to NK cell cytolytic activity in this model. Our results demonstrate that NK cells, activated and recruited independently of NKT cells, contribute to PEA-induced T cell-dependent liver injury in mice.** ***The Journal of Immunology,*** **2004, 172: 3034–3041.**

Human viral or autoimmune hepatitis is accompanied by overstimulation of the immune system (1, 2). Further insight into the pathophysiology of inflammatory liver disease is necessary for identification of new targets for hepatoprotective treatment. Therefore, several animal models of T cell-dependent liver failure have been developed (3, 4). We recently described a mouse model of T cell-mediated hepatic injury induced by the injection of *Pseudomonas aeruginosa* exotoxin A (PEA)[4] (5). In this model, liver damage depends on T cells that activate Kupffer cells (KCs) to produce TNF-α (5, 6). TNF-α is involved in the disease process by triggering cooperative cell death signaling through TNFR1 and TNFR2 on hepatocytes (7). Other known mediators of PEA-induced liver injury include IL-18 (8) and perforin (5).

IL-18 is known to activate NK and NKT cells (9). Both of these cell types are abundant in liver tissue (10, 11). Upon activation, they produce large amounts of cytokines, such as IFN-γ and IL-4 (10, 11), and exert cytotoxicity mediated by release of perforin/granzyme B and by expression of Fas ligand (11, 12). NKT cells are a subset of T cells expressing both an invariant Vα14 TCR and typical NK cell markers (11). They were shown to be rapidly activated upon stimulation in vivo. In vivo activators of NKT cells include the artificial ligand α-galactosylceramide (α-GalCer) and general T cell activators such as Con A or agonistic anti-CD3 mAb. Injections of α-GalCer or Con A are known to induce NKT cell-dependent liver injury in mice (13, 14). However, NK cells seem not to play a critical role in Con A-mediated liver injury (13), whereas α-GalCer was shown to induce NKT cell-dependent NK cell activation (15, 16). Other authors showed that NK cells can cause hepatocyte apoptosis in virus-infected livers of mice (17).

Hence, the aim of this study was to investigate the involvement of NK and NKT cells in PEA-induced liver injury, thereby further elucidating the pathogenetic mechanisms of immune-mediated liver injury. We show that NKT cell-independent activation of NK cells, which are recruited to liver tissue in dependence of TNF-α and VCAM-1, contribute to PEA-induced liver injury in mice.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany). B6.*cd1d*° mice were generated, as described (18). 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 were fed a standard laboratory chow (Altromin 1313; Altromin, Lage, Germany) ad libitum. For the experiments, 8- to 10-wk-old male mice weighing 20–25 g were used.

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[4] Abbreviations used in this paper: PEA, *Pseudomonas aeruginosa* exotoxin A; α-GalCer, α-galactosylceramide; Cl_2MBP liposome, liposome-encapsulated dichloromethylene-bisphosphonate; KC, Kupffer cell; MFI, mean fluorescence intensity.

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Animal treatments

PEA (Sigma-Aldrich, Taufkirchen, Germany) was injected i.v. in pyrogene-free saline containing 0.1% human serum albumin at a dose of 500 μg/kg in a total volume of 250 μl/25 g mouse. For depletion of KCs, C57BL/6 mice were injected with 100 μl of liposome-encapsulated dichloromethylene-bisphosphonate (Cl_2MBP liposomes) i.v. 48 h before challenge, as described previously (6, 19). Cl_2MBP used for preparation of Cl_2MBP liposomes was a gift of Roche Diagnostics (Mannheim, Germany). In control experiments, C57BL/6 mice were pretreated with saline instead of Cl_2MBP liposomes. Saline liposomes were not used because liposomes themselves block macrophage phagocytosis for certain periods of time (20). Depletion of NK cells was achieved by i.v. injection of 80 μl/mouse of rabbit anti-asialo GM1 polyclonal Ab solution (Wako Chemicals, Neuss, Germany) 4 days before challenge, depletion of both NK and NKT cells by i.p. injection of 200 μg/mouse of anti-NK1.1 mAb (clone PK136; BD PharMingen, Heidelberg, Germany) to C57BL/6 wild-type or *cd1d*° mice at days −4 and −2 before PEA injection. For in vivo neutralization of TNF, mice were injected i.v. with 200 μg/mouse polyclonal sheep anti-mouse TNF-α IgG in saline/0.1% human serum albumin 15 min before challenge. In vivo block of VCAM-1 was achieved by i.v. injection of 4 mg/kg purified rat anti-mouse CD 106 (VCAM-1) mAb (clone 429; BD PharMingen) 15 min before challenge. Control animals received injections of irrelevant Abs: rabbit IgG (Sigma-Aldrich), rat IgG1 mAb (clone R3-34; BD PharMingen), or mouse IgG2a (Sigma-Aldrich). To elucidate the effect of LPS treatment on MHC class I expression, mice were treated i.p. with 25 μg/mouse of LPS from *Salmonella abortus equi* (Metalon, Ragow, Germany) solved in saline and injected in a total volume of 250 μl. Mice were sacrificed 48 h after LPS treatment.

Sampling of material

Mice were lethally anesthetized with 150 mg/kg i.v. pentobarbital, containing 15 mg/kg heparin. Blood was withdrawn by cardiac puncture for analysis of plasma transaminases. 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. For flow cytometric analysis of liver leukocytes and cytotoxicity assays, complete fresh livers were used.

Analysis of transaminases

Liver injury was quantified 12 h after PEA administration by determination of plasma enzyme activities of alanine aminotransferase and aspartate aminotransferase, according to Bergmeyer (21), using an automated procedure.

Immunofluorescent staining and confocal laser imaging

The expression of VCAM-1 in liver tissue as well as the efficiency of KC depletion were analyzed by immunofluorescent staining and confocal laser imaging. Cryostat sections (10 μm) of livers were thawed onto glass slides, air dried, and fixed in acetone/methanol (1/1) at 4°C for 10 min. After washing in PBS, the sections were blocked with 3% BSA/PBS at room temperature for 30 min. Incubation was continued with rat anti-mouse VCAM-1 mAb (clone 429; BD PharMingen; 1/100) or rat mAb directed against murine macrophages (clone BM8; Dianova, Hamburg, Germany; 1/100) dissolved in 3% BSA/PBS overnight at 4°C. After rinsing with PBS, binding sites were detected using Texas Red-labeled goat anti-rat IgG Ab (Dianova; 1/200) diluted in 3% BSA/PBS at room temperature for 1 h. After rinsing with PBS, sections were coverslipped with 10% glycerol/PBS, pH 8.6, and examined by confocal laser-scanning microscopy (Axiovert 100M; Carl Zeiss, Oberkochen, Germany).

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

RNA was isolated from pieces of ~25 mg of liver tissue by use of a RNA purification kit (Qiagen, Hilden, Germany). A total of 1 μg 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, Mannheim, Germany). The following oligonucleotide pairs were used: β-actin, 728–751 and 1076–1052 in GenBank X03765 and VCAM-1, 516–536 and 1034–1015 in GenBank NM011693. 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. VCAM-1: 95°C, 1 s; 57°C, 7 s; 72°C, 22 s. mRNA levels were calculated using the comparative C_t method (22) and normalization to β-actin. To confirm amplification specificity, 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.

Isolation of liver leukocytes and hepatic NK cells

Leukocytes were isolated from livers essentially as described previously (17). Briefly, livers were passed through 100-μm nylon meshes in HBSS solution. After centrifugation for 5 min at 500 × *g*, the cell pellet was resuspended in isotonic 37% Percoll solution containing 100 U/ml heparin. The cell suspension was centrifuged for 20 min at 800 × *g*, and the remaining cell pellet was resuspended in RBC lysis solution containing 139 mM NH_4Cl and 19 mM Tris. After incubation for 10 min, the cells were washed twice with HBSS containing 5% basal medium supplement (Biochrom, Berlin, Germany). For further isolation of NK cells, the MACS system and anti-NK cell (DX 5) MicroBeads were used according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Isolation of hepatocytes and spleen cells

The two-step collagenase perfusion method of Seglen (23) was used to isolate hepatocytes. After hepatocyte isolation, the spleen was removed and spleen cells were isolated. Briefly, the spleen was pressed through a 100-μm nylon mesh in FACS buffer containing 1% BSA and 0.05% NaN_3

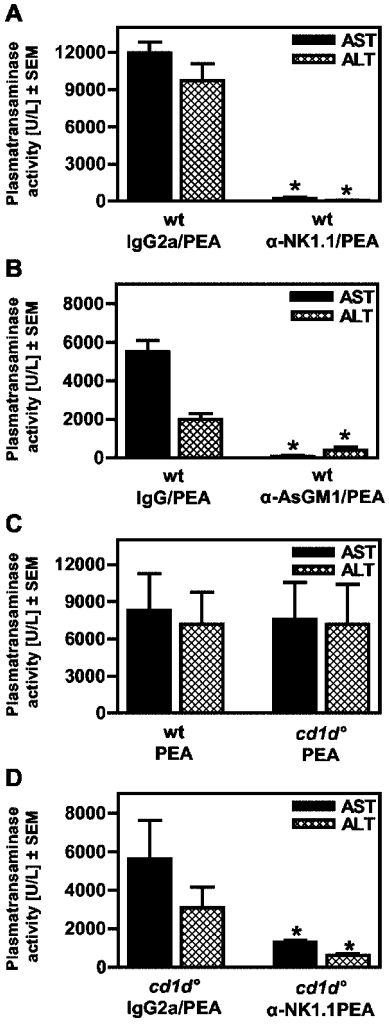

FIGURE 1. PEA hepatotoxicity in NK and NKT cell-deficient mice. C57BL/6 wild-type mice received 500 μg/kg PEA i.v. after pretreatment with 200 μg/mouse of anti-NK1.1 mAb on days −4 and −2 for NK and NKT cell depletion (*A*) or 4 days after pretreatment with 80 μl/mouse of rabbit anti-asialo GM1 Ab for depletion of NK cells (*B*). Control animals were pretreated with irrelevant mouse IgG2a or rabbit IgG, respectively. *C*, C57BL/6 wild-type and *cd1d*° mice were injected with 500 μg/kg PEA i.v. *D*, *cd1d*° mice were pretreated with 200 μg/mouse of anti-NK1.1 4 and 2 days before i.v. injection of 500 μg/kg PEA. Control animals received mouse IgG2a instead of anti-NK1.1 mAb. Blood was withdrawn for transaminase determination 12 h after challenge with PEA. Data are expressed as the mean ± SEM ($n = 3$). *, $p \leq 0.05$ vs control or wild type.

in PBS. After centrifugation, the cell pellet was resuspended in RBC lysis solution and incubated for 10 min. Subsequently, the cells were washed in FACS buffer and resuspended in FACS buffer for flow cytometric analysis.

Flow cytometric analysis of liver leukocytes, spleen cells, and hepatocytes

For flow cytometric analysis, 10^6 cells were stained using a standard protocol. The following Abs were used: FITC-labeled Armenian hamster anti-mouse CD3ϵ mAb (clone 145-2C11; BD PharMingen), PE-labeled mouse anti-mouse NK1.1 mAb (clone PK136; BD PharMingen), PE-labeled rat anti-mouse CD19 (clone 1D3; BD PharMingen), PE-labeled rat anti-mouse Ly-6G mAb (clone RB6-8C5; BD PharMingen), rat anti-mouse MHC class I (clone ER-HR 52; Biomedicals AG, Augst, Switzerland), isotype-matched control IgG (Biozol, Eching, Germany), and PE-labeled goat anti-rat IgG (Dianova). Flow cytometric analysis was performed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Krefeld, Germany) or on a FACScan flow cytometer (BD Biosciences, Heidelberg, 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.

Cytotoxicity assay

NK cytotoxicity was determined by using a standard 4-h ^{51}Cr release assay. YAC-1 cells (American Type Culture Collection, Manassas, VA) were used as target cells, and liver leukocytes or isolated hepatic NK cells were used as effector cells. Target cells were incubated with effector cells at various E:T ratios, and specific lysis (%) was determined as follows: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

Statistical analysis

The results were analyzed by one-way ANOVA, followed by the Dunnett or the Student-Newman-Keuls multiple comparison tests. All data in this study are expressed as the mean ± SE. Value of $p < 0.05$ was considered significant.

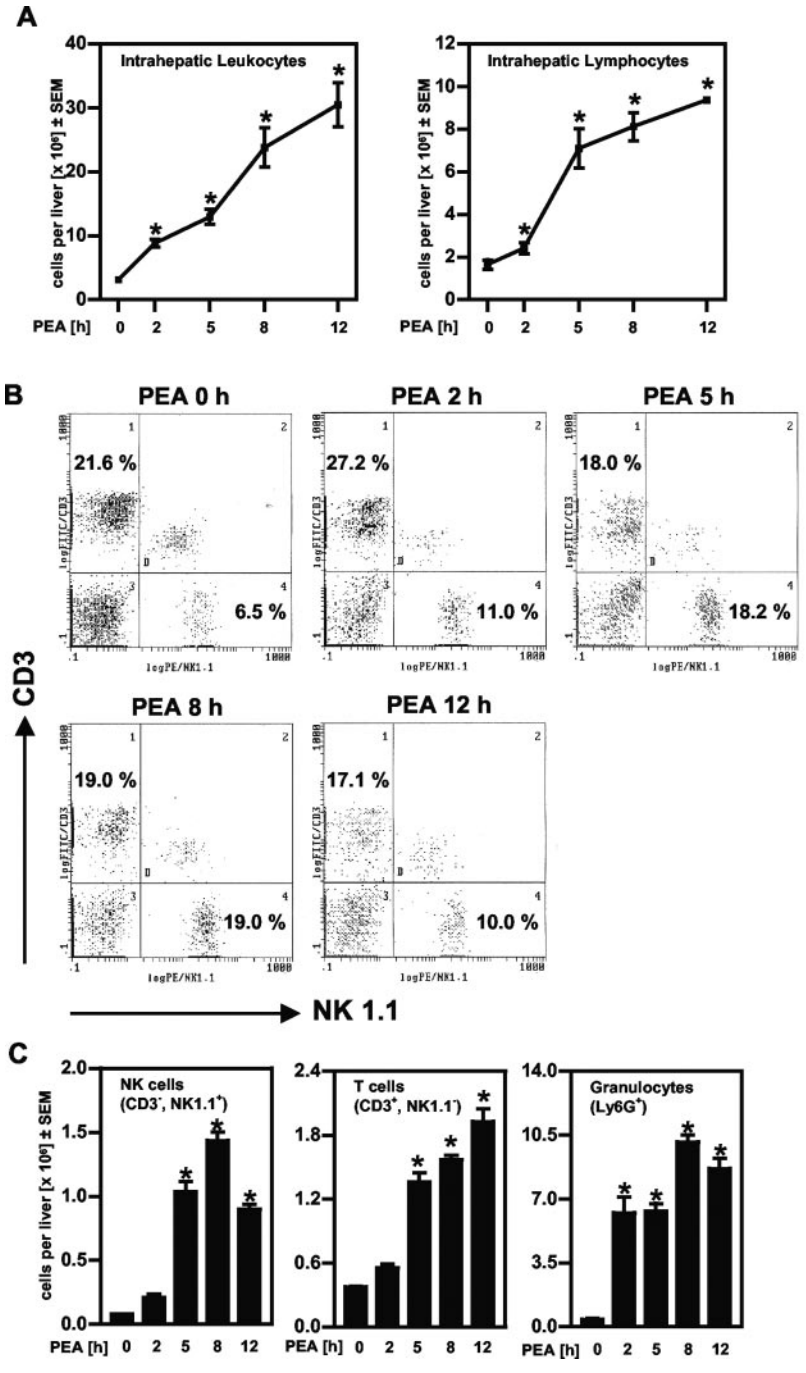

FIGURE 2. Time course of leukocyte recruitment to liver parenchyma in PEA-treated C57BL/6 mice. C57BL/6 mice were treated with 500 μg/kg PEA i.v. At the indicated times, intrahepatic leukocytes were isolated and subjected to immunofluorescent staining and flow cytometric analysis using Abs specific for CD3ϵ and NK1.1 or Ly-6G. *A*, Leukocyte yield per liver and absolute numbers of lymphocytes per liver, calculated by multiplying the total number of isolated leukocytes with the percentage of cells in the lymphoid gate. *B*, NK1.1 vs CD3ϵ staining on lymphoid-gated cells. *C*, Absolute numbers of NK and T cells as well as granulocytes per liver. The proportion of NK and T cells within hepatic lymphocytes was combined with the total lymphocyte yield per liver to present absolute numbers, as shown. Absolute numbers of granulocytes were calculated by multiplication of the percentage of Ly-6G-positive cells with the total number of leukocytes per liver. Data are expressed as the mean ± SEM ($n = 3$). ∗, $p \leq 0.05$ vs 0 h.

Results

PEA-induced liver injury depends on NK cells, but not on NKT cells

Previous studies from our laboratory showed that treatment of mice with PEA results in highly increased plasma transaminase activities 12 h after i.v. injection, which depended on T lymphocytes and KCs (5, 6). To analyze the role of NK and NKT cells for PEA-induced liver damage, we first depleted both NK and NKT cells by administration of anti-NK1.1 mAb to C57BL/6 mice. As shown in Fig. 1*A*, depletion of both cell types significantly protected mice from PEA-induced liver failure. To discriminate between NK and NKT cells, we either depleted NK cells by injection of polyclonal anti-asialo GM1 Ab or we used NKT cell-deficient *cd1d*° mice (18). Ab-mediated depletion of NK cells alone also significantly inhibited the release of transaminases to plasma after PEA administration compared with animals treated with control Ab (Fig. 1*B*). However, NKT cell-deficient *cd1d*° mice were as sensitive as control animals toward the toxin (Fig. 1*C*), indicating that NKT cells, in contrast to NK cells, are not important for PEA-induced liver injury. Depletion of NK cells by injection of anti-NK1.1 Ab to *cd1d*° mice again significantly reduced the release of alanine aminotransferase and aspartate aminotransferase after PEA treatment (Fig. 1*D*). In control experiments, *cd1d*° mice were injected i.v. with 20 mg/kg Con A. It has been described previously that Con A induces T cell-dependent hepatitis in mice (3) that depends on NKT cells, which has been shown by resistance of *cd1d*° mice (24). In these control experiments, *cd1d*° mice failed to release IL-4 and to develop liver injury upon Con A injection (data not shown).

PEA induces infiltration of NK cells, T cells, and neutrophils into liver parenchyma

The strong accumulation of leukocytes in the livers of PEA-treated mice (Fig. 2*A*) prompted us to further characterize the infiltrating leukocyte populations by flow cytometry. We found a strong increase in the numbers of lymphocytes, which were calculated by multiplying the total number of isolated leukocytes with the percentage of cells in the lymphoid gate (Fig. 2*A*). Within lymphocyte populations, we observed a strong increase in NK cell percentages and a decrease in the percentages of other populations such as T cells (Fig. 2*B*). As an increase in the percentage of one population causes a decrease in the percentage of other populations, although their total number may not have changed, we checked for changes in absolute numbers of cells per liver. Therefore, we combined the proportions of cells within hepatic lymphocytes with the total lymphocyte yield per liver (for NK, NKT, T, and B cells). For calculation of the total number of granulocytes, the percentage of Ly-6G-positive cells within leukocytes was multiplied with the total number of leukocytes per liver. We observed a strong time-dependent increase in the absolute numbers of NK cells ($NK1.1^+$, $CD3^-$), T cells ($NK1.1^-$, $CD3^+$), and neutrophils ($Ly\text{-}6G^+$) in a time frame of 12 h following PEA injection (Fig. 2*C*), whereas numbers of NKT ($NK1.1^+$, $CD3^+$) and B cells ($CD19^+$) were not significantly increased (data not shown).

PEA augments cytotoxicity of hepatic NK cells

Next, we investigated whether the infiltrating NK cells after PEA administration show differences in cytotoxicity compared with hepatic NK cells from untreated mice. Therefore, we enriched NK cells from liver leukocytes of untreated and PEA-treated mice using the MACS system. In ^{51}Cr release assays, we measured the cytotoxicity of these NK cells against YAC-1 cells, a typical NK target cell. Five hours after challenge with PEA, the cytotoxicity of intrahepatic NK cells significantly increased compared with NK cells from untreated mice (Fig. 3*A*).

Contribution of KCs and NKT cells to NK cell infiltration and cytotoxicity after PEA injection

In additional experiments, we investigated whether the absence of KCs or NKT cells alters the accumulation of NK cells or the increase in NK cell cytotoxicity after PEA treatment. In these experiments, KCs were depleted using clodronate liposomes, or NKT cell-deficent mice (*cd1d*°) were used. The efficiency of KC depletion was verified by immunofluorescent staining and subsequent confocal laser imaging (Fig. 4). Both the NK cell infiltration into liver parenchyma (Fig. 5) and the increase in NK cell cytotoxicity (Fig. 3, *B* and *C*, respectively) after PEA treatment depended on KCs, but were independent of NKT cells.

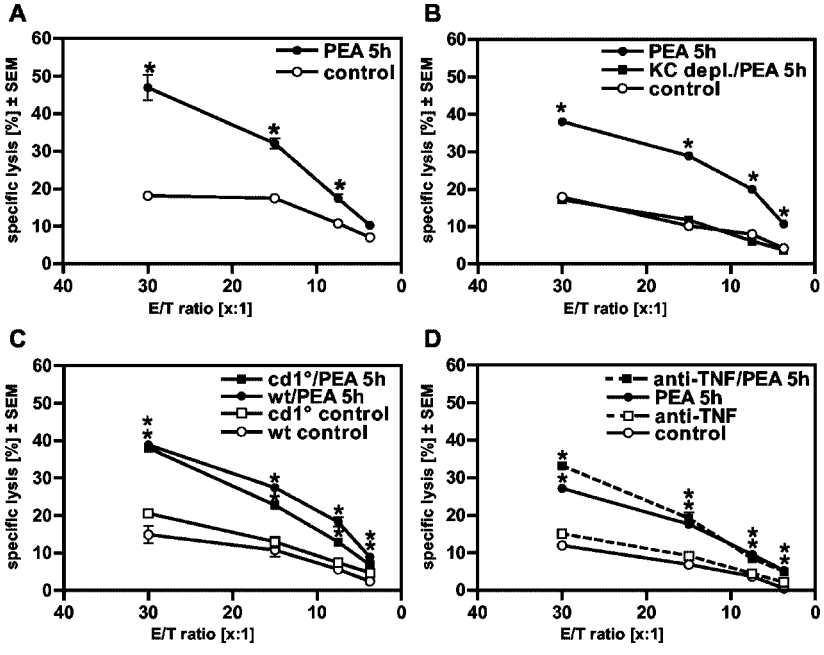

FIGURE 3. Cytotoxicity of hepatic NK cells against YAC-1 target cells. C57BL/6 wild-type (*A–D*) and *cd1d*° (*C*) mice were treated with 500 μg/kg PEA or saline. In some experiments, C57BL/6 mice were pretreated with neutralizing anti-TNF Ab (*D*) or injected with Cl_2MBP liposomes for KC depletion (*B*). Hepatic leukocytes were isolated 5 h after PEA injection, and leukocytes from three to six mice were pooled. NK cells were enriched by labeling with anti-DX5 microbeads and positive selection using the MACS system. Data are expressed as the mean ± SEM ($n = 3$). *, $p < 0.05$ vs solvent.

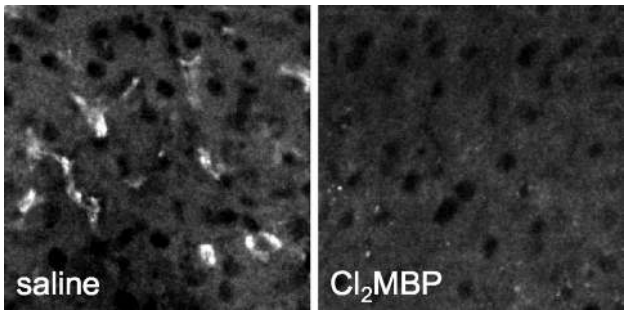

FIGURE 4. KC depletion by Cl_2MBP liposomes. C57BL/6 mice were treated with 100 μg/mouse of liposomal encapsulated Cl_2MBP for depletion of KCs. Control animals received saline. Liver sections were subjected to immunofluorescent staining and subsequent confocal laser imaging 48 h after treatment. KCs were stained with anti-macrophages mAb clone BM8, and binding sites were detected using goat anti-rat IgG tagged with Texas Red.

Role of TNF-α in hepatic NK cell recruitment and cytotoxicity following PEA injection

As KCs are the main source of intrahepatic TNF-α, which plays a major role in PEA-induced liver toxicity (5), we also analyzed the role of TNF-α in PEA-induced NK cell recruitment and cytotoxicity in the liver. In vivo neutralization of TNF-α revealed that PEA-induced infiltration of NK cells depends significantly on TNF-α (Fig. 5). The observed increase in cytotoxicity of hepatic NK cells against YAC-1 cells after PEA administration was shown to be independent of TNF-α, as treatment with anti-TNF-α Ab had no effect on the increase of NK cell cytotoxicity (Fig. 3*D*).

TNF-α-dependent up-regulation of hepatic VCAM-1 expression in PEA-treated mice

VCAM-1 is known to play a role in NK cell infiltration into the liver (25), and TNF-α is capable of inducing expression of VCAM-1 in liver sinusoids and portal veins (26). To elucidate PEA-induced changes in the expression of VCAM-1, we quantified the intrahepatic expression of VCAM-1 mRNA and protein. As shown in Fig. 6, PEA strongly increased the expression of VCAM-1 mRNA within liver tissue. Immunofluorescent staining and subsequent confocal laser imaging revealed increased intrahepatic expression of VCAM-1 protein after PEA injection (Fig. 7). This induction clearly depended on TNF-α, because pretreatment of mice with anti-TNF-α Ab attenuated PEA-induced VCAM-1

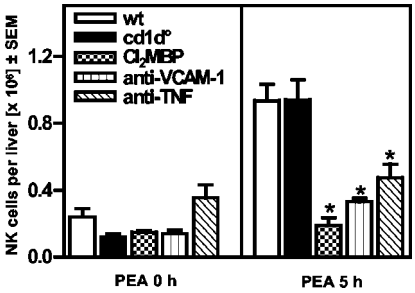

FIGURE 5. Dependence of PEA-induced NK cell infiltration. Intrahepatic leukocytes from C57BL/6 wild-type or *cd1d*° mice were isolated, stained for CD3ϵ and NK1.1, and analyzed by flow cytometry at the indicated time points after injection of PEA. Depletion of KC was achieved by pretreatment with Cl_2MBP liposomes; VCAM-1 was blocked or TNF was neutralized in vivo by injection of anti-VCAM-1 or anti-TNF Abs, respectively. Data are expressed as the mean ± SEM ($n = 3$). *, $p < 0.05$ vs PEA 0 h or PEA 5 h.

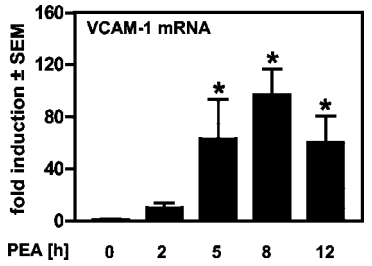

FIGURE 6. PEA-induced expression of VCAM-1 mRNA in liver tissue. Intrahepatic VCAM-1 mRNA was quantified by means of real-time RT-PCR at the indicated time points after injection of PEA, as described in *Materials and Methods*. Data are expressed as the mean ± SEM ($n = 3$). *, $p < 0.05$ vs 0 h.

expression (Fig. 7). Neutralization of VCAM-1 by use of blocking anti-VCAM-1 Ab significantly attenuated PEA-induced NK cell infiltration into liver parenchyma (Fig. 5), indicating that NK cell recruitment in this model depends on a TNF-α-dependent up-regulation of VCAM-1 expression in the liver.

Down-regulation of MHC class I expression on hepatocytes by PEA treatment

Flow cytometry revealed strong expression of MHC class I on spleen cells isolated from untreated mice (Fig. 8*A*), whereas primary hepatocytes from the same mice were only weakly positive (Fig. 8*B*). In vivo PEA further down-regulated MHC class I levels on hepatocytes 5 and 9 h after treatment (Fig. 8, *C* and *D*), respectively. In control experiments, LPS treatment of mice up-regulated hepatocyte MHC class I expression (Fig. 8*E*).

Discussion

This study clearly demonstrated that NK, but not NKT cells are required for liver injury following PEA injection to mice. Depletion of NK cells by anti-asialo GM1 Ab protected mice from PEA-induced liver injury, providing evidence for a major role of NK cells in this animal model. To rule out the possibility that the effect of anti-asialo GM1 was due to depletion of asialo-GM1-expressing T cells (27), we further showed that simultaneous depletion of both NK and NKT cells by an Ab specific for the NK1.1 Ag, which is not expressed by conventional T cells, protected mice from PEA-induced liver injury. Although NKT cells are very frequent cells in the liver (10, 11), which are rapidly activated by appropriate stimulation (11, 28) and in turn activate NK cells (11, 15, 16), they do not seem to be important in this model of immune-mediated liver injury, because NKT cell-deficient *cd1d*° mice were as susceptible to PEA-induced liver disease as wild-type mice. In contrast, it was previously shown that NKT cells are important in other in vivo models of immune-mediated liver injury, e.g., caused by administration of α-GalCer or the plant lectin Con A (13, 14). In our control experiments, this finding was confirmed, as *cd1d*° mice in contrast to wild-type mice failed to develop liver injury and did not show IL-4 release upon injection with Con A (data not shown).

PEA induced a strong increase in the number of liver leukocytes with mainly NK cells, T cells, and neutrophils infiltrating into liver parenchyma. The strong increase in the frequency of neutrophils among liver leukocytes as early as 2–4 h after PEA injection is not unexpected, as neutrophils are known to be recruited early to inflammatory sites and PEA induces a rapid intrahepatic production of proinflammatory cytokines such as TNF-α (5, 6). The observed infiltration of T cells into liver parenchyma was also not surprising, as T cells were previously shown to be important in PEA-mediated liver injury by inducing intrahepatic TNF-α production by KCs

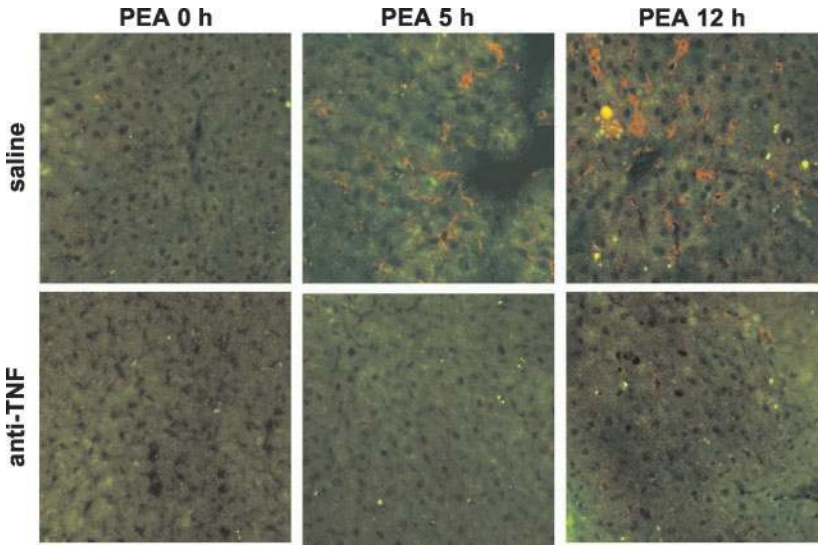

FIGURE 7. Changes in the intrahepatic expression of VCAM-1 protein following PEA injection. Ten-micrometer liver sections were subjected to immunofluorescent staining and confocal laser imaging at different stages following injection of PEA to C57BL/6 mice. In vivo neutralization of TNF was achieved by pretreatment with anti-TNF Ab. Control animals received saline instead of Ab. VCAM-1 was stained with anti-VCAM-1 mAb (clone 429), and binding sites were detected with goat anti-rat IgG tagged with Texas Red.

(5). Our findings are consistent with previous studies reporting that activated T cells migrate into the liver, where they are able to induce a transient cellular immune attack, which seems to be followed by T cell tolerance (29). Moreover, NK cells whose numbers are enriched in liver tissue after PEA treatment were also previously reported to recruit T cells to the liver through a multistep cytokine/chemokine cascade (29). Pretreament with anti-TNF-α Ab showed that PEA-induced TNF-α not only induces apoptosis in the liver, as it was described previously (5), but also plays an important role in NK cell infiltration after PEA injection. This was further supported by the block of NK cell infiltration in KC-depleted mice, as KCs are the main source of PEA-induced hepatic TNF-α (6). The expression of VCAM-1 protein in liver sinusoids and portal veins increased TNF-α dependently after PEA administration, and this augmented VCAM-1 expression contributed to NK cell infiltration, indicating that PEA mediates NK cell recruitment to the liver by TNF-α-dependent up-regulation of VCAM-1. These results are consistent with previous studies showing that expression of adhesion molecules such as VCAM-1 in lung, kidney, and liver endothelium of mice is induced by TNF-α (26, 30) and that VCAM-1 mediates NK cell infiltration into lung and liver parenchyma (25).

Increasing numbers of hepatic NK cells alone are not an explanation for the NK cell dependence of PEA-induced hepatic injury, as nonactivated NK cells do not produce high amounts of cytokines and the lytic activity of NK cells dramatically increases only after activation. However, PEA not only induced infiltration of NK cells and other cell types such as neutrophils and T cells to the liver, but also activated NK cells, as cytotoxicity of hepatic NK cells was augmented after PEA treatment. KC depletion inhibited both the recruitment and the increase in cytotoxicity of hepatic NK cells after PEA challenge. But, in contrast to the TNF-α-dependent augmentation of NK cell numbers in the liver, NK cell activation seems not to be mediated by TNF-α produced by KCs. Hence, another yet unidentified factor such as IL-18 (8, 9) seems to augment NK cell cytotoxicity in the liver.

NKT cells have been shown to be potent activators of NK cells (15, 16) and to exibit lytic activity (11). It has recently been shown that NKT cells can down-modulate surface markers such as NK1.1 or TCR upon activation, and therefore staining with anti-CD3 and anti-NK1.1 Abs is no reliable method for identification of activated NKT cells (31, 32). Our observed increase in the numbers of T cells could be interpreted as augmentation of activated NKT cells that have down-regulated NK1.1. However, our experiments with

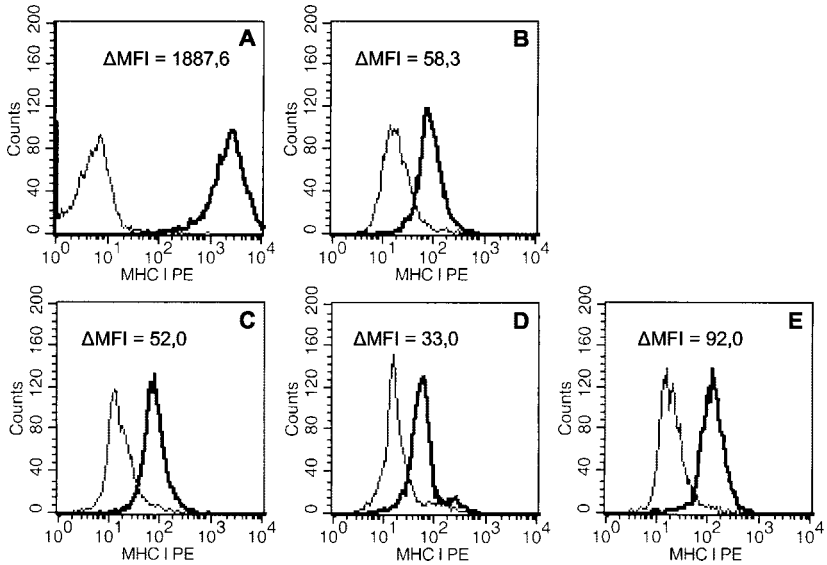

FIGURE 8. Expression of MHC class I molecules on splenocytes and hepatocytes and alteration of MHC class I expression on hepatocytes isolated from PEA- or LPS-treated mice. MHC class I profiles of splenocytes (*A*) or hepatocytes (*B*) isolated from untreated C57BL/6 mice, hepatocytes from C57BL/6 mice 5 h after PEA-treatment (*C*), 9 h after PEA treatment (*D*), or 48 h after LPS treatment (*E*), as measured by flow cytometry. Spleen cells or hepatocytes were isolated, stained with rat Ab against mouse MHC class I or isotype-matched control rat IgG, followed by PE conjugated anti-rat IgG Ab, and subjected to flow cytometric analysis. The difference in mean fluorescence intensity (ΔMFI) indicated in each plot was calculated by subtracting MFI of isotype control-stained samples (thin lines) from MFI of anti-MHC class I-stained samples (bold lines) for each treatment.

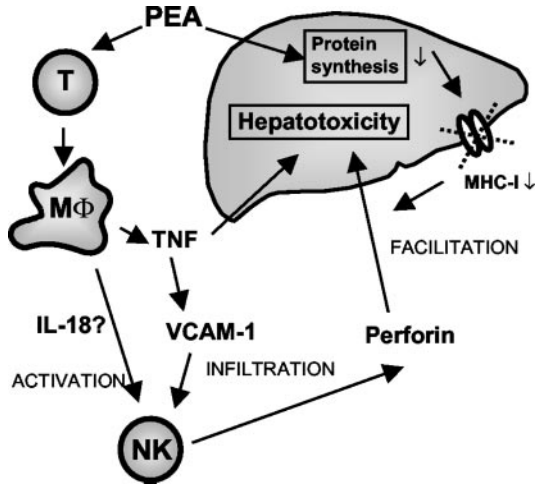

FIGURE 9. Proposed mechanism of PEA-induced liver injury in mice. PEA induces T cell-dependent TNF production by KC. TNF exerts pro-apoptotic effects on hepatocytes, and induces NK cell infiltration into the liver by up-regulation of VCAM-1. PEA also augments KC-dependent hepatic NK cell cytotoxicity. This effect might be due to KC-derived production of IL-18. As PEA inhibits protein synthesis in the liver, a down-regulation of MHC class I molecules on liver cells could facilitate perforin-mediated cytolytic activities of NK cells toward hepatocytes.

cd1d° mice clearly showed that NKT cells are not important for pathogenicity of PEA-induced liver injury and that they neither contributed to elevation of NK cell frequencies nor to NK cell cytotoxicity following PEA treatment. Hence, NKT cell activation and subsequent down-regulation of surface markers after PEA treatment are highly unlikely. Taken together, our experiments show that NK cells can be activated in dependence of KCs and independent of NKT cells.

NK cells are able to discriminate between self and nonself by recognition of MHC class I molecules expressed by other cells. MHC class I binding inhibits the lytic functions of NK cells (33). PEA was previously shown to be a ligand for CD91 (= α_2-macroglobulin receptor/low density lipoprotein receptor-related protein), a scavenger receptor predominantly located on hepatocytes and macrophages, e.g., KCs (34, 35), but also found on anti-CD3-activated T cells (36). After binding to this receptor via its N-terminal domain, PEA subsequently enters the cell by receptor-mediated endocytosis and is capable of blocking protein synthesis by adenosine-diphosphate ribosylation of the elongation factor 2 (34, 37). Thus, it seemed likely that it might reduce the expression of MHC class I molecules on the surface of hepatocytes, which could facilitate cytotoxic action of NK cells toward hepatocytes. Interestingly, our data demonstrated that MHC class I molecules are only weakly expressed on hepatocytes from untreated mice, whereas high expression is detected on spleen cells of the same mice. Moreover, PEA treatment further down-regulated MHC class I levels on hepatocytes. As MHC class I molecules serve as ligands for inhibitory receptors on NK cells (33), a diminished MHC class I expression results in susceptibility toward cytotoxic actions of activated NK cells. It was previously shown that in vivo LPS treatment enhances MHC class I expression (38). Consistent with these data, we observed MHC class I up-regulation on hepatocytes of LPS-treated mice, presumably due to increased amounts of IFN-γ that were found in plasma (data not shown). Although the amount of IFN-γ in plasma is also augmented in PEA-treated mice, MHC class I is not up-regulated, but down-regulated on hepatocytes, most likely due to an inhibition of protein synthesis in hepatocytes. Taken together, PEA causes a down-regulation of MHC class I Ags on parenchymal liver cells, which renders the hepatocytes susceptible to lytic NK cell functions.

This study provides new insights into the mechanisms and involvement of cells in PEA-induced liver failure in mice (Fig. 9). The characterization of pathways and mediators in small animal models of immune-mediated liver injury such as the PEA model described in this work is helpful for development and investigation of potential therapeutic strategies against hepatic injury. Our results presented in this work, showing a NKT cell-independent activation of NK cells following an immune activation in the liver, suggest that NK cells might be a suitable target for therapy of immune-mediated hepatotoxicity in humans.

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