Involvement of CD95 (Apo-1/Fas) Ligand Expressed by Rat Kupffer Cells in Hepatic Immunoregulation

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Background & Aims: CD95 (Apo-1/Fas) ligand suppresses inflammatory responses in immune-privileged organs. In this study, modulation of the hepatic CD95 receptor/ligand system by interferon gamma and cyclosporin A was investigated. *Methods:* CD95 receptor and ligand expression were measured at the messenger RNA level by using quantitative reverse-transcription polymerase chain reaction and immunocytochemistry in primary cultures of rat Kupffer cells, hepatocytes, and T lymphocytes. Soluble CD95 in culture supernatants was detected by enzyme-linked immunosorbent assay and apoptosis by the TUNEL method. *Results:* Interferon gamma treatment led to an increase in CD95 ligand messenger RNA levels in Kupffer cells followed by an overexpression of the soluble CD95 receptor. Supernatants derived from 24-hour but not from 48-hour interferon gamma–treated Kupffer cells killed lymphocytes by a CD95-dependent mechanism. Cyclosporin A inhibited CD95 ligand expression in Kupffer cells and lymphocyte killing. In liver parenchymal cells, interferon gamma increased messenger RNA levels of the transmembrane CD95 isoform and sensitivity of these cells toward CD95-mediated apoptosis. *Conclusions:* The expression pattern of CD95 receptor and ligand in response to interferon gamma points to a coordinated interplay between Kupffer cells, hepatocytes, and T lymphocytes in which Kupffer cells may regulate programmed cell death of T lymphocytes and hepatocytes.

CD95 (Apo-1/Fas) belongs to the tumor necrosis factor (TNF) receptor/nerve growth factor receptor family and acts as an inducer of apoptosis in CD95-expressing cells when activated by agonistic CD95 antibodies or the CD95 ligand (CD95L).[1,2] The CD95/CD95L system has been widely studied for its role in modulation of the immune response,[3,4] tissue growth,[5] and elimination of malignant and damaged cells.[6–8]

Components of the CD95/CD95L system are two CD95 receptor isoforms and their natural ligand.[9,10] The transmembrane CD95 isoform (CD95tm) and soluble CD95 isoform (CD95sol) are generated by alternative splicing and are functionally different. CD95tm transduces the death signal in response to CD95L cross-linking, whereas CD95sol is highly active in neutralizing CD95L and may protect from apoptosis.[11–14] CD95L is present on the cell surface but may be also shed from the cell surface by matrix metalloproteinases.[15,16] On one hand, CD95 ligation may transduce a costimulatory signal in T lymphocytes (TLCs).[17] On the other hand, CD95L is a highly efficient mediator of apoptosis of activated TLCs, thus creating an immunoprivileged site. Immune privilege induced by CD95L was shown in the anterior chamber of the eye[18] and in the Sertoli cells of testis.[19] The immunologic features along the hepatic sinusoids may also represent an immune privilege of the liver.[20,21] Here, Kupffer cells (KCs; liver macrophages) may play an important role in intrahepatic immunosuppression and induction of tolerance.[22,23] Because KCs also act as antigen-presenting cells (APCs) toward intrahepatic lymphocytes, they hold a key position in the regulation of the hepatic immune response.[24–26]

Interferon gamma (IFN-γ) is a critical cytokine in hepatic damage.[27] It is secreted by TLCs, and the cytotoxic effect of IFN-γ on hepatocytes involves apoptosis.[27–30] IFN-γ increases CD95L-dependent apoptosis in lymphoid and CD95 expression in nonlymphoid tissue.[28] Interestingly, TLCs are stimulated by lipopolysaccharide-activated KCs to secrete IFN-γ.[29,30] Therefore, IFN-γ plays an important role in the TLC-KC interaction. Treatment with the immunosuppressive agent cyclosporin A (CsA) impairs CD95L expression and CD95L-

Abbreviations used in this paper: CD95L, CD95 ligand; CD95tm, transmembrane CD95 isoform; CD95sol, soluble CD95 isoform; CsA, cyclosporin A; ELISA, enzyme-linked immunosorbent assay; HPRT, hypoxanthine guanine phosphoribosyltransferase; IFN-γ, interferon gamma; KC, Kupffer cell; PC, parenchymal cell; PCR, polymerase chain reaction; PHA, phytohemagglutinin; RT, reverse-transcription; TLC, thymic lymphocyte; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine phosphate nick-end labeling.

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dependent apoptosis in TLCs, whereas CD95 messenger RNA (mRNA) levels are not affected.[31,32]

However, little is known about the role of CD95 receptor and ligand in regulation of the immune response in the liver by IFN-γ. This was investigated in the present study. The data show a sequential expression of CD95 ligand and receptor isoforms in KCs in response to IFN-γ. CD95L was functionally active because TLCs and liver parenchymal cells (PCs) were killed by supernatants derived from IFN-γ–treated KCs. CsA inhibits the expression of CD95L in KCs. The data point to a timely coordinated interplay between KCs and both PCs and TLCs in IFN-γ–induced activation of the apoptosis machinery.

Materials and Methods

Reagents and Antibodies

Polybead microspheres (2.5% solid latex; 1-μm diameter) were obtained from Polysciences Ltd. (St. Goar, Germany) and RPMI 1640 medium and fetal bovine serum from Biochrom (Berlin, Germany). IFN-γ of rat origin and CsA, which both modulate the secretion of various cytokines,[33,34] were purchased from Laboserv (Staufenberg, Germany) and Merck (Darmstadt, Germany), respectively. Phytohemagglutinin (PHA) was from Seromed (Berlin, Germany), and the salts required for preparation of the Krebs–Henseleit buffer were from Merck. Williams E medium, anti-rabbit–peroxidase immunoglobulin G, and all other chemicals were from Sigma (Deisenhofen, Germany). Oligonucleotides were synthesized by Birsner and Grob (Freiburg, Germany). Rabbit polyclonal antibodies used for immunocytochemical procedures raised against a peptide corresponding to amino acids 308–327 at the carboxy terminus of transmembrane CD95 (Fas/Apo-1) receptor of rat origin and against a peptide corresponding to amino acids 100–178 at the amino terminus of CD95 receptor ligand (FasL) of rat origin were from Santa Cruz Biotechnology (Santa Cruz, CA). Agonistic anti-CD95 (clone CH-11) antibody was purchased from Immunotech (Marseille, France). Recombinant CD95:Fc-immunoglobulin fusion molecule was obtained from Alexis (San Diego, CA). The soluble domain of CD95 (amino acids 1–154) is fused to the Fc portion of immunoglobulin G1. BB-3103, an inhibitor of matrix metalloproteinases, was a gift from British Biotech Pharmaceuticals (Oxford, England).

CD95-Soluble Enzyme-Linked Immunosorbent Assay

For quantitative assessment of CD95sol levels in supernatants from KC cultures, a soluble CD95–enzyme-linked immunosorbent assay (ELISA) from Alexis was used. For each condition, 10^6 KCs from three independent preparations were incubated with 300 μL culture medium in 24-well plates. Ten microliters of each supernatant was collected, and the ELISA was performed following the manufacturer's protocol. Each determination was performed in duplicate. Contents of CD95sol were calculated using an automated ELISA-reader (Anthos, Cologne, Germany) at a wavelength of 450 nm, based on the results of a standard dilution curve.

Cell Isolation and Culture Conditions

Male Wistar rats (300–400 g body wt) were raised in the local institute for laboratory animals, held, and fed ad libitum on stock diet, according to the rules of the local ethical guidelines. Liver PCs were prepared by collagenase perfusion as described previously.[35] Cells were plated on collagen-coated, cluster-six dishes (Costar, Cambridge, MA) at a density of about 1×10^6 cells/mL and were then cultured in Williams E medium, 37°C, 5% CO_2, pH 7.4, plus glucose (5 mmol/L), supplemented with 5% fetal bovine serum for 24 hours. Thereafter, cells were maintained under various test conditions for 6, 24, or 48 hours.

KCs were prepared by collagenase-pronase perfusion and separated by a single Nycodenz gradient and centrifugal elutriation as described previously.[36,37] The phagocytotic activity of KCs was measured by the ability to ingest latex particles.[38] KCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum on cluster-six dishes (Costar) for 48 hours. The experiments were performed during the following 48 hours using RPMI 1640 medium containing 1% heat-inactivated fetal bovine serum. In one set of experiments, IFN-γ–treated KCs were incubated simultaneously with 1 μmol/L of the matrix metalloproteinase inhibitor BB-3103 (British Biotech Pharmaceuticals).

Rat lymphocytes were isolated as described previously.[39] The cells were cultured for 24 hours in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and activated with 2.4 μg/mL PHA. Thereafter, cells were maintained under various test conditions for 24 hours. In some experiments, 1 μmol/L CsA was added 30 minutes before IFN-γ.

The viability of PCs, KCs, and TLCs was $> 95\%$ as assessed by trypan blue exclusion at the end of cell isolations and after 2 days of cell culture. The purity of PCs, KCs, and TLCs was $>98\%$ after 1 day of culture and was assessed as described previously.[39]

In Vivo Studies

For in vivo studies of CD95L expression, 3 rats were injected intraperitoneally either with 500 μL 0.9% NaCl or 200,000 U rat IFN-γ dissolved in 500 μL of 0.9% NaCl. After 6 hours, the rat livers were prepared, and total RNA was extracted from freshly isolated KCs for reverse-transcription polymerase chain reaction (RT-PCR) analysis. The purity of freshly isolated KCs was $89\% \pm 2\%$ ($n = 6$) as assessed by ED-2 immunostaining. In addition, parts of the liver samples were used for immunohistochemistry.

Quantitative Competitive RT-PCR

Total RNA from rat KCs, liver PCs, and TLCs was isolated using a total RNA extraction kit (Qiagen, Hilden, Germany) and reverse-transcribed using a first-strand comple-

mentary DNA (cDNA) synthesis kit (Boehringer Mannheim, Mannheim, Germany). The mRNA levels of CD95L, CD95 isoforms, and hypoxanthine guanine phosphoribosyltransferase (HPRT) were determined using quantitative competitive RT-PCR. HPRT mRNA levels are not changed by various stimuli, including treatment with IFN-γ,[39,40] and were used for standardization. The cDNA to be assayed was coamplified with known amounts of an internal DNA standard, which was apart from a deletion of four nucleotides identical to the corresponding fragment of the assayed cDNA. DNA standards were essentially constructed as described by Pannetier et al.[41] and were kindly provided by Drs. Patrice Douillard and Emmanuelle Gilbert (Immunointervention dans les Allo-et Xénotransplantations, INSERM Unité 437, Nantes, France). For construction of DNA standards, the respective 5′ and 3′ PCR primers (see below) were used to amplify a specific fragment in a rat spleen lymphocyte-derived cDNA. A 1000-fold dilution of this product was reamplified using the respective 3′ PCR primer (see below), and an additional construct primer containing a four-nucleotide deletion was compared with the wild-type sequence. For quantification of the transcripts for CD95L, CD95tm, CD95sol, and HPRT, a constant amount of cDNA, corresponding to 50 ng reverse-transcribed total RNA, was mixed with 10^8, 10^7, . . . 10^3, or 0 copies of the respective standard, respectively, and then amplified to saturation (40 cycles of 94°C for 20 seconds, 58°C for 45 seconds, and 72°C for 45 seconds with 10 minutes extension time at 72°C on cycle 40).

The primers used for quantitative competitive PCR were 5′-ATGGAACTGCTTTGATCTCTGG and 5′-AGATTCCTCAAAATTGATCAGAG for CD95L, 5′-GATATGCTGTGGATCATGGC and 5′-AACTTTTCGTTCACCAG or 5′-TTCATGTGAACCTACCAGGC for CD95tm or CD95sol, or 5′-TGCTGGATTACATTAAAGCGC and 5′-CTTGGCTTTTCCACTTTCGC for HPRT.

The readout of the amplification involved one additional fluorescent dye labeled oligonucleotide, which allows for discrimination between wild-type and standard DNA species. PCR amplification products were specifically labeled in run-off reactions, loaded on an acrylamide gel, and analyzed by an automated sequencer (ABI 373A; Applied Biosystems, Foster City, CA). The fluorescent dye-labeled (FAM) oligonucleotides used in run-off reactions were 5′-TGGCTTAGGGGCTGGCTGTT for CD95L, 5′-GGACTGATAGCATCTTTGAGG for both CD95 isoforms, or 5′-GTTGACTGGTCATTACAGTAGC for HPRT. The fluorescent profiles were recorded, and the profile areas were analyzed using the software Immunoscope,[41] which was kindly provided by Dr. C. Pannetier (Unité de Biologie Moléculaire du Géne, INSERM Unité 277, Institut Pasteur, Paris, France).

Immunocytochemical Procedures

For in situ labeling of rat liver macrophages, a polyclonal mouse anti-rat ED-2 antibody[42] was used from Serotec (Oxford, England). The specificity of immunostaining was verified using normal rabbit or mouse immunoglobulin G1 instead of the primary antibody.

KCs, PCs, and TLCs were subjected to immunocytochemical analysis as described previously.[39] The number of cells staining positive for CD95 and CD95L was determined by light microscopy. CD95 receptor and ligand protein expression in cell culture was evaluated semiquantitatively using an immunocytochemistry score, and 150 cells were counted out for each experiment (three separate experiments for each condition). Both the extent of the encompassed cell population and the intensity of staining were taken into account. The following values were used to evaluate the extent: 0, no cells stained positive for CD95 (CD95L); 1, less than one third of the cells stained positive for CD95 (CD95L); 2, between one third and two thirds of the cells stained positive for CD95 (CD95L); and 3, more than two thirds of the cells stained positive for CD95 (CD95L). The intensity was assessed as 0, absence of staining; 1, weak staining; 2, strong staining; and 3, very strong staining. Total scores were calculated as averages of the two single scores.

Cryosectioning and staining of cryosections for immunofluorescence were performed as described previously by Kubitz et al.[43] The specificity of the second antibodies was tested, and no cross-reactivity was observed.

Detection of Apoptosis in Liver PCs and TLCs

The relevance of the observed CD95tm/CD95sol expression in rat liver parenchymal cells (PC) was tested by using a monoclonal agonistic antibody anti-CD95 (Immunotech), which induces apoptosis in CD95-sensitive cells.[10] Apoptosis of PCs was measured after incubation with concentrations of the antibody as indicated for 24 hours under the various test conditions.

In another set of experiments, it was examined whether supernatants derived from IFN-γ–treated rat KCs can stimulate apoptosis in rat TLCs or PCs. Cultured KCs were incubated without (control) or with 100 U/mL IFN-γ or with IFN-γ plus 1 μmol/L CsA for either 24 or 48 hours. The supernatants obtained from the 24- or 48-hour period, respectively, were then added to TLCs or PCs for 24 hours. In another set of experiments, TLCs cocultured with KCs were incubated for 24 hours under the conditions described above, and apoptosis was measured during the following 24 hours. To determine whether apoptosis in TLCs and PCs is mediated by CD95, supernatants from KCs were supplemented with 100 μg/mL of the CD95/Fc-immunoglobulin fusion molecule (Alexis), which specifically blocks CD95L-mediated apoptosis.

Apoptosis was detected by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method[44] using an in situ cell death detection kit (Boehringer Mannheim). After two rinsings in phosphate-buffered saline, cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature and washed again in phosphate-buffered saline for 5 minutes. The manufacturer's protocol was then followed. The incorporated fluorescein was detected by

measuring the cell-associated fluorescence. The excitation wavelength was 490 nm.

Statistics

Data are expressed as means ± SEM (n = number of cell preparations from different animals). Statistical analysis was performed using the Student *t* test. *P* values of <0.05 were considered statistically significant.

Results

Effect of IFN-γ on the Expression of CD95 (Apo-1/Fas) Receptor and CD95L in Rat Liver Macrophages (KCs) and PCs

The mRNA levels for the CD95 (Apo-1/Fas) receptors (CD95tm and CD95sol) and CD95L were measured using the quantitative RT-PCR technique as described in Materials and Methods. As shown in Figure 1*A*, addition of 100 U/mL IFN-γ to KC cultures led to a 15-fold increase of the CD95L mRNA levels compared with untreated cells. A small increase in CD95L mRNA abundance in untreated controls is probably caused by the change of the medium. Under control conditions only a few of the KCs stained positive for CD95L, but most KCs stained positive for CD95L after 24 hours of IFN-γ treatment (Table 1). The positive staining for CD95L in KCs after IFN-γ treatment was more intense when cells were incubated simultaneously with the matrix metalloproteinase inhibitor BB 3103, indicating that, in the absence of BB-3103, CD95L was shed in part from the cell surface into the culture supernatant (Figure 2).

The quantities of CD95 receptor and CD95L protein expression cannot be estimated precisely by immunocyto-

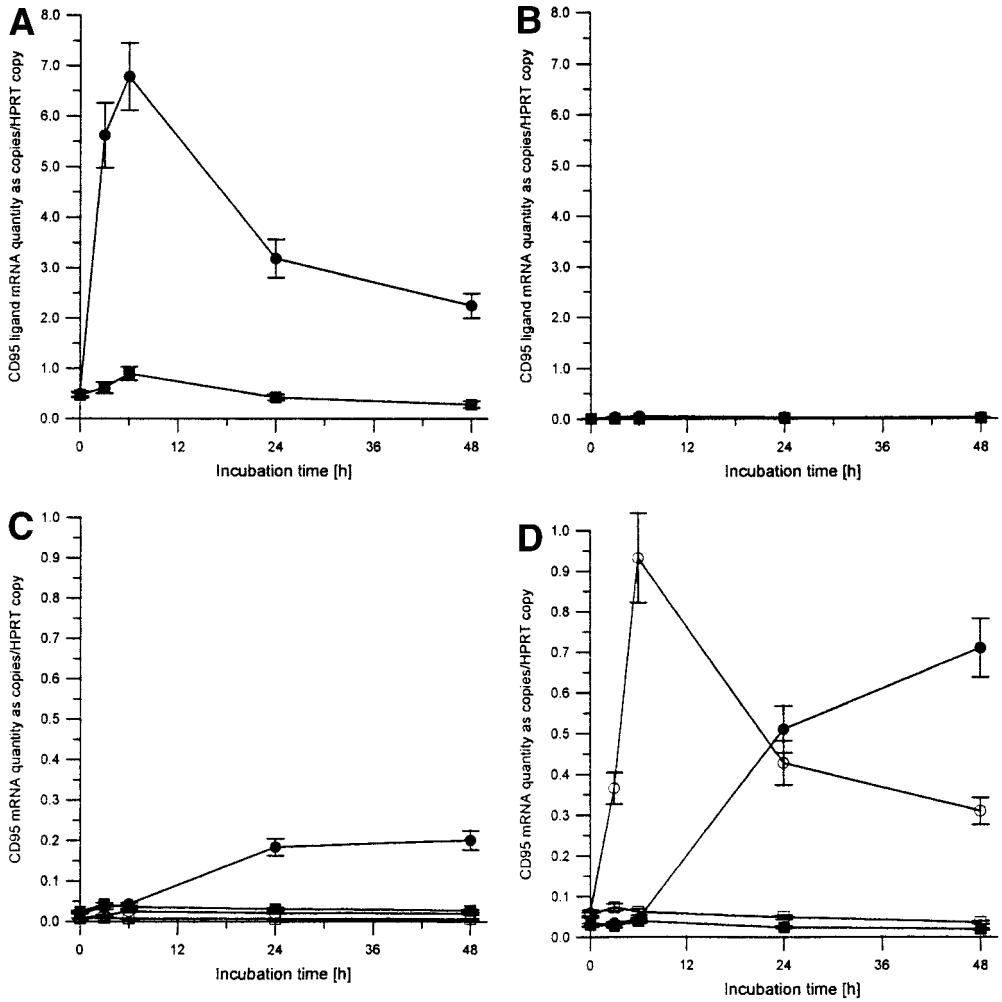


Figure 1. Modulation of CD95L, CD95tm, and CD95sol mRNA expression in response to IFN-γ in KCs and liver PCs. Total RNA was extracted from IFN-γ–treated (100 U/mL) and untreated (control) rat KCs or liver PCs at the time points indicated. The RNA was then reverse-transcribed and quantified by PCR as described in Materials and Methods. (*A* and *B*) CD95L mRNA expression in IFN-γ–treated (100 U/mL; ●) and untreated (control; ■) (*A*) KCs or (*B*) PCs. (*C* and *D*) mRNA expression of the CD95 isoforms in IFN-γ–treated (100 U/mL; ● and ○) and untreated (control; ■ and □) (*C*) KCs or (*D*) PCs. ○ and □, CD95tm; ● and ■, CD95sol. Results are expressed as the ratio of the number of CD95L or CD95 isoform transcripts obtained with the indicated primers to the number of HPRT transcripts. Data are expressed as means ± SEM of 3 separate experiments for each condition.

chemistry because a fraction of CD95 receptor represents the soluble form (as shown in the present study by RT-PCR) and antibodies raised against CD95 receptor do not distinguish between the two isoforms. Moreover, immunostaining for CD95L does not detect the fraction of CD95L that has been shed from the cell surface into the supernatant, unless CD95L shedding has been blocked by inhibitors of matrix metalloproteinases, such as BB-3103.

The mRNA levels for CD95tm and CD95sol were very low in unstimulated KCs (Figure 1*C*). Stimulation of KCs by IFN-γ had no effect on CD95tm but increased the mRNA levels for CD95sol about 10-fold after a lag phase of at least 6 hours (Figure 1*C*). Thus, the induction of CD95L mRNA precedes the induction of CD95sol mRNA by IFN-γ. On the other hand, IFN-γ treatment had little effect on the number of KCs staining positive for CD95 (Table 1).

In contrast to KCs, cultured hepatocytes stained positive for CD95 under control conditions. On stimulation with IFN-γ, the mRNA levels for the CD95 isoforms were up-regulated, and hepatocytes stained intensively positive for CD95 (Figures 1*D* and 3 and Table 1). Interestingly, a switch from CD95tm to CD95sol occurred after about 6–24 hours. The CD95tm/CD95sol mRNA ratio in PCs was 21.0 at 6 hours and 0.4 at 48 hours.

Only 1%–5% of the cells in the hepatocytes culture stained positive for CD95L, and the mRNA levels for CD95L were very low under control conditions. They increased from only 0.01 ± 0.01 to 0.06 ± 0.01 copies/HPRT copy (n = 3) in response to IFN-γ within 6 hours (Table 1 and Figure 1*B*). The possibility must be considered that the IFN-γ–induced increase in CD95L

Table 1. Immunocytochemical Evaluation of CD95 Receptor and CD95L Expression in Rat KCs, Liver PCs, and TLCs

Protein	CD95	CD95L
KCs		
Control	0.3	1.2[b]
IFN-γ (100 U/mL)	0.8	2.2[a]
IFN-γ + BB 3103 (1 μmol/L)	ND	3.0[a,b]
IFN-γ + CsA (1 μmol/L)	0.7	1.3[b]
PCs		
Control	1.2	0.3
IFN-γ (100 U/mL)	2.7[a]	0.5
IFN-γ + BB 3103 (1 μmol/L)	ND	0.5
IFN-γ + CsA (1 μmol/L)	2.3[a]	0.3
TLCs		
Control	1.8	1.7[b]
PHA (2.4 mg/mL)	2.8[a]	2.7[a]
PHA + BB 3103 (1 μmol/L)	ND	3.0[a]
PHA + CsA (1 μmol/L)	2.7[a]	1.5[b]

NOTE. Cells were incubated for 24 hours with the addition indicated. CsA was added 30 minutes before IFN-γ or PHA incubation. Immunocytochemical procedures and semiquantitative evaluation by an immunocytochemistry score were performed as described in Materials and Methods. The cells staining positive for CD95 and CD95L were detected by light microscopy. Data are from 3 separate experiments for each condition, and SEM was always <0.3.
ND, Not determined.
[a]Significantly different ($P < 0.05$) from control.
[b]Significantly different ($P < 0.05$) compared with the IFN-γ or PHA condition without CsA.

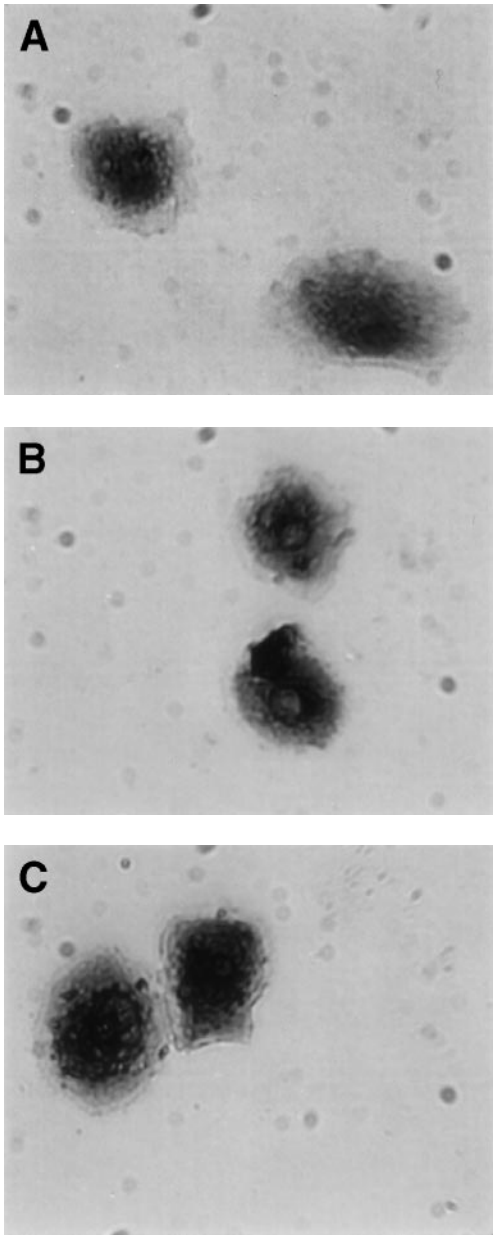


Figure 2. Effect of IFN-γ on the expression of CD95L in rat KCs. Cultured rat KCs were incubated (*A*) without or (*B*) with 100 U/mL IFN-γ or (*C*) with IFN-γ plus 1 μmol/L of the matrix metalloproteinase inhibitor BB 3103 for 24 hours. Immunocytochemical procedures were performed as described in Materials and Methods. Cells staining positive for CD95L were determined by light microscopy and were stained darkly.

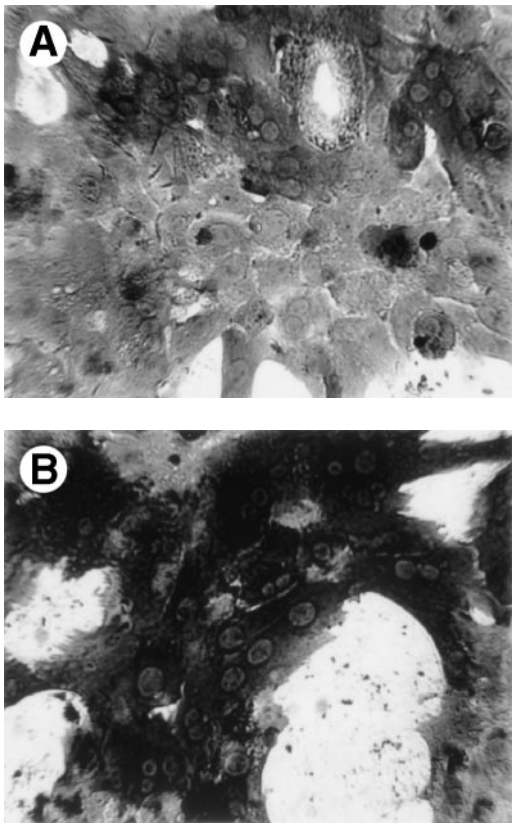


Figure 3. Effect of IFN-γ on the expression of CD95 receptor in rat PCs. Cultured rat PCs were incubated (*A*) without or (*B*) with 100 U/mL IFN-γ for 24 hours. Immunocytochemical procedures were performed as described in Materials and Methods. Cells staining positive for CD95 (Apo-1/Fas) were determined by light microscopy and were stained darkly.

mRNA is caused by contaminating KCs because KCs showed a strong increase in CD95L mRNA in the presence of IFN-γ (Figure 1*A*). The number of CD95L mRNA copies/HPRT mRNA copies in PCs was only 1% of that found in KCs. Taking into account a 1% contamination of the PC cultures with KCs, as assessed by morphological examination and by the ability to ingest latex particles,[38] the low level of CD95L expression in PC cultures can be fully attributed to this contamination.

Effect of CsA on the CD95 Receptor and CD95L Expression in IFN-γ–Treated Rat Liver Macrophages (KCs) and PCs

The immunosuppressive agent CsA modulated the IFN-γ–induced stimulation of CD95L expression in KCs. When 1 μmol/L CsA was added 30 minutes before the IFN-γ incubation periods, the IFN-γ–induced increase in CD95L mRNA was inhibited by about 80% in KCs, i.e., from 6.78 ± 0.67 to 1.22 ± 0.17 copies/HPRT mRNA copies (n = 3) after 6 hours and 2.25 ± 0.24 to 0.62 ± 0.10 copies/HPRT mRNA copies (n = 3) after 48 hours, respectively. The CD95L mRNA levels under control conditions (i.e., IFN-γ absent) were 0.89 ± 0.14 and 0.28 ± 0.07 copies/HPRT mRNA copies (n = 3), respectively. In line with this, CsA also diminished the IFN-γ–induced increase in the number of KCs staining positive for CD95L (Table 1).

In IFN-γ–treated PCs, a decrease in CD95L mRNA levels was measured in response to CsA, but as outlined above, this may reflect the effect on contaminating KCs. In the 6- and 48-hour incubations, CsA inhibited the IFN-γ–induced increase in CD95L mRNA between 50%–80%, i.e., from 0.06 ± 0.01 to 0.02 ± 0.01 and 0.02 ± <0.01 to 0.01 ± <0.01 copies/HPRT mRNA copies (n = 3), respectively. The mRNA levels were 0.01 ± <0.01 copies/HPRT mRNA (n = 3) under both control conditions. The number of hepatocytes staining positive for CD95L was very low under all conditions (Table 1). However, CsA had no significant effect on the CD95 receptor expression in PCs and KCs as detected at the mRNA (not shown) or protein level (Table 1).

Expression of CD95 (Apo-1/Fas) Receptor and CD95L in Rat TLCs

Because this study focused on the interaction between antigen-activated TLCs and antigen-presenting KCs, we determined the CD95 and CD95L expression in both cell types. Stimulation of TLCs by 2.4 μg/mL PHA, which is a commonly used model of activated TLCs,[31,32] led to a biphasic mRNA expression of CD95L and the CD95 isoforms. The mRNA levels for CD95L and CD95tm increased 2–6-fold within 4 hours and decreased thereafter (Figure 4). PHA treatment also led to an increase in positive TLC staining for CD95L and CD95 after 24 hours (Table 1). The positive staining for CD95L in TLCs after PHA treatment was slightly enhanced when cells were incubated simultaneously with BB 3103, indicating that CD95L was released from these cells into the supernatant (Table 1). After a lag phase of 4 hours, the increase in mRNA levels for CD95sol followed the up-regulation of CD95L mRNA levels and the CD95tm mRNA levels in activated TLCs (Figure 4). The CD95tm/CD95sol mRNA ratio was 0.9 at 4 hours and 0.1 at 48 hours. Addition of 1 μmol/L CsA to the PHA-treated TLCs had no effect on the CD95 expression but largely inhibited the PHA-induced increase in CD95L expression at both the mRNA level and immunocytochemistry (Figure 4 and Table 1).

Sensitivity of Liver PCs Toward Anti-CD95 Antibody–Induced Apoptosis in Response to IFN-γ and CsA Treatment

In experiments using the agonistic antibody anti-CD95 (CH-11), the IFN-γ–induced increase in CD95 receptor expression led to a shift of the dose-response curve (Figure 5). This may suggest that the CD95 receptor, which was induced by IFN-γ within 24 hours, was functionally active. When PCs were incubated with antibody concentrations of 10 ng/mL for 24 hours, about 65% of the PCs became apoptotic in the presence of IFN-γ, whereas the rate was only 10% in the absence of IFN-γ (Figure 5). Addition of 1 μmol/L CsA to IFN-γ–treated PCs resulted in a 20% lower rate of apoptotic cells compared with the IFN-γ–treated cells without CsA (Figure 5), although CD95 expression was not significantly altered by CsA.

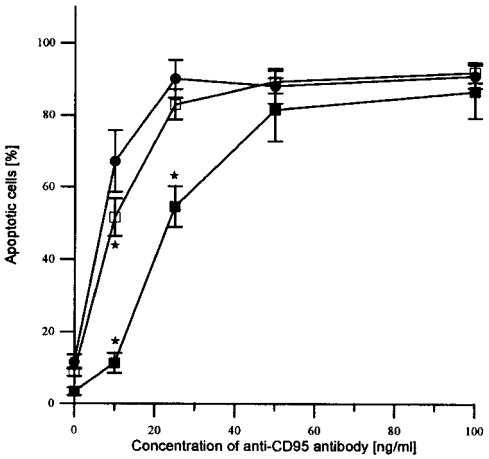
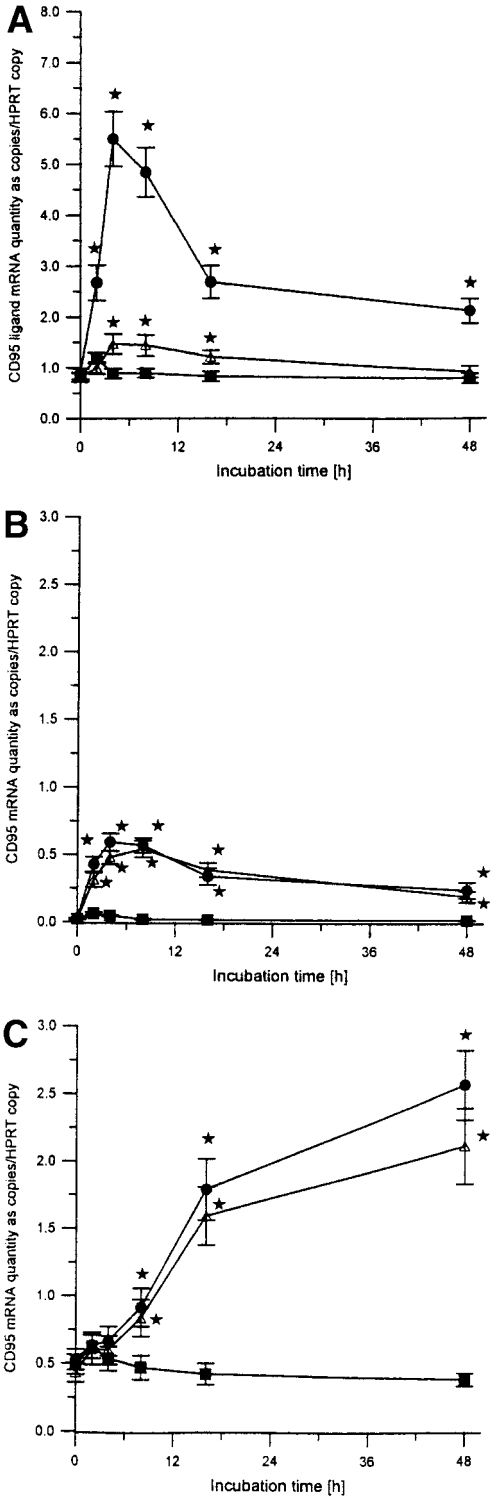


Figure 5. Effect of IFN-γ and CsA on anti-CD95 antibody–induced apoptosis of rat PCs. Cultured rat PCs were incubated without (■) or with 100 U/mL IFN-γ (●) or with IFN-γ plus 1 μmol/L CsA (□) and various concentrations of anti-CD95 antibody (Immunotech) for 24 hours. Apoptosis was measured as described in Materials and Methods. Data are expressed as means ± SEM of 3 separate experiments for each condition. *Significantly different from the control ($P < 0.05$).



Induction of Apoptosis in Liver PCs and TLCs in Coculture With KCs and by Supernatants Derived From KC Cultures

To determine whether IFN-γ–stimulated KCs can induce apoptosis in rat liver PCs and TLCs, cultured KCs were incubated with 100 U/mL IFN-γ for 24 or 48 hours.

About one fourth of TLCs cocultured in the presence of untreated KCs underwent apoptosis, which could in large

Figure 4. Modulation of CD95L, CD95tm, and CD95sol mRNA expression in response to PHA in rat TLCs. Rat TLCs were incubated without or with 2.4 μg/mL PHA or with PHA plus 1 μmol/L CsA. Total RNA was extracted at time points indicated, reverse-transcribed, and quantified by PCR as described in Materials and Methods. mRNA expression of (*A*) CD95L, (*B*) CD95tm, and (*C*) CD95sol in untreated (control; ■), with PHA (●), and with PHA plus CsA–treated (△) lymphocytes. Results are expressed as the ratio of number of CD95L or CD95 isoform transcripts obtained with the indicated primers to the number of HPRT transcripts. Data are expressed as means ± SEM of 3 separate experiments for each condition. *Significantly different from the control ($P < 0.05$).

part be blocked by the addition of CD95/Fc-immunoglobulin (Table 2), indicating that apoptosis induced in TLCs was largely mediated by the CD95L-dependent pathway. In comparison, KCs that had been stimulated for 24 hours by IFN-γ killed about 70% of cocultured TLCs. This was also in large part caused by CD95 ligation because the addition of CD95/Fc-immunoglobulin rescued 75% of cocultured TLCs (Table 2). However, the effect of IFN-γ on the induction of apoptosis in cocultured TLCs by KCs was restricted to the first 24 hours of stimulation. KCs stimulated by IFN-γ for 48 hours did not induce apoptosis in TLCs significantly above control levels (Table 2).

When supernatants from 24-hour IFN-γ–treated KCs were added to cultured rat PCs or TLCs for 24 hours, apoptosis was induced in these cells (Table 3). When PCs or TLCs were cultured with supernatants derived from untreated KCs, only a few apoptotic cells were detected, whereas 50%–60% of the cells, respectively, underwent apoptosis after treatment with supernatants derived from 24-hour IFN-γ–stimulated KCs (Table 3). The supernatant obtained from 10^6 KCs was added to 10^6 PCs or TLCs, resulting in an effector/target cell ratio of one. The induction of apoptosis was diminished when supernatants from KCs were supplemented with 100 μg/mL CD95/Fc-immunoglobulin, which specifically neutralizes CD95L, suggesting that IFN-γ–dependent induction of apoptosis in these cells was in large part mediated by CD95L (Table 3). In line with this, only a few apoptotic cells were detected when the cells were exposed to supernatants derived from 24-hour IFN-γ–stimulated KCs that were treated simultaneously with 1 μmol/L BB 3103 (Table 3), an inhibitor of the shedding of CD95L. When PCs or TLCs were preincubated for 6 hours with 500 μmol/L of the interleukin 1β–converting enzyme (ICE) inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethylketone before incubation with KC-derived supernatants, the induction of apoptosis was largely prevented. Because apart from CD95L other mediators of apoptosis such as TNF-α or TNF-related apoptosis–inducing ligand (TRAIL) use ICE-dependent pathways, the high inhibitory activity of acetyl-Tyr-Val-Ala-Asp-chloromethylketone suggests that, beyond CD95L, other ICE-dependent factors are involved in PC or TLC killing by KCs in response to IFN-γ (Table 3).

A marked decrease of the apoptotic response was also observed when supernatants from 24-hour IFN-γ–treated KCs that were incubated simultaneously with 1 μmol/L CsA were used (Table 3).

Interestingly, supernatants derived from KCs, which were maintained under various test conditions for 48 hours, diminished apoptosis in PCs and TLCs in part even below control level (Table 3), suggesting that these

Table 2. Induction of Apoptosis in Rat TLCs Cocultured With KCs

	Apoptotic TLCs (%)
24-Hour coculture	
Control	23 ± 3
Fas-Fc	11 ± 2[a]
IFN-γ	70 ± 6[a]
IFN-γ + Fas-Fc	19 ± 2
48-Hour coculture	
Control	13 ± 2
Fas-Fc	12 ± 2
IFN-γ	18 ± 4
IFN-γ + Fas-Fc	16 ± 2

NOTE. Cultured rat KCs were incubated without (control) or with 100 U/mL IFN-γ (IFN-γ) for 24 or 48 hours. Autologous TLCs that had been activated with 2.4 μg/mL PHA were added to the KC cultures at an effector/target cell ratio of 1. In some experiments, KCs were preincubated for 24 hours with 100 μg/mL CD95/Fc-immunoglobulin (Fas-Fc). Apoptosis was detected by the TUNEL method as described in Materials and Methods. The cells staining positive for apoptosis were detected by fluorescence microscopy. Data are expressed as means + SEM of 3 separate experiments for each condition.
[a]Significantly different ($P < 0.05$) from control.

Table 3. Induction of Apoptosis in Rat Liver PCs and TLCs by Supernatants Derived From KC Cultures

	Apoptotic cells (%)	
	PCs	TLCs
24-Hour supernatants		
Control	7 ± 2	16 ± 2
Fas-Fc	4 ± 1	5 ± 2[a]
Ac-YVAD-cmk	1 ± 1	2 ± 1[a]
IFN	49 ± 4[a]	63 ± 7[a]
IFN + Fas-Fc	13 ± 2[a]	22 ± 3
IFN + Ac-YVAD-cmk	6 ± 1	7 ± 1[a]
IFN + BB 3103	10 ± 2[a]	3 ± 1[a]
IFN + CsA	25 ± 3[a]	26 ± 2[a]
48-Hour supernatants		
Control	15 ± 2	13 ± 3
Fas-Fc	10 ± 3	7 ± 2[a]
Ac-YVAD-cmk	4 ± 1[a]	5 ± 2[a]
IFN	10 ± 1[a]	15 ± 3
IFN + Fas-Fc	10 ± 2	19 ± 3
IFN + Ac-YVAD-cmk	11 ± 1[a]	10 ± 1
IFN + BB 3103	6 ± 1[a]	13 ± 2

NOTE. Cultured rat KCs were incubated without (control) or with 100 U/mL IFN-γ (IFN-γ) and/or with 1 μmol/L matrix metalloproteinase inhibitor BB 3103 (BB 3103) and/or 1 μmol/L CsA (CsA) for 24 hours. The supernatants were then added to liver PCs or TLCs for 24 hours. To determine whether apoptosis induced by the supernatants was caused by CD95 ligation, some of these supernatants were supplemented with 100 μg/mL CD95/Fc-immunoglobulin (Fas-Fc). In some experiments, PCs or TLCs were preincubated for 6 hours with 500 μmol/L acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk). Apoptosis was detected by the TUNEL method as described in Materials and Methods. The cells staining positive for apoptosis were detected by fluorescence microscopy. Data are expressed as means ± SEM of 3 separate experiments for each condition.
[a]Significantly different ($P < 0.05$) from control.

supernatants prevented PCs and TLCs from undergoing CD95-specific apoptosis (Table 3). This was probably caused by the formation of CD95sol by KCs during the 24–48 hours after the IFN-γ addition (Figure 1*C*). The content of CD95sol in these supernatants was quantified by an ELISA technique as described in Materials and Methods. Supernatants from KC cultures kept for 24 hours under control conditions and treated with IFN-γ contained 8.6 ± 1.2 and 12.1 ± 3.6 pg/mL CD95sol (n = 3); supernatants from KC cultures kept for 48 hours under these conditions contained 15.9 ± 1.2 and 80.1 ± 10.9 pg/mL (n = 3), respectively. Consistent with the decreased capacity to induce apoptosis, the content of CD95sol in supernatants derived from 48-hour IFN-γ–treated KCs was 6-fold higher than in supernatants from 24-hour IFN-γ–treated KCs.

CD95L Expression in KCs in Response to IFN-γ Treatment In Vivo

To identify CD95L-expressing cells in rat liver in vivo, immunostains of liver cryosections from NaCl-injected rats were performed as described in Materials and Methods. By immunofluorescence, rat liver sections were double stained for CD95L and ED2, which are specifically expressed by KCs in the liver.[42]

About 80% of CD95L-expressing cells in rat liver are $ED2^+$ KCs (Figure 6*A* and *B*). All KCs coexpress ED2 and CD95L. According to their morphological aspect, the remaining $CD95L^+ED2^-$ cells are other nonparenchymal liver cells, such as hepatic stellate cells or endothelial cells. No staining for CD95L was detectable on rat liver PCs.

We then examined whether a similar response to IFN-γ could be obtained in vivo when it was injected intraperitoneally into the animals, as described in Materials and Methods. The distribution of CD95L expression among the different cell types in rat liver was similar when rats were injected intraperitoneally with 200,000 U IFN-γ and livers were prepared 6 hours later (Figure 6*C* and *D*). In view of the fact that IFN-γ–induced upregulation of CD95L mRNA expression reaches near-maximal level already after 3 hours of incubation under cell culture conditions (Figure 1*A*), immunostains of rat livers from 6 hours IFN-γ–treated rats are consistent with mRNA data (Figures 1*A* and 6*C* and *D*).

Because KCs account for a large part of CD95L-expressing cells in the rat liver in vivo (Figure 6), CD95L mRNA levels in freshly isolated KCs from IFN-γ– and NaCl-injected control rats were quantified. In KCs from IFN-γ–injected rats, CD95L mRNA levels were 6-fold

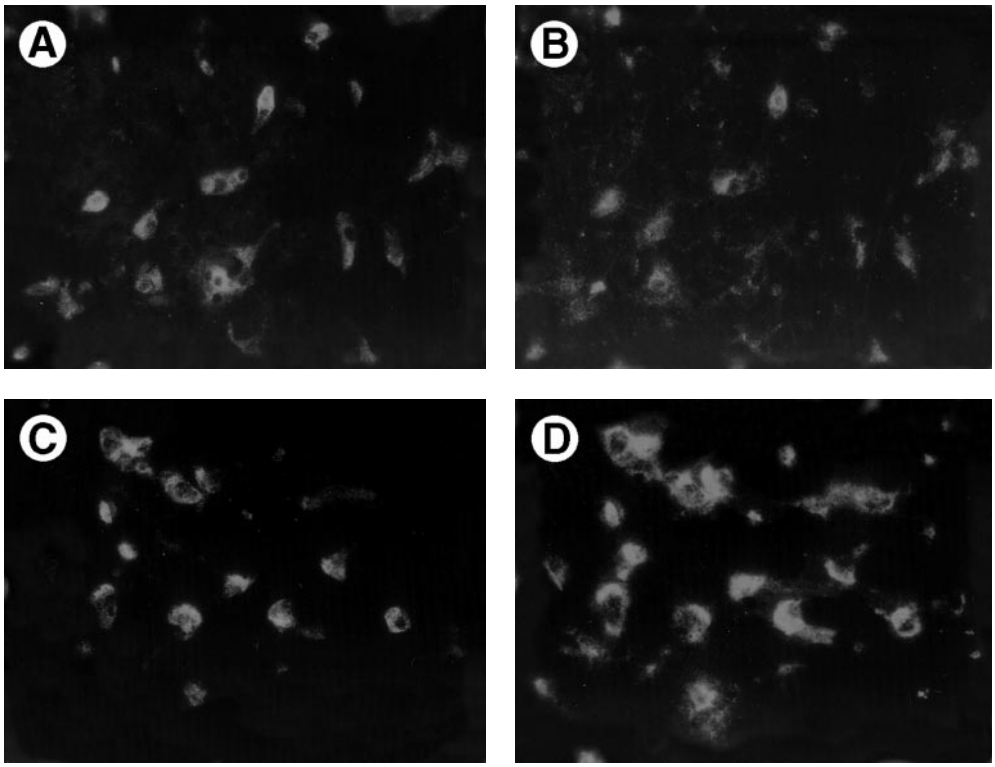


Figure 6. CD95L expression in rat liver in vivo. Cryosections from rat livers were performed and double stained as described in Materials and Methods. $ED2^+$ KCs are shown in *A* and *C*, $CD95L^+$ cells on the same section in *B* and *D*. Rat liver cryosections were obtained from (*A* and *B*) NaCl and (*C* and *D*) IFN-γ–injected rats 6 hours after the injections.

higher than in NaCl-injected control rats (14.95 ± 3.14 copies/HPRT copy and 2.43 ± 1.59 copies/HPRT copy, respectively; n = 3), indicating that CD95L overexpression in KCs in response to IFN-γ also occurs in vivo. However, compared with CD95L mRNA baseline levels under cell culture conditions. CD95L mRNA levels in freshly isolated KCs from NaCl-injected control rats were 5-fold higher (see Figure 2). This might possibly be caused by cell preparation–related stress.

Discussion

This study addressed the role of CD95 receptor and ligand in the regulation of the immune response in the liver with respect to KC interactions with both TLCs and PCs. Because activated TLCs express IFN-γ at high levels,[29] in the present study KCs were exposed to IFN-γ. This resulted in a rapid 15-fold increase in CD95L mRNA expression in KCs and after a lag phase in an increase in mRNA expression of the CD95sol. CD95L was functionally active because apoptosis was induced in TLCs and PCs by supernatants derived from IFN-γ–treated KCs, which was diminished by CD95/Fc-immunoglobulin and the shedding inhibitor BB 3103.

The interaction between KCs and TLCs could have an impact on antigen-specific immune response. The expansion of specifically reacting TLCs requires the presence of antigen-presenting cells,[45] a role fulfilled by KCs, the resident liver macrophages.[24–26] Here, KCs process and present antigenic epitopes as a class II major histocompatibility complex–antigen complex that is recognized by T-cell receptor ligation of a specifically reacting TLC clone. In addition, T-cell receptor–major histocompatibility complex cross-linking leads to TLC activation and overexpression of the transmembrane CD95, which renders TLCs more susceptible to CD95-mediated apoptosis, called activation-induced cell death.[3,46] Thus, KCs may participate in both activation of specific T-cell clones and their apoptotic removal. Conceivably, IFN-γ–induced overexpression of CD95L in KCs could then terminate the immune response against antigens that are delivered via portally drained viscera. This phenomenon, designated portal venous tolerance, has also been suggested as a potential therapeutic approach in transplantation medicine.[47,48]

On the other hand, CD95L seems to be required to maintain homoeostasis and balance in KC-TLC interaction. KCs express cytokines like interleukin 1, which themselves can activate TLCs.[29,30] Another important mediator in this interaction is the recently identified IFN-γ–inducing factor, which is secreted by KCs when stimulated by lipopolysaccharide or *Propionibacterium acnes*.[29,30] IFN-γ–inducing factor was shown to be essential for lipopolysaccharide-induced liver damage, whereas inhibition of IFN-γ–inducing factor prevented lipopolysaccharide-treated mice from hepatic failure.[29,30] TLCs exposed to IFN-γ–inducing factor show an increased IFN-γ production, which itself is active as a KC stimulator.[29] It is conceivable that the expression of CD95L in IFN-γ–activated KCs is critical to terminate the interleukin 1–induced activation of TLCs and to limit TLC cytotoxic activity by simultaneous activation of CD95-mediated apoptosis. However, because CD95L cross-linking to CD95-bearing T cells might result in costimulation[17] rather than in apoptotic removal of these cells under yet unknown conditions, CD95L may modulate the immune response in several ways.

Besides its significance in T-cell killing, CD95L may also induce liver damage in mice and humans.[49,50] In the present study, PCs became more susceptible to CD95-specific apoptosis on IFN-γ exposure and could be killed as bystander cells. In connection with the IFN-γ–induced increase in CD95L in KCs, CD95tm-bearing bystander cells like PCs require protection against severe damage. This could be the role of the isoform switch observed in the CD95 expression (Figure 1), which, however, shows some delay compared with CD95L expression.

Induction of CD95-specific apoptosis in TLCs and PCs by supernatants derived from IFN-γ–treated KCs probably was an early event and was restricted to the first 24 hours after KC stimulation. In contrast, supernatants from 48-hour IFN-γ–treated KCs protected TLCs and PCs from CD95-specific apoptosis rather than induced killing of these cells (Table 3). This was paralleled by the switch in the expression of CD95 from CD95tm to CD95sol that occurred in TLCs, KCs, and PCs after stimulation for 16–24 hours (Figures 1 and 4) and by increased amounts of CD95sol protein in the 48-hour supernatants. Because CD95tm is required to transduce the death signal and because CD95sol can neutralize CD95L,[11–14] TLCs were probably less susceptible to CD95-specific apoptosis in this situation. The loss of susceptibility to apoptosis in TLCs is reflected by an altered CD95tm/CD95sol ratio from 0.9 at 4 hours to 0.1 at 48 hours. In a similar way, PCs may acquire albeit only after a lag phase of at least 24 hours, secondary resistance to CD95-specific killing: CD95tm/CD95sol ratio was 21.0 at 6 hours and 0.4 at 48 hours at mRNA level in PCs. During the first 24 hours, however, PCs were not protected against CD95L expressed by IFN-γ–primed KCs.

The immunosuppressive agent CsA impaired CD95L expression and CD95L-dependent apoptosis in human and murine lymphocytes, whereas CD95 mRNA levels

were not affected.[31,32] Furthermore, CsA inhibited the IFN-γ production in PHA-stimulated human peripheral blood mononuclear cell cultures.[51] In the present study, CsA decreased CD95L expression in PHA-activated rat lymphocytes as well as in IFN-γ–treated KCs (Figure 4 and Table 1). In line with the reduced CD95L expression in KCs, apoptosis of PCs and TLCs was diminished when supernatants from KCs were used that were incubated for 24 hours with IFN-γ plus 1 μmol/L CsA (Table 3). Further studies are required to clarify the question of whether these effects of CsA on CD95L expression are similar in vivo.

Together the findings suggest a delicate balance of host defense and tissue damage in the acute immune response, which involves a timely coordinated interplay between KCs, TLCs, and liver PCs in the CD95/CD95L system.

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