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Deficient Liver Regeneration after Carbon Tetrachloride Injury in Mice Lacking Type 1 but Not Type 2 Tumor Necrosis Factor Receptor

Yasuhiro Yamada and Nelson Fausto

From the Department of Pathology, University of Washington School of Medicine, Seattle, Washington

Signaling by tumor necrosis factor type 1 receptor (TNFR-1) is required for the initiation of liver regeneration after partial hepatectomy. Using knockout mice that lack either TNFR-1 or TNFR-2, we determined whether signaling through TNF receptors is important for liver injury and hepatocyte proliferation induced by carbon tetrachloride (CCl_4). Lack of TNFR-1 inhibited hepatocyte DNA synthesis after CCl_4 injection. At 44 hours after the injection, replication of hepatocytes in TNFR-1 was 50% to 90% lower than in wild-type (WT) animals, depending on the dose injected. In WT animals, hepatocyte replication was essentially completed by 4 days after CCl_4 injection, but replication at a low level persisted in TNFR-1 mice for at least 2 weeks. TNFR-1 knockout mice had little detectable NF-κB and STAT3 binding during the first 5 hours after CCl_4, high plasma TNF, and reduced levels of plasma interleukin (IL)-6 and liver IL-6 mRNA. Injection of IL-6 30 minutes before CCl_4 administration corrected the deficiency of hepatocyte replication at 44 hours and restored STAT3 binding to normal levels. In contrast, mice lacking TNFR-2 did not differ significantly from WT mice in NF-κB and STAT3 binding, IL-6 and TNF levels, or hepatocyte replication. Although AP-1 binding was induced in WT TNFR-1 and TNFR-2 knockout mice, binding in TNFR-2 knockouts was lower than in WT mice. C/EBP binding was much lower in TNFR-1 and TNFR-2 knockout mice than in WT mice. As assessed by morphological analysis and alanine aminotransferase levels, the acute injury caused by CCl_4 appeared to be similar in the three groups of animals, but subsequent regeneration was impaired in mice lacking TNFR-1. We conclude that a TNFR-1 signaling pathway involving NF-κB, IL-6, and STAT3 is an important component of the hepatocyte mitogenic response induced by CCl_4 injury in mouse liver. *(Am J Pathol 1998, 152:1577–1589)*

Tumor necrosis factor (TNF) acts by binding to type 1 or type 2 receptors (TNFR-1 and TNFR-2, respectively). Although most of the effects of TNF in various cells are mediated primarily through TNFR-1, TNFR-2 functions as the main transducer for membrane-anchored (nonsoluble) TNF and is the signaling receptor for TNF-induced thymocyte proliferation.[1–5] Work with knockout mice lacking either TNFR-1 or TNFR-2 demonstrated that signaling through the type 1 receptor is required[6] for the initiation of liver regeneration after partial hepatectomy (PH). More than 50% of the mice lacking a functional TNFR-1 died during the first 3 days after PH. The surviving animals had deficient DNA synthesis and delayed gain of liver mass. Activation of the transcription factors NF-κB and STAT3, which occurs during the first hours after PH, was not detectable in these animals. A deficiency in STAT3 binding after PH was also found in mice that lack a functional interleukin (IL)-6 gene.[7] The mortality of IL-6 knockout mice after PH is very high, and the surviving animals have reduced DNA synthesis and slow liver growth. In both TNFR-1 and IL-6 knockout mice a single injection of IL-6 before PH restored STAT3 binding, prevented mortality, and completely corrected the defects in DNA replication and liver growth.[6,7] Work with these two animal models, as well as that using anti-TNF antibodies,[8,9] has established that a required pathway for the initiation of liver regeneration involves signaling through TNFR-1 and the downstream sequence TNFR-1→NF-κB→IL-6→STAT3.

In contrast to the drastic consequences caused by the absence of functional TNFR-1, lack of TNFR-2 had no effect on DNA replication or restoration of hepatic mass after PH (Y. Yamada and N. Fausto, submitted).[10] Delays in AP-1 and C/EBP binding as well as in the increase of c-jun and c-myc mRNAs after PH occurred in TNFR-2 knockout mice, but they did not alter the timing and extent of DNA synthesis during liver regeneration and were not associated with increased mortality. An important question that emerged from these experiments is whether the pathway requiring signaling through TNFR-1 is specific for the initiation of liver regeneration after PH or

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Address reprint requests to Dr. Nelson Fausto, Department of Pathology, Box 357705, University of Washington School of Medicine, K-078 Fialkow Biomedical Research Pavilion, Seattle, WA 98195-7705. E-mail: nfausto@u.washington.edu.

whether it also plays a role in other types of hepatic proliferative responses. In particular, we were interested in determining whether lack of either TNFR-1 or TNFR-2 would interfere with liver regeneration induced by CCl_4 injury. Both PH and CCl_4 elicit a synchronized wave of hepatocyte replication, but these processes differ from each other in one significant way.[11] After PH, the remaining liver is entirely normal, the compensatory growth being a response to tissue resection. In contrast, hepatocyte replication after CCl_4 administration is a response to massive cell death. As a consequence, the proliferative response takes place in a severely damaged liver. In this paper we present a study of CCl_4-induced liver regeneration in TNFR-1 and TNFR-2 knockout mice and report that regeneration is impaired in mice lacking TNFR-1.

Materials and Methods

Animals

TNFR-1 knockout mice (p55−/−), TNFR-2 knockout mice (p75−/−), and C57BL/6 control mice weighing 20 to 25 g were used in these experiments. TNFR-1 knockout mice were derived in a C57BL/6 background.[6,12,13] In the TNFR-2 knockout mice the mutation was moved into the C57BL/6 background by five successive crosses. Animals were kept in a temperature-controlled room with alternating 12-hour dark/light cycles. For most experiments mice were injected intraperitoneally with 0.25 ml/kg CCl_4 dissolved in olive oil. In a few experiments, as indicated in the text, mice received 1 ml/kg CCl_4 dissolved in olive oil. Human recombinant IL-6 (R&D Systems, Minneapolis, MN) was injected subcutaneously at a dose of 1 μg/g in 0.9% NaCl. The experiments were performed in accordance with the institutional guidelines of the University of Washington School of Medicine.

ALT Activity

Plasma alanine aminotransferase (ALT) activity was measured by the SIGMA Diagnostics transaminase kit (Sigma Chemical Co., St. Louis, MO). Mice were bled by cardiac puncture, and plasma was collected with 7.5% EDTA (pH 7.4) at the indicated times. For each determination, 10 μl of plasma was used.

Nuclear Extracts

Mice were killed from 0.5 to 5 hours after CCl_4 injection as indicated. Tissue was homogenized and nuclear extracts prepared with solutions containing protease inhibitors as previously described.[6,14] The extracts were kept at −80°C until use. Protein concentrations were measured by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA).

Probes for Electrophoretic Mobility Shift Assays (EMSAs)

The DNA probes were NF-κB binding sequence from the class 1 major histocompatibility enhancer element (H2K) as previously described[6,14]; AP-1, consensus oligonucleotide probe (Santa Cruz Biotechnology, Santa Cruz, CA); STAT3, oligonucleotide corresponding to the binding site for the Sis-inducible factor (Santa Cruz Biotechnology); and C/EBP, oligonucleotide corresponding to nucleotides −112 to −86 of the rat albumin gene promoter.[15] These probes were end labeled by ^{32}P as previously described.[6,14]

EMSA Technique

For each assay, 10 μg of nuclear protein was incubated with 0.2 ng of ^{32}P-end-labeled double-stranded oligonucleotide probes as previously described.[6,14] After 30 minutes of incubation at room temperature, samples were elecrophoresed through 5% polyacrylamide Tris/glycine/EDTA gels. For supershift analysis, 1 μg of the antibody (all from Santa Cruz Biotechnology) was added to the extracts after 30 minutes of incubation with the labeled probe. Samples were electrophoresed after an additional 30 minutes of incubation at room temperature. The antibodies were anti-p50 and -p65, specific polyclonal antibodies for the NF-κB components; c-Jun/AP-1, polyclonal antibody reactive with c-Jun, JunB, and JunD; c-Jun/AP-1, polyclonal antibody specific for c-Jun; JunB-specific polyclonal antibody; c-Fos-specific polyclonal antibody; and C/EBP polyclonal antibody specific for C/EBPα and C/EBP polyclonal antibody specific for C/EBPβ. Dried gels were exposed to Kodak X-AR film from 2 hours to 2 days.

RNA Preparation and Northern Blot Hybridization

RNA was purified by ultracentrifugation through cesium chloride after liver homogenization in 4 mol/L guanidine thiocyanate as previously described.[6,14] RNA samples (20 μg/lane) were separated by electrophoresis through 1.1% formaldehyde-agarose gels and transferred to a nylon membrane (MagnaGraph, Micron Separations, Westborough, MA). After 2 to 4 hours of prehybridization at 42°C, the filters were hybridized with the following probes labeled with [α-^{32}P]dCTP by random priming: TNFR-1, 240-bp *Spe*I-*Bgl*II fragment from murine TNFR-1 cDNA; TNFR-2, 450-bp *Xba*I-*Sal*I fragment from murine TNFR-2 cDNA; c-fos 915-bp *Eco*RI-*Sph*I fragment from human c-fos cDNA; c-jun, 1800-bp *Eco*RI fragment from murine c-jun cDNA; jun B, 1700-bp *Eco*RI fragment from jun B cDNA; and β_2-microglobulin (loading control), 350-bp *Pst*I fragment from mouse cDNA. After hybridization at 42°C for 12 to 24 hours, the filters were washed and exposed to Kodak X-AR film with intensifying screens at −80°C. After each hybridization, probes were removed by washing with 50% formamide, 6X SSC at 65°C for 30 minutes.

Plasma Cytokine Assays

Blood was taken by cardiac puncture with 7.5% EDTA (pH 7.4) and centrifuged, and supernatant was stored at −80°C. TNF and IL-6 activity was measured by enzyme-linked immunosorbent assay (ELISA) using the manufacturer's protocol (PharMingen, San Diego, CA). Purified anti-mouse TNF and IL-6 antibodies were diluted in 0.1 mol/L $NaHCO_3$ (pH 9.5) to 4 μg/ml and 2 μg/ml, respectively.

Determination of IL-6 mRNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Assay

To measure the expression of liver IL-6 mRNA, we used RT-PCR.[6] cDNA was prepared from 1 μg of total RNA from each liver sample by the Gene Amp RNA PCR kit (Perkin Elmer, Branchburg, NJ), in a buffer containing 2.5 U of MuLV reverse transcriptase, and 2.5 μmol/L oligo d(T) primer. The RNA was incubated at 42°C for 15 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. A sample of cDNA corresponding to 50 ng of input RNA was amplified. The PCR reaction contained, in the same buffer as the reverse transcriptase reaction, 0.4 μmol/L IL-6 primers (Clontech Laboratories, Palo Alto, CA) and 2.5 U of Ampli*Taq* DNA polymerase. It was performed at 94°C for 1 minute, 60°C for 1 minute, and 74°C for 1.5 minutes for 30 cycles. To determine optimal conditions for PCR analysis, the reaction was initially performed using 10 to 40 cycles. PCR product was not detected after 10 or 20 cycles and was saturated at 40 cycles. Amplified products obtained with 30 cycles were electrophoresed in 2% agarose gels and stained with ethidium bromide. Quantitation of IL-6 mRNA was done by competitive PCR using the PCR Mimic Protocol (Clontech) as previously described.[6] IL-6 competitor primer yielding a product size of 435 bp was used for reaction in concentrations of 10 to 10^{-6} amol.

DNA Labeling and Histological Analysis

Bromodeoxyuridine (BrdU) was injected intraperitoneally at 30 μg/g 2 hours before killing (at least three animals from each experimental group at each time point). Livers were fixed in methyl Carnoy's fluid for 4 to 6 hours, processed for histological analysis, and subsequently stained using the Amersham cell proliferation kit (Amersham, Arlington Heights, IL). Approximately 800 nuclei were counted per slide.

Results

Liver Injury after CCl_4 Injection

WT and TNFR-1 and TNFR-2 knockout mice injected with 0.25 ml/kg CCl_4 were bled by cardiac puncture at various times between 1 hour and 14 days after the injection (at least three mice from each group for each time point).

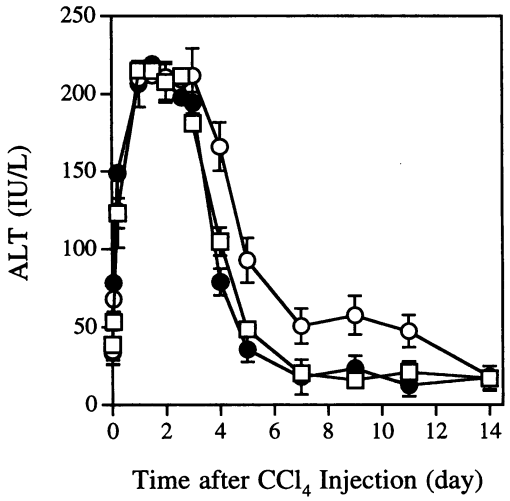

Figure 1. ALT levels after CCl_4 injection. Blood was taken by cardiac puncture (three mice per group), and the plasma was collected with 7.5% EDTA (pH 7.4) 1 to 14 days after intraperitoneal injection of 0.25 ml/kg CCl_4 dissolved in olive oil. ALT activity was measured using the Sigma Diagnostics transaminase kit. □, WT mice; ○, TNFR-1 knockout mice; ●, TNFR-2 knockout mice. The bars indicate the SD of the means.

Plasma from blood collected in 7.5% EDTA was used to determine ALT activity in these animals (Figure 1). ALT activity rose by approximately 10-fold during the first 24 hours after CCl_4 injection in mice from the three groups and began to decline at 96 hours. From 4 to 14 days after the injection, ALT activity followed an identical pattern of reduction in WT and TNFR-2 knockout mice. However, although ALT activity also decreased in TNFR-1 knockout mice during this period, enzyme levels were approximately twice as high (Figure 1) as those of the other two groups from days 4 to 9 (values from TNFR-1 knockouts were significantly higher than WT at the 0.005 level for days 4 and 5 and at the 0.001 level for days 7 and 9). Histological examination of the liver of these animals showed the pattern of cell swelling and midzonal necrosis expected to occur after CCl_4 injection.[16] The extent and timing of the damage was very similar in the livers of WT and TNFR-1 and TNFR-2 knockout mice. Practically all lobules contained necrotic hepatocytes in centrolobular and midzonal areas. Necrotic hepatocytes with eosinophilic cytoplasm and other typical features of coagulation necrosis (Figure 2, A and B) were first detected at 24 hours and became clearly delineated from the rest of the parenchyma by 36 to 48 hours after CCl_4 injection. Damaged hepatocytes were necrotic and did not display features of apoptosis (cell shrinkage, chromatin condensation, apoptotic bodies, etc). In WT and TNFR-2 knockout mice restoration of normal morphology was completed between 5 and 7 days after the injection. Livers of TNFR-1 knockout mice also had normal overall morphology at 5 to 7 days. However, in these animals, at 5 to 12 days after CCl_4 injection, clusters of inflammatory cells (mostly lymphocytes) were present around central veins and areas of healed necrosis (Figure 2, C and D). These infiltrates were not seen in livers of WT or TNFR-2 knockout mice. Thus, enzyme and morphological data indicate that the acute injury caused by CCl_4 at the dose used

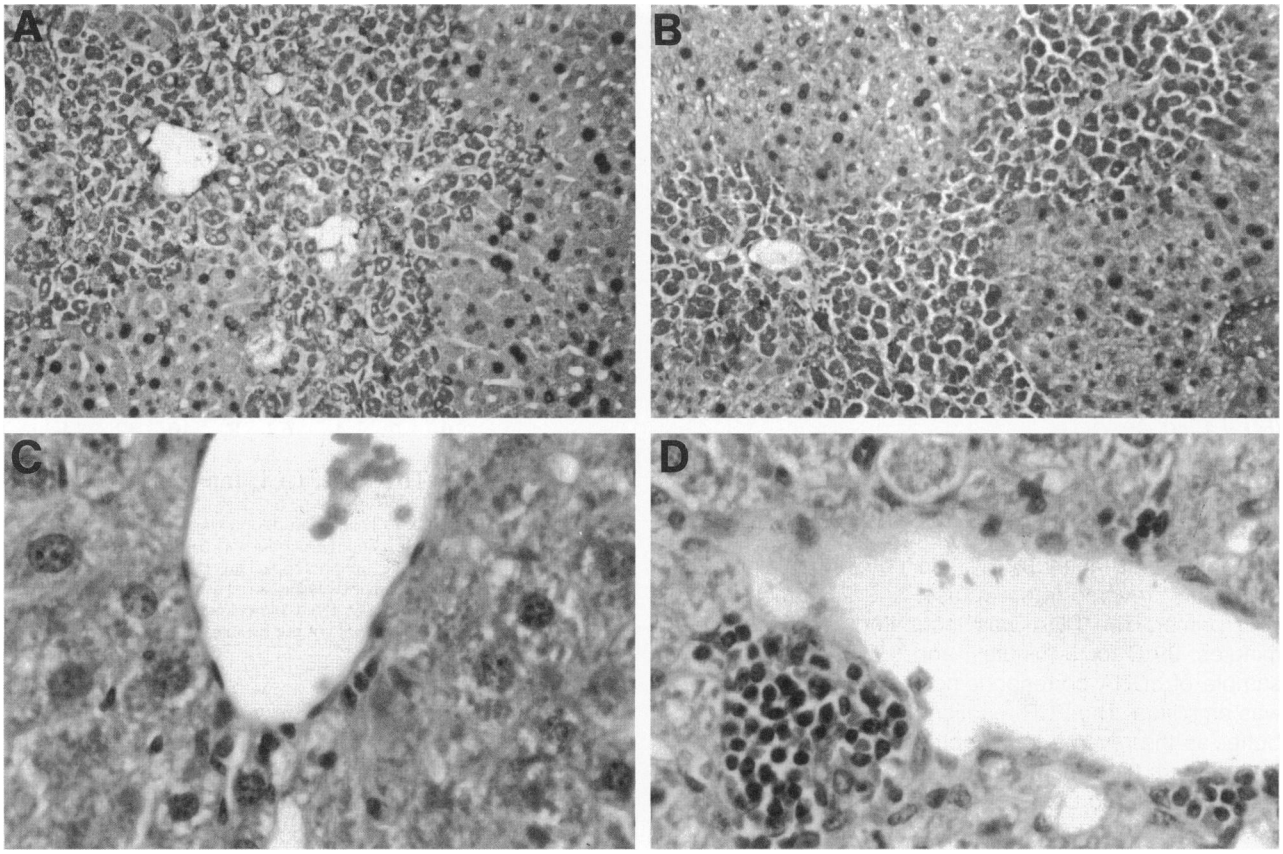

Figure 2. Liver morphology after CCl_4 injection. **A** and **B** demonstrate centrolobular liver necrosis in WT and TNFR-1 knockout mice, respectively, 62 hours after CCl_4 injection. Magnification, ×100. **C** and **D** show centrolobular areas of livers of WT and TNFR-1 mice, respectively, 7 days after CCl_4 injection. Magnification, ×400. Note similar extent of necrosis in **A** and **B** and the mononuclear cell infiltrate in **D**.

was not significantly modified in mice that lack either TNFR-1 or TNFR-2 but that recovery from injury was delayed in TNFR-1 knockouts. To determine whether this pattern of injury would be modified if higher doses of CCl_4 were used, we injected mice with 1 ml/kg CCl_4 and determined ALT activity. Enzyme levels in these animals reached a peak 2 to 3 days after the injection (Figure 3) and were approximately fivefold higher than the maximal level of activity measured in mice injected with the lower dose of CCl_4. However, the pattern of change of ALT activity after CCl_4 was similar regardless of dosage. WT and TNFR-2 knockout mice had an identical pattern whereas ALT levels decreased more slowly in TNFR-1 knockouts.

Expression of TNFR-1 and TNFR-2 mRNAs

We investigated the expression of TNFR-1 and TNFR-2 mRNAs in livers of WT and TNFR-1 and TNFR-2 knockout mice from 0.5 to 24 hours after CCl_4 injection (Figure 4). As expected, TNFR-1 mRNA was not detectable in TNFR-1 knockouts, and TNFR-2 mRNA could not be demonstrated in TNFR-2 knockout mice. In WT mice, expression of TNFR-1 mRNA increased by 1 hour after CCl_4 injection and was highest at 4 to 5 hours. A similar pattern of TNFR-1 mRNA expression occurred in livers of TNFR-2 knockout mice, although the changes were of lower magnitude. TNFR-2 mRNA expression increased 0.5 to 1 hour after CCl_4 injection and reached a maximum at 3 to 5 hours. In TNFR-1 knockout mice, TNFR-2 mRNA also increased but reached maximal levels 4 to 5 hours after the injection. Thus, expression of the mRNAs for

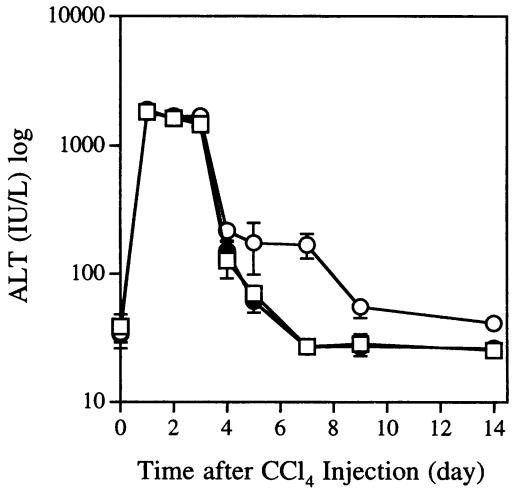

Figure 3. ALT levels after high-dose injection of CCl_4. ALT activity was measured, as described in Figure 1, 1 to 14 days after injection of 1 ml/kg CCl_4 (three mice per group). □, WT mice; ○, TNFR-1 knockout mice; ●, TNFR-2 knockout mice. The bars indicate the SD of the means.

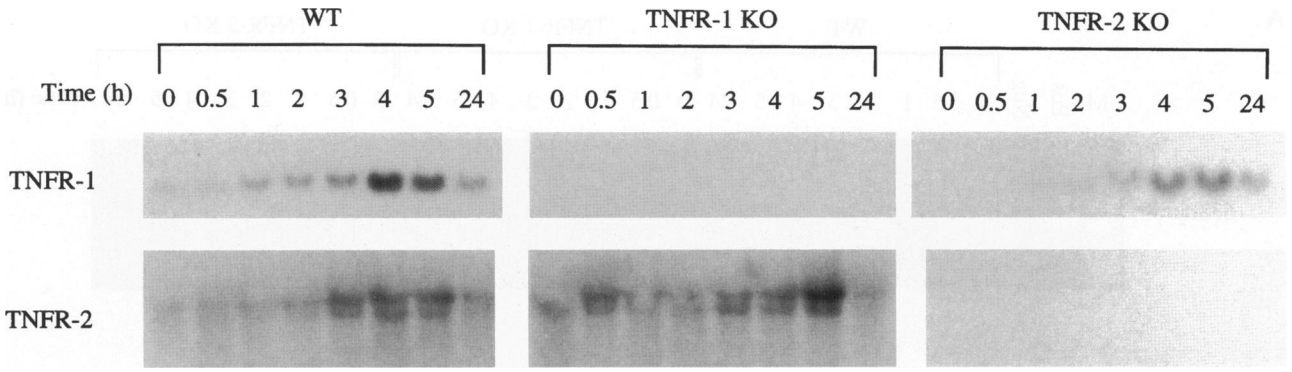

Figure 4. Expression of TNF receptor mRNAs after CCl_4 injection. Northern blot analysis of TNFR-1 and TNFR-2 mRNA expression (three mice per group) from livers of WT and TNFR-1 and TNFR-2 knockout (KO) mice. Filters were hybridized with [α-^{32}P]dCTP-labeled probes. After each hybridization, probes were removed from filters with 50% formamide as described in Materials and Methods. The time after CCl_4 injection is indicated at the top of the figure (0 refers to saline-injected mice). β_2-microglobulin mRNA hybridization (shown in Figure 10) was used as a control for loading.

both TNF receptors increased rapidly in livers of WT mice after CCl_4 injection. TNFR-1 and TNFR-2 mRNA was increased in livers of TNFR-2 and TNFR-1 knockout mice, respectively, with some variations in the timing and extent of the changes compared with WT animals.

TNF and IL-6 Levels after CCl_4 Injection

We measured TNF and IL-6 concentrations in plasma as well as IL-6 mRNA levels in the liver in WT and TNFR-1 and TNFR-2 knockout mice 30 minutes to 5 hours after CCl_4 injection (Figure 5, A and B). At least three animals from each experimental group were used at each time point. Plasma TNF concentrations increased markedly by 30 minutes and reached a maximum at 1 hour after CCl_4 injection (Figure 5A). In WT and TNFR-2 knockout mice there was a slow decrease in TNF levels from 1 to 5 hours after the injection. In contrast, in TNFR-1 knockout mice, TNF levels remained high and constant for at least 5 hours after CCl_4 injection. The pattern of change of plasma IL-6 concentration after CCl_4 injury was similar in WT and TNFR-2 knockout mice (Figure 5B). A very large increase occurred during the first hour, and the highest levels remained at least until 3 hours after the injection. In TNFR-1 knockout mice, the increase in plasma IL-6 during the first hour after CCl_4 injection was less than 50% of that detected in WT and TNFR-2 knockout mice. In TNFR-1 knockout mice the IL-6 concentrations remained constant from 1 to 5 hours after the injection. In all animals (WT and TNFR-1 and TNFR-2 knockout mice) plasma TNF and IL-6 concentrations had not returned to normal at 5 hours after CCl_4 injection (Figure 5, A and B).

To determine whether hepatic IL-6 mRNA concentrations after CCl_4 injury would be lower in TNFR-1 knockout mice than in WT or TNFR-2 knockout animals, we used a quantitative competitive RT-PCR assay to measure liver IL-6. Using 1 μg of total RNA and extracts from two mice per experimental group as the starting material, we established that 30 amplification cycles was the optimal condition for this assay. Conventional RT-PCR assays showed that liver IL-6 mRNA increased in all experimental groups but that the increase was much smaller in TNFR-1 knockout mice (Figure 6A). Quantitation of the

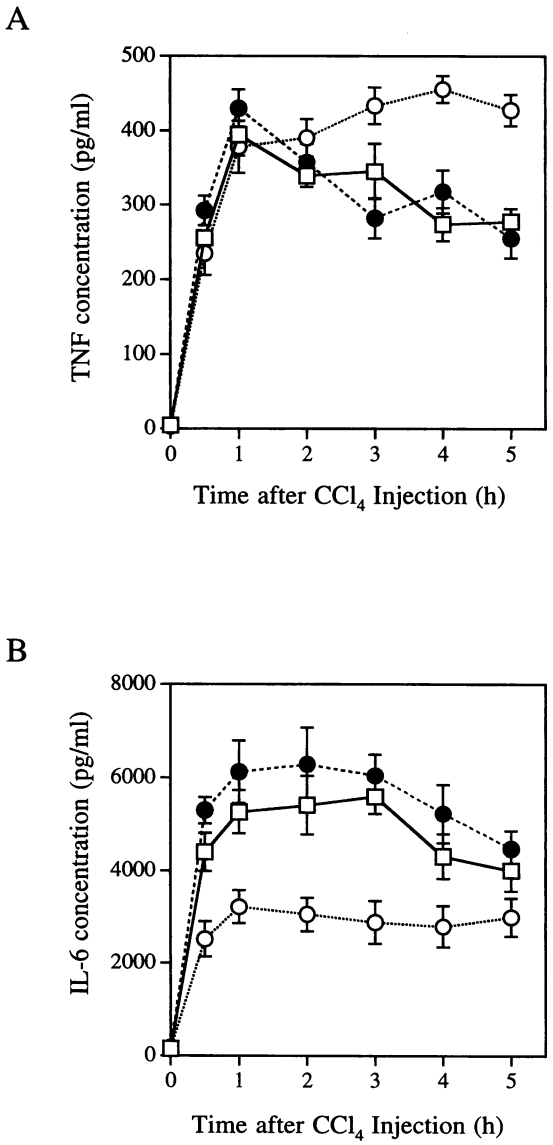

Figure 5. Plasma TNF and IL-6 concentrations after CCl_4 injection. Plasma TNF and IL-6 concentrations were measured by ELISA as described in Materials and Methods (three mice per group). Mice were killed 0.5 hour after injection of 0.25 ml/kg CCl_4. □, WT mice; ○, TNFR-1 knockout mice; ●, TNFR-2 knockout mice. The bars indicate the SD of the means.

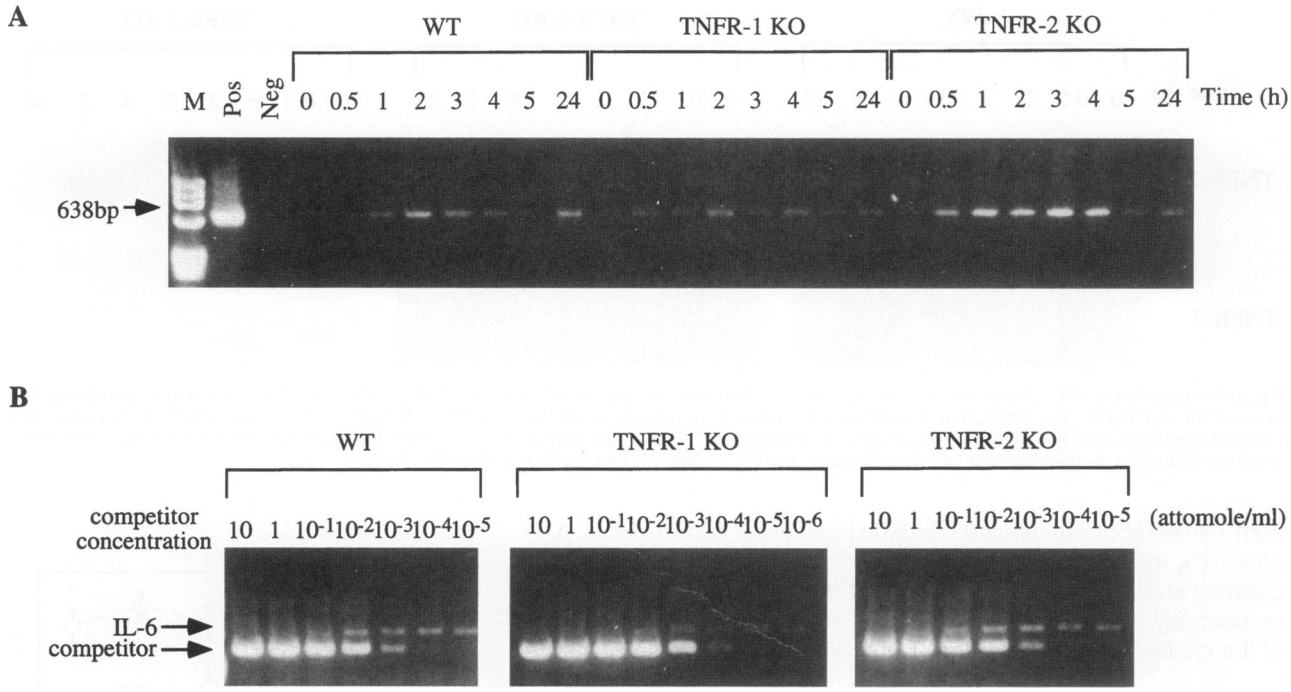

Figure 6. Liver IL-6 mRNA expression after CCl_4 injection. Hepatic IL-6 mRNA was measured by RT-PCR. Total RNA (1 μg) was translated to DNA and amplified by 30 cycles of PCR using unsaturated conditions as described in Materials and Methods. **A:** WT and TNFR-1 and TNFR-2 knockout (KO) mice were killed from 30 minutes to 24 hours after CCl_4 injection as indicated at the top of the figure. M, molecular weight marker; Pos, IL-6 mRNA positive control; Neg, no mRNA added at RT step. IL-6 mRNA is detected as a 638-bp fragment. **B:** Quantitation of hepatic IL-6 mRNA expression by competitive PCR using the PCR Mimic Protocol (Clontech) The RNA sample used was obtained 4 hours after CCl_4 injection. The competitor is detected as a 435-bp fragment. Competition was completed at 10^{-3} to 10^{-4} amol/μl for WT mice, 10^{-3} amol/μl for TNFR-2 knockouts, and 10^{-4} to 10^{-5} amol/μl for TNFR-1 knockouts.

changes by competitive RT-PCR (Figure 6B) showed that competition was completed at 10^{-3} to 10^{-4} amol/μl for WT mice, at 10^{-3} amol/μl for TNFR-2 knockouts, and at 10^{-4} to 10^{-5} amol/μl for TNFR-1 knockouts. Thus, IL-6 mRNA amounts were at least 10 times lower in the liver of TNFR-1 knockout mice than in WT or TNFR-2 knockout mice.

Hepatocyte DNA Synthesis

To analyze hepatocyte replication we injected BrdU into WT and TNFR-1 and TNFR-2 knockout mice at various times between 24 hours and 14 days after CCl_4 administration at a dosage of 0.25 ml/kg (Figure 7). DNA synthesis in the liver was negligible until 32 hours after CCl_4. Between 32 and 40 hours after CCl_4 there was a drastic increase in hepatocyte replication in WT and TNFR-2 knockout mice. At 40 hours, more than 60% of hepatocytes were labeled in livers of WT and TNFR-2 knockouts. Incorporation of BrdU returned to normal in these animals at approximately 5 days after CCl_4 administration. In contrast, less than 30% of hepatocytes were labeled at 40 hours in livers of TNFR-1 knockout mice. The distribution of labeled hepatocytes throughout the lobule was similar in WT and TNFR-1 and TNFR-2 knockout mice. The inhibition of hepatocyte DNA synthesis in TNFR-1 knockout hepatocytes compared with those of WT and TNFR-2 animals persisted until approximately 96 hours after the operation. Between 5 and 14 days after CCl_4, hepatocyte replication was negligible in the livers of WT and TNFR-2 knockout mice. However, approximately 2% of hepatocytes were labeled in livers of TNFR-1 knockout mice during this period of time (Figure 7). In mice injected with a higher dosage of CCl_4 (1 ml/kg), only approximately 5% of hepatocytes were labeled by BrdU at 44 hours after CCl_4 injections in TNFR-1 knockouts whereas labeling was similar in WT and TNFR-2 knockout mice (data not shown). In sum, hepatocyte replication in TNFR-1 knockout mice was decreased by more than 50% during the first 3 days after an injection of 0.25 ml/kg CCl_4 in comparison with WT and TNFR-2 knockout animals. From

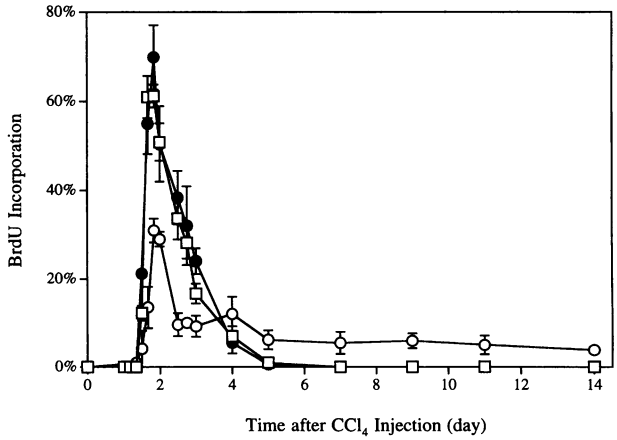

Figure 7. DNA synthesis after CCl_4 injection. Hepatocyte DNA synthesis 1 to 14 days after CCl_4 injection (0.25 ml/kg) was measured by BrdU incorporation (three mice per group) in WT and TNFR-1 and TNFR-2 knockout mice. BrdU (30 μg/g) was injected intraperitoneally 2 hours before killing. □, WT mice; ○, TNFR-1 knockout mice; ●, TNFR-2 knockout mice. The bars indicate the SD of the means.

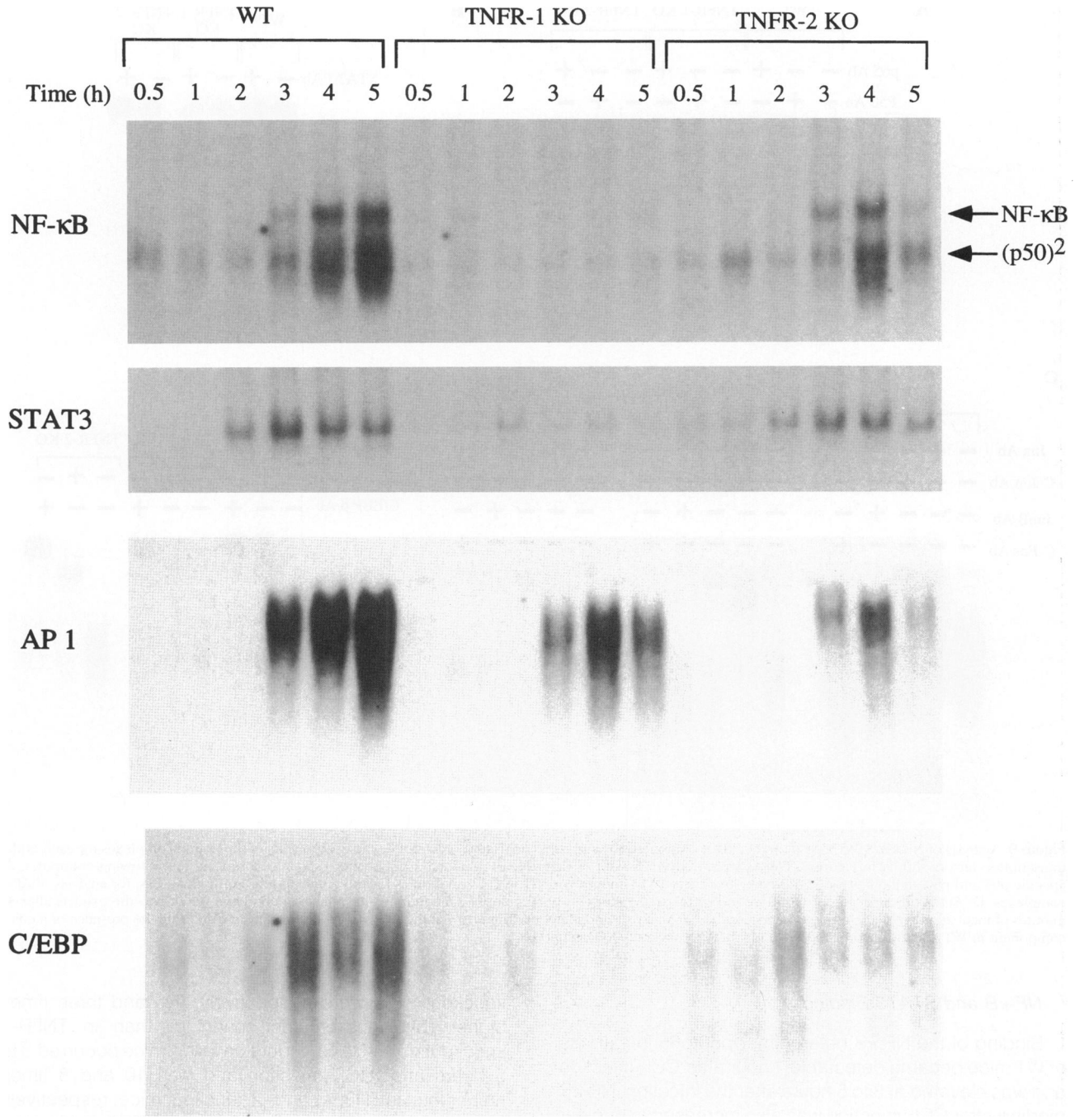

Figure 8. Transcription factor binding after CCl_4 injection. WT and TNFR-1 and TNFR-2 knockout mice were injected intraperitoneally with 0.25 ml/kg CCl_4 dissolved in olive oil and sacrificed (three mice per group) 0.5 to 5 hours after the injection as indicated at the top of the figure. Nuclear extracts and EMSAs were performed using 10 μg of nuclear protein in each lane and ^{32}P-end-labeled oligonucleotide probes for NF-κB, STAT3, AP-1, and C/EBP as described in Materials and Methods. Reticulocyte lysate was used as a marker to determine the position of the p50/p65 NF-κB heterodimer; $(p50)^2$ is the p50 homodimer.

days 4 to 14 there was a small amount of DNA replication in hepatocytes of TNFR-1 knockout mice, but replication was no longer detectable in WT and TNFR-2 knockout mice.

Transcription Factor Binding

TNF is a potent stimulator of NF-κB in normal and regenerating liver after PH.[6,13] Absence of TNFR-1 signaling after PH completely abolishes binding of NF-κB and STAT3 and blocks hepatocyte replication.[6] To investigate binding of the transcription factors NF-κB, STAT3, AP-1, and C/EBP in CCl_4 injury, we isolated nuclei from livers of WT and TNFR-1 and TNFR-2 knockout mice 30 minutes to 5 hours after CCl_4 injection (Figure 8). At least two animals from each group were used at each time point. The findings were consistent and reproducible among the animals or in repeated EMSAs from the same extracts.

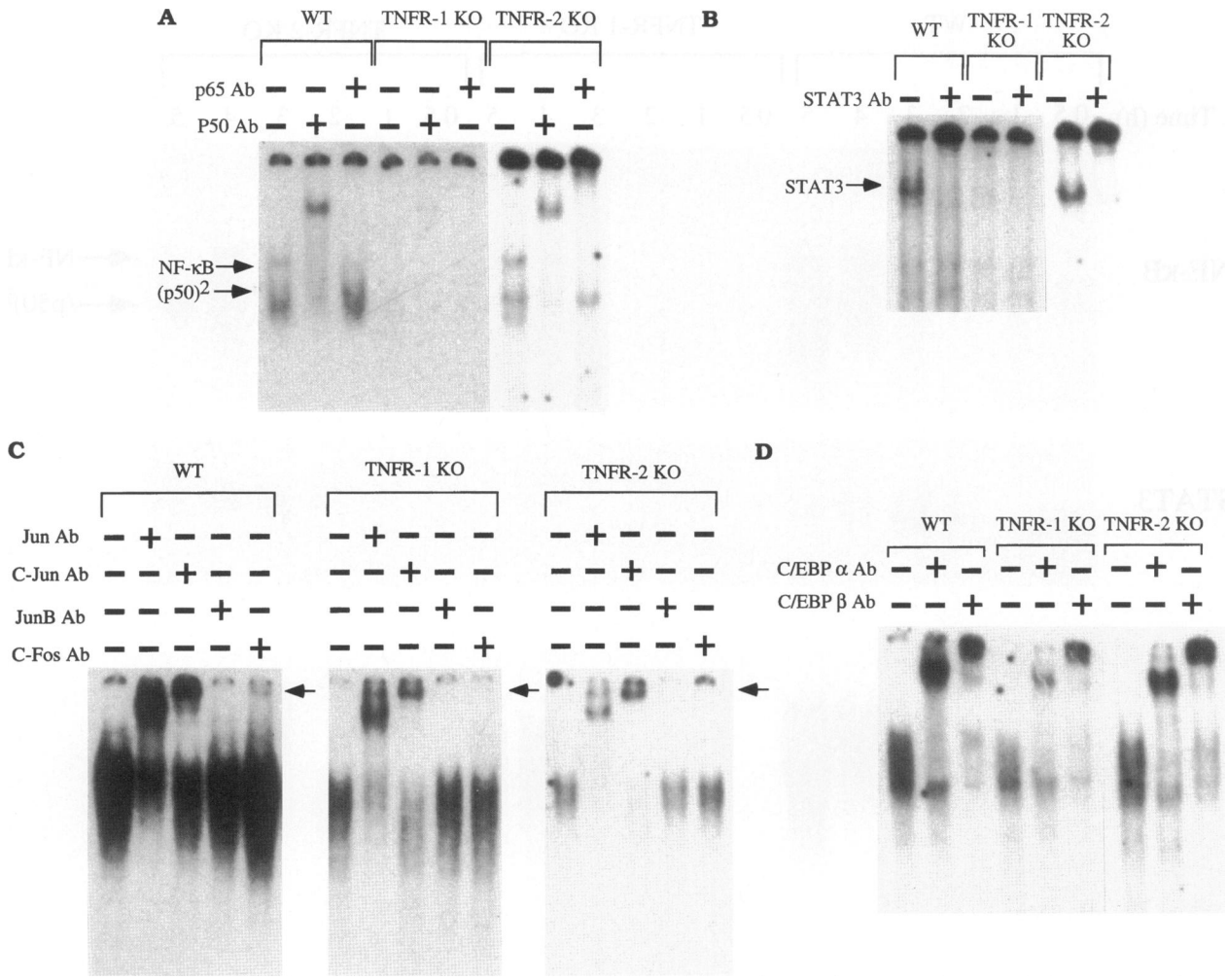

Figure 9. Analysis of NF-κB, STAT3, AP-1, and C/EBP components by supershift analysis. To determine the components of the bound complexes for each of the transcription factors shown in Figure 8, EMSAs were performed with nucleoproteins obtained 4 hours after CCl_4 injection using the following antibodies. **A:** Specific p65 and p50 antibodies for NF-κB complexes. **B:** STAT3 antibody for STAT3. **C:** Antibodies for Jun family, c-Jun, JunB, and c-Fos, for analysis of AP-1 complexes. **D:** Antibodies specific for C/EBP-α and -β for analysis of C/EBP complexes. One microgram of each antibody was added to the extracts after 30 minutes of incubation with labeled probes as described in Materials and Methods. The **arrow** at the right side of the panels in **C** indicate the presence of a c-Fos component in WT and absence in TNFR-1 and TNFR-2 knockout (KO) extracts.

NF-κB and STAT3 Binding

Binding of the NF-κB heterodimer p50/p65 in extracts of WT mice became detectable 1 hour after CCl_4 injection and was elevated at 3 to 5 hours after the injection. NF-κB binding after CCl_4 injection was also increased in nuclear extracts of TNFR-2 knockout mice although the increase was of a smaller magnitude than that of control mice. In contrast, p50/p65 NF-κB binding was not detectable in nuclear extracts of TNFR-1 knockouts during the first 5 hours after CCl_4 injection. STAT3 binding increased 2 to 5 hours after CCl_4 administration in WT and TNFR-2 knockout mice. However, STAT3 binding was not detectable in nuclear extracts from TNFR-1 knockout animals during the first 5 hours after CCl_4 administration.

AP-1 and C/EBP Binding

AP-1 binding was induced at 3 to 5 hours in all three groups of animals, but the increase in WT and TNFR-1 knockout mice were, respectively, five and three times higher (by densitometric scanning) than in TNFR-2 knockout mice. C/EBP induction in WT mice occurred 3 to 5 hours after CCl_4 injection and was 10 and 8 times higher than in TNFR-1 and -2 knockout mice, respectively (densitometric scanning for AP-1 and C/EBP EMSAs not shown).

Supershift Analysis

The binding of the four transcription factors in nuclear extracts prepared 4 hours after CCl_4 injection was analyzed using supershift assays with specific antibodies (Figure 9). Antibodies to the p50 and p65 components of NF-κB displaced these components in gel shifts of nuclear extracts from WT and TNFR-2 knockout mice but showed no reactivity in nuclear extracts from TNFR-1 knockouts (Figure 9A). The STAT3 band was completely shifted by STAT3 antibody in WT and TNFR-2 knockout

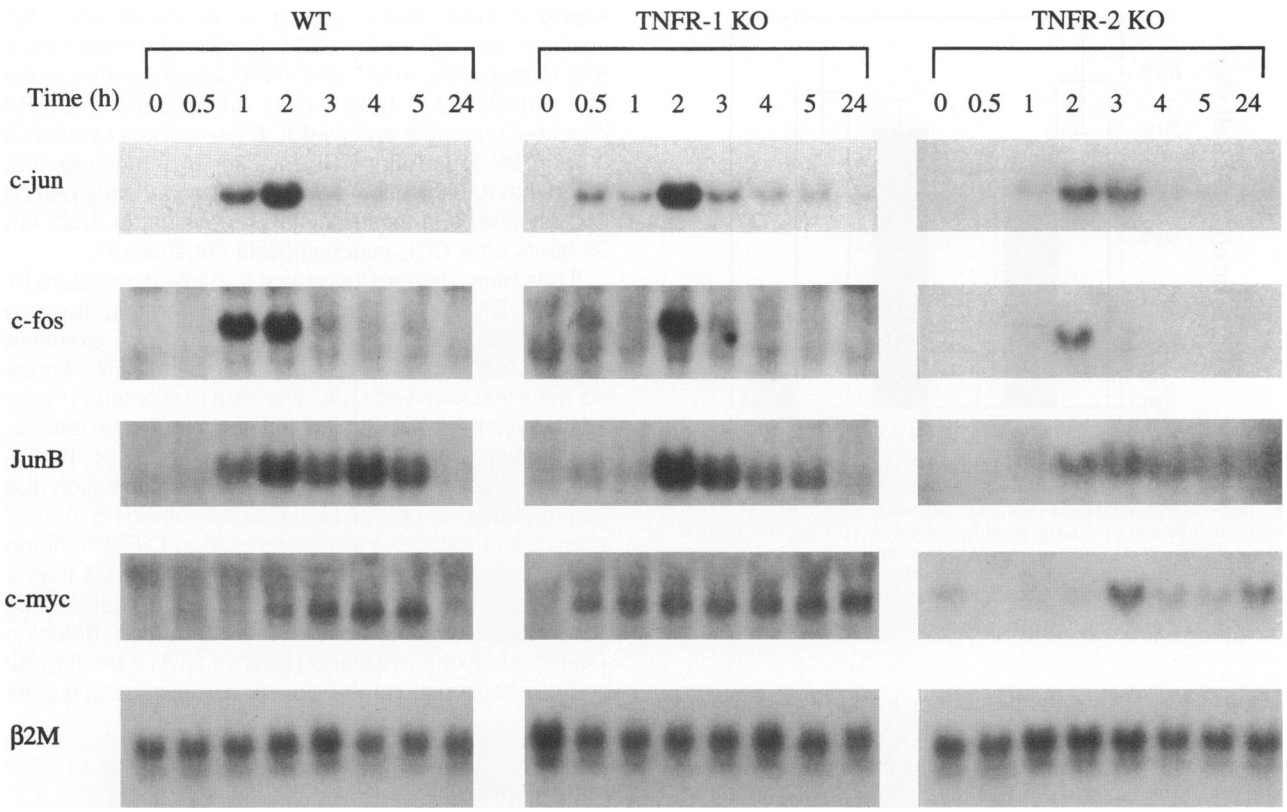

Figure 10. Expression of proto-oncogene mRNAs after CCl_4 injection. We measured the expression of c-jun, c-fos, junB, and c-myc mRNAs in WT and TNFR-1 and TNFR-2 knockout (KO) mice by Northern blot analysis. Animals were killed 0.5 to 24 hours after injection of CCl_4, and 20 μg of total RNA was separated by electrophoresis and hybridized with [α-^{32}P[dCTP probes as described in Materials and Methods. Hybridization with β2M probe (bottom of figure) was used as control for loading of the samples.

mice extracts but had no reactivity in extracts from TNFR-1 knockout mice (Figure 9B). For analysis of AP-1 components, four antibodies were used: 1) Jun, to detect Jun family components, 2) c-Jun, 3) JunB, and 4) c-Fos to specifically detect each of these proteins. The AP-1 band in nuclear extracts of WT mice contained c-Jun, JunB, and c-Fos, as each of these components was displaced by the appropriate antibody (Figure 9C). However, in extracts from TNFR-1 or TNFR-2 knockout mice, band displacement was obtained with the Jun and c-Jun antibodies but not with antibodies to JunB or c-Fos (indicated by the arrow at the right side of each panel in Figure 9C). The results of this gel shift analysis indicate that, in addition to the inhibition of overall AP-1 binding, the c-Fos and JunB components were not detectable in the AP-1 complex from TNFR-1 and TNFR-2 knockout mice 4 hours after CCl_4 injection. To analyze the components of C/EBP in nuclear extracts of TNFR-1 and TNFR-2 knockout mice and compare them with those of WT animals, film exposure was greatly increased for gels containing extracts from TNFR-1 and TNFR-2 knockout mice because of the low level of C/EBP binding in these extracts (Figure 9D). The supershift analysis revealed that the C/EBP complex in extracts of WT and TNFR-1 and TNFR-2 knockout mice contained both C/EBP-α and -β and that the ratio between these components was similar for the three groups of animals.

Proto-Oncogene mRNA Expression after CCl_4 Injection

We and others have shown that the expression of the mRNAs from several proto-oncogenes increases shortly after CCl_4 administration.[17–20] Because of the defect in DNA replication in TNFR-1 knockout mice and the alterations in transcription factor binding found in both TNFR-1 and TNFR-2 knockout mice, we studied the expression of c-jun, c-fos, JunB, and c-myc mRNAs by Northern blot analysis in livers from WT and TNFR-1 and TNFR-2 knockout mice obtained 30 minutes to 24 hours after CCl_4 injection. β_2-Microglobulin mRNA expression was used as a control for loading of the samples (Figure 10). As previously reported, c-fos, c-jun, and c-myc mRNAs increased in the liver of WT mice after CCl_4 injection.[17–20] The changes in c-fos and c-jun mRNA levels were very short-lived and were detected only at 1 and 2 hours after the injection. JunB and c-myc mRNA elevation occurred between 1 and 5 hours after CCl_4 injection, but these mRNAs were no longer detected at 24 hours. In TNFR-1 and TNFR-2 knockout mice, increased expression of all mRNAs tested also occurred, but the extent and timing of the changes differed from those in WT mice. c-jun mRNA had a prolonged increase (0.5 to 5 hours) in TNFR-1 knockout mice although expression was

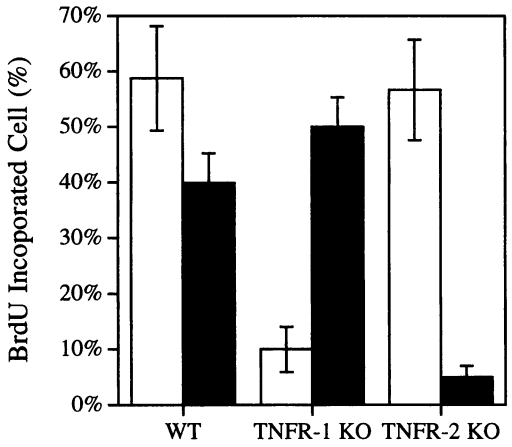

Figure 11. DNA synthesis after CCl_4 injection preceded by IL-6 injection. Hepatocyte DNA synthesis was assessed at 44 hours after CCl_4 injection in WT and TNFR-1 and TNFR-2 knockout (KO) mice injected with IL-6 (subcutaneous injection of 1 μg/g human recombinant IL-6 in 0.9% NaCl) 30 minutes before the CCl_4 injection. BrdU was injected intraperitoneally 2 hours before killing. The bars indicate the SD of the mean. □, CCl_4 injection; ■, mice injected with IL-6 before CCl_4 injection. Mouse lines are indicated at the bottom of the figure.

delayed by approximately 1 hour in TNFR-2 knockout mice. c-fos mRNA was detectable only at 2 hours in TNFR-1 and TNFR-2 knockout mice. JunB mRNA expression was decreased and delayed in TNFR-2 knockout mice. Expression of c-myc mRNA remained constant from 30 minutes to 24 hours after the injection in TNFR-1 knockout mice and was detectable from 3 to 24 hours in TNFR-2 knockout mice. In summary, in TNFR-1 knockout mice, there was a prolonged expression of c-jun and c-myc mRNAs whereas c-fos expression was short-lived. In TNFR-2 knockout mice, expression of c-jun, c-fos, c-myc, and junB mRNAs were lower than in WT mice.

Effect of IL-6 on Hepatocyte Proliferation and STAT3 Binding

TNFR-1 knockout mice had lower levels of plasma IL-6 and liver IL-6 mRNA than WT or TNFR-2 knockout mice in liver regeneration induced by CCl_4 injection. We have shown that a single injection of IL-6 can correct the defect in DNA synthesis after PH in TNFR-1 knockout mice.[6] To determine whether IL-6 would also reverse the DNA synthesis deficiency of TNFR-1 knockout mice in CCl_4-induced liver regeneration, we injected IL-6 subcutaneously (1 μg/g human recombinant IL-6) 30 minutes before CCl_4 administration. WT and TNFR-1 and TNFR-2 knockout mice (three animals per group) that received either CCl_4 alone (0.25 ml/kg) or IL-6 and CCl_4 were killed at 44 hours (the peak of DNA replication) to determine the percentage of replicating hepatocytes. In this experiment, the proportion of labeled hepatocytes at 44 hours after CCl_4 injection was lower than that of data shown in Figure 7. IL-6 injection corrected the DNA synthesis impairment of CCl_4-injected TNFR-1 knockout mice (Figure 11) and increased the percentage of labeled hepatocytes from 10% to 50%. In contrast, IL-6 injection had an inhibitory effect on DNA synthesis of WT and TNFR-2 knockout mice. BrdU labeling, at 40 hours after CCl_4 injection, decreased from 58% to 40% and from 55% to 5%, respectively, in WT and TNFR-2 knockout mice that had received IL-6 before CCl_4 administration. TNFR-2 knockout mice that received IL-6 were also examined at 30 and 35 hours after CCl_4 injection. The analysis indicated that IL-6 injection did not produce an early peak of DNA synthesis in these animals as evaluated at 30 and 35 hours after CCl_4 injection (data not shown).

It has been demonstrated that IL-6 injection before PH restores STAT3 binding in the regenerating livers of TNFR-1 and IL-6 knockout mice.[6,7] We examined whether IL-6 would have a similar effect in TNFR-1 knockout mice that received CCl_4. The data presented in Figure 12 demonstrate that IL-6 injection completely restored the binding of STAT3 in nuclear extracts of TNFR-1 knockout mice obtained 4 hours after CCl_4 injection. IL-6 had no effect on p50/65 NF-κB or AP-1 binding in these animals but caused a small increase in C/EBP binding, which, however, remained at much lower levels than in WT mice (Figure 12). In sum, IL-6 injection before CCl_4 administration corrected the DNA synthesis defect in TNFR-1 knockout mice and restored STAT3 binding but had no effect on the binding of other transcription factors.

Discussion

We show in this paper that lack of a functional TNFR-1 gene causes a deficiency in hepatocyte replication in CCl_4-induced liver regeneration. At 44 hours after CCl_4 injection, the time of maximal hepatocyte DNA synthesis in this type of injury, replication of hepatocytes in TNFR-1 knockout mice is 50% to 90% lower than in WT mice. TNFR-1 knockout mice showed little detectable NF-κB and STAT3 binding in nuclear extracts obtained during the first 5 hours after CCl_4. These animals had persistent high levels of plasma TNF but much lower levels of plasma IL-6 and liver IL-6 mRNA than WT mice. A single injection of IL-6 30 minutes before CCl_4 administration corrected the DNA synthesis defect and restored STAT3 binding to normal levels. TNF and IL-6 have pleiotropic effects and are the major mediators of the acute-phase response to inflammatory stimuli.[21,22] Release of these cytokines in CCl_4 injury might have been expected to be a component of the cell injury caused by the chemical. On the contrary, the absence of TNF signaling through TNFR-1 had no apparent effect on the extent of early injury caused by CCl_4 and inhibited hepatocyte replication. These results are entirely consistent with the observations of Bruccoleri et al[20] who demonstrated that antibodies to TNF delayed the repair of liver injury and inhibited protooncogene and AP-1 activation in CCl_4 injected mice. Bruccoleri et al[20] also showed that TNF and TNF mRNA increase in mouse liver after CCl_4 injection.

It has been reported that administration of soluble TNF receptor (sTNFR) to rats before an injection of CCl_4 can prevent liver injury as assessed by histological examination and serum enzyme levels.[23] These experiments did not include determinations of TNF and IL-6 levels in liver and plasma in rats that received sTNFR. This is an im-

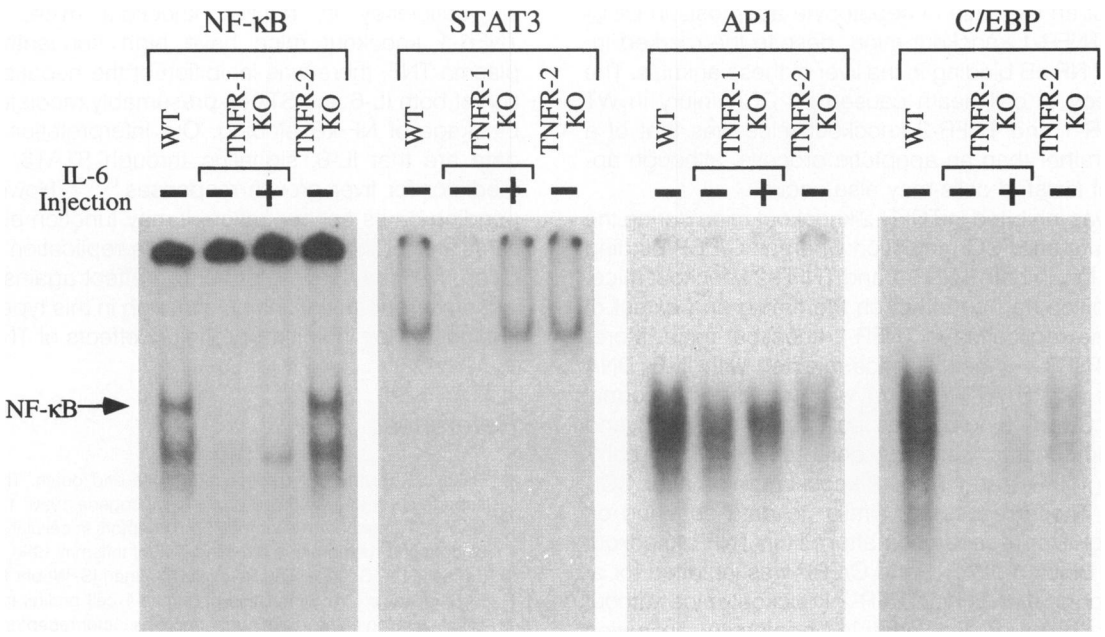

Figure 12. Transcription factor binding in TNFR-1 mice injected with CCl_4 and IL-6. EMSAs are shown of NF-κB, STAT3, AP-1, and C/EBP in TNFR-1 knockout (KO) mice injected with IL-6 30 minutes before CCl_4 administration (same as in Figure 1). EMSAs of extracts from WT and TNFR-2 knockout mice injected only with CCl_4 are shown for comparison (10 μg of protein per lane). All animals were killed 4 hours after CCl_4 injection.

portant consideration because sTNFR can have agonist rather than inhibitory effects depending on its dosage.[24] Nevertheless, it is possible that the mechanisms of CCl_4 damage may differ in the rat and mouse or that the functional deficiency of TNFR-1 might have different consequences than TNF blockage. From our experiments, we conclude that signaling through TNFR-1 during CCl_4-induced injury in mice is an important component of the mitogenic response to the injury. As is the case for liver regeneration after PH, the TNFR-1 signaling pathway in CCl_4 injury also involves NF-κB, IL-6, and STAT3. In both models of liver regeneration, IL-6 reversed the inhibitory effect created by absence of functional TNFR-1, demonstrating that this pathway is important for hepatic compensatory growth responses induced by loss of tissue or massive cell death.[6,7,25] Lack of TNFR-1 signaling after PH was lethal for a majority of animals, and regeneration in the surviving mice was deficient. CCl_4 injection into TNFR-1 knockout mice at the doses used in our experiments, did not cause mortality and led to similar increases in ALT activity in WT mice and TNFR-2 knockout mice. Although lack of TNFR-2 by itself had no apparent effect on the injury and subsequent regeneration caused by CCl_4 we do not know whether intact TNFR-2 signaling in TNFR-1 knockout mice may have a protective effect in preventing more extensive injury. Experiments with double knockout mice that lack both TNF receptors may clarify this issue.

The inhibition of DNA synthesis at 44 hours after injection in TNFR-1 knockout mice varied from 50% to 80% in two sets of experiments using 0.25 ml/kg CCl_4 (Figures 7 and 11) and was approximately 90% in mice injected with 1 ml/kg. In both sets of animals a small but detectable amount of hepatocyte DNA synthesis persisted for at least 2 weeks in TNFR-1 knockout mice whereas the proliferative response was completed at approximately 5 days after CCl_4 injection in WT and TNFR-2 knockout mice. ALT activity remained higher than normal in TNFR-1 knockout mice between 4 and 9 days after CCl_4, and a mononuclear cell infiltrate was present around central veins and in small areas of resolving necrosis. The data indicate that lack of TNFR-1 signaling caused a deficiency in hepatocyte DNA replication and of functional recovery after CCl_4 injury. The delayed recovery in TNFR-1 knockout mice is most likely caused by persistent injury as indicated by elevated ALT activity in these animals for approximately 10 days after CCl_4 injection. Additional studies are required to examine the possibility that the mononuclear infiltrates present in livers of TNFR-1 knockout mice at approximately 7 days after CCl_4 injection cause or contribute to the injury. It is also plausible to speculate that, in the absence of TNFR-1, TNF signaling leads to cell injury rather than hepatocyte replication. In TNFR-1 knockout mice, plasma TNF after CCl_4 injection remains elevated for a much longer period of time than in WT or TNFR-2 knockout mice.

It has been reported that administration of an aqueous vitamin E preparation prevents injury after CCl_4 injection and blocks the increase in NF-κB.[26] The data presented in the present report as well as in other work using TNFR and IL-6 knockout mice suggest that both the prevention of injury as well as the inhibition of NF-κB binding by vitamin E may have been caused by the efficient capture of CCl_4-generated reactive molecular species. However, it is unlikely that NF-κB inhibition *per se* would have prevented CCl_4 damage. On the contrary, deficiency of NF-κB binding is associated with inhibition of DNA replication after PH and may cause hepatocyte apoptosis. Although apoptosis caused by blockage of NF-κB binding in hepatocytes has been demonstrated,[27,28] we did

not detect an increase in hepatocyte apoptosis in CCl_4-injected TNFR-1 knockout mice, despite the marked inhibition of NF-κB binding in the liver of these animals. The morphology of cell death caused by CCl_4 injury in WT and TNFR-1 and TNFR-2 knockout mice was that of a necrotic rather than an apoptotic process, although apoptosis of isolated cells may also occur.

AP-1 was inhibited in TNFR-2 knockout mice during the first 5 hours after CCl_4 injection whereas C/EBP binding was very low in both TNFR-1 and TNFR-2 knockout mice. This inhibition had no effect on the timing and extent of hepatocyte replication in TNFR-2 knockout mice. Moreover, in TNFR-1 knockout mice injected with IL-6, DNA synthesis and STAT3 binding were restored to normal whereas C/EBP binding was not increased. JunB and c-Fos components were not detectable in AP-1 complexes in TNFR-1 and TNFR-2 knockout mice after CCl_4 injection. These results are similar to data obtained on analysis of liver regeneration after PH in TNFR knockout mice.[6,10] Binding of AP-1 and C/EBP was inhibited for at least 4 hours after PH in TNFR-2 knockout mice without causing a delay or deficiency in hepatocyte replication (Y. Yamada and N. Fausto, submitted).[10] In addition, the c-Fos component of AP-1 was not detectable in nuclear extracts of TNFR-1 and TNFR-2 knockout mice after PH, even in animals injected with IL-6 (Y. Yamada and N. Fausto, submitted).[6] The data indicate that regeneration of the liver induced by either CCl_4 injection or PH is not altered in major ways by the lack of c-Fos component in AP-1 and that delayed activation of AP-1 and C/EBP binding at the start of regeneration does not interfere with the wave of hepatocyte replication that takes place many hours later. The pattern of expression of several proto-oncogene mRNAs in TNFR-1 and TNFR-2 knockout mice during the first 5 hours after CCl_4 injection differed from that of WT mice. However, the data did not reveal any obvious relationships between expression patterns of proto-oncogene mRNAs and the timing or extent of hepatocyte replication.

Plasma TNF and IL-6 levels and liver IL-6 mRNA were severalfold higher in CCl_4-injected WT and TNFR-2 knockout mice than those detected during liver regeneration after PH.[6] Two other observations regarding IL-6 are also of interest. Despite the lack of TNFR-1 and inhibition of NF-κB binding, IL-6 mRNA levels in the liver were reduced but not eliminated. This finding suggests that there are alternative pathways for IL-6 production in the liver. These pathways may involve IL-1 or perhaps signaling through TNFR-2 when TNFR-1 function is deficient. Another interesting aspect of hepatic IL-6 production in livers of TNFR knockout mice was the very high level of IL-6 mRNA detected in TNFR-2 knockout mice. This is a puzzling observation in view of the finding that plasma IL-6 levels in TNFR-2 knockout mice after CCl_4 injection are similar to those of WT mice. In both WT and TNFR-2 knockout mice, IL-6 injection preceding CCl_4 administration had a marked inhibitory effect on hepatocyte replication. This was particularly the case for TNFR-2 knockout mice, presumably because these animals have the highest levels of hepatic IL-6 mRNA after CCl_4 injection. In contrast, IL-6 injection corrected the DNA synthesis deficiency in TNFR-1 knockout mice. Although TNFR-1 knockout mice have high concentrations of plasma TNF, there was inhibition of the hepatic production of both IL-6 and STAT3 presumably mediated by the blockage of NF-κB binding. Our interpretation of these data are that IL-6, signaling through STAT3, is a key mediator for liver growth responses.[6,7,29] However, depending on its concentration, it may function either as a stimulator or inhibitor of hepatocyte replication.[30] In addition, IL-6 may have a protective effect against hepatic ischemia/reperfusion injury, although in this type of injury it might act by inhibiting pro-injury effects of TNF.[31]

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