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Blockade of Neutrophil Elastase Attenuates Severe Liver Injury in Hepatitis B Transgenic Mice

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Serine proteinases produced by polymorphonuclear neutrophils play important roles in neutrophil-mediated tissue injury at inflammatory sites. Although neutrophil recruitment to the liver has been shown to be involved in the exacerbation of liver inflammation, the function of neutrophil elastase (NE) in liver injury remains unclear. Here, we found that administration of an NE inhibitor (NEI) reduced serum alanine aminotransferase (sALT) activity and inflammatory cell infiltration into the liver from 8 to 24 h after injection of antigen-specific cytotoxic T lymphocytes (CTLs) into hepatitis B virus transgenic mice. Furthermore, the NEI treatment reduced the expressions of inflammatory cytokines and chemokines in the liver and tumor necrosis factor alpha production by macrophages. In addition, the NEI treatment suppressed the mRNA expressions of CC chemokine ligand 3 (CCL-3), CCL-4, and macrophage inflammatory protein 2 (MIP-2) in neutrophils in the liver at 8 h after the CTL injection. In support of these results, we confirmed that administration of anti-CCL-3, anti-CCL-4, and anti-MIP-2 monoclonal antibodies suppressed sALT activity and leukocyte migration into the liver. In conclusion, the present results suggest that NE contributes to the early step of the inflammatory cascade in acute viral hepatitis and that NEIs may have potential as therapeutic drugs against acute severe viral hepatitis.

Leukocyte recruitment into tissues is an essential defense mechanism in the body's armament against invading pathogens. Leukocyte migration from the vascular lumen into the surrounding extravascular tissue is a characteristic feature of the inflammatory response and has been associated with the pathogenesis of numerous inflammatory conditions (16, 17, 31, 32). Some previous reports have suggested that neutrophils make a significant contribution to liver injury in experimental models of endotoxin shock and ischemia/reperfusion (12, 14, 15). Rolling of leukocytes is an important prerequisite for adhesion and migration into tissues, and a two-step paradigm for leukocyte recruitment has been established (7, 27, 30). In addition to adhesion molecules, leukocyte proteases have also been implicated in the process of leukocyte migration through the vessel wall, due to their abilities to disrupt endothelial cell junctional complexes (34) and degrade key components of the basement membrane. However, the interactions between leukocyte proteases and liver inflammation remain unknown.

Serine proteinases have diverged evolutionarily from a single gene product, undergoing duplication and mutations that have yielded enzymes with diverse biologic functions such as digestive enzymes of exocrine glands and clotting factors, as well as leukocyte granule-associated proteinases such as neutrophil elastase (NE) (5). NE is a 30-kDa glycoprotein chymo-trypsin-like serine proteinase that has potent catalytic activity, dictated by a catalytic triad of His, Asp, and Ser residues that form a charge-relay system, and broad substrate specificity (18). As a result, excessive release of NE degrades collagens, laminins, and other extracellular matrix components of the endothelium, thereby leading to subsequent organ damage through endothelial cell injury (28, 29).

Fulminant hepatitis is a clinical syndrome consisting of sudden and severe liver dysfunction that results in hepatic encephalopathy and acute liver failure (33, 42). The rate of mortality from fulminant hepatitis patients remains very high, although intensive medical care and implementation of the latest therapies, including liver transplantation, are progressing. Fulminant hepatitis develops in about 1% of patients with acute hepatitis B (26) and involves an excessive immune response from the host defense (21). A fulminant hepatitis model has been created in mice by adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTLs) into hepatitis B virus (HBV) transgenic mice (6, 11). To date, this mouse model has provided us with the opportunity to develop and evaluate drug therapies for curing HBV infection (11, 19, 22, 23).

The present study used these HBV transgenic mice to examine the role of NE in liver injury. We found that NE plays an important role in exacerbating CTL-induced liver injury and that an NE inhibitor (NEI) could attenuate liver damage and inflammatory cell recruitment to the liver. These results suggest that targeted therapy of proteases, including NE, may be useful for severe liver injury and that NEIs have potential as drugs for improving mortality.

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MATERIALS AND METHODS

Mice. The HBV transgenic mouse lineage 107-5 used in this study has been described previously (2) and was generously provided by Francis V. Chisari (The Scripps Research Institute, La Jolla, CA). In all experiments, the mice were matched for age (8 weeks), sex (female), and serum hepatitis B surface antigen (HBsAg) level before experimental manipulation. All animals were housed in pathogen-free rooms under strict barrier conditions and received humane care according to the guidelines of the Animal Care Committee of Gifu University School of Medicine.

CTL clones. HBV transgenic mice were injected with an HBsAg-specific, H-2d-restricted, CD8$^+$ CTL clone (designated 6C2) that recognizes an epitope (IPQSLDSWWTSL) located between residues 28 and 39 of HBsAg (2). At 5 days after the last stimulation, the cells were washed, counted, and injected intravenously into HBV transgenic mice.

Experimental procedure. The NEI {ONO-5046.Na; N-[2-[4-(2,2-dimethyl propionyloxy)phenylsulfonylamino]benzoyl]aminoacetic acid; silevestat, PubChem CID107706} was generously supplied by Ono Pharmaceutical Co., Ltd. (Osaka, Japan) (20). ONO-5046.Na was dissolved in sterile physiological saline and administered intravenously at 10 mg/kg. Mice were treated with ONO-5046.Na at 1 h before the CTL injection and then injected every 6 h until sacrifice. Other mice were injected with an antibody cocktail consisting of a rat anti-mouse CC chemokine ligand 3 (CCL-3) monoclonal antibody (MAb), rat anti-mouse CCL-4 MAb, and rat anti-mouse macrophage inflammatory protein 2 (MIP-2) MAb (all from R&D Systems, Minneapolis, MN) at 200 μg/mouse at 1 h before the CTL injection. The mice were sacrificed at the indicated time points after the CTL injection, and their intrahepatic leukocytes (IHLs) were analyzed.

RNA analyses. Total RNA was isolated from frozen livers (left lobe) and isolated cells and then analyzed for inflammatory cytokine or chemokine mRNAs by an RNase protection assay (RPA) as described previously (22). Specific signals were detected using a BAS-2500 Imaging analyzer (Fuji Film, Nakanuma, Japan) and a FLA-3000 phosphorimager (Fuji Film). The mRNA expression levels were calculated as relative percentage values of L32 housekeeping gene expression.

Biochemical and histological analyses. The extent of hepatocellular injury was monitored both histologically and biochemically at multiple time points after the CTL injection. For biochemical analysis, the serum alanine aminotransferase (sALT) activity was measured with a standard clinical automatic analyzer. For histological analysis, liver tissue was fixed in 10% zinc-buffered formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin.

Immunohistochemistry. Immunohistochemical staining with a rat anti-mouse Ly-6G (Gr-1) immunoglobulin G2b (IgG2b) MAb (clone RB6-8C5; BD PharMingen, San Diego, CA) was performed using an avidin-biotin-peroxidase complex technique. Briefly, tissue slices were incubated with the anti-mouse Gr-1 MAb overnight, followed by treatment with 3% H$_2$O$_2$ in absolute methanol to inhibit endogenous peroxidase activity. The slices were then incubated with a biotinylated secondary antibody for 10 min, washed, and incubated with an avidin-biotin-peroxidase complex reagent. Finally, the slides were treated with 0.06% diaminobenzidine and 0.01% H$_2$O$_2$ in Tris buffer (0.05 M; pH 7.6) for 10 min, counterstained with hematoxylin, dehydrated, and mounted. The numbers of positive cells were counted in at least 50 high-power fields (magnification, ×400), and the results were expressed as percentage ratios of positive cells versus total cells.

TUNEL. Apoptotic cells were estimated by the terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assay assay, which relies on the incorporation of labeled dUTP at DNA break sites. All the reagents, including the buffers, were part of a TUNEL assay kit (Apop Tag; Oncor, Gaithersburg, MD), and the procedure was performed according to the manufacturer's instructions.

Isolation of IHLs and PBCs. To isolate IHLs, single-cell suspensions were prepared from liver perfused with phosphate-buffered saline (PBS) via the inferior vena cava and digested in 10 ml of RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 0.02% (wt/vol) collagenase IV (Sigma-Aldrich, St. Louis, MO) and 0.002% (wt/vol) DNase I (Sigma-Aldrich) for 40 min at 37℃. Cells were overlaid on 24% (wt/vol) metrizamide (Sigma-Aldrich) in PBS. To isolate peripheral blood cells (PBCs), peripheral blood (0.4 ml) was obtained by cardiac puncture under ether anesthesia. After density separation (Lympholyte-Mouse; Cedarlane Westbury, NY), cell counts and immunofluorescence analyses were performed.

Peptide-HLA class 1 tetramer. PE-labeled tetrameric peptide-HLA class 1 complexes, among which the Ld tetramer contained the peptide HBs28-39, were purchased from Proimmune, Ltd. (Oxford, United Kingdom).

Detection of intracellular cytokines. IHLs were harvested from mice at the indicated times after the CTL injection and cultured ex vivo for 4 h in brefeldin A (BD PharMingen) to allow sequestration of the intracellular cytokines in the Golgi apparatus. Cells were then surface stained with anti-CD11b-allophycocya-

nin and anti-Gr-1–fluorescein isothiocyanate (FITC) MAb, washed in fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% fetal calf serum), and fixed in 2% paraformaldehyde for 30 min at room temperature. After fixation, the cells were permeabilized in 25 μl of PBS containing 0.5% saponin for 30 min. Next, an anti-mouse tumor necrosis factor (TNF-α)-phycoerythrin (PE) or isotype control (anti-rat IgG1–PE) MAb was added at a final dilution of 1:100, and the cells were incubated for 30 min at room temperature. Samples were acquired using a FACScalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA), and the data were analyzed using CELLQuest software (BD Immunocytometry Systems).

Isolation of Gr-1$^+$ cells. Gr-1-positive (Gr-1$^+$) cells were purified by positive selection using BD IMag anti-mouse Gr-1 Particles-MSC (BD PharMingen) and a magnetic cell separation system column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's directions.

Data analysis. All data are expressed as means ± standard deviation [SD]. The significance of differences among mean values was evaluated according to the Mann-Whitney U test.

RESULTS

Injection of the NEI reduces liver injury and cell recruitment after the CTL injection. Three groups (three mice/group) of age-, sex-, and serum HBsAg-matched transgenic mice from lineage 107-5 received a single intravenous injection of CTLs (4×10^6 cells/mouse) with or without the NEI (ONO-5046.Na). The mice were sacrificed, and their livers were harvested at 4, 8, 12, and 24 h after the injection. As shown in Fig. 1A, similar levels of sALT activity were detected at 4 h in the presence or absence of the NEI, but the activity was significantly reduced from 12 h to 5 days in the NEI-treated mice compared with the untreated mice ($P < 0.05$). These results suggest that the NEI partially suppressed liver injury in this model.

To further evaluate the effect of the NEI on inflammatory cell recruitment in the same livers, we quantified the absolute numbers of IHLs recovered and determined the phenotypes of the recruited inflammatory cell subsets by FACS analysis. As shown in Fig. 1B, the absolute numbers of IHLs at 4 h after the CTL injection were similar in the presence or absence of the NEI. However, a significant reduction in the total number of IHLs was observed at 8 h after the CTL injection in the NEI-treated mice compared with the untreated mice ($P < 0.05$). In addition, we found that the Gr-1$^+$/CD11b$^+$ neutrophil population was significantly decreased at 8 h, whereas the Gr-1$^-$/CD11b$^+$ macrophage population was significantly decreased at 12 h, indicating that the NEI had the ability to control the recruitment of these cells to the liver. Interestingly, T cells, NK cells and NK T cells had also infiltrated the livers to lesser extents.

In addition, as shown in Fig. 1C, the total number of PBCs was decreased at 8 h but not at 4 h in the NEI-treated mice after the CTL injection. The numbers of neutrophils and NK cells among the PBCs were reduced at 8 h in the NEI-treated mice, indicating that the NEI reduced the recruitment of neutrophils to the liver as well as PBCs.

Next, to determine whether the NEI treatment affected the recruitment of HBV-specific CTLs to the liver, we calculated the absolute numbers of transferred CTLs at 24 h after the injection. NEI administration had no effect on the numbers of transferred CTLs in the liver (see Fig. 3D), since CD8$^+$/HBs28-39 tetramer-positive cells were detected at the same level in both groups. These results demonstrate that NEI treatment did not affect the recruitment of CTLs to the liver.
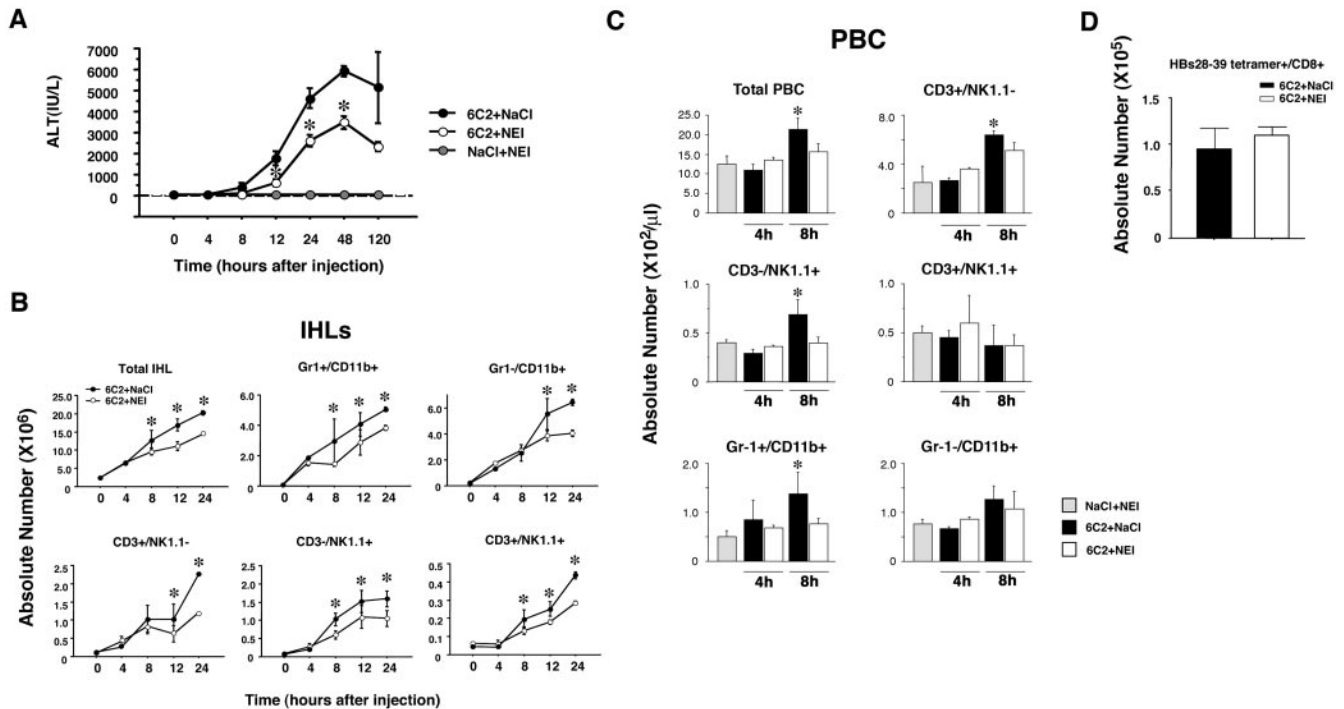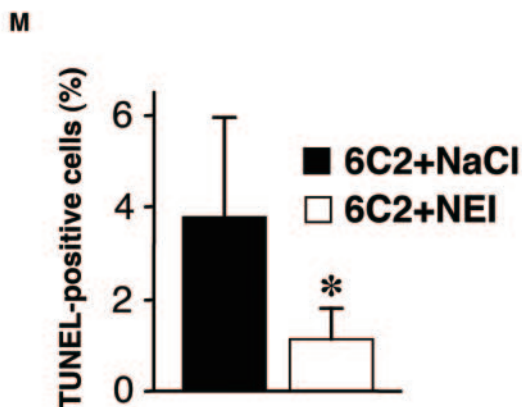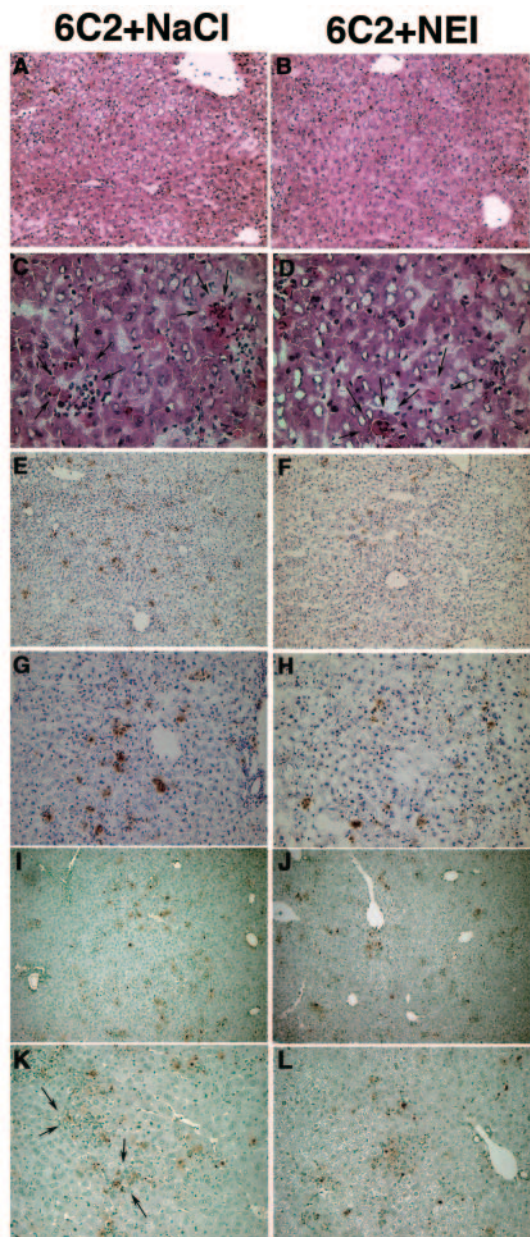
FIG. 1. NEI treatment suppresses CTL-induced liver injury and cell recruitment. (A) Three HBV transgenic mice per group were injected intravenously with 4×10^6 6C2 cells or NaCl. The mean sALT activity measured at the time of autopsy is indicated for each group and expressed as international units per liter (mean ± SD). ∗, $P < 0.05$ compared with NaCl injection. (B and C) IHLs and PBCs were isolated from the animals studied for panel A, and the effects of the NEI treatment were analyzed. The numbers in each subset of cells were calculated by multiplying the total number of IHLs or PBCs by the frequency of each subset in the IHL or PBC population by FACS analysis (mean ± SD). ∗, $P < 0.05$ compared between the presence and absence of NEI treatment. (D) To calculate the number of HBs28-39-specific CTLs in the liver, IHLs were isolated from three mice per group at 24 h after the CTL injection and stained with Ld-restricted tetramer HBs28-39-PE and CD8-FITC. The numbers were calculated as described above and are expressed as means ± SD.

Collectively, the results suggest that the NEI had no effect on the migration of the transferred CTLs to the liver but had large effects on the migration of other antigen-nonspecific inflammatory cells and PBCs to the liver.

Histology. To determine the histological changes in livers with or without NEI treatment after the CTL injection, we stained liver tissues with hematoxylin and eosin or an anti-mouse Gr-1 MAb. A histological analysis revealed widely scattered necroinflammatory foci, containing mostly lymph mononuclear cells and a few apoptotic hepatocytes in the liver parenchyma at 24 h after the CTL injection (Fig. 2A and C). On the other hand, although a few apoptotic hepatocytes were also detected in the parenchyma, the lymph mononuclear cell infiltration was reduced in the NEI-treated mice (Fig. 2B and D). In addition, NEI-treated livers showed a marked decrease in the number of Gr-1-positive cells after the CTL injection (Fig. 2F and H). Furthermore, to determine whether NEI treatment affected the induction of apoptosis in hepatocytes after the CTL injection, we investigated the liver tissue by TUNEL staining. Although the CTL-treated livers showed a marked increase in the number of TUNEL-positive cells (Fig. 2I and K), TUNEL-positive hepatocytes were decreased after NEI treatment (Fig. 2J and L). The numbers of apoptotic cells in the two groups were quantified (Fig. 2M). These results suggest that, without NEI treatment, the liver underwent massive apoptosis of hepatocytes after CTL injection, in associa-

tion with the highly elevated levels of sALT activity. Importantly, we showed that the infiltrating inflammatory cells, as well as CTLs, could induce apoptosis of hepatocytes at this time point (Fig. 2K). In contrast, NEI administration provided partial protection against the hepatocyte apoptosis (Fig. 2L and M).

NEI treatment suppresses cytokine and chemokine expressions in the liver after the CTL injection. To evaluate the effects of the NEI on cytokine and chemokine expressions in the liver after the CTL injection, we performed an RPA using the same liver samples. As shown in Fig. 3A for two representative mice per group, the CTL injection induced sequential activation of various cytokines and chemokines in the liver as described previously (11). NEI treatment had no effect on the cytokine and chemokine expressions in the liver at 4 h after the CTL injection, whereas the mRNA expressions of gamma interferon (IFN-γ), lymphotoxin-β, interleukin 1β (IL-1β), and TNF-α were clearly suppressed at 8 and 12 h compared with the untreated livers. Similarly, NEI treatment also reduced the expressions of inflammatory chemokines at 8 and 12 h after the CTL injection, consistent with the cell recruitment results in Fig. 1B. To quantify the differences in the mRNA expressions at various time points, we evaluated the mRNA expression levels by calculating their relative percentage values to the L32 housekeeping gene expression. As shown in Fig. 3A (right), the cytokine and chemokine

6C2+NaCl   6C2+NEI

M

FIG. 2. Histological analysis. (A to D) Liver sections were obtained from mice sacrificed at 24 h after the injection of CTLs and stained with hematoxylin and eosin. Note that in the CTL-injected mice at 24 h, small inflammatory foci containing mostly lymph mononuclear cells are present and apoptotic hepatocytes are detected in the liver (arrows). In CTL-injected mice with NEI treatment, lymph mononuclear cells and apoptotic hepatocytes are reduced in the parenchyma (arrows). (E to H) To evaluate the neutrophil distribution in the liver after the CTL injection with or without NEI treatment, liver sections were stained with an anti-mouse Gr-1 MAb. NEI-treated livers show a marked decrease in the number of Gr-1^{+} cells after the CTL injection (F and H). (I to L) To evaluate the induction of apoptosis, liver sections were stained by the in situ TUNEL assay. The CTL-treated livers demonstrate a marked increase in the number of TUNEL-positive cells after the injection (I and K). In contrast, TUNEL-positive hepatocytes are decreased after NEI treatment (J and L) compared with NaCl treatment. (M) The relative percentages of apoptotic hepatocytes among the total hepatocytes were determined by TUNEL staining. Data are expressed as means ± SD for three mice. *, $P < 0.01$ compared with NaCl injection. Original magnification, ×100 (A, B, E, F, I, and J) and ×400 (C, D, G, H, K, and L).

mRNA expression levels in the liver were clearly reduced by NEI at 8 and 12 h but not at 4 h after CTL injection.

Cytokine productions by macrophages and neutrophils. To determine whether the NEI affected the cytokine productions by macrophages and neutrophils, we stained intrahepatic macrophages and neutrophils with an anti-TNF-α antibody. As shown in Fig. 3B, we found that the macrophages mainly produced TNF-α, while the neutrophils only produced a small amount of TNF-α after the CTL injection. Consistent with the RPA analysis, we found that NEI treatment decreased TNF-α production by macrophages and neutrophils in the liver at 8 h but not at 4 h after the CTL injection.

Chemokine production derived from Gr-1^{+} cells. Since the absolute number of neutrophils showed a more prominent increase (about 20 fold) than the other populations within 4 h and the NEI treatment could suppress cell recruitment and inflammatory cytokine and chemokine expression in the liver, we hypothesized that neutrophils played a prominent role in this inflammatory event. To investigate the effect of the NEI against neutrophils, we performed an RPA analysis using isolated Gr-1^{+} cells. As shown in Fig. 4, the NEI treatment had no effect on the chemokine expressions in the Gr-1^{+} cells at 4 h after the CTL injection. The mRNA expressions of CCL-3, CCL-4, MIP-2, and CXC chemokine ligand 10 (CXCL10) were reduced at 8 h, but the reductions were small compared with the levels in the whole liver (Fig. 3A). These results suggest that the suppressive effect of the NEI for liver inflammation is due to a decrease in the number of recruited neutrophils rather than reductions in the chemokine productions by the neutrophils themselves.

Roles of CCL-3, CCL-4, and MIP-2 in CTL-induced liver injury. The above results revealed that NEI treatment reduced the neutrophil-derived CCL-3, CCL-4, and MIP-2. To confirm the roles of these chemokines in this liver injury, mice were injected with CTLs (4×10^6) and an antibody cocktail (anti-CCL-3, anti-CCL-4, and anti-MIP-2 MAb) or rat IgG as a control, and then they were sacrificed at 8 h after the injection. As shown in Fig. 5A, administration of the antibody cocktail significantly reduced sALT activity at 8 h after the CTL injection com-
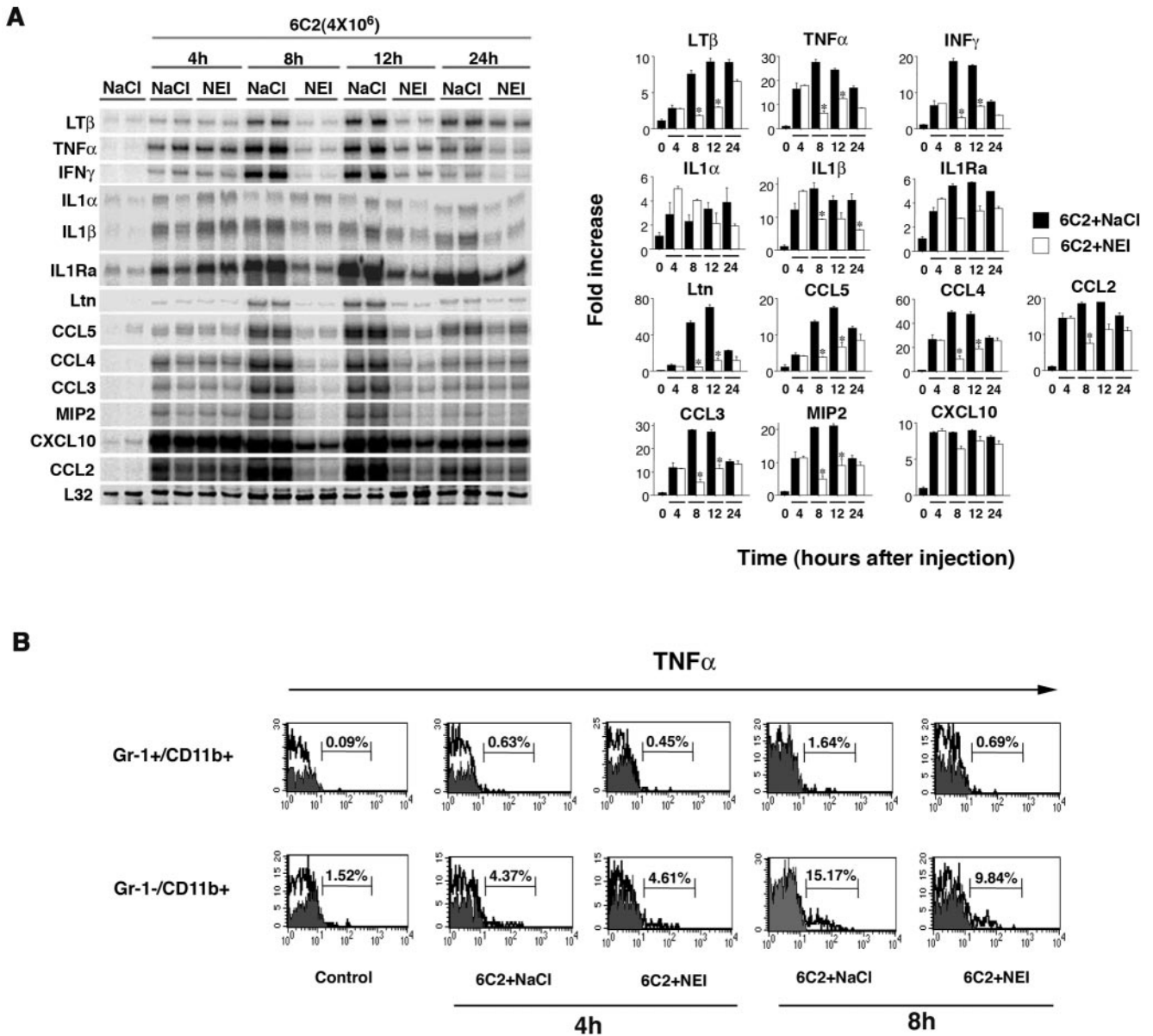
FIG. 3. Inflammatory cytokine and chemokine expressions and intracellular cytokine expressions in macrophages in the liver. (A) Three HBV transgenic mice per group were injected intravenously with 4×10^6 6C2 cells and the NEI or NaCl. (Left) Total hepatic RNA (20 µg) was isolated from the livers at various time points and analyzed for cytokine and chemokine expressions by RPA. (Right) To quantify the differences in the mRNA expressions at various time points, the mRNA expression levels were calculated as the relative percentage values of the L32 housekeeping gene expression. *, $P < 0.05$ compared with NaCl treatment. (B) The intracellular cytokine expressions in IHLs were examined at 4 and 8 h after injection of either the NEI or NaCl into CTL-injected mice. IHLs were isolated from the liver as described in Materials and Methods and stained with anti-CD11b-allophycocyanin or anti-Gr-1–FITC and anti-mouse TNF-α–PE or anti-rat IgG$_1$-PE. The expression levels of TNF-α (open histograms) and the isotype control (filled histograms) are shown at the indicated time points. Representative results of three independent experiments are shown.

pared to the rat IgG treatment ($P < 0.05$). Furthermore, as shown in Fig. 5B, the absolute number of IHLs was significantly decreased in the presence of the antibody cocktail compared to the rat IgG treatment ($P < 0.05$). In addition, neutrophils, macrophages, and other inflammatory cells were also significantly decreased, indicating that these chemokines play pivotal roles in the liver injury and cell recruitment at this time point. Consistent with these results, the antibody cocktail treatment suppressed the expressions of IFN-γ and TNF-α in the liver (Fig. 5C). Importantly,

the antibody cocktail showed the same degree of effect as the NEI treatment, demonstrating that the role of the NEI against liver inflammation was due to the suppression of these chemokines.

DISCUSSION

In this study, we have shown that NEI effectively attenuated the severe liver injury induced by HBV-specific CTLs as well as the hepatic inflammatory responses, including the enhanced
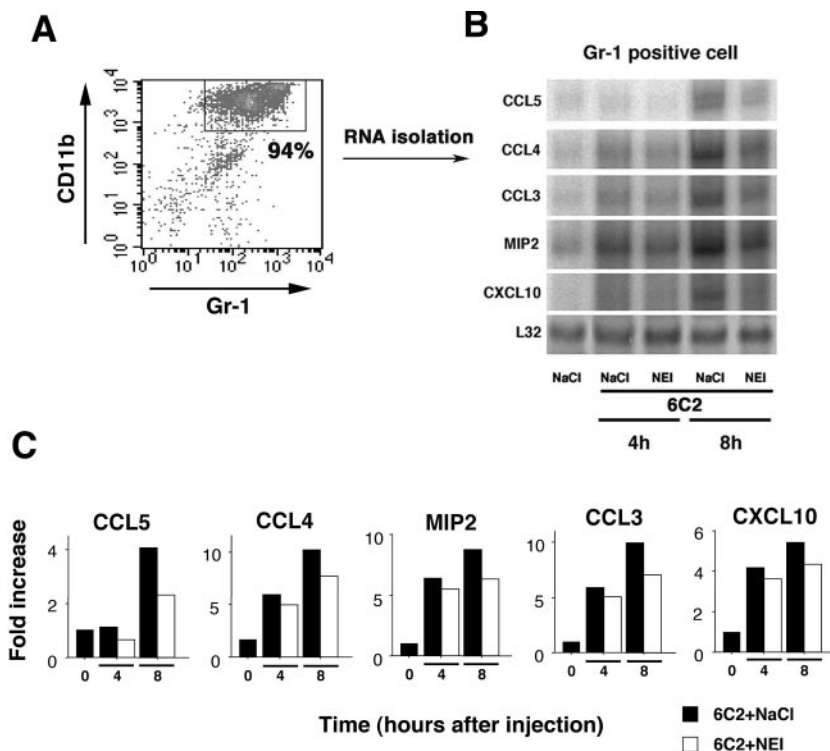
FIG. 4. Chemokine expressions by neutrophils after the CTL injection. (A) Gr-1$^+$ cells from IHLs were purified by positive selection using BD IMag anti-mouse Gr-1 Particles-MSC. (B) After separation of the Gr-1$^+$ cells, total RNA was isolated, and the chemokine mRNA expression levels were analyzed by RPA. (C) To quantify the differences in the mRNA expressions at 4 and 8 h, the mRNA expression levels were calculated as the relative percentage values of the L32 housekeeping gene expression.

recruitment of leukocytes in the liver. These observations imply that NE plays an important role in liver inflammation at an early phase after the CTL injection, although its role in acute viral hepatitis remains unknown. This notion is supported by the recent finding that Gr-1$^+$ cells contribute to liver inflammation, since administration of an anti-Gr-1 antibody suppressed liver injury, although these cells are not involved in the down-regulation of HBV replication in the livers of transgenic mice (40). The current observations suggest that several important interactions occur among serine proteases, NE, and liver injury at local inflammatory sites.

First, it is of note that the protection conferred by the NEI against hepatocellular injury in this model was partial, rather than complete. As previously demonstrated (2), this liver injury consists of three steps after the CTL injection. Briefly, the first step begins within 1 h of the administration and involves antigen recognition by the CTLs and delivery of a signal that results in hepatocyte death by apoptosis. The second step begins at 4 to 12 h after the administration, when the CTLs recruit many host-derived inflammatory cells to the liver, resulting in the formation of necroinflammatory foci and the extension of hepatocellular apoptosis and lysis. Finally, the third step begins at 24 to 72 h after the administration, when the liver displays massive hepatocellular necrosis and the inflammatory cell infiltrate consists principally of host-derived lymph mononuclear cells. As shown in Fig. 1A, the suppressive effect of the NEI against liver damage was detected at 8 h but not at 4 h after the CTL injection, suggesting that (i) NE was

not involved in the first step of the liver injury and (ii) the NEI had no effect on the migration and cytotoxic activity of the CTLs to the hepatocytes. In fact, we confirmed that the levels of CTL recruitment to the liver were similar in the presence or absence of the NEI treatment, since the numbers of HBs28-39 tetramer-positive CD8$^+$ T cells in the IHLs did not differ significantly between the two groups (Fig. 1D). Furthermore, cytotoxicity of the CTLs against HBs-transfected target cells (2) did not differ between the presence or absence of the NEI in vitro (data not shown). Importantly, the effect of the NEI with regard to the liver injury and cell recruitment was observed at 8 h, and this result is consistent with a previous observation that neutrophils begin to participate in hepatocellular injury at the second step (2).

It has been well established that neutrophils can be induced to express a number of mediators that can influence inflammatory and immune responses. These mediators include reactive oxygen species, complement components, and proteases, as well as a variety of cytokines (e.g., TNF-α, IL-1, and IL-12) and chemokines (including CCL3, CCL4, and IL-8) (38). Murine MIP-2 is a chemokine that is considered to be functionally analogous to human IL-8 and rat neutrophil chemoattractant (35) and is primarily induced by TNF-α (41). Here, we have shown that NEI administration suppressed the mRNA expressions of inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) and chemokines (CXCL10, CCL4, CCL2, CCL3, and MIP-2) in the liver after the CTL injection (Fig. 3A). In addition, we found that macrophages, but not neutrophils, mainly produced
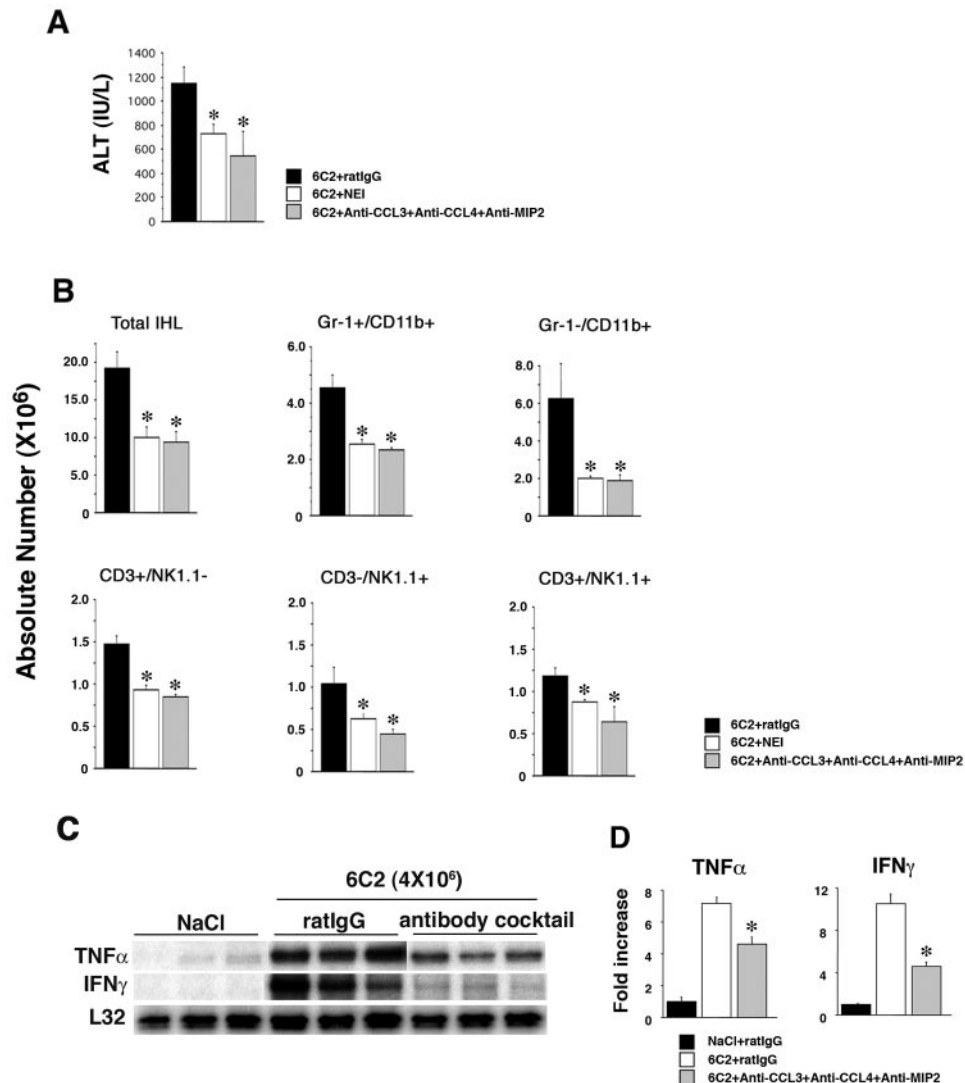
FIG. 5. Roles of CCL3, CCL4, and MIP-2 in the liver injury. Three HBV transgenic mice per group were injected with anti-CCL3, anti-CCL4, and anti-MIP-2 MAb or rat IgG as a control prior to the injection of CTLs. (A) Mean sALT activity was measured at 8 h after the CTL injection. (B) IHLs were isolated, and the effects of the antibodies and the NEI were analyzed. The numbers in each subset of cells in the liver were calculated by multiplying the total number of IHLs by the frequency of each subset in the IHL population by FACS analysis (mean ± SD). *, $P < 0.05$ compared with IgG injection. (C) Total hepatic RNA was analyzed for cytokine mRNA expressions by RPA as described in the legend for Fig. 3A. (D) The mRNA expression levels were calculated as the relative percentage values of L32 housekeeping gene expression. *, $P < 0.05$ compared with 6C2 plus rat IgG treatment.

TNF-α and that NEI administration significantly reduced TNF-α production by macrophages (Fig. 3B). Our hypotheses to explain these results are that (i) the NEI directly suppressed the activation of macrophages and (ii) the NEI induced the inactivation and recruitment of neutrophils; consequently, macrophages became inactivated due to the suppressed inflammatory response. Considering the findings that proinflammatory monocytes express significant amounts of NE (36) and that monocyte migration requires surface-bound NE in lung diseases (39), we suppose that the NEI may have had a direct effect on macrophages. However, we consider that a major reason for the suppression of macrophage activity was the reduced cytokine and chemokine productions by neutrophils and other inflammatory cells (NK cells, T cells, and NK T

cells), since we found that chemokine expression by neutrophils was reduced (Fig. 4). In support of this hypothesis, IFN-γ, a stimulator of macrophages, was reduced at 8 h after the CTL injection with NEI treatment (Fig. 3A).

Furthermore, we have demonstrated that the NEI treatment reduced the mRNA expressions of CCL2, CCL3, and MIP-2 by neutrophils in the liver after CTL administration (Fig. 4). A previous study reported that NE induces IL-8 expression via an IL-1 receptor-associated kinase-signaling pathway (8). Furthermore, these chemokines can be induced by several stimuli in vitro, such as TNF-α (41), IL-1β (41), and IFN-γ (9). Collectively, we consider that the mechanism by which the NEI suppressed the CTL-induced liver damage is as follows. After antigen recognition by the CTLs, which secrete IFN-γ, TNF-α,

and chemokines, these mediators induce inflammatory cell recruitment to the liver and the infiltrating neutrophils secrete NE. This secreted NE induces inflammatory chemokine (MIP-2, CCL3, and CCL4) expression by neutrophils. Due to NEI-mediated blocking of the expressions of MIP-2 and other chemokines by neutrophils, the recruitment of neutrophils, macrophages, and other cells was partially protected against and the inflammatory cascade was suppressed.

The hepatic sinusoid is characterized by a discontinuous endothelium and the absence of a basement membrane (1), meaning that CTLs can easily attack hepatocytes and cause liver inflammation. Knolle et al. previously suggested that damage to liver sinus endothelial cells (SECs) is an early event in T-cell-mediated liver injury, since recruitment of T lymphocytes from the sinusoidal circulation and loss of the SEC barrier function subsequently expose the underlying hepatocytes to further attack by activated T lymphocytes (24, 25). It is of note to mention that NEI administration down-regulates adhesion molecules on hepatic microvessels during endotoxemia (13). Furthermore, it has been demonstrated that NEI treatment decreases the expression of CD11b in circulating leukocytes in rats subjected to hepatic ischemia-reperfusion (4). Based on these observations, we hypothesize that NEI treatment down-regulates adhesion molecules on hepatic microvessels, thereby limiting the interaction between CTLs and the endothelium during liver inflammation, which may in turn protect against the loss of the SEC barrier.

NE was reported to be required for maximal intracellular killing of gram-negative bacteria by neutrophils (3), although it had not generally been considered a significant antimicrobial agent. Currently, we are trying to analyze whether NE has an antiviral effect against HBV replication in mice, despite an observation that NE treatment did not affect HBV replication in an HBV replicate cell line (37). In addition, we need to further examine the lineage of the HBV transgenic mice (107-5) used in this study, since HBsAg-positive hepatocytes of this lineage were previously reported to be sensitive to the hepatocytotoxic effects of IFN-γ (10). These findings raise the question of whether NEI treatment is effective for human HBV-mediated liver disease, and we intend to perform clinical studies to clarify the possibility that NEI administration may be therapeutic for HBV-induced fulminant hepatitis in the future. Finally, it is noteworthy to mention that NEI treatment can suppress CTL-induced liver damage at the second step due to the protection of cytokine and chemokine induction and cell recruitment. It has already been demonstrated that adoptively transferred CTLs can inhibit HBV replication noncytopathically and that this antiviral effect is mediated by IFN-γ and TNF-α, which were secreted by CTLs at the first step (11). Based on these findings, NEI treatment is thought to be an ideal drug therapy, since the effect of CTLs against HBV replication is not affected by NEIs, as demonstrated in this study.

In summary, the current study provides important information regarding the role of NE in the inflammatory liver injury induced by administration of HBV-specific CTLs. Our data indicate that NE contributes to the accumulation of neutrophils at inflamed sites and the secretion of inflammatory chemokines by neutrophils. Thus, NEIs may have potential as drugs for severe viral hepatitis virus-induced liver injury.

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