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Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice

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Abstract: Using hepatitis B virus (HBV) transgenic mice as recipients of virus-specific cytotoxic T lymphocytes (CTLs), we recently showed that polymorphonuclear neutrophils (PMNs) and the matrix-degrading metalloproteinases (MMPs) they produce are necessary for the intrahepatic recruitment of antigen nonspecific mononuclear cells that amplify the liver damage initiated by the CTLs. We now report that the high-mobility group box 1 protein (HMGB1) is also involved in this process. Transfer of CTLs in HBV transgenic mice induces the translocation of HMGB1 from the nucleus to the cytoplasm of hepatocytes surrounding CTL-containing necroinflammatory liver foci, without significant net synthesis of HMGB1. Treatment of CTL-injected HBV transgenic mice with either recombinant Box-A or glycyrrhizin, two functional inhibitors of extracellular HMGB1, significantly decreases the intrahepatic recruitment of PMNs and all other inflammatory cells, in the face of intact homing of virus-specific CTLs into the liver. The inhibition of PMN chemoattraction explains the mode of action of glycyrrhizin, which has long been used in Japan for the treatment of hepatitis, and suggests that new and more potent inhibitors of HMGB1 may be useful for the treatment of patients chronically infected with HBV. *J. Leukoc. Biol.* 81: 000–000; 2007.

Key Words: *immunopathology · tissue damage · liver infection · inflammation*

INTRODUCTION

Nonlytic viruses like hepatitis B virus (HBV) infect the hepatocyte without causing direct cell damage [1]. Thus, both liver disease and viral clearance after this infection is thought to be mediated by the cellular immune response to viral antigens [1]. Accordingly, transgenic mice that replicate HBV in the liver show no signs of liver disease [2] until the adoptive transfer of HBV-specific CTLs, whereupon they develop a necroinflammatory liver disease that is histologically similar to acute viral hepatitis in man [3–7]. The first step in the disease process is antigen recognition by the CTLs, which rapidly induce hepatocellular apoptosis [8] via Fas- and perforin-dependent pathways [9]. CTL killing in vivo is an inefficient process that requires direct physical contact between the CTLs and the infected cells [8]. Thus, it is not surprising that in this model, the initial apoptotic process affects a small number of hepatocytes [8]. This event is followed by the increased activity of serum alanine aminotransferase (sALT) (a hepatocellular enzyme that is released into the circulation by necrotic hepatocytes) and the concomitant recruitment of many host derived, antigen nonspecific, polymorphonuclear, and mononuclear cells (i.e., NK and NKT cells, T and B lymphocytes, monocytes, macrophages, dendritic cells). The intrahepatic recruitment of these antigen nonspecific cells contribute to the formation of necroinflammatory foci that are scattered throughout the liver parenchyma and amplify the tissue damage initiated by the CTLs [5]. The recruitment process is a chemokine-dependent event, since it has been shown that blocking the IFN-γ-inducible chemokines CXCL9 and CXCL10 reduces the migration of antigen nonspecific lymphomononuclear cells and the severity of liver disease after CTL injection [5]. Recent studies also showed that the severity of CTL-induced liver disease in this model is ameliorated by the depletion of Gr-1+ cells (Gr-1 is an antigen highly expressed by polymorphonuclear neutrophils, PMNs), which, secondarily, abolishes the intrahepatic recruitment of all antigen nonspecific Gr-1-mononuclear cells despite the strong induction of chemokine gene expression [7]. Those results suggest that in addition to chemokine expression, additional CTL-induced functions are necessary for mononuclear cell recruitment to occur. These functions likely include the production of matrix metalloproteinases (MMPs) by PMNs (such as MMP-8 and MMP-9), since these enzymes are rapidly activated into the liver after CTL transfer, and their inhibition reduces the intrahepatic recruitment of antigen nonspecific mononuclear cells and much of the attendant liver disease [6]. Finally, it is important to note that most of the antiviral potential of CTLs in this model is mediated by noncytolytic mechanisms that involve the intrahepatic production of IFN-γ by these cells [3] and similar CTL-dependent

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antiviral mechanisms contribute to viral clearance during acute viral hepatitis in chimpanzees [10, 11].

Whether hepatocellular damage itself contributes to sustain a detrimental inflammatory response during viral hepatitis is poorly understood. Recent studies have shown that high-mobility group box 1 (HMGB1) protein, an abundant nuclear protein acting as an architectural chromatin binding factor [12–14], can be passively released by necrotic or damaged cells and behave as a mediator of inflammation [13, 15, 16]. HMGB1 can also be secreted by activated monocytes and macrophages [17, 18] and binds with high affinity to RAGE (the receptor for advanced glycation end products) [19, 20]. In keeping with this, HMGB1 has been involved in the pathogenesis of sepsis induced by LPS [17] and has been shown to chemoattract neutrophils [21], smooth muscle cells [22], and vessel-associated stem cells [23]. HMGB1 has two separate and characteristic DNA binding domains, HMG boxes A and B, each containing ~80 amino acid residues [14, 16]. Interestingly, Box A can exert an antagonistic, anti-inflammatory effect when injected in, mice [14, 24, 25], perhaps by binding unproductively to RAGE and competing with functional HMGB1 [14]. Along these lines, we have recently recognized glycyrrhizin as another HMGB1 inhibitor: glycyrrhizin binds directly to both HMG boxes in HMGB1, inhibiting its chemoattractant functions on fibroblasts and smooth muscle cells (unpublished results). Of note, glycyrrhizin is a natural compound that is commonly used in Japan to treat patients with chronic hepatitis [26–28].

The current study was undertaken to determine whether HMGB1 plays a pathogenetic role in the acute hepatitis that results from transferring HBV-specific CTLs into HBV transgenic mice. First, we monitored the expression and localization of HMGB1 within the inflamed liver parenchyma. Second, we injected HBV transgenic mice with either recombinant Box-A or glycyrrhizin, and we monitored the capacity of these HMGB1 inhibitors to modulate the ability of passively transferred CTLs to home to the liver, induce antiviral activity, activate intrahepatic chemokine expression, recruit inflammatory cells and, ultimately, mediate liver disease. The results indicate that inhibitors that target HMGB1 can reduce the severity of hepatitis.

MATERIALS AND METHODS

Mice

HBV transgenic mouse lineage 1.3.32 used in this study has been described previously [2]. Lineage 1.3.32 (inbred C57BL/6, H-2^b) was bred one generation against B10D2 mice (H-2^d) to produce H-2^{bxd} F_1 hybrids before injection of H-2^d-restricted HBsAg-specific CTL lines. In all experiments, the mice were matched for age (8 weeks), sex (females), and HBeAg levels in their serum before experimental manipulations. All animals were housed in pathogen-free rooms under strict barrier conditions. These studies were approved by the Animal Research Committee of The Scripps Research Institute.

Injection of HBV-specific CTLs

HBsAg-specific CTL lines were derived from spleen cells of nontransgenic B10.D2 male mice immunized as described previously [29]. Briefly, 8-wk-old B10.D2 male mice received a single intramuscular injection of a plasmid that expresses the middle and major ENV proteins (preS2/S) of HBV [29]. After 2 wk, the animals were boosted with a single intravenous injection of vaccinia virus encoding preS2/S [29], and 3 wk later, they were killed and spleen cells were collected. The CTL lines were stimulated weekly as described [29]. After 2 wk of in vitro stimulation, the cells were tested for Ag specificity by flow cytometry using a recombinant soluble dimeric H-2 Ld:Ig Fusion Protein (BD PharMingen, San Diego, CA) that was incubated with the immunodominant peptide epitope (Env 28-39) of HBsAg [30], according to the manufacturer's instructions. Over 95% of cells were $CD8^+$ and specific for Env 28-39. At this time, the cells were washed, counted, suspended in HBSS containing 2% fetal calf serum, and injected intravenously (1×10^7/mouse) into transgenic female mice from lineage 1.3.32. Groups of mice were killed at different time points after CTL injection, and their livers were perfused and harvested for histological and flow cytometry analyses, or they were snap frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses (see below).

Injection of Box-A or glycyrrhizin

Recombinant Box-A was a gift from HMGBiotech (Milan, Italy); it was produced and purified as described previously [31]. Recombinant Box-A (400 μg/mouse) or glycyrrhizin (Minophagen Pharmaceutical, Tokyo, Japan; 10 mg/mouse) were administered intraperitoneally into groups of HBV transgenic mice (4 mice per group) 1 h before the injection of either HBV-specific CTLs or saline (NaCl). Control mice were injected either with diluent obtained from the purification process of an empty plasmid preparation or with NaCl.

Tissue DNA and RNA analyses

Total liver DNA was isolated from frozen livers (left lobe), extracted, and analyzed for HBV DNA by Southern blot analysis as described previously [2]. Total liver RNA isolated from the same lobe was analyzed for HMGB1 and GAPDH RNA by Northern blot analysis and for cytokine and chemokine mRNAs by RNase protection assay (RPA), exactly as described previously [2, 3, 5–7]. The relative abundance of specific DNA and RNA molecules was determined by phosphor imaging analysis, using the Optiquant image analysis software (Packard, Meriden, CT).

Liver disease

The extent of hepatocellular injury was monitored by measuring serum alanine aminotransferase (sALT) activity at different time points. sALT activity was measured in a Paramax chemical analyzer (Baxter Diagnostics, McGaw Park, IL) exactly as described previously [3].

Liver HMGB1 expression and intracellular distribution

The hepatic content of HMGB1 and β-actin was assessed on liver extracts using a polyclonal rabbit anti-HMGB1 as described [23]. The intracellular distribution of HMGB1 was assessed immunohistochemically. Briefly, paraffin-embedded sections in 10 mM citrate-buffer (pH 6.0) were boiled for 5 min in a microwave oven and washed twice in PBS. After blocking the sections with normal goat serum for 30 min at room temperature, a rabbit polyclonal anti-HMGB1 antibody (BD/PharMingen) was applied at 1:100 dilution o/n at 4°C. After washing twice with PBS, a secondary antiserum consisting of biotin conjugated goat anti-rabbit IgG $F(Ab')_2$ (Sigma, St. Louis, MO) was applied at a 1:100 dilution for 60 min. The antibody-coated slides were washed twice with PBS, treated with streptavidin-HRP conjugate (Extravidin, Sigma) at a 1:600 dilution for 60 min, stained with 3-amino-9-ethyl carbazole (AEC, Shandon-Lipshaw, Pittsburgh, PA) and counterstained with Mayer's hematoxylin before mounting.

Detection of HMGB1 in plasma

Blood was obtained by retroorbital bleeding of anesthetized mice, placed on ice into a test tube with citrate anticoagulant (3.8% Na citrate), and immediately centrifuged at 4°C. Plasma was mixed with SDS-PAGE buffer, denatured at 95°C for 10 min, diluted with an equal volume of PBS, and centrifuged through a YM-100 Microcon column (Millipore Corp., Billerica, MA). The flowthrough was centrifuged through a YM-10 Microcon column (Millipore), and the retentate was loaded onto an SDS-PAGE gel and subjected to Western blot analysis with anti-HMGB1 polyclonal rabbit antibodies (BD/PharMingen). Reference curves were built by spiking recombinant HMGB1 (HMGBiotech)

into normal mouse blood plasma and subjecting the sample to the same processing described above.

Isolation and analysis of intrahepatic leukocytes

Mouse livers were weighed at the time of autopsy. Single cell suspensions were prepared from two liver lobes of known weight and analysis of the intrahepatic leukocyte (IHL) population was performed by flow cytometry, exactly as described [5]. The cells were surface stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC)-labeled anti-CD4, anti-CD8, anti-CD3, anti-TCR, anti-NK1.1, anti-Gr-1, anti-CD11b, and anti-CD11c Abs (PharMingen, San Diego, CA) for the detection of Gr-1$^+$/CD11b$^+$/CD11c$^-$ cells (mostly PMNs), CD4$^+$/TCR$^+$ (mostly T helper cells), CD8$^+$/TCR$^+$ cells (mostly CTLs), NK1.1$^+$/CD3$^-$ cells (NK cells), NK1.1$^+$/CD3$^+$ cells (NKT cells) and Gr-1$^-$/CD11b$^+$/CD11c$^-$ (mostly macrophages). To quantify the number of transferred CTLs, a recombinant soluble dimeric H-2L^d:Ig Fusion Protein (BD/PharMingen) complexed with immunodominant peptides derived from HBsAg (Env28) was used as described [4]. Samples were acquired on a FACSCalibur flow cytometer, and the data were analyzed using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistical methods

Data are expressed as means ± SD. Statistical significance was assessed using the two-tailed Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Intrahepatic HMGB1 mRNA and protein content are stable after transfer of HBV-specific CTLs into HBV-transgenic mice

To define the kinetics of HMGB1 expression in the liver of CTL-injected HBV transgenic mice, total hepatic RNA and proteins isolated at different time points after CTL transfer from the same mice described in Kakimi et al. [5] were analyzed for HMGB1 RNA and protein content. As shown in **Fig. 1, A and B** and when compared with NaCl-injected controls, the content of all 3 HMGB1 mRNA species, as well as the content of HMGB1 protein did not significantly change, even at those time points (24 and 48 h after CTL transfer) in which the liver disease is relatively severe and reaches its peak [5].

HMGB1 translocates from the nucleus to the cytoplasm of hepatocytes surrounding CTL-containing necroinflammatory foci

As shown in Fig. 1C, most hepatocellular nuclei (but not cytoplasm), as well as cells resembling the resident macrophages of the liver (i.e., Kupffer cells, arrowhead) stained positive for HMGB1 in mice that were injected with saline (NaCl). Interestingly, hepatocytes surrounding CTL-containing necroinflammatory foci (Fig. 1D, arrow) became negative for HMGB1 in the nucleus and positive in the cytoplasm (Fig. 1D), suggesting that CTL-induced liver inflammation resulted in the intracellular translocation of this nuclear protein. Because the total hepatic content of HMGB1 RNAs and protein in these livers were similar to those observed in NaCl-injected mice (Fig. 1, A and B), HMGB1 translocation occurred independently of its transcription and/or translation. In the plasma of mice that were injected with either NaCl or CTLs and bled at

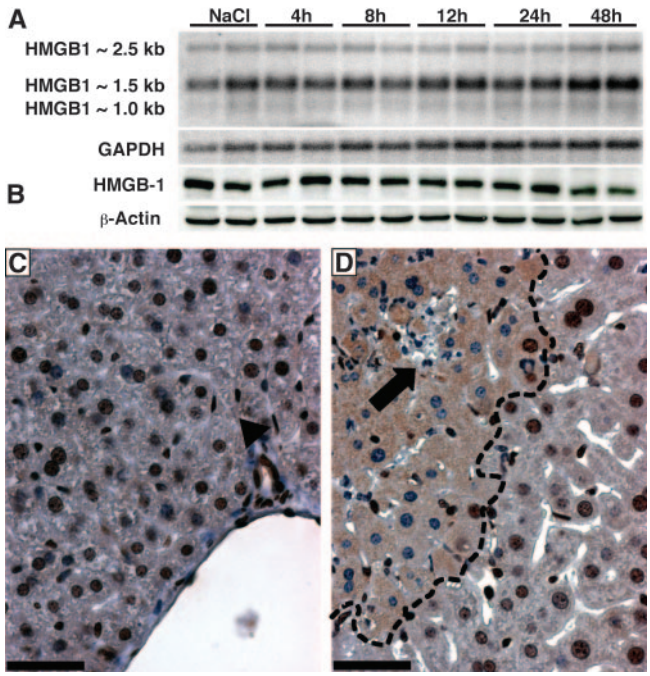

Fig. 1. HMGB1 translocates from the nucleus to the cytoplasm of hepatocytes surrounding CTL-induced necroinflammatory foci, independently of changes in transcription and/or its overall intracellular abundance. (A and B) Five groups of age- and HBeAg-matched HBV transgenic from lineage 1.3.32 mice were injected with HBsAg-specific CTLs and killed 4, 8, 12, 24, and 48 h later. The intrahepatic content of the 3 HMGB1 mRNA species and protein was measured by Northern and Western blot analylsis, on total RNA and on protein extracts, respectively. The results were compared with an additional group of transgenic mice that was injected with saline (NaCl). Immunohistochemical staining of mouse HMGB1 in the livers of HBV transgenic mice that were killed 2 days after injection of saline (C) or HBV-specific CTLs (D). (C) HMGB1 stains the nuclei (but not cytoplasms) of hepatocytes, as well as of cells resembling the resident macrophages of the liver (arrowheads). (D) Hepatocytes surrounding necroinflammatory foci (arrow) are negative for HMGB1 in the nucleus and positive in the cytoplasm and delimited by the broken line. Scale bar = 40 μm.

24 h and 48 h after injection, we did not detect extracellular HMGB1 at higher concentration than the detection limit (5 ng/ml) of our assay (see Material and Methods) (not shown). Conversely, we detected plasma HMGB1 concentrations as high as 200 ng/ml in mice that had undergone cecal ligation and puncture (not shown).

Box-A administration ameliorates the severity of liver disease without affecting either the intrahepatic recruitment or the antiviral functions of HBV-specific CTLs

To define the role of HMGB1 in the liver disease induced by HBV-specific CTLs, we monitored the ability of Box-A to modulate this process. Groups of age- and serum HBeAg-matched transgenic mice from lineage 1.3.32 (4 mice per group) were treated with either Box-A or control diluent before the transfer of HBV-specific CTLs. Two additional groups of control mice were injected either with control diluent or Box-A alone but were not injected with CTLs. Two days later mice were bled, killed, perfused, and the livers were harvested.

Liver disease was monitored biochemically by quantifying sALT activity. As shown in **Fig. 2A** and when compared with

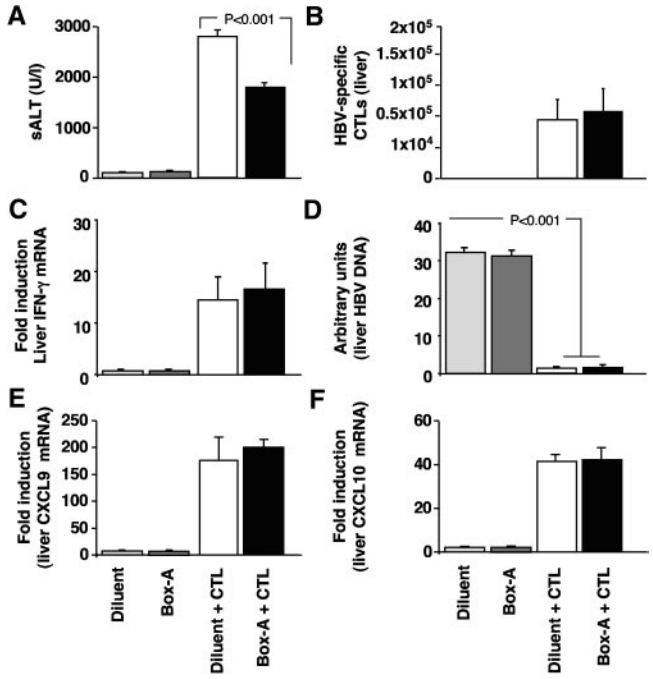

Fig. 2. Box-A administration ameliorates the severity of liver disease without affecting either the intrahepatic recruitment or the antiviral functions of HBV-specific CTLs. Groups of age- and serum- HBeAg-matched transgenic mice from lineage 1.3.32 (4 mice per group) were injected with either diluent (open bars) or Box-A (solid bars) and 1×10^7 HBV-specific CTLs. Mice were bled and killed, and livers were harvested at day 2 after CTL transfer. Two additional groups of control mice that received either diluent (light gray bars) or Box-A alone (dark gray bars) were also analyzed at the same time points. (A) The mean sALT activity (±SD) measured at the time of autopsy is indicated for each group and is expressed in units/liter. (B) Absolute number (mean ± SD) of HBV-specific CTLs recovered from the entire liver 2 days after CTL transfer. (C) Total hepatic RNA derived from the same mice described above was analyzed by RNA protection assay (RPA) for the expression of IFN-γ. The housekeeping mRNA encoding the ribosomal protein L32 was used to normalize the amount loaded in each lane, and quantitative phosphor imaging analysis was performed. (D) Total hepatic DNA from the same mice was analyzed for HBV replication by Southern blot analysis and quantitative phosphor imaging analysis. The relative content of HBV replicative intermediates is expressed as arbitrary units calculated after normalization to the band corresponding to the integrated transgene. (E and F) RPA analysis for the intrahepatic expression of CXCL9 and CXCL10. L32 was used to normalize the amount loaded in each lane; NaCl-injected controls were set as 1.

mice injected with NaCl (light gray bars) or Box-A alone (dark gray bars), sALT activity was markedly elevated in CTL-injected mice that received control diluent (white bars), while it was comparatively reduced in CTL-injected mice treated with Box-A ($P<0.001$). As shown in Fig. 2B, the Box-A-dependent reduction in liver disease severity was not associated with a decrease in the intrahepatic recruitment of transferred CTLs, since similar numbers of Env28-specific CTLs were recovered from the liver of CTL-injected mice that received either control diluent or Box-A. Accordingly, the relative levels of intrahepatic IFN-α mRNA (a marker of antigen recognition by CTLs [3, 7, 32]) in the two groups of mice (Fig. 2C) closely reflected the number of CTLs recovered from their livers (Fig. 2B). Because HBV replication is highly sensitive to the antiviral activity of IFN-α produced by the transferred CTLs [3, 7, 32], it is not surprising that, when compared with mice that received either NaCl or Box-A alone, both groups of CTL-injected animals showed a marked inhibition of HBV replication in their livers (Fig. 2D). Because IFN-α in our model induces both CXCL9 and CXCL10, two chemokines involved in the CTL-dependent recruitment of host-derived mononuclear cells into the liver [5], it is also not surprising that their messages were induced at similar high levels in the liver (Fig. 2, E and F).

These results indicate that the reduced liver disease severity observed in mice treated with Box-A was due to neither impaired CTL homing nor impaired CTL activation. The results also indicate that Box-A does not have a direct antiviral activity against HBV and that its administration does not affect the potential of HBV-specific CTLs to inhibit viral replication.

Box-A administration significantly reduces the intrahepatic recruitment of PMNs and other antigen nonspecific inflammatory cells

To determine the characteristics of the intrahepatic inflammatory infiltrate in the same livers described above, the phenotype of the recruited inflammatory cell subsets was determined by FACS analysis. When compared with NaCl-injected controls, the total number of Gr-1^+/CD11b^+ cells (mostly PMNs) and IHLs increased 18- and 5.2-fold, respectively, in the liver of CTL-treated mice that received control diluent (**Fig. 3, A and B**). Importantly, Box-A treatment before CTL transfer significantly decreased the total intrahepatic number of PMNs, as well as IHLs (Fig. 3, A and B), which included mononuclear cells such as $CD4^+$/TCR^+ cells (T helper cells, Fig. 3C), $CD8^+$/TCR^+ cells (CTLs, Fig. 3D), NK1.1^+/$CD3^-$ (NK cells, Fig. 3E), NK1.1^+/$CD3^+$ cells (NKT cells, Fig. 3F), $CD11b^+$/$CD11c^-$ (mostly macrophages, Fig. 3G), and $CD11b^-$/$CD11c^+$ (mostly lymphoid dendritic cells, Fig. 3H) (in all comparisons, $P < 0.05$).

These results indicate that Box-A treatment reduces the intrahepatic recruitment of antigen nonspecific polymorphonuclear and mononuclear inflammatory cell subsets. The reduced recruitment of mononuclear cells occurred regardless of the strong induction in CXCL9 and CXCL10 expression (Fig. 2, E and F), which is consistent with the notion that in our system, these chemokines are not sufficient to recruit mononuclear cells when PMNs are either absent or malfunctioning [6, 7].

Glycyrrhizin administration ameliorates the severity of CTL-induced liver disease

To independently confirm a role for HMGB1 in contributing to CTL-induced liver disease, groups of age- and serum HBeAg-matched transgenic mice from lineage 1.3.32 (4 mice per group) were treated with either glycyrrhizin or NaCl before CTL transfer. Two additional groups of control mice were injected either with NaCl or glycyrrhizin but no CTLs. Mice were bled, killed, and perfused and livers were harvested on day 2.

As shown in **Fig. 4A**, when compared with mice that received NaCl alone, mice that received glycyrrhizin before CTL transfer showed a 60% reduction of sALT activity ($P < 0.01$). As occurred after treatment with Box-A, the glycyrrhizin -dependent reduction in liver disease severity was not associated with a decrease in the intrahepatic recruitment of transferred

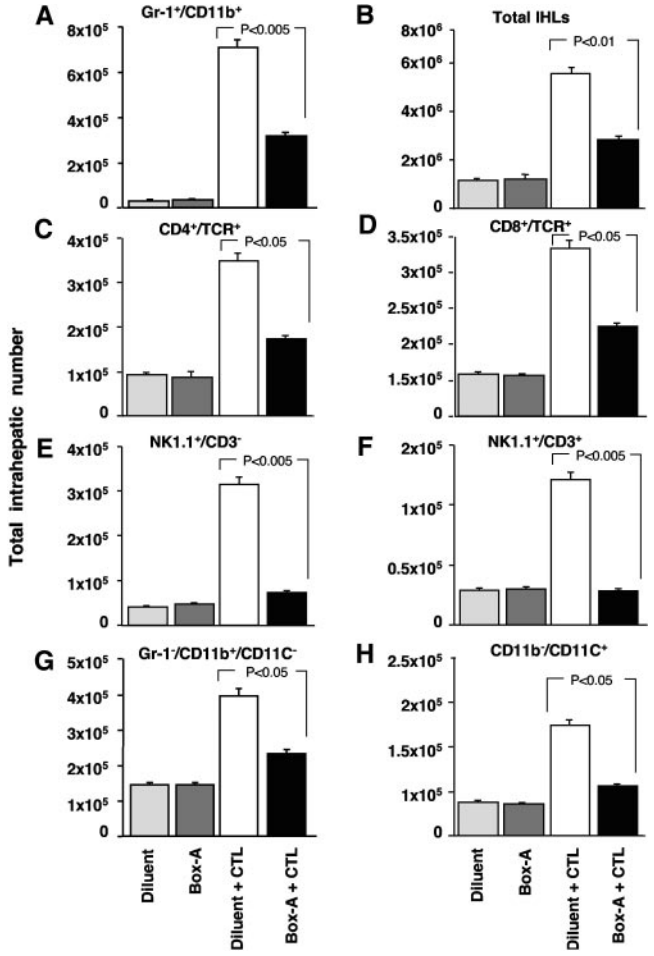

Fig. 3. Box-A administration significantly reduces the intrahepatic recruitment of PMNs and other antigen nonspecific inflammatory cells. Intrahepatic leukocytes (IHLs) analysis in the same animals described in the legend to Fig. 2. Livers were weighed at the time of autopsy. IHLs were isolated from two liver lobes of a known weight and analyzed by flow cytometry. The indicated numbers of Gr-1$^+$/CD11b$^+$ cells (PMNs), total IHLs, and different cell subsets represent the numbers detected in the whole liver.

CTLs, as similar numbers of Env28-specific CTLs were recovered from the liver of CTL-injected mice that received either saline or glycyrrhizin (Fig. 4B). Consistently, the intrahepatic IFN-γ, CXCL9, and CXCL10 mRNA, in the two groups of CTL-injected mice was similarly induced (Fig. 4, C, E, and F), and HBV replication was comparably inhibited (Fig. 4D). Glycyrrhizin treatment, however, significantly decreased the total intrahepatic number of PMNs, as well as IHLs (**Fig. 5, A and B**), which included mononuclear cells such as CD4$^+$/TCR$^+$, CD8$^+$/TCR$^+$, NK1.1$^+$/CD3$^-$, NK1.1$^+$/CD3$^+$, CD11b$^+$/CD11c$^-$, and CD11b$^-$/CD11c$^+$ cells (Fig. 5, C–H).

Treatment with Box-A or glycyrrhizin prior to CTL transfer does not inhibit HMGB1 translocation from the nucleus to the cytoplasm of hepatocytes surrounding necroinflammatory foci

To determine whether the Box-A- or glycyrrhizin-induced decrease of IHL recruitment and liver disease severity was associated with reduced HMGB1 translocation from the nucleus to the cytoplasm of hepatocytes, immunohistochemical staining for HMGB1 was performed on liver sections obtained from mice described in Figs. 2 and 4 that were killed at day 2 after CTL transfer. As shown in **Fig. 6**, neither Box-A nor glycyrrhizin administration inhibited the CTL-induced capacity of HMGB1 to translocate from the nucleus to the cytoplasm of hepatocytes surrounding CTL-containing necroinflammatory foci (Fig. 6, B and C, arrows).

DISCUSSION

In this study, we showed that following the passive transfer of HBV-specific CTLs into transgenic mice that replicate HBV at high levels in their liver [2], HMGB1 translocated from the nucleus to the cytoplasm of hepatocytes surrounding CTL-containing necroinflammatory foci. HMGB1 translocation occurred independently of changes in HMGB1 transcription and/or its overall intracellular abundance. Importantly, treatment of these mice with two different inhibitors (Box-A and glycyrrhizin) of HMGB1 [14, 24, 25; also unpublished data] diminished recruitment of antigen-nonspecific inflammatory cells into the liver and diminished the severity of the liver disease. This occurred in the face of intact homing and anti-

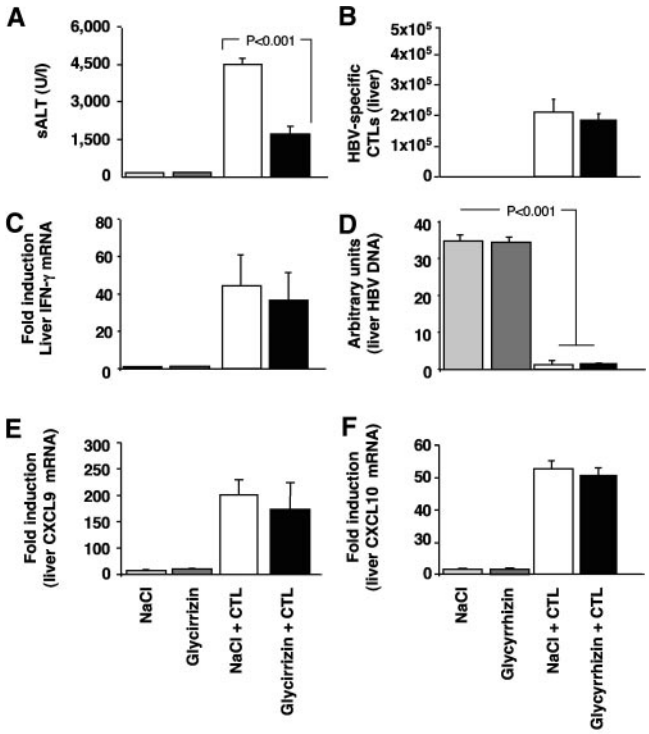

Fig. 4. Glycyrrhizin administration ameliorates the severity of CTL-induced liver disease. Groups of age- and serum- HBeAg-matched transgenic mice from lineage 1.3.32 (4 mice per group) were injected with either glycyrrhizin (solid bars) or NaCl (open bars) and 1×10^7 HBsAg-specific CTLs. Mice were bled and killed, and livers were harvested at day 2 after CTL transfer. Two additional groups of control mice that received either NaCl (light gray bars) or glycyrrhizin alone (dark gray bars) were also analyzed at the same time points. (A) The mean sALT activity (±SD), measured at the time of autopsy, is indicated for each group and is expressed in units/liter. (B) Absolute number (means ± SD) of HBV-specific CTLs recovered from the entire liver 2 days after CTL transfer. The intrahepatic content of IFN-γ (C), HBV-DNA (D), CXCL9 (E), and CXCL10 (F) were measured as described in the legends to Fig. 2.

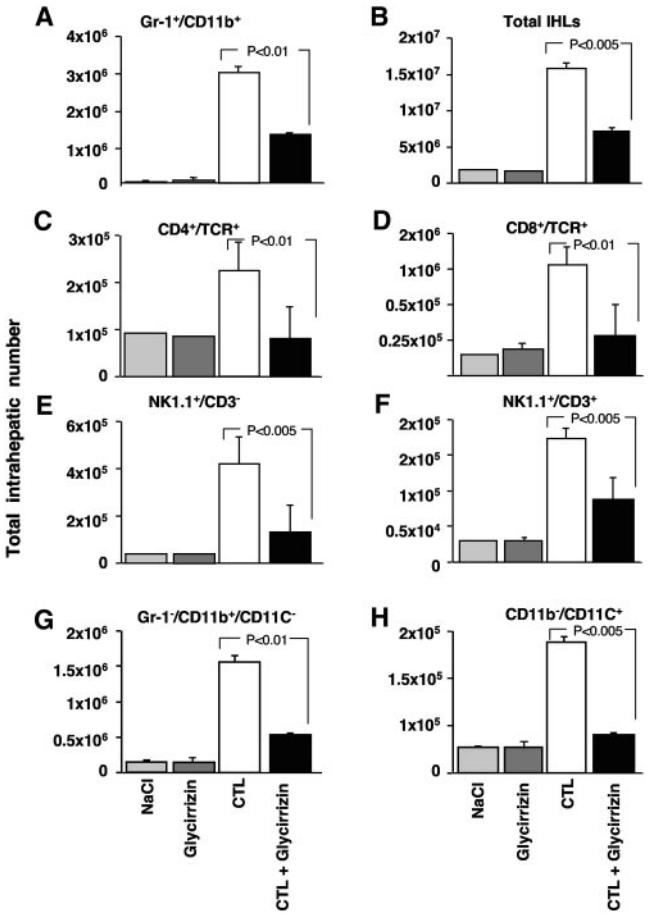

Fig. 5. Glycyrrhizin administration significantly reduces the intrahepatic recruitment of PMNs and other antigen nonspecific inflammatory cells. IHL analysis was performed in the same animals as described in Fig. 4. IHLs were isolated from two liver lobes of a known weight and analyzed by flow cytometry. The indicated numbers of Gr-1$^+$/CD11b$^+$ cells (A), total IHLs (B), and different cell subsets represent the numbers contained in the whole liver (C–H).

viral functions of HBV-specific CTLs, as well as strong induction of CXCL9 and CXCL10.

The results are consistent with the notion that HMGB1 represents an important chemoattractant for PMNs, as previously shown in the case of acute lung inflammation induced by intratracheal delivery of HMGB1 [21]. The results are also consistent with the observations that in our system depleting PMNs or inhibiting the function of PMN-dependent MMPs prior to CTL transfer renders antigen nonspecific mononuclear cells unresponsive to their chemoattractants (such as CXCL9 and CXCL10) and unable to amplify the liver damage initiated by CTLs [5–7].

Moreover, it is of note that treatment with either Box-A or glycyrrhizin did not affect the CTL-induced translocation of HMGB1 from the nucleus to the cytoplasm of hepatocytes surrounding necroinflammatory foci. This suggests that the abundant cytoplasmic localization of HMGB1 is not sufficient per se to promote PMN recruitment, but it probably reflects a phenotype associated with increased release/secretion of this protein into the extracelllular space by hepatocytes. If this is correct, it is likely that HMGB1 exerts its chemotactic function toward PMNs extracellularly, and treatment with Box-A or glycyrrhizin may hinder this process. Box-A is believed to compete with HMGB1 for the occupation of membrane receptors [24], whereas glycyrrhizin binds directly to both HMG boxes in HMGB1 (unpublished data). The known ability of HMGB1 to bind endothelial surface proteins and components of the extracellular matrix [33, 34], along with the fact that circulating HMGB1 is rapidly cleaved proteolytically [35], may explain why we did not detect HMGB1 in the plasma of our mice.

In conclusion, we found that HMGB1 is not involved in the migratory and antiviral activity of virus-specific CTLs, but it is

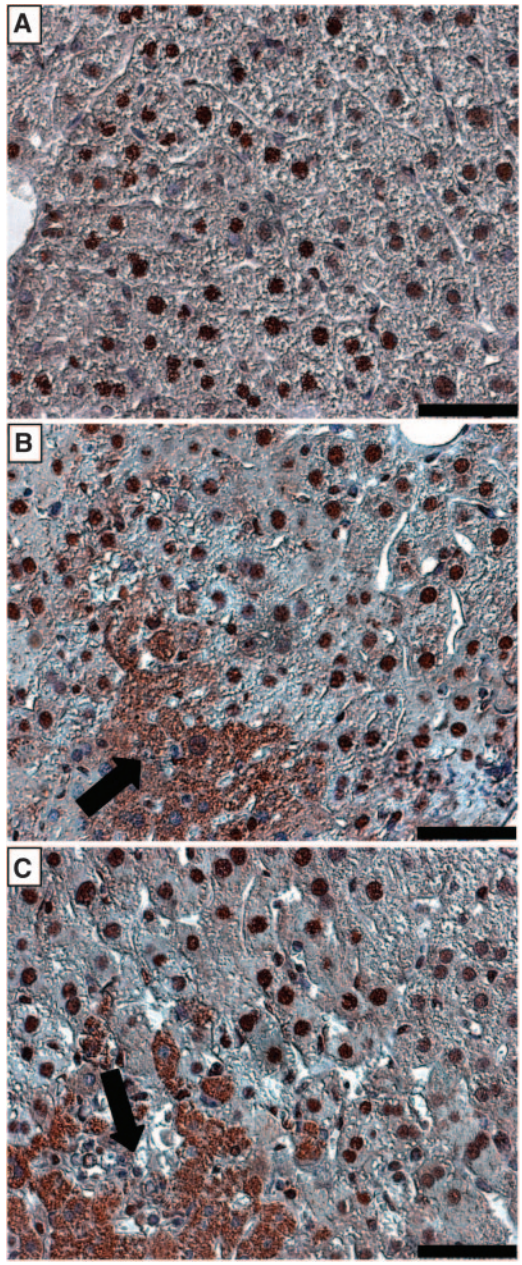

Fig. 6. Treatment with Box-A or glycyrrhizin before CTL transfer does not inhibit HMGB1 translocation from the nucleus to the cytoplasm of hepatocytes surrounding necroinflammatory foci. Immunohistochemical staining of mouse HMGB1 was performed on liver sections obtained from mice described in Figs. 2 and 4 that were killed at day 2 after injection of saline (A) or HBV-specific CTLs and either Box-A (B) or glycyrrhizin (C). Scale bar = 40 μm.

necessary for the intrahepatic recruitment of antigen nonspecific cells that amplify the CTL-dependent liver damage. A similar role of HMGB1 could be played in the pathogenesis of viral hepatitis in man, in which the intrahepatic number of HBV-specific T cells is also outnumbered by recruited nonvirus-specific T cells and other inflammatory cells [36]. Because the treatment of mice with Box-A or glycyrrhizin is associated with maintenance of CTL-dependent antiviral effects but diminished tissue damage, the design of new and more potent inhibitors of HMGB1 may be useful for the treatment of chronic HBV infection. Along these lines, it is noteworthy that our results shed new light onto the mechanism whereby glycyrrhizin, a natural compound long used in Japan to treat patients with chronic hepatitis [26–28], may carry out its beneficial effect.

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