Activated Intrahepatic Antigen-Presenting Cells Inhibit Hepatitis B Virus Replication in the Liver of Transgenic Mice

Kiminori Kimura, Kazuhiro Kakimi, Stefan Wieland, Luca G. Guidotti and Francis V. Chisari

*J Immunol* 2002; 169:5188-5195; doi: 10.4049/jimmunol.169.9.5188

http://www.jimmunol.org/content/169/9/5188

**References** This article cites 49 articles, 32 of which you can access for free at: [http://www.jimmunol.org/content/169/9/5188.full#ref-list-1](http://www.jimmunol.org/content/169/9/5188.full#ref-list-1)

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions** Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Activated Intrahepatic Antigen-Presenting Cells Inhibit Hepatitis B Virus Replication in the Liver of Transgenic Mice

Kiminori Kimura, Kazuhiro Kakimi, Stefan Wieland, Luca G. Guidotti, and Francis V. Chisari

In this study we evaluated the ability of activated intrahepatic APCs to inhibit hepatitis B virus (HBV) replication in transgenic mice. Intrahepatic APCs were activated by administration of an anti-CD40 agonistic mAb (aCD40). We showed that a single i.v. injection of aCD40 was sufficient to inhibit HBV replication noncytopathically by a process associated with the recruitment of dendritic cells, macrophages, T cells, and NK cells into the liver and the induction of inflammatory cytokines. The antiviral effect depended on the production of IL-12 and TNF-α by activated APCs; however, it was mediated primarily by IFN-γ produced by NK cells, and possibly T cells, that were activated by IL-12. Collectively, these results suggest that activated APCs can directly produce antiviral cytokines (IL-12, TNF-α) and trigger the production of other cytokines (i.e., IFN-γ) by other cells (e.g., NK cells and T cells) that do not express CD40. These results provide insight into a hitherto unsuspected antiviral function of intrahepatic APCs, and they suggest that therapeutic activation of APCs may represent a new strategy for the treatment of chronic HBV infection. The Journal of Immunology, 2002, 169: 5188–5195.

In recent years a growing body of evidence has shown that the antiviral potential of the effector cells of the innate and adaptive immune system (NK, NKT, and T cells) includes their ability to produce antiviral cytokines at the site of the infection. For example, we have previously shown that hepatitis B virus (HBV)-specific CD8+ (1) or CD4+ (2) T cells can inhibit HBV replication noncytopathically in the livers of transgenic mice, and that this antiviral effect is mediated by the intrahepatic induction of IFN-γ and TNF-α. Using this same transgenic mouse model, it was shown that IL-12 inhibits HBV replication via an IFN-γ-dependent pathway (3), and similar cytokine-dependent mechanisms are responsible for the antiviral activity observed after activation of intrahepatic NK cells and NKT cells (4), or after infection with unrelated hepatotropic viruses (5, 6), in which case the antiviral effect is also triggered by IFN-α/β, the antiviral activity of which was confirmed by administration of the IFN-α/β inducer, polyinosinic-polycytidylic acid complex (7, 8). Finally, we have shown that HBV replication is also controlled noncytopathically in acutely HBV-infected chimpanzees, and this is temporally associated with the induction of IFN-γ in the liver of these animals (9). Like the case of HBV, other viruses, including adenovirus (10, 11), mouse hepatitis virus (12, 13), coxsackievirus (14, 15), and measles virus (13), are susceptible to the antiviral activity of cytokines produced by CTLs or other immune cells, and it has long been known that IFN-α/β inhibits noncytopathically the replication of these and other viruses, including retroviruses, influenza viruses, vesicular stomatitis virus, HSV, vaccinia virus, and reovirus (16, 17).

While it is widely accepted that cytokines produced by NK, NKT, and T cells play an indispensable role in the control of viral infections, very little is known about the ability of APC-derived cytokines to contribute to this process. This is germane because APCs can become activated and produce antiviral cytokines after appropriate stimulation (18–20), suggesting that activated APCs might also participate in the control of viral infections by similar mechanisms.

Very large numbers of APCs are present in the liver (21, 22), and they can be activated by the administration of an agonistic anti-CD40 mAb (aCD40) (23, 24). CD40 is a member of the TNF receptor family, found on professional APCs (B cells, dendritic cells (DCs), and activated macrophages) and other cell types (fibroblasts, epithelial cells, and endothelial cells) (25–27). CD40 activation is a critical step in the final maturation of DCs into fully competent APCs and is required for the generation of CTLs (28). CD40 ligation on monocytes and DCs enhances their survival and their ability to secrete TNF-α, IL-1-α, IL-1-β, IL-6, IL-8, IL-10, IL-12, IL-18, and IFN-γ and to produce NO (18, 20, 26, 29, 30).

In the current study we took advantage of our HBV transgenic mouse model to determine whether systemic administration of aCD40 could activate intrahepatic APCs to produce antiviral cytokines and inhibit HBV replication.

Materials and Methods

Mice

The HBV transgenic mouse linesages 1.3.32 and 1.3.46 used in this study have been previously described (31). Both lineages of mice replicate HBV at high levels in the liver and kidney without any evidence of cytopathology. Lineage 1.3.32 was expanded by repetitive backcrossing (>20 generations) against the C57BL/6 parental strain and then bred one generation against BALB/c mice to produce the F1 hybrids used in this study. Transgenic mice from lineages 1.3.36 were backcrossed against mice genetically deficient for the IFN-α/βR−/− (32) exactly as previously described (7).
The Journal of Immunology

May 15, 1995

5189

The knockout mice were provided by M. Aguet (IFN-αR−/−; Genentech, South San Francisco, CA). In all experiments the mice were matched for age (8 wk), sex (male), and serum hepatitis B e Ag (HBcAg) levels before experimental manipulations. All animals were housed in pathogen-free rooms under strict barrier conditions.

Anti-CD40 and anti-cytokine Abs

The FGK45 hybridoma producing rat IgG2a mAb against mouse CD40 (aCD40) was provided by Dr. A. Rolink (Basel Institute for Immunology, Basel, Switzerland) (33). aCD40 was purified from FGK45 culture supernatants by affinity chromatography using a protein G column and was tested for LPS levels with the Limulus amebocyte lysate test (0.16 endotoxin units/ml) using a commercially available kit (Sigma-Aldrich, St. Louis, MO). The mice were injected i.v. either with 100 μg of aCD40 or 100 μg of purified rat IgG2a (BD PharMingen, San Diego, CA) control Ab.

The mice were sacrificed at different time points after injection. Their livers were perfused via the inferior vena cava with 10 ml of PBS (Invitrogen, Carlsbad, CA), and they were harvested for histological, histochemical, and FACS analyses or were snap-frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses (see below).

Twenty-four hours after aCD40 injection, mice were injected i.p. (250 μg/mouse) with 1 hamster mAb H22 specific for murine IFN-γ (34), 2 hamster mAb TN3 19.12 specific for murine TNF-α (35) (both of which were provided by Dr. R. Schreiber (Washington University, St. Louis, MO), 3) control hamster IgG (Jackson ImmunoResearch, West Grove, PA), 4) goat polyclonal Ab specific for murine IL-12 (36) (provided by Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ), or 5) control goat IgG (Sigma-Aldrich).

In vivo depletion of macrophages, DCs, CD4+ T cells, CD8+ T cells, and NK cells

To deplete macrophages and DCs in the liver, mice were injected i.v. (100 μl/mouse) with liposome-encapsulated dichloromethylene dipophosphate (37, 38) (L-MDP; provided by Dr. M. Naito, Niigata University School of Medicine, Niigata, Japan) 24 h before aCD40 injection. Liposome-encapsulated PBS was used as a negative control (37, 38). To deplete CD4+ and CD8+ T cells, mice were injected i.v. (2 mg/mouse) with rat anti-mouse CD4 (YTS191.1) and rat anti-mouse CD8 (YTS169.4) mAb (39) (provided by Dr. R. Zinkernagel, University of Zurich, Zurich, Switzerland). YTS191.1 or YTS169.4 was injected twice, 3 and 1 days before aCD40 injection. Purified rat IgG2a (BD PharMingen) was used as a negative control.

To deplete NK cells in the liver, mice were injected i.v. with either rabbit anti-mouse asialoGM1 Ab (50 μg/mouse; Wako Pure Chemical, Osaka, Japan) or anti-mouse NK1.1 Ab (200 μg/mouse; BD PharMingen) 24 h before aCD40 injection. Purified rabbit IgG (Sigma-Aldrich) was used as a negative control.

Tissue DNA and RNA analyses

Frozen liver was mechanically pulverized under liquid nitrogen, and total genomic DNA and RNA were isolated for Southern and Northern blot analyses for HBV DNA and 2,5′-oligoadenylyl synthetase (2,5′-OAS), respectively, exactly as previously described (31). Quantification of various cytokine and chemokine RNAs was performed by RNase protection assay (RPA) exactly as previously described (1, 40, 41).

Biochemical and histological analyses

The extent of hepatocellular injury was monitored histologically and biochemically by measuring serum alanine aminotransferase (ALT) activity at multiple time points after infection. Serum ALT activity was measured in a Paramax chemical analyzer (Baxter Diagnostics, McGaw Park, IL) exactly as previously described. For histological analysis, liver tissue was fixed in 10% zinc-buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with H&E.

Intrahepatic leukocyte preparation

Single-cell suspensions were prepared from liver that was perfused with PBS via the inferior vena cava and pressed through a 70-μm cell strainer (BD Biosciences, Mountain View, CA). Total liver cells were digested with 10 ml of RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 0.02% (w/v) collagenase IV (Sigma-Aldrich) and 0.002% (w/v) DNase I (Sigma-Aldrich) for 40 min at 37°C. Cells were washed with RPMI 1640 and then underlaid with 24% (w/v) metrizamide (Sigma-Aldrich) in PBS. After centrifugation for 20 min at 1500 × g, intrahepatic leukocytes (IHLs) were isolated at the interface.

Flow cytometry

Single-cell suspensions of IHLs were washed in PBS (containing 1% BSA and 0.02% sodium azide), and incubated for 20 min on ice with culture supernatant from the hybridoma cell line 2.4G2 (American Type Culture Collection, Manassas, VA) to block FcR. The cells were surface-stained with fluorochrome-conjugated mAb for 20 min on ice. The following Abs were used: anti-CD3, anti-CD4, anti-CD8, anti-NK1.1, anti-DX-5, anti-CD11b, anti-CD11c, anti-B220, anti-Gr-1, anti-CD86, and anti-CD86 (all from BD PharMingen). Samples were acquired on a FACSCalibur flow cytometer, and the data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Detection of intracellular cytokines

IHLs were harvested from HBV transgenic mice at the indicated times after injection of aCD40, and they were cultured ex vivo for 4 h in brefeldin A (BD PharMingen) to allow the intracellular cytokines to be sequestered in the Golgi apparatus. Cells were then surface-stained with anti-CD3-FITC, anti-NK1.1-PE, anti-CD11b-FITC, and anti-CD11c-PE mAb; washed in FACS buffer (PBS with 1% FCS); and fixed in 2% paraformaldehyde for 30 min at room temperature. After fixation, cells were permeabilized for 30 min in 25 μl of PBS plus 0.5% saponin. Mouse anti-mouse IFN-γ and TNF-α, all lyophilocycin or isotype control-lyophilocycin mAb were added at a final dilution of 1/100, and cells were incubated for 30 min at room temperature. Cells were washed and resuspended in 1 ml of FACS buffer for analysis on a FACScan flow cytometer as described above.

Results

Injection of aCD40 inhibits HBV replication

To determine whether aCD40 injection can inhibit HBV replication in the liver of transgenic mice and to relate this effect to the kinetics of cytokine and chemokine expression and liver disease, eight groups (three mice per group) of age-, sex-, and serum HBcAg-matched transgenic mice from lineage 1.3.32 received a single i.v. injection of aCD40 (100 μg/mouse). Mice were bled and sacrificed, and livers were harvested at 4, 8, and 12 h and 1, 3, 5, 7, and 14 days after injection. The results were compared with those observed in livers from age-, sex-, and serum HBcAg-matched transgenic littermates (three mice) injected with control rat IgG2a (rat IgG) and sacrificed 1 day after injection.

As shown in the Southern blot at the top of Fig. 1, for two representative mice per group, HBV replicative intermediates disappeared from the liver between 12 and 24 h after injection of aCD40, remained virtually undetectable for at least 7 days, and returned to baseline by day 14 (Fig. 1). As shown by RPA in the lower portion of Fig. 1, aCD40 injection induced sequential activation of various cytokines and chemokines in an orderly, hierarchical, and biphasic manner. The first wave of genes to be induced included TNF-α, IL-12p40, and the chemokine CXCL10 (chemokine responsive to γ-2/IFN-γ-inducible protein), whose mRNA levels were induced in the liver as early as 4 h after aCD40 injection, reached peak levels by 12 h, and slowly subsided thereafter. The second peak was observed on day 5, which rapidly decreased to the baseline level on day 7 (Fig. 1). The second wave of responsive genes included 2′,5′-OAS (a well-known IFN-α/β-inducible gene) and IFN-γ, whose mRNA were first detectable by 8 h, peaked by 12 h, and followed the same biphasic time course as that described above (Fig. 1). Interestingly, the IL-12 p40 and p35 mRNAs were not coordinated, since p40 gene expression was maximal by 12 h, and p35 gene expression was maximal on day 5 (Fig. 1). CXCL9 (monokine induced by IFN-γ), whose mRNA was also first induced at 8 h, peaked by 12 h, declined, and reached a transient second peak on day 5. The chemokine receptor for monocytes/macrophages CCL5 (RANTES) was induced by 8–12 h and remained constantly elevated for 5–7 days (Fig. 1). Collectively, these results indicate that a single injection of aCD40 inhibits HBV replication in the liver of transgenic mice.
and 5 days (C and D) after injection of αCD40-injected were stained with H&E. Note that at 12 and 24 h, small inflammatory foci containing mostly lymphomononuclear cells and few apoptotic hepatocytes (asterisks) were observed in the liver (see insets for higher magnification). On day 5 larger foci containing much more lymphomononuclear cells and few polymorphonuclear cells were detected in both the portal tracts and the parenchyma. Original magnification: A–C, ×200; D and insets in the upper panels, ×400.

Injection of αCD40 recruits inflammatory cells into the liver

To determine the characteristics of the intrahepatic inflammatory cell infiltrate in the same livers, we quantified the absolute number of IHLs recovered, and we determined the phenotype of the recruited inflammatory cell subsets by FACS analysis.

The IHLs detected in control transgenic mice (three mice) that had been injected with rat IgG and sacrificed 24 h later served as a baseline for this experiment. Those results are represented as the day 0 point (Fig. 3).

The absolute number of IHLs began to increase 4 h after αCD40 injection and gradually rose thereafter until it had increased 6-fold above baseline by day 5, after which it rapidly decreased (Fig. 3, top panel), corresponding well with the histological analysis shown in Fig. 2. Based on these results, the absolute number of each IHL subset at each time point was calculated by multiplying the total number of IHLs by the frequency of the subset determined by FACS analysis (Fig. 3). The CD11b+/CD11c− macrophage population was the first to increase, rising as early as 4 h after αCD40 injection and reaching a peak at ~20-fold induction by day 1, where it remained until day 5. The next populations to change included the NKT cells (CD3+/NK1.1+), which decreased by 12 h, and the NK cells (CD3+/NK1.1−), which increased by 12 h, with the latter remaining elevated for 5 days, and the former remaining depressed for the duration of the experiment. CD3+/ NK1.1− cells (T cells) also increased by 12 h after injection, reached peak levels on day 5, and returned to preinjection levels by day 7 (Fig. 3). While CD11b+ /CD11c+ DCs (mostly lymphoid
dendritic cells) showed a modest increase by days 3–5, CD11b⁺/CD11c⁻ DCs (mostly myeloid dendritic cells) were more strongly induced by 24 h, peaked by day 5, and returned to baseline levels by day 7 (Fig. 3). The proportion of B cells (B220⁺/CD3⁻/CD11c⁻) in the liver decreased slightly by 12 h and returned to the baseline by day 7 (data not shown). These results demonstrated that macrophages expand first in the liver following αCD40 injection, followed by increase in the NK and T cell populations, the disappearance of NKT cells, and, finally, a slower, but progressive, accumulation of both myeloid and lymphoid DCs.

The antiviral activity of αCD40 requires APCs and is independent of T cells

To evaluate the role of APCs in the αCD40-induced inhibition of HBV replication, macrophages and DCs were depleted from the liver of age- (8–10 wk), sex- (male), and serum HBeAg-matched transgenic mice by L-MDP, which induces apoptosis of macrophages and DCs in vivo and in vitro (37, 43). One day later the mice were injected i.v. with αCD40 or rat IgG, and they were sacrificed 24 h later. The results were compared with those observed in the liver of transgenic littermates injected with liposome-encapsulated PBS (L-PBS) prior to the injection of either αCD40 or rat IgG.

As shown in Fig. 4, αCD40 inhibited HBV replication in the liver of L-PBS-treated mice, but not in L-MDP-treated mice, indicating that intrahepatic APCs are required for the αCD40-induced antiviral effect. Compared with that in L-PBS-treated mice, the induction of TNF-α, IFN-α/β, and, to a lesser extent, IFN-γ was diminished in L-MDP-treated mice (Fig. 4), again suggesting that these cytokines are likely to play a role in the αCD40-dependent inhibition of HBV replication. Furthermore, no elevation of sALT was observed in the L-MDP-treated mice (Fig. 4), indicating that APCs are necessary for the initial cytopathic effect of αCD40. The induction of CCL5 and CXCL10 was also diminished in the APC-depleted animals (Fig. 4), suggesting that less recruitment of inflammatory cells should occur in these livers. Consistent with this, αCD40-induced expansion of all the intrahepatic inflammatory cell subsets was blocked in L-MDP-treated mice (Fig. 4, bottom). The lack of CCL5 expression in these mice also confirms the efficacy of the APC depletion, since it has been previously shown that the hepatic expression of this chemokine is dramatically reduced in L-MDP-treated mice following LPS injection (44). Surprisingly, the expression of CXCL9 was not particularly affected by APC depletion (Fig. 4), thus suggesting that this chemokine...
may not contribute substantially to the recruitment of inflammatory cells in this system.

Next, to determine whether the antiviral effect of αCD40 requires the activation of conventional T cells, age-(8–10 wk), sex-(male), and serum HBeAg-matched transgenic mice (three mice per group) from lineage 1.3.32 were treated with rat anti-mouse CD4 and CD8 mAb, injected i.v. with αCD40 or control rat IgG, and sacrificed 24 h later. Importantly, the total number of CD4 and CD8 cells that were recoverable from the liver of the mAb-treated animals decreased 44- and 61-fold, respectively, compared with CD8 cells that were recoverable from the liver of the αCD40 or control rat IgG, and sacrificed 24 h later. Nonetheless, αCD40 treatment inhibited HBV replication in the liver on day 1 regardless of the presence or the absence of T cells (Fig. 5), suggesting that intrahepatic CD4+ and CD8+ T cell populations did not contribute to the antiviral activity of αCD40 at this time point. Importantly, TNF-α, IFN-γ, and IFN-α/β were induced to a similar degree in the liver of both groups of animals (Fig. 5). This suggests that either the remaining undepleted intrahepatic T cells, representing 1.5–2.5% of the original T cell population, are responsible for the production of these antiviral cytokines on day 1 after αCD40 injection or that other cells, e.g., NK cells, were responsible for this effect. The same is true for the mild elevation of sALT activity, since similar values were detected in the blood of both groups of animals (Fig. 5). Consistent with the idea that αCD40 directly activates APCs, and the activation products of the activated APCs trigger the recruitment and activation of other cells in the liver, T cell depletion did not affect the recruitment of NK cells, macrophages, and DCs into the liver by αCD40 (Fig. 5, bottom). This indicates that T cells are not necessary for the recruitment of other inflammatory cells after αCD40 injection. This interpretation is supported by the fact that the chemokines CCL5, CXCL10, and CXCL9 were similarly induced in animals in which T cells were either depleted or not (Fig. 5).

Unfortunately, we could not determine the role of NK cells in the antiviral effect of αCD40, because treatment of the mice with anti-mouse asialoGM1 or anti-mouse NK1.1 Ab that reduced the intrahepatic NK cell population ~10-fold also triggered the production of IFN-γ and TNF-α, which inhibited HBV replication (data not shown). Intrahepatic APCs are activated and produce high levels of TNF-α after αCD40 injection

To determine which cells produced IFN-γ and TNF-α after αCD40 injection, we stained the intrahepatic macrophage (CD11b+/CD11c−), DC (CD11b+/CD11c−), CD11b+CD11c−, NK cell (CD3−/NK1.1+), and T cell (CD3+/NK1.1−) subsets with Abs to IFN-γ and TNF-α 12 h after αCD40 injection. As shown in Fig. 6, macrophage and DC subsets expressed high levels of TNF-α (Fig. 6), but little or no IFN-γ (data not shown). In

**FIGURE 5.** The antiviral activity of αCD40 is independent of T cells. Age-, sex-, and serum HBeAg-matched lineage 1.3.32 HBV transgenic mice were injected with anti-mouse CD4 (αCD4), anti-mouse CD8 (αCD8), or control (rat IgG) Abs before αCD40 injection and were sacrificed 1 day later. Total hepatic DNA and RNA were analyzed as described in Fig. 1. The mean sALT activity, measured at the time of autopsy, is indicated for each group and is expressed in units per liter. IHLs from these animals were isolated, and the effect of T cells in αCD4 and αCD8 or control Ab-treated mice was analyzed with αCD40 or rat IgG. The number of each cell subset in the liver was calculated by multiplying the total number of IHLs by the frequency of the subset in the IHL populations by FACS analysis.

**FIGURE 6.** Intrahepatic APCs are activated and produce high levels of TNF-α after αCD40 injection. Intracellular cytokine expression of IHLs isolated from livers injected with either αCD40 or control Abs. IHLs were stained with anti-CD3-FITC, anti-NK1.1-PE, anti-CD11b-FITC, anti-CD11c-PE, and anti-mouse IFN-γ- or TNF-α. The cytokine levels of the indicated gated populations are plotted in histogram format.
contrast, IFN-γ was found to be produced at high levels by NK cells and at lower levels by T cells (Fig. 6) at this time point, even though these cells do not express CD40 (25). Collectively, these results suggest that cross-linking of CD40 directly triggers the production of TNF-α by APCs and, indirectly, IFN-γ by NK cells and T cells. Thus, activated APCs may directly and/or indirectly contribute to the inhibition of HBV replication via the production of these antiviral cytokines.

The antiviral effect of αCD40 is mediated by IFN-γ, TNF-α, and IL-12

To determine whether the αCD40-dependent inhibition of HBV replication is mediated by IFN-γ or TNF-α, we monitored the ability of hamster mAb specific for either cytokine to modulate this process. Groups (three mice per group) of age-, sex-, and serum HBsAg-matched transgenic mice (lineage 1.3.32) were injected with either αIFN-γ or αTNF-α mAb or irrelevant hamster IgG prior to the injection of αCD40 and were sacrificed 24 h later. The results were compared with those observed in livers from age-, sex-, and serum HBsAg-matched transgenic littermates (three mice) injected with an irrelevant hamster IgG.

As shown in Fig. 7, administration of either αIFN-γ or αTNF-α mAb completely blocked the antiviral effect of αCD40 injection, suggesting that both these cytokines must be induced for αCD40 to inhibit HBV replication. Neutralization of these cytokines reduced their own intrahepatic induction as well as the induction of 2′,5′-OAS mRNA, which is primarily induced by IFN-α/β (Fig. 7). In addition, both treatments blocked the cytopathic effect of αCD40 (see the sALT activity values; Fig. 7), suggesting that they function cooperatively in this regard as well. Consistent with the observation, both Abs effectively diminished the induction of CCL5, CXCL10, and CXCL9 (Fig. 7) as well as the recruitment of NK cells, macrophages, and DCs into the liver (data not shown).

Since IL-12 is known to be produced by activated APCs (45), and it was rapidly induced in the liver after αCD40 injection (Fig. 1), experiments were performed to determine the relative contribution of this cytokine to the antiviral effect of αCD40. Groups (three mice per group) of age-, sex-, and serum HBsAg-matched transgenic mice (lineage 1.3.32) were injected with eIL-12 polyclonal Ab (36) and sacrificed 24 h later. The results were compared with those observed in livers from age-, sex-, and serum HBsAg-matched transgenic littermates (three mice) injected with irrelevant hamster IgG.

As shown in Fig. 8, the administration of αIL-12 polyclonal Ab completely blocked the antiviral effect of αCD40, indicating that the IL-12 pathway plays an important role in this model. The αCD40-induced liver injury along with the induction of 2′,5′-OAS, IFN-γ, TNF-α, CCL5, CXCL10, and CXCL9 was diminished in these animals, indicating that these effects require IL-12 as well. In keeping with the diminished cytokine and chemokine expression, the administration of eIL-12 also blocked cell recruitment of inflammatory cells into the liver (data not shown).

To determine whether IFN-α/β plays a role in this system, either αCD40 or rat IgG was injected into groups (three mice per group) of age- (8–10 wk), sex- (male), and serum HBsAg-matched animals from lineage 1.3.46 that were heterozygous (IFN-α/β+/-) or homozygous (IFN-α/β-/-) for the IFN-α/β null mutation (7). Age-, sex-, and serum HBsAg-matched mice injected with control IgG and serum HBeAg-matched animals from lineage 1.3.46 that were heterozygous (IFN-α/β+/-) or homozygous (IFN-α/β-/-) for the IFN-α/β null mutation (7). The animals were sacrificed 24 h later.

HBV replication was inhibited in IFN-α/β+/- and IFN-α/β-/- mice that were injected with αCD40. Indeed, the content of HBV replicative forms was similarly reduced in both groups of mice (as measured by phosphor imaging analysis using the transgene band for normalization; not shown) compared with the respective rat IgG-injected controls (note that IFN-α/β+/- mice replicate HBV at higher levels than IFN-α/β+/- mice) (7). As expected, no αCD40-dependent induction of 2′,5′-OAS was observed in the IFN-α/β-/- animals, although IFN-γ and TNF-α were induced in these mice at control levels (data not shown). These results indicate that IFN-α/β-mediated signaling is not necessary for αCD40 to exert its antiviral effect. Given that blocking IFN-γ, TNF-α, and IL-12 strongly suppressed 2′,5′-OAS induction (Figs. 7 and 8), the results do not rule out the possibility that IFN-α/β may contribute to the antiviral activity of αCD40. Indeed, there is very strong evidence from other experiments that HBV replication is strongly inhibited by IFN-α/β (7, 8).

Rather, the current results suggest that the induction of IFN-α/β by αCD40 is dependent on IFN-γ, TNF-α, and IL-12, and these
cytokines can inhibit HBV replication in the absence of IFN-α/β-mediated signaling. The αCD40-induced liver injury was not inhibited in IFN-α/βR−/− mice, and the transcripts of IFN-γ, TNF-α, CCL5, CXCL10, and CXCL9 were well induced (data not shown), indicating that these effects do not require IFN-α/β-mediated signaling. Consistent with these results, there was no difference between IFN-α/βR−/− and IFN-α/βR−/− mice with regard to the αCD40-induced cell recruitment of NK cells, macrophages, and DCs in liver (data not shown).

Discussion

In this study we showed that a single injection of αCD40 into transgenic mice that replicate HBV at high levels in their hepatocytes rapidly and profoundly inhibited viral replication. This effect was associated with the recruitment of APCs, T cells, and NK cells into the liver, the intrahepatic induction of various cytokines and chemokines, and mild inflammatory liver disease. Follow-up experiments were performed to determine the relative contributions of these events to the antiviral activity of αCD40.

The results indicate that the antiviral effect of αCD40 is dependent on APCs, since depletion of these cells abrogated the antiviral activity of αCD40. Given that αCD40 has the potential to activate other CD40-bearing cells (fibroblasts, epithelial cells, and endothelial cells) (25–27), the results suggest that those cells did not contribute to the antiviral effect of αCD40. Depletion of APCs also abrogated the in vivo recruitment of inflammatory cells, most of the cytokine and chemokine induction, and the liver disease, suggesting that all these events are downstream of APC activation. It remains to be determined whether the αCD40-induced antiviral activity depends on activation of the resident intrahepatic APC population or recruitment and activation of additional APCs into the liver. It is noteworthy that macrophages were recruited in high numbers into the liver as early as 4 h following αCD40 injection, while intrahepatic DCs increased with delayed kinetics. This suggests that if there is an antiviral role for the recruited APCs, it would most likely involve the macrophages. Future experiments will attempt to monitor the antiviral effect of αCD40 in animals in which the recruitment of APCs into the liver will be inhibited by the administration of anti-CXCL9 and anti-CXCL10 Abs, a treatment that we have previously shown to inhibit the intrahepatic recruitment of APCs after the transfer of virus-specific CTLs (42) or the activation of intrahepatic NKT cells in these mice (46). Resident and/or recruited activated macrophages are also likely to be involved in the antiviral activity against HBV that is observed in these animals during the blood stage of malaria infection (47). Under these conditions, the resident macrophages of the liver are initially activated by phagocytosis of infected erythrocytes, and this is associated with the intrahepatic recruitment of macrophages, NK cells, NKT cells, and T cells, all of which are likely to produce the inflammatory cytokines that eliminate HBV from the hepatocyte (47).

Importantly, APCs isolated from the liver of αCD40-injected animals were found to be activated and produce high levels of TNF-α, but little or no IFN-γ, suggesting that it was produced by other cells. The importance of TNF-α, IFN-γ, and IL-12 in the antiviral activity of αCD40 was underscored by experiments showing that the inhibition of HBV replication was blocked by Ab neutralization of these cytokines. These results are reminiscent of several studies that previously defined the antiviral activity of TNF-α, IFN-γ, and IL-12 in this model (1, 3, 7). Furthermore, the results obtained in IFN-α/βR−/− mice do not exclude the possibility that IFN-α/β may contribute to the antiviral activity of αCD40, although its induction was found to be dependent on IFN-γ, TNF-α, and IL-12 (Figs. 7 and 8). This idea is supported by previous studies showing that HBV replication can be abolished in transgenic mice in response to IFN-α/β that is produced in the liver during lymphocytic choriomeningitis virus (5, 7) and adenovirus (6, 7) infections or after polyinosinic-polycytidylic acid complex injection (7, 8) as long as they express the IFN-α/β receptor.

While APCs may represent the major source of TNF-α in this system, IFN-γ was found to be mostly produced by NK cells and to a much lesser extent by T cells 12 h after αCD40 injection. Lack of an important role for T cells either in the early cytokine-dependent antiviral activity of αCD40 or in the accompanying inflammatory processes was indicated by the experiment in which T cells were depleted before αCD40 injection. In this setting HBV replication was abolished, and both IFN-γ and several IFN-γ-dependent chemokines (CXCL9 and CXCL10) were fully induced, again suggesting that non-T cells, especially NK cells, were the most abundant source of this cytokine. It is unfortunate that we could not directly prove that NK cells contribute to the antiviral effect of αCD40, but our attempts to deplete NK cells with anti-mouse asialo-GM1 or anti-mouse NK1.1 resulted in the inhibition of HBV replication, probably because the Ab treatment not only killed NK cells, but also activated them to produce IFN-γ in the liver. Future experiments using HBV transgenic mice genetically deficient for NK cells will be required to prove that NK cells play a central role in this system. Finally, the high levels of IL-12 mRNA detected in the liver of αCD40-injected animals together with the results of the IL-12 neutralization experiments demonstrated a critical role for this cytokine as a mediator of the αCD40-induced antiviral effect. Future studies will be necessary to identify the cell(s) that produces this cytokine in our system, but we expect that these studies will confirm published reports that macrophages and DCs produce IL-12 following activation by αCD40 (20, 48). Collectively, these results suggest that activated APCs can directly produce antiviral cytokines (IL-12, TNF-α) and trigger the production of other cytokines (i.e., IFN-γ) by other cells (e.g., NK cells and T cells) that do not express CD40 (25).

Injection of αCD40 was accompanied by a very mild liver disease, particularly at the time points that just preceded (12 h) or were concomitant with (24 h) the disappearance of HBV replicative intermediates from the liver and the first peak of intrahepatic cytokine and chemokine expression. These results indicate that the antiviral activity of αCD40 relies primarily on noncytotoxic mechanisms. A more severe liver disease was observed in these animals on day 5, when the influx of T cells, NK cells, and myeloid DCs into the liver was maximal and when we observed a rebound in cytokine and chemokine expression. It must be noted, however, that even at this time point the overall severity of liver disease as well as the elevation of sALT activity were quite modest compared with those in previously published models of liver cell injury (1, 49). We should note that we do not understand the reason for the transient and delayed second peak of liver inflammation, but the inflammatory rebound may have contributed to the prolonged duration of the antiviral effect of a single injection of αCD40.

In conclusion, these results demonstrate that activation of intrahepatic APCs, especially macrophages, can initiate a cascade of events that begins with the production of IL-12 and TNF-α, followed by activation of intrahepatic NK cells and probably NKT cells and T cells to produce IFN-γ, and subsequent induction of IFN-α/β, all of which contribute to the inhibition of HBV replication in the hepatocyte and the recruitment of additional inflammatory cells into the liver. The cost of this process is a mild inflammatory liver disease that is probably induced by the same mediators. Nonetheless, the disease is very modest, in contrast to the major impact of these events on viral replication. Thus, pharmacological activation of the resident intrahepatic macrophage...
population may represent a novel therapeutic approach for the treatment of chronic HBV infection.

Acknowledgments

We thank Dr. Antonius Rolink (Basel Institute for Immunology) for providing anti-mouse agonistic CD40 mAb; Dr. Rolf Zinkernagel (University of Zurich) for providing anti-mouse CD4 and CD8 mAb; Drs. Makoto Naito and Hisami Watanabe (Niigata University School of Medicine) for providing L-MDP; Dr. Robert Schreiber (Washington University), for providing anti-mouse IFN-γ and TNF-α mAb; Dr. Maurice Gatley (Hoffman-La Roche) for providing anti-mouse IL-12 Ab; Drs. Monte Hobbs and Iain Campbell (The Scripps Research Institute) for providing the probe sets used in the RPA; and Alan Altage, Rick Koch, Amber Morris, Heike Mendez, and Margie Chadwell for excellent technical assistance.

References