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Activation of a Nonclassical NKT Cell Subset in a Transgenic Mouse Model of Hepatitis B Virus Infection

Jody L. Baron,[1,2,4] Leon Gardiner,[2] Stephen Nishimura,[3] Kanade Shinkai,[2,4] Richard Locksley,[1,2,4] and Don Ganem[1,2,4,5]
[1]Howard Hughes Medical Institute
Departments of [2]Microbiology/Immunology, [3]Pathology, and [4]Medicine
University of California Medical Center
San Francisco, California 94143

Summary

NKT cells are specialized cells of the immune system that bear both T cell and NK cell markers. Classical NKT cells display TCRs of restricted heterogeneity (Vα14-Jα281) and recognize lipid antigens (e.g., α-galactosyl ceramide) presented by nonpolymorphic CD1 molecules. Recently, other nonclassical NKT subsets have been recognized, including NKT cells not reactive with CD1d-α-galactosyl ceramide complexes. The biological functions of these cells are unknown. Here, we show that nonclassical NKT cells that are CD1d restricted but nonreactive to α-GalCer are activated in response to hepatocytes expressing hepatitis B viral antigens in a transgenic mouse model of acute hepatitis B virus infection. Our results document the first in vivo function for such nonclassical NKT cells and suggest a role for these cells in the host response to HBV infection.

Introduction

One of the challenges in understanding viral pathogenesis is the elucidation of the full repertoire of immune responses that control the replication of the invading pathogen. Generally speaking, such control mechanisms can be either noncytocidal or cytocidal. Noncytocidal responses can result from the release of cytokines or other substances with antiviral potential, without necessarily injuring the infected cell. By contrast, cytocidal responses aid in suppressing the pathogen but can also contribute to tissue injury and disease via the killing of infected cells.

Many of these notions derive in part from studies of hepatitis B virus (HBV) pathogenesis. Human HBV is an important cause of acute and chronic liver injury in vivo and is strongly linked to the development of hepatocellular cancer. Interestingly, its replication affects neither the viability nor growth of infected cells in culture (Ganem, 1996). Correlative clinical and pathological studies suggest that liver injury does not result from viral replication directly, but from host immune responses to viral antigens displayed by infected cells (Chisari and Ferrari, 1995). Experimental study of such immune responses, long frustrated by the inability to transmit HBV to immunologically tractable animal hosts, is now possible owing to pioneering studies by Chisari and colleagues, who developed transgenic mice expressing complete or partial copies of the HBV genome in the liver (Chisari and Ferrari, 1995; Chisari et al., 1987, 1986, 1985; Guidotti et al., 1995). Transgenic mice are tolerant to viral gene products and do not develop liver disease (Wirth et al., 1995). However, adoptive transfer of lymphocytes from syngeneic, HBV-immunized donor mice into such recipients results in both suppression of viral replication and the induction of liver injury that recapitulates many features of human hepatitis. These studies not only demonstrated the potential of host immune cells to elicit tissue damage but have also implicated inflammatory cytokines released from these cells in the control of viral replication (Cavanaugh et al., 1997; Gilles et al., 1992a; Guidotti and Chisari, 1999; Guidotti et al., 1996; Guilhot et al., 1993; Tsui et al., 1995).

Prior studies have focused on the role of virus-specific cytotoxic T lymphocytes (CTLs) isolated from previously immunized donors as effectors of liver injury in these models. We sought to examine the potential of other immune cells to contribute to liver injury and in particular wished to explore those cell-mediated responses that arise when naive (unimmunized and nontolerized) immune systems are exposed to infected hepatocytes, as occurs in acute hepatitis B in vivo. To this end, we introduced two modifications into the adoptive transfer model of Chisari to mimic more closely primary HBV infection. First, HBV-transgenic animals rendered immunodeficient by mutations in the recombinase-activating gene (Rag-1) or the T cell receptor (TCR C-α) were used as recipients; this eliminates most or all tolerized immune elements from recipient mice (Mombaerts et al., 1992b, 1992a). (Rag-1$^{-/-}$ mice lack T and B cells, while TCR C-α$^{-/-}$ mice lack primarily T cells; in both lines, NK cells and other components of the innate immune system are intact). Second, we reconstituted these mice with splenocytes from *nonimmunized* syngeneic donors. In this way, bias introduced by immunization or by selection for particular immune effector subpopulations is avoided. Using this system, we here describe the rapid development of an acute hepatitis that is dependent upon a nonclassical subset of NKT cells.

Results

Acute and Chronic Hepatitis after Adoptive Transfer of Naive Splenocytes

Transgenic mice expressing the large, middle, and small HBV surface (envelope) antigens (HBV-Env) in the liver (Chisari et al., 1987) were backcrossed to immune-deficient (Rag-1$^{-/-}$) (Mombaerts et al., 1992b) or T cell receptor C-α chain-deficient (TCR C-α$^{-/-}$) mice (Mombaerts et al., 1992a). Following adoptive transfer of naive splenocytes into these recipients, the ensuing immune responses and pathologic outcomes were monitored. As shown in Figure 1A, transfer was followed by the rapid onset of liver injury, as evidenced by a rise in the serum level of the enzyme alanine aminotransferase (ALT), which is released from injured or necrotic hepato-

[5]Correspondence: ganem@cgl.ucsf.edu

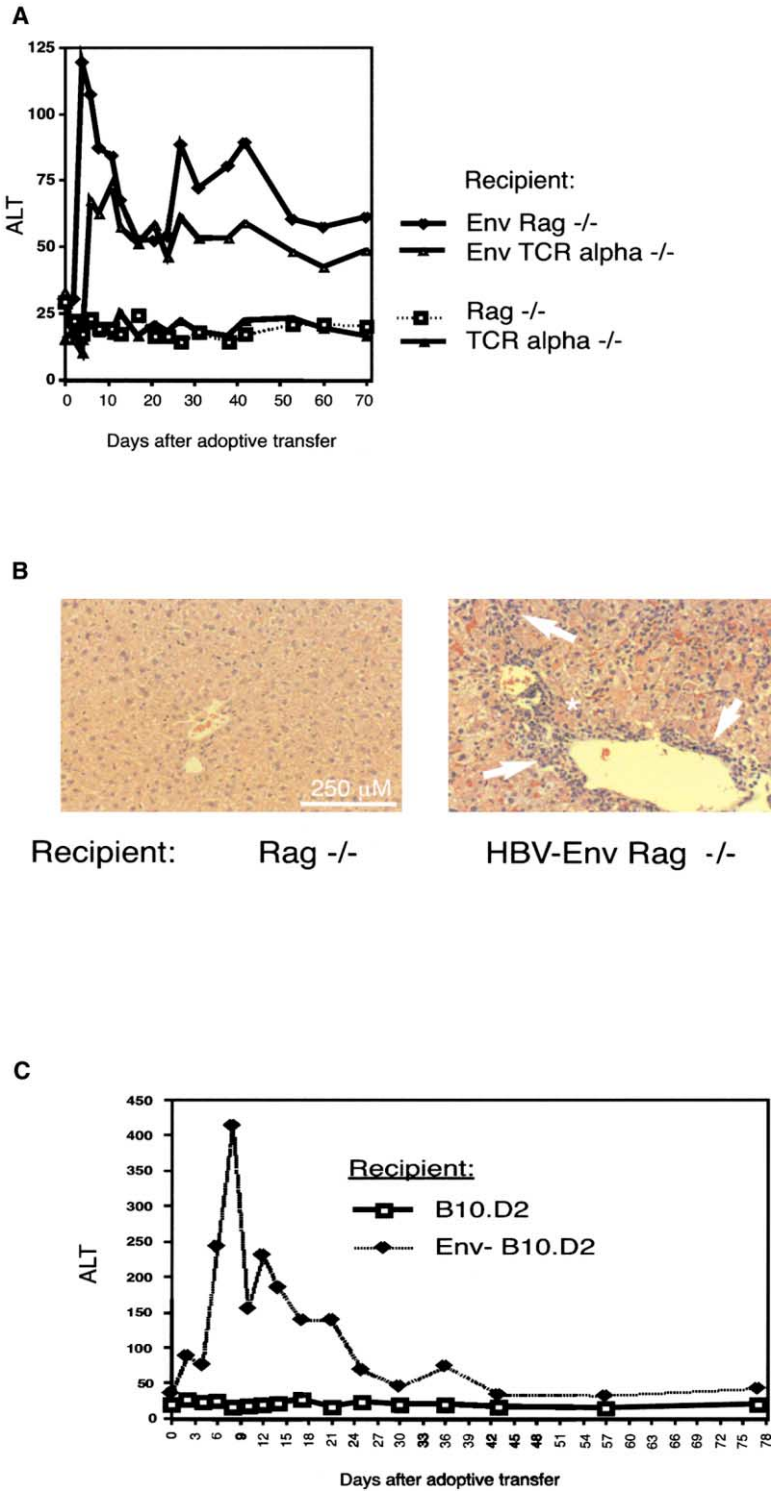


Figure 1. Acute and Chronic Hepatitis after Adoptive Transfer of Naive Splenocytes

(A) HBV-Env × Rag-1$^{-/-}$ mice and HBV-Env × TCR C-$\alpha^{-/-}$ mice develop an acute and chronic hepatitis after adoptive transfer of syngeneic, naive splenocytes. HBV-Env-transgenic mice on a B10.D2 genetic background (Chisari et al., 1987) were crossed with either B10.D2 Rag-1$^{-/-}$ mice or B10.D2 TCR-$\alpha^{-/-}$ mice to generate mice that carry the HBV-Env transgene in their liver in the absence of endogenous B cells and T cells (Rag-1$^{-/-}$) or in the absence of endogenous T cells (TCR C-$\alpha^{-/-}$). Naive, syngeneic splenocytes (1 × 10^8 cells, minus red blood cells) were adoptively transferred into male mice (6–10 weeks of age) intravenously. Animals were bled at regular intervals, and serum alanine aminotransferase (ALT) was measured as an indicator of liver injury. As a control for HBV specificity and for alloreactivity, naive, syngeneic splenocytes were also adoptively transferred into either Rag-1$^{-/-}$ or TCR C-$\alpha^{-/-}$ mice without the HBV transgene. Each experimental group had at least five mice.

(B) Histologic evidence of acute hepatitis in Rag-1$^{-/-}$ × HBV-Env mice but not Rag-1$^{-/-}$ mice, 3 days after adoptive transfer of syngeneic splenocytes. Liver tissue was fixed in 10% neutral-buffered formalin, paraffin embedded, sectioned (5 μm), and stained with hematoxylin and eosin. Size bar = 250 μm. Arrows point to inflammatory infiltrate surrounding portal triads with necrotic hepatocytes (asterisk).

(C) HBV-Env × Rag-1$^{+/+}$ mice develop an acute and chronic hepatitis after adoptive transfer of syngeneic, naive splenocytes (1 × 10^8 cells).

cytes. The ALT rise began as early as 2–3 days after adoptive transfer; these rapid kinetics suggest that an innate immune response to the target cells is responsible for at least a component of the injury. Liver tissue harvested at this time revealed histologic evidence of lobular inflammation and hepatocellular damage (Figure 1B). Subsequently, the recipient animals develop a chronic hepatitis, characterized by sustained serum ALT elevation (Figure 1A) and by persistent histologic signs of inflammation (data not shown). These kinetics suggest that this second phase of the illness may be due to adaptive immune responses to HBV. Both forms of injury were dependent on viral antigen expression, since transfer of splenocytes into Rag-1$^{-/-}$ mice or TCR C-$\alpha^{-/-}$ without the HBV envelope transgene did not induce liver injury (Figure 1). Compilation of ALT results

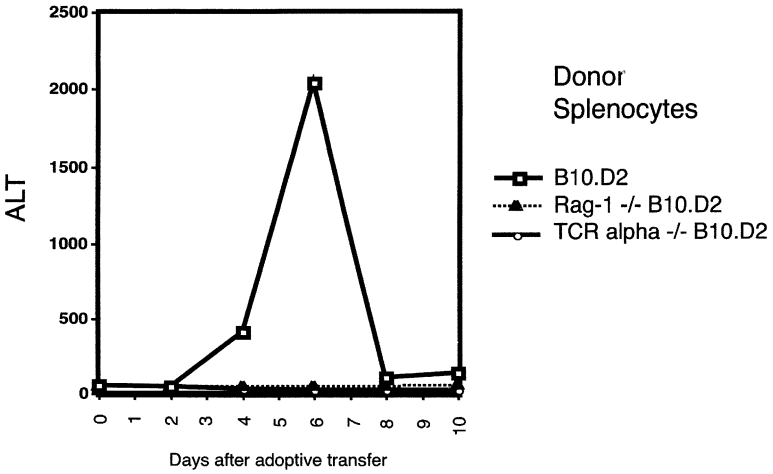


Figure 2. Acute Hepatitis Is Induced Only When Transferred Cells Have a Rearranged α/β T Cell Receptor

HBV-Env $\times$ TCR C-$\alpha^{-/-}$ mice develop acute hepatitis following adoptive transfer of donor splenocytes (1×10^8) from syngeneic wild-type (B10.D2) but do not develop acute hepatitis when the donor splenocytes are from syngeneic Rag-1$^{-/-}$ mice (Rag$^{-/-}$ B10.D2) or syngeneic TCR C-$\alpha^{-/-}$ mice (TCR C-$\alpha^{-/-}$ B10.D2).

from three experiments after adoptive transfer of syngeneic, naive splenocytes demonstrates that these results are highly significant: 16/18 HBV-Env $\times$ TCR C-$\alpha^{-/-}$ mice and 13/13 HBV-Env $\times$ Rag-1$^{-/-}$ mice developed hepatitis as compared to 0/11 TCR C-$\alpha^{-/-}$ mice or 0/12 Rag-1$^{-/-}$ mice (Fisher's exact test: two-sided P value is <0.0001) (data not shown).

The acute hepatitis was not the result of the expansion of effector populations in the recipient liver owing to enhanced "replication space" resulting from the Rag$^{-/-}$ mutation. As shown in Figure 1C, when splenocytes were transferred from unimmunized B10D2 mice into Rag$^+$ Env$^+$ B10.D2 recipients, hepatitis developed with similar kinetics and severity as in the Rag$^{-/-}$ animals. As before, this lesion was dependent upon the presence of viral antigens, as nontransgenic B10.D2 recipients experienced no ALT elevation throughout the course of the experiment.

Acute Hepatitis Is Induced Only When Transferred Cells Have a Rearranged TCR

Additional transfer experiments (Figure 2) revealed that Rag$^{-/-}$ donor cells, which contain normal levels of NK cells but lack T cells, B cells, NK1.1 T cells, and $\gamma\delta$ cells, did not produce injury (0/5 mice developed acute hepatitis). Similarly, TCR C-$\alpha^{-/-}$ donor cells, which contain normal levels of NK cells, B cells, and $\gamma\delta$ cells but lack α/β T cells and NK1.1 T cells, also did not produce liver injury (0/7 mice developed acute hepatitis) (Figure 2). These experiments, taken together with the rapid kinetics of disease onset, suggest natural killer T cells (NK1.1$^+$ T cells, or NKT cells) are likely effector(s) of this acute hepatitis.

The Number of NKT Cells Is Greatly Increased in HBV-Transgenic Mice with Acute Hepatitis but Not in Mice that Do Not Carry the HBV Transgene

To explore further the potential role of NKT cells in mediating the early liver injury, we characterized lymphocytes eluted from the livers of transgenic animals with hepatitis. Two days after adoptive transfer of naive spleen cells into HBV-transgenic Rag-1$^{-/-}$ or nontransgenic Rag1$^{-/-}$ mice, flow cytometric analysis revealed a significant increase in the proportion of NK1.1 T cells in the livers of the HBV-transgenic Rag-1$^{-/-}$ recipients, as compared to that observed in the intrahepatic lymphocyte pool from nontransgenic Rag-1$^{-/-}$ recipients (45% versus 11%; $p < 0.05$) (Figures 3A and 3B). Most of these recovered NK1.1$^+$ T cells express the Vβ8 TCR (Figure 3C), consistent with the prominent usage of Vβ8 among NKT cells compared to conventional $\alpha\beta$ T cells in this mouse strain (Bendelac et al., 1997; Benlagha et al., 2000). Furthermore, the NKT cells eluted from the livers of HBV-Env $\times$ Rag-1$^{-/-}$ mice with acute hepatitis were clearly activated, since ELISpot assays (Figures 6B and 6C), as well as intracellular cytokine staining (data not shown), revealed that the eluted cells were specifically producing interferon-γ and IL-4. In contrast, fewer than 0.1% of the cells eluted from the livers of Rag$^{-/-}$ mice 3 days after adoptive transfer of syngeneic, naive splenocytes were producing either IFN-γ or IL-4. Together, these data support the hypothesis that NKT cells are specifically activated in the mice which carry the HBV-Env transgene, since the Rag-1$^{-/-}$ mice that received identical cells had many fewer NKT cells in their livers, and the great majority of these cells were not activated.

Sorted NKT Cells Mediate Acute Hepatitis

We next fractionated donor spleen cells into NK1.1$^+$ and NK1.1$^-$ populations and transferred these cells into HBV-transgenic Rag-1-deficient recipients. Consistent with a role for NKT cells, depletion of NK1.1$^+$ cells ablates the early acute hepatitis, while enrichment for these cells augments tissue damage (Figure 4A). Specifically, the mice that received 1×10^8 splenocytes depleted of NK1.1$^+$ cells had only a trivial increase in their baseline ALT (21%) 2 days after adoptive transfer, while mice that received 1×10^8 unsorted syngeneic splenocytes or 2×10^6 NK1.1-positive sorted splenocytes had a 146% and 391% increase over baseline ALT, respectively.

Although the NK1.1-enriched population includes both NK cells and NK1.1 T cells, our prior studies with Rag$^{-/-}$ and TCR$^{-/-}$ donors (Figure 2) suggested that NK cells, which are not affected by these gene deletions, did not contribute to liver injury. To confirm these findings, we separated cells bearing both NK1.1 and TCR markers (NKT cells) from cells positive for NK1.1 alone

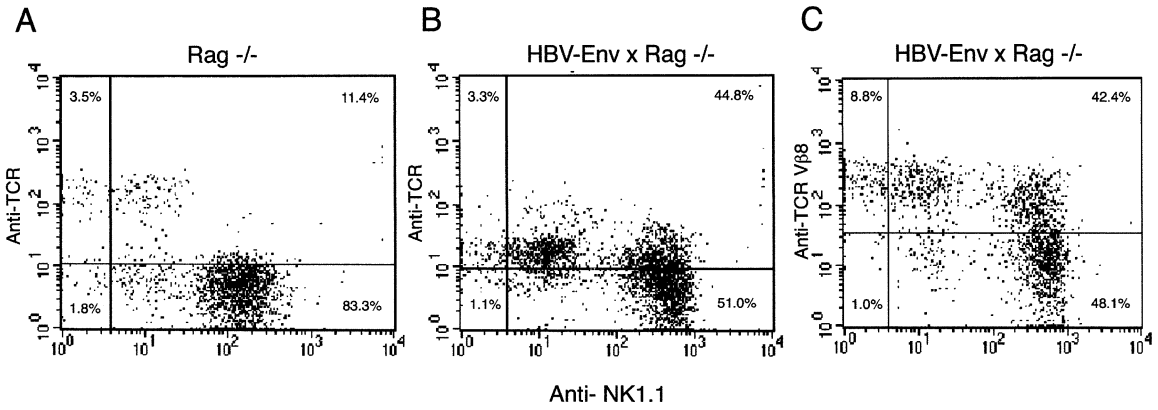


Figure 3. The Number of NKT Cells Is Greatly Enriched In HBV-Transgenic Mice with Acute Hepatitis

Rag-1$^{-/-}$ × HBV-Env mice have increased numbers of NK1.1 T cells in their livers (45%), 2 days after adoptive transfer of syngeneic splenocytes (B), as compared to similarly adoptively transferred Rag-1$^{-/-}$ mice (11%) (A). The majority of these NK1.1 T cells use Vβ8 as their TCR β chain (C). Mice were injected with naive, syngeneic splenocytes as described. Lymphocytes were isolated from the animals by perfusion and gradient isolation as described. Each experimental group of cells (e.g., Rag$^{-/-}$ or HBV-Env × Rag$^{-/-}$) represents pooled lymphocytes from the livers of five mice. Cells were stained by standard protocol with fluorescent-tagged antibodies (NK1.1-FITC, H57-PE, and F23.1-PE). The figure represents one of three experiments. Note that infiltrating NKT cells display a bimodal distribution of NK1.1 levels. This likely reflects the fact that levels of NK1.1 decrease when NKT cells are activated (Chen et al., 1997).

(NK cells) using sterile cell sorting and tested each subpopulation by adoptive transfer into HBV-Env-transgenic Rag$^{-/-}$ recipients. Mice that received 1.2 × 10^6 NKT cells developed a severe hepatitis as evidenced by a large increase in serum ALT (2600% over baseline ALT; Figure 4B) and by histologic evidence of severe lobular inflammation and hepatocellular necrosis (Figure 4C). By contrast, the mice that received 1.2 × 10^6 NK cells displayed no increase in serum ALT (Figure 4B) and little or no abnormality in liver histology (Figure 4C). Taken together, these data demonstrate that NKT cells are both necessary and sufficient to induce acute hepatitis in this model.

NKT Cells that Mediate Acute Hepatitis Do Not Bind CD1d-α-Galactosylceramide Tetramers

We next sought to characterize the NKT cells involved in mediating the acute hepatitis. The largest group of NKT cells express an invariant Vα14$^+$ T cell receptor specific for the lipoglycan α-galactosylceramide (α-GalCer), presented by CD1d. Surprisingly, the vast majority of NK1.1$^+$ TCR$^+$ cells eluted from the livers of mice with acute hepatitis were CD1d-α-GalCer tetramer negative, as assessed using CD1d tetramers loaded with α-GalCer (Benlagha et al., 2000; Matsuda et al., 2000) (Figures 5A and 5B). Furthermore, the majority of NKT cells eluted from the livers were both CD4 and CD8 negative (data not shown). In contrast, 24% of lymphocytes eluted from the livers of wild-type B10.D2 mice were tetramer-positive cells, consistent with prior studies (Benlagha et al., 2000; Matsuda et al., 2000) (Figure 5C); this indicates that our techniques for α-GalCer/CD1 binding have the appropriate sensitivity.

It is possible that the NKT cells eluted from the livers of HBV-Env × Rag-1$^{-/-}$ with acute hepatitis were tetramer positive before activation but lost staining due to TCR downregulation. To test this possibility, we separated naive splenocytes into CD1d-α-GalCer tetramer-positive and -negative populations using sterile cell sorting and tested each subpopulation by adoptive transfer into HBV Env-transgenic Rag$^{-/-}$ recipients. As shown in Figure 5D, acute hepatitis segregated with the CD1d-α-GalCer tetramer-negative population (5/5 mice developed acute hepatitis) and not with the CD1d-α-GalCer tetramer-positive population (0/5 mice developed acute hepatitis). Thus, the NKT cells that mediate the acute hepatitis do not recognize α-GalCer presented by CD1d.

Acute Hepatitis Is CD1d Dependent but Not Mediated by TCR Vα14 NKT Cells

To test whether the NKT cells involved in the acute hepatitis used the invariant Vα14$^+$ T cell receptor, we used mice transgenic for the Vα14$^+$ T cell receptor, crossed with the TCR C-α$^{-/-}$ mice, as splenocyte donors in adoptive transfer experiments into HBV-Env-transgenic × Rag-1$^{-/-}$ recipients. By using these mice as splenocyte donors, we increased the number of Vα14$^+$ NKT cells transferred by at least 7-fold and eliminated any other T cells or NKT cells that do not express the Vα14 TCR (Bendelac et al., 1996). Despite the vast increase in Vα14$^+$ NKT cells in the adoptive transfer, the HBV-Env-transgenic Rag$^{-/-}$ recipients developed little or no early acute hepatitis (1/8 mice developed mild hepatitis), as compared to the accompanying transfer of WT splenocytes (8/8 mice developed acute hepatitis) (Fisher's exact test: two-sided P value = 0.001) (Figure 6). Thus, canonical Vα14$^+$ NKT cells are not sufficient to induce acute hepatitis in HBV-Env-transgenic × Rag-1$^{-/-}$ mice. This result confirmed our earlier suspicion that the NKT cells involved in the acute hepatitis are predominantly TCR Vα14 negative and are therefore distinct from classical NKT cells.

In view of the importance of CD1d molecules in presentation of antigens to NKT cells, we sought to determine whether CD1d functioned in any role in mediating the acute hepatitis. HBV-Env-transgenic Rag$^{-/-}$ recipi-

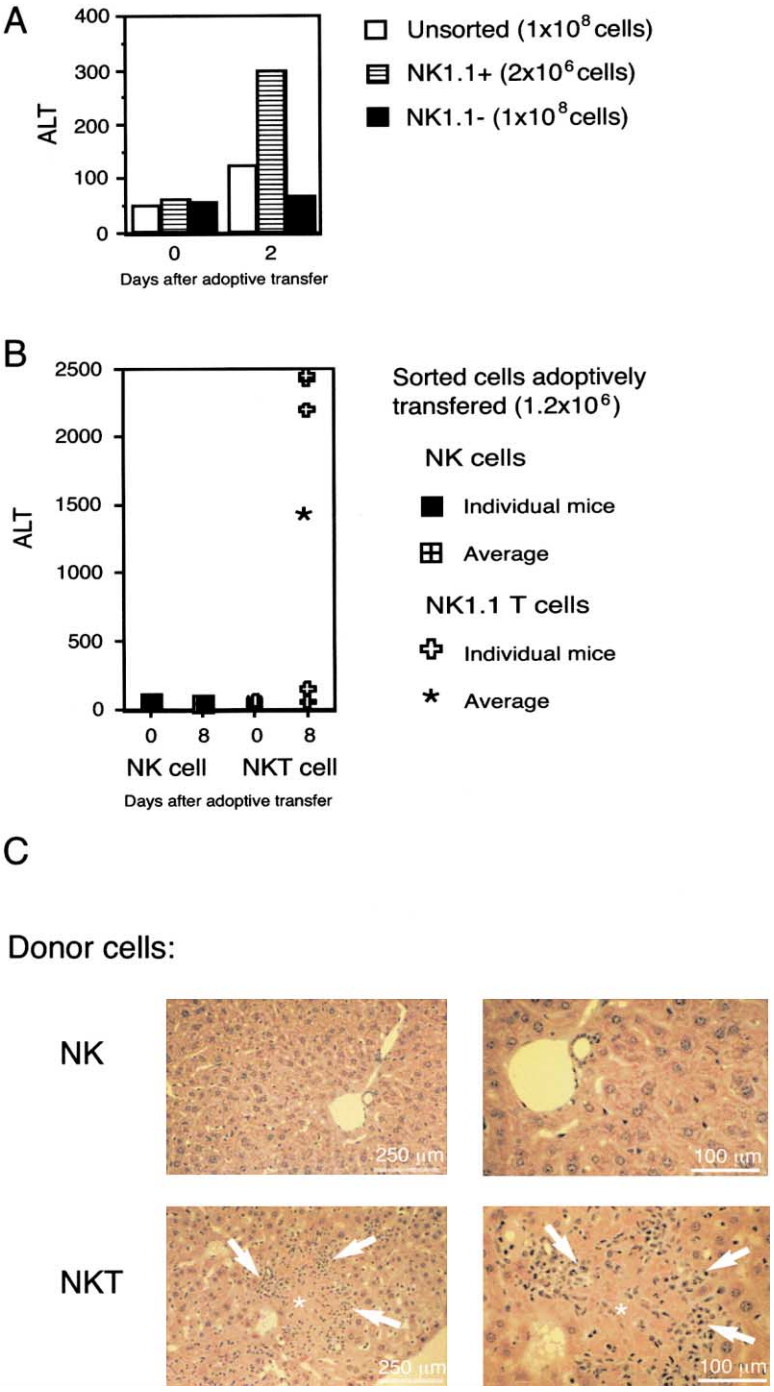


Figure 4. NKT Cells but Not NK Cells Mediate Acute Hepatitis

(A) Rag-1$^{-/-}$ × HBV-Env mice develop acute hepatitis when the donor splenocytes are from syngeneic wild-type mice (1 × 10^8 cells) or syngeneic splenocytes positively sorted for NK1.1 (2 × 10^6 cells) but do not develop acute hepatitis when syngeneic donor splenocytes have been depleted of NK1.1-positive cells (1 × 10^8 cells). B10.D2 splenocytes were isolated, red blood cells were lysed, and cells were stained with an FITC-conjugated antibody to NK1.1. Cells were then fractionated, using a MoFlo FACS, into NK1.1$^+$ and NK1.1$^-$ populations. These two populations were immediately transferred intravenously into Rag-1$^{-/-}$ × HBV-Env mice. ALT values represent an average of five mice.

(B) Rag-1$^{-/-}$ × HBV-Env mice develop acute hepatitis when donors cell are NK1.1 T cells but do not develop acute hepatitis when donor cells are NK cells. Splenocytes were isolated as above and stained with antibodies against NK1.1 and the T cell receptor chains Vβ8, Vβ7, and Vβ2 (these TCRs represent approximately 85% of the TCR β chains used by NK1.1 T cells). Populations of cells were collected that were either singly positive for NK1.1 (NK cells) or doubly positive for NK1.1 and TCR (NK1.1 T cells). These cells were immediately transferred intravenously into Rag-1$^{-/-}$ × HBV-Env mice. The ALTs of individual mice and also the mean of the experimental groups are represented.

(C) Histologic features of normal liver (animals receiving NK cells) or acute hepatitis (animals receiving NK1.1 T cells) 8 days after adoptive transfer are shown using a hematoxylin and eosin stain.

ents were rendered CD1d null by crossing them to CD1d$^{-/-}$ mice (Mendiratta et al., 1997), and animals were reconstituted with syngeneic, naive splenocytes. As shown in Figure 6A, HBV-Env- transgenic Rag$^{-/-}$ mice deficient in CD1d do not develop acute hepatitis (0/8 mice developed acute hepatitis), as compared to similarly treated HBV-Env-transgenic Rag$^{-/-}$ mice (8/8 mice developed acute hepatitis following adoptive transfer). Furthermore, production of interferon-γ and IL-4 by lymphocytes eluted from livers of HBV-Env × Rag$^{-/-}$ mice after adoptive transfer of syngeneic splenocytes is dependent on the presence of CD1d, since the production of these cytokines seen during acute hepatitis is almost absent if these mice are rendered CD1d null (Figures 6B and 6C). Thus, the NKT cells that mediate the acute hepatitis are not functional in the absence of CD1d.

Acute Hepatitis Develops in HBV-Env Rag$^{-/-}$ Mice after Adoptive Transfer of Syngeneic Lymphocytes Eluted from Wild-Type Livers

To test whether the NKT cells that mediate acute hepatitis are present in the liver despite the fact that they do not express the Vα14 TCR, we used lymphocytes eluted from the livers of WT B10.D2 mice in adoptive transfer

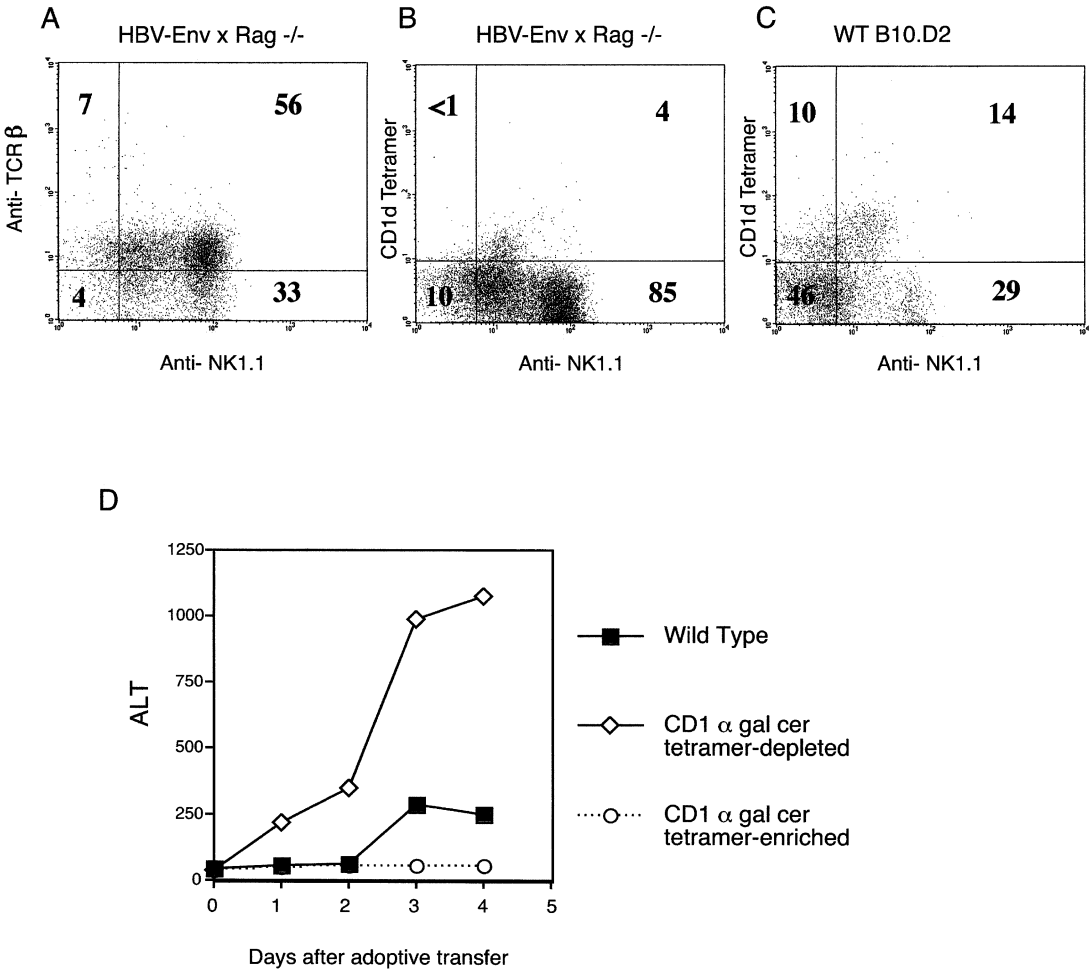


Figure 5. The NK1.1 T Cells that Mediate Acute Hepatitis Are CD1d-α-Galactosylceramide Tetramer Negative

NK1.1 T cells eluted from Rag-1$^{-/-}$ × HBV-Env mice 2 days after adoptive transfer of syngeneic splenocytes are CD1d-α-galactosylceramide tetramer negative.

(A) 56% of the cells eluted from the livers of Rag-1$^{-/-}$ × HBV-Env mice with acute hepatitis, 2 days after adoptive transfer of syngeneic splenocytes, are NK1.1, T cell receptor positive.

(B) Only 4% of these NK1.1 T cells are CD1d-α-galactosylceramide tetramer positive.

(C) 24% of lymphocytes eluted from wild-type B10.D2 livers are CD1d-α-galactosylceramide tetramer positive. Lymphocytes were isolated from the animals as described. Each experimental group of cells (e.g., HBV-Env × Rag$^{-/-}$ or WT B10.D2) represents pooled lymphocytes from the livers of five mice. Cells were stained by standard protocol with fluorescent-tagged antibodies (NK1.1-FITC, H57-PE) and the CD1d-α-galactosylceramide tetramer. The figure represents one of four experiments.

(D) Acute hepatitis is mediated by CD1d tetramer-depleted splenocytes but not by CD1d tetramer-enriched splenocytes. B10.D2 splenocytes were isolated, red blood cells were lysed, and cells were stained with a PE-labeled CD1d-α-galactosylceramide tetramer. Cells were then fractionated using a MoFlo FACS into tetramer^{+} and tetramer^{-} populations. These two populations were immediately transferred intravenously into Rag-1$^{-/-}$ × HBV-Env mice: 1 × 10^{8} tetramer^{-}, or total splenocytes cells/mouse; 2 × 10^{6} tetramer^{+} cells. ALT values represent an average of five mice.

experiments. As seen in Figure 6D, acute hepatitis develops in HBV-Env Rag$^{-/-}$ mice after adoptive transfer of approximately 100-fold fewer syngeneic lymphocytes eluted from WT livers as compared to the standard number lymphocytes transferred from WT spleens in all the previous experiments (1 × 10^{8}). Thus, cells that can mediate acute hepatitis are present in significant numbers in the liver.

Acute Hepatitis in HBV-Replication × Rag$^{-/-}$ Mice after Adoptive Transfer of Syngeneic Splenocytes

In order to assess the relevance of our observations to responses to authentic HBV infection, we characterized the responses to splenocytes adoptively transferred into mice bearing intact, replication-competent HBV transgenes. We initiated these experiments because the HBV-Env-transgenic mice express only a subset of viral genes, do not display viral replication, and as such do not allow exploration of the antiviral aspects of the immune responses under study. In addition, since they overexpress one of the three isoforms of HBV envelope proteins (the large or L protein) which is retained in the endoplasmic reticulum (ER), these mice display an increased sensitivity to IFN-γ (Gilles et al., 1992b; Guidotti et al., 1995) and thus may have exaggerated ALT rises in response to production of this cytokine. In fact,

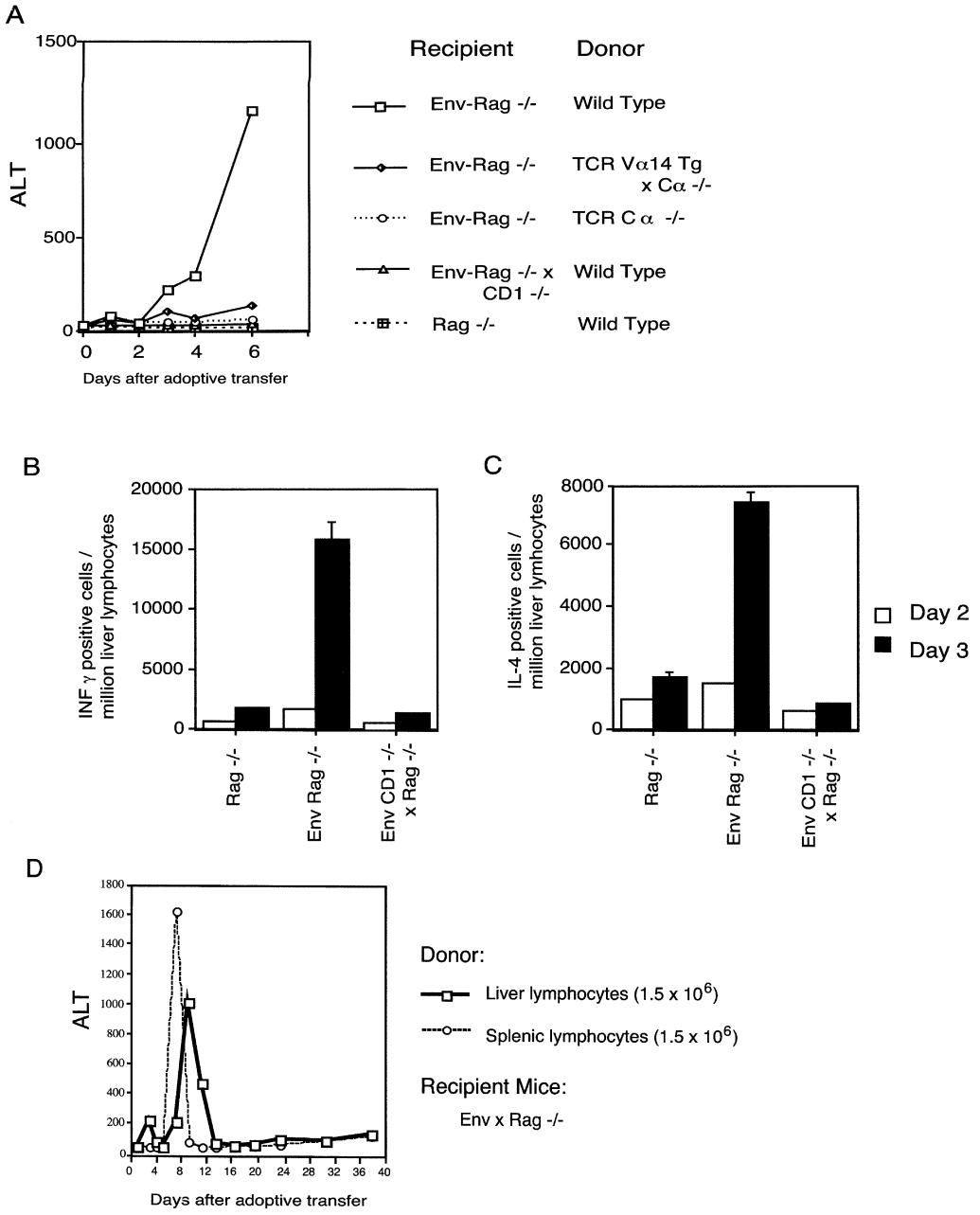


Figure 6. The NKT Cells that Mediate Acute Hepatitis Are CD1d Restricted and Present in High Frequency in the Liver but Are Not TCR Vα14 Positive

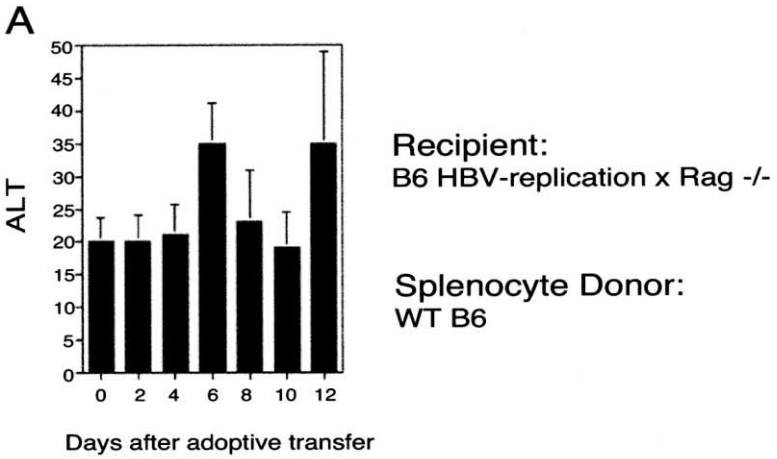
(A) HBV-Env × Rag-1$^{-/-}$ mice develop an acute hepatitis after adoptive transfer of syngeneic, naive splenocytes from WT B10.D2 mice but do not develop acute hepatitis if splenocyte donors are B10.D2, TCR Vα14 Tg × TCR C-α$^{-/-}$, or B10.D2, TCR C-α$^{-/-}$. HBV-Env × Rag-1$^{-/-}$ × CD1d$^{-/-}$ or Rag-1$^{-/-}$ mice on a B10.D2 background do not develop acute hepatitis after adoptive transfer of syngeneic, naive splenocytes from WT B10.D2 mice. ALT values represent an average of at least seven mice.

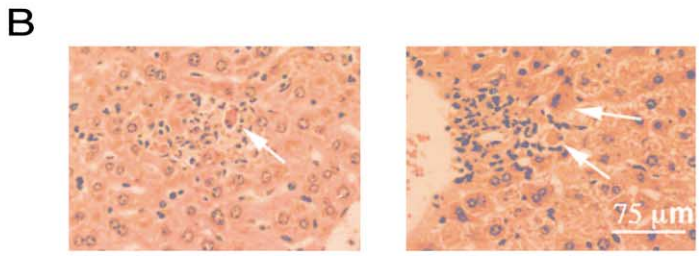
(B and C) ELISpot analysis of eluted liver lymphocytes reveals that HBV-Env × Rag$^{-/-}$ mice have a 10-fold increase in the percent of γ interferon-producing cells and a 7-fold increase in the percent of IL-4-producing cells in their livers 3 days after adoptive transfer of syngeneic splenocytes, as compared to similarly adoptively transferred Rag-1$^{-/-}$ mice or HBV-Env × Rag-1$^{-/-}$ mice which are CD1d null (CD1d$^{-/-}$).

(D) HBV-Env × Rag$^{-/-}$ mice develop acute hepatitis after adoptive transfer of 1.5 × 10^6 syngeneic lymphocytes eluted from WT livers, as compared to adoptive transfer of lymphocytes from WT spleens.

we initiated our studies of HBV immune responses in these mice precisely because this sensitivity gave us a simple readout (ALT release) that would reflect the intrahepatic production of this cytokine. In contrast, the HBV-replication-transgenic mice display intrahepatic HBV replication, express all viral proteins at their proper stoichiometry, and do not display enhanced sensitivity to the toxic effects of IFN-γ (Guidotti et al., 1995).

As shown in Figure 7, adoptive transfer of syngeneic splenocytes into the HBV-replication × Rag$^{-/-}$ mice re-





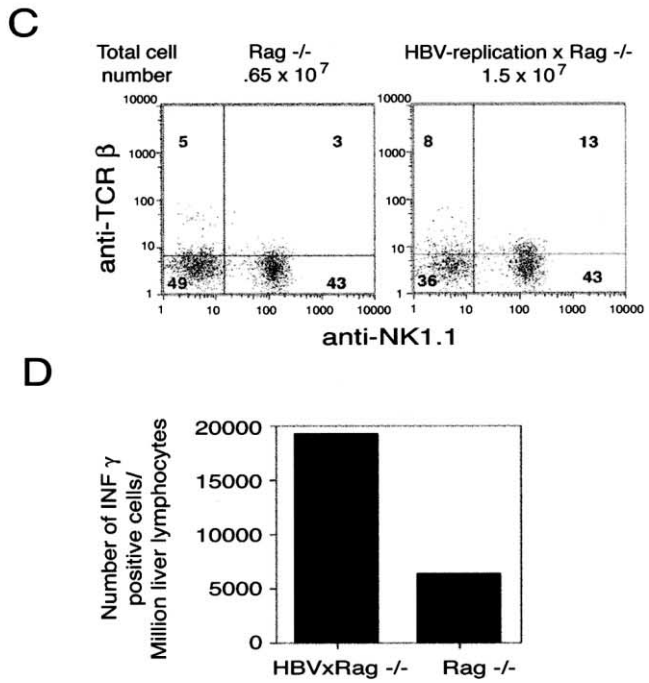


Figure 7. Adoptive Transfer of Syngeneic Splenocytes into C57/BL6 HBV-Replication × Rag$^{-/-}$ Mice Results in Acute Hepatitis as well as Accumulation and Activation of NKT Cells in the Liver

(A) C57/BL6 HBV-replication × Rag$^{-/-}$ mice develop acute hepatitis following adoptive transfer of donor splenocytes from syngeneic wild-type mice (1 × 10^8 cells).

(B) Histologic evidence of acute hepatitis in HBV-replication × Rag$^{-/-}$ mice, 2 days after adoptive transfer of syngeneic splenocytes. The figure presents representative hepatic lesions from two mice. Size bar = 75 μm. Arrows point to inflammatory infiltrate with necrotic hepatocytes.

(C) HBV-replication × Rag$^{-/-}$ mice have increased numbers of NK1.1 T cells in their livers (13%), 2 days after adoptive transfer of syngeneic splenocytes, as compared to similarly adoptively transferred Rag-1$^{-/-}$ mice (3%). There was a >2-fold increase in the absolute number of cells eluted from HBV-replication × Rag$^{-/-}$ livers. Each experimental group of cells represents pooled lymphocytes from the livers of three mice.

(D) ELISpot analysis of eluted liver lymphocytes reveals that HBV-replication × Rag$^{-/-}$ mice have a 3-fold increase in the percent of γ interferon-producing cells in their livers 3 days after adoptive transfer of syngeneic splenocytes, as compared to similarly adoptively transferred Rag-1$^{-/-}$ mice.

sults in acute hepatitis as well as accumulation and activation of NKT cells in the liver. Specifically, a similar biphasic ALT rise that is seen in the HBV-Env $\times$ Rag$^{-/-}$ mice is again observed (Figure 7A), but as expected, the ALT rise is much more modest than that seen in the Env^{+} mice—typically, serum transaminases were elevated no more than 2-fold above background. Liver tissue harvested at this time revealed histologic evidence of lobular inflammation and necrosis in 12/12 mice beginning 2 days after adoptive transfer; 10/10 Rag-1$^{-/-}$ mice lacking the HBV transgene showed no evidence of inflammation or necrosis 2 days after adoptive transfer of syngeneic splenocytes (Fisher's exact test: two-sided P value is < 0.0001). Figure 7B shows representative hepatic lesions from two HBV-replication $\times$ Rag$^{-/-}$ mice, 2 days after adoptive transfer. When the eluted cells were examined by flow cytometry for NKT cells, there was a 4-fold increase in the fraction of cells in the HBV-transgenic liver that displayed NKT cell markers. Moreover, there was a 2-fold increase in the absolute number of cells eluted from these livers (Figure 7C), indicating a significant increase in the total number of infiltrating NKT cells in this setting. Furthermore, when cells were eluted from the livers of these mice at day 3 post adoptive transfer, a clear increase in the number of IFN-γ-producing cells was observed, as determined by ELISpot analysis; this increase was dependent upon the presence of the viral genome (Figure 7D). Thus, similar processes are at work in the context of replicating HBV genomes, though, as expected, the severity of the immediate liver injury in this context is lower.

Discussion

NKT cells are a specialized subpopulation of T cells that coexpress receptors of the natural killer lineage (e.g., NK1.1) and TCRs. Classical NKT cells express a restricted TCR repertoire; most express a canonical Vα14 receptor that is conserved between mice and humans, paired with a limited number of Vβ chains, primarily Vβ8 (Bendelac et al., 1997). Classical NKT cells are selected by the nonpolymorphic MHC-like class I molecule, CD1d (CD1.1 in mouse), which contains a large hydrophobic binding cleft capable of presenting lipid or other hydrophobic antigens (Porcelli and Modlin, 1999). Indeed, NKT cells can be activated by CD1-restricted display of a variety of lipids, including cell wall antigens of mycobacteria and galactosylceramide from sea sponges. NKT cells display an activated phenotype and are capable of rapid expression of cytokines, including IFN-γ and IL-4, providing early help for other effector cells and potentially regulating differentiation of some adaptive immune responses. Classical NKT cells are enriched in the intrahepatic lymphocyte pool (where they represent 30%–50% of resident lymphocytes, as compared to 0.5% of splenocytes and 0.1% of peripheral lymph nodes) (Bendelac et al., 1997).

Recent evidence suggests that subsets of NKT cells likely exist. Using CD1 tetramers loaded with α-GalCer, subpopulations of noncanonical NKT cells have been identified that fail to recognize this complex (Behar and Cardell, 2000), in contrast to classical NKT cells which directly bind this glycolipid in the context of CD1d. In addition, NK1.1-negative populations that bind α-GalCer/CD1 tetramers have been described. Beyond these phenotypic characterizations, however, little is known of the in vivo roles of any of these newly described NKT subsets in immune regulation or disease pathogenesis.

The results presented in this paper demonstrate a role for NKT cells—a population of cells which straddle the innate and adaptive immune system—in the response to infection by a human viral pathogen. In addition, they reveal that the NKT cells involved in this response represent a subpopulation of NKT cells (CD1d restricted, Vα14 negative, and unable to recognize α-GalCer), whose existence has been recognized only recently and whose biological functions have hitherto been obscure. Our findings raise several provocative mechanistic questions and provide an experimental system in which they can be addressed. First, what is the nature of the molecule being presented by CD1d and recognized by these NKT cells in HBV-positive livers? While NKT cells are known to recognize hydrophobic ligands, the exact nature of the target(s) in the liver is unknown. Two classes of possibilities exist. First, the target might be virus encoded —candidates would include the myristate moiety of the large envelope protein or hydrophobic peptides from the HBV envelope protein. This would be similar to the ability of nonpolymorphic MHC Ib H2-M3 molecules to bind and present N-formylated peptides of bacterial origin (Chiu et al., 1999). Alternatively, HBV gene expression could lead to enhanced presentation of endogenous hydrophobic ligands—e.g., host lipids or glycolipids—by CD1, or sensitize NKT cells to activation by a given quantity of such ligands. The latter model would be consistent with the observation that many NKT cells are autoreactive (Bendelac et al., 1997). Other viral infections are known to influence activation of innate immune effectors—for example, CMV infection leads to the upregulation of MIC (a distant homolog of MHC class I which is a ligand for the NK cell stimulatory receptor NKG2D) on infected cells (Groh et al., 2001). In either case, the infected cell would be presenting an activation signal alerting the immune system that an infection has occurred and mobilizing an immediate response, in much the same way that interferon is released upon viral infection, mobilizing antiviral defenses in surrounding uninfected cells.

In both lineages of transgenic mice we have examined, the NKT cell response results in liver cell injury and could thus contribute to viral pathogenesis. While classical NKT cells have also been implicated in experimental liver cell injury mediated by the experimental administration of concanavalin A (Kaneko et al., 2000) or α-GalCer-activated cells (Kakimi et al., 2000), this is the first demonstration that NKT cells are activated by a naturally occurring liver pathogen in vivo and can play a role in liver injury mediated by such a pathogen. Furthermore, this in vivo activation and liver injury is mediated by a novel, nonclassical NKT cell population.

In addition to their role in injury, activated NKT cells are known to release large quantities of IFN-γ, a cytokine known to inhibit HBV replication in vivo. Such release may also contribute to the noncytolytic clearance of virus from surrounding hepatocytes and recruitment of lymphomononuclear inflammatory cells, as earlier demonstrated for cytokines released from intrahepatic CTLs

or adoptively transferred α-GalCer-activated classical NKT cells (Guidotti et al., 1996; Kakimi et al., 2001). In fact, given the modest degree of liver injury observed in the mice harboring replicating HBV genomes, this may be the primary role of this response in authentic hepatitis B. In a recent study of acute HBV infection in primates, levels of intrahepatic viral DNA decline by 90% well in advance of the peak influx of antigen-specific CTLs in the liver (Guidotti et al., 1999). Such a drop would be compatible with the rapid mobilization of NKT cells, as modeled here in mice. Although our results are limited to work with HBV, the enrichment of NKT cells in the lymphocyte populations of normal mouse and human liver leads us to speculate that NKT cells may have evolved to play a more general role in the defense against hepatic pathogens. Consistent with this, NKT cells in the liver have been found to expand or disappear (presumably due to activation-induced cell death) in animal models of Listeria, malaria, and lymphocytic choriomeningitis virus infection (Emoto et al., 1997; Hobbs et al., 2001; Pied et al., 2000). Furthermore, in our experimental model of hepatitis B virus infection, acute hepatitis develops in HBV-Env Rag$^{-/-}$ mice after adoptive transfer of approximately 100-fold fewer syngeneic lymphocytes eluted from WT livers as compared to lymphocytes from WT spleens, suggesting that functional NKT effectors are enriched in this organ.

Beyond its immediate role as an antiviral defense mechanism designed to restrict HBV replication, mobilization of NKT cells in HBV-infected liver may modulate the nature of the later adaptive response through cytokine production. The fact that the early NKT-mediated injury evolves in this model into a more chronic hepatitis allows investigations of the influence of the early NKT cell response on the subsequent evolution of hepatitis. By allowing access to this and other questions, the system described here should not only inform the study of the pathogenesis of chronic hepadnaviral infection but also enhance our understanding of the fundamental biology of NKT cells.

Experimental Procedures

Mice

B10.D2 and C57/Bl 6 mice were purchased from Jackson Laboratory, Bar Harbor, ME. HBV-transgenic mice used include HBV-Env: mouse lineage 107-5D (official designation Tg[Alb-1.HBV] Bri66; inbred B10.D2, H-2^d) (Chisari et al., 1987) and HBV-replication: lineage 1.3.46 (official designation, Tg [HBV 1.3 genome] chi46) (Guidotti et al., 1995). The HBV-Env mice contain the entire HBV envelope coding region (subtype ayw) under the constitutive transcriptional control of the mouse albumin promoter. These mice express the HBV small, middle, and large envelope proteins in their hepatocytes. The HBV-replication mice contain a terminally redundant HBV DNA construct (Guidotti et al., 1995). These mice have high-level viral replication in their hepatocytes and in the proximal convoluted tubules of their kidneys. The replication level seen in these animals is comparable to that observed in the infected livers of patients with chronic persistent HBV hepatitis, but the mice show no evidence of cytopathology (Guidotti et al., 1995).

Rag-1 KO mice (Mombaerts et al., 1992b) and TCR-α KO mice (Mombaerts et al., 1992a) were obtained on a C57BL/6 background. In addition, mice were backcrossed for at least ten generations with B10.D2 mice. Heterozygous B10.D2 HBV-Env mice were crossed with homozygous Rag-1 KO mice or homozygous TCR-α KO mice on a B10.D2 background. The offspring of this cross, heterozygous HBV-Env × heterozygous Rag-1 KO or TCR-α KO mice, were backcrossed with homozygous RAG-1 KO or TCR-α KO mice to obtain mice heterozygous for the Envelope transgene and homozygous for the Rag-1 KO or TCR-α KO mutation on a B10.D2 background. Heterozygous HBV-replication mice were similarly crossed with Rag-1 KO mice on a C57/BL6 background to obtain heterozygous HBV-replication × homozygous Rag-1 KO mice. CD-1d KO mice on a C57/BL6 background (generously provided by Luc Van Kaer, Vanderbilt University) (Mendiratta et al., 1997) were crossed with B10.D2 mice to obtain mice on a B10.D2 background (H-2^d). These mice were then crossed with the B10.D2 heterozygous HBV-Env × homozygous Rag-1 KO mice. The offspring of this mating were bred to obtain the B10.D2 heterozygous HBV-Env × homozygous Rag-1 KO × homozygous CD1d KO mice. Vα14-transgenic mice crossed with TCR C-α−/− mice on a C57/BL6 background (Bendelac et al., 1996) were crossed with B10.D2 mice to obtain mice on a B10.D2 background (H-2^d). All mice are kept in a specific pathogen-free facility at UCSF.

Cell Sorting and Cell Transfer Experiments

Donor splenocytes from male mice 6–10 weeks old were obtained by rupturing the lymphoid organ following conventional procedures. Red blood cells were lysed with ammonium chloride, and the remaining cells were washed with PBS, counted, filtered through a nylon membrane, and then injected intravenously. NK1.1$^+$ cells were stained and purified first using Fc block, 1:5 (Unkeless, 1979), to block non-antigen-specific binding of immunoglobulins Fcγ II and III receptors, then using an FITC-labeled antibody to NK1.1 (Yokoyama and Seaman, 1993) and a MoFlo cell sorter (Cytomation, Inc., Ft. Collins, CO). Sorted populations were >95% pure. NK1.1 T cells were separated from NK cells using Fc block, then an FITC-labeled antibody to NK1.1 and PE-labeled antibodies to the T cell receptor β chains V_β 8, 7, and 2. (NK1.1 [Yokoyama and Seaman, 1993], F23.1 [Staerz et al., 1985], TR310 [Okada et al., 1990], and B20.6 [Necker et al., 1991], PharMingen, San Diego, CA). Cells were >93% pure. CD1d-α-GalCer tetramers were generously provided by Mitch Kronenberg (LIAI, La Jolla, CA). Tetramer staining of lymphocytes was done according to standard protocol (Matsuda et al., 2000), and staining and cell sorting experiments were performed with a PE-labeled tetramer using the MoFlo cell sorter. Cell populations were >98% pure. Cells were adoptively transferred as mentioned.

Disease Model

Various mice (male, 6–10 weeks of age) were intravenously injected with cell populations described. Mice were bled by tail vein at described intervals, and serum was collected. The mouse serum was used to obtain ALT values as follows: Figure 1: 25 μl of mouse serum was used in a manual Sigma Diagnostics assay for ALT. The enzymatic reaction was run at room temperature (23°C). Note: the values obtained from this assay are lower than those in all other figures, which were run by a standardized, automated machine at 37°C. Figures 2, 4, 5, and 6: 100 μl of mouse serum was run on a Beckman synchon LX 20 to determine serum ALT. Mice were sacrificed at the described time points and livers were collected for histology.

Preparation of Liver Lymphocytes

Intrahepatic lymphocytes were isolated as previously described (Dao et al., 1998). In brief, animals were perfused via the portal vein with digestion medium (RPMI medium containing 0.2 mg/ml collagenase and 0.02 mg/ml DNase, and 5% FCS). After perfusion, we homogenized the livers by forcing them through a metal strainer; the livers were then digested with digestion buffer at 37°C for 45 min. The cell preparation was then centrifuged to remove hepatocytes (30 RCF for 3 min), and the remaining cells were centrifuged in a metrizamide gradient to obtain the lymphocytes. Cells were stained by standard protocol (Colligan et al., 1999) first with Fc-block 1:5 (Unkeless, 1979) and then with fluorescent-tagged antibodies (NK1.1 [Yokoyama and Seaman, 1993], H57 [Kubo et al., 1989], F23.1 [Staerz et al., 1985]; PharMingen, San Diego, CA) or CD1d-α-GalCer tetramers (Matsuda et al., 2000). Fluorescently labeled isotype-matched nonbinding antibodies were used as negative controls in all experiments since the level of TCR and NK1.1 is low, especially on activated cells. FACS analysis was done on a Beckman FACSCal-

ibur or a MoFlo cell sorter (Cytomation, Inc., Ft. Collins, CO). Analysis of data was done using Cellquest software.

ELISpot Assay

Liver lymphocytes were eluted from various mice 2 and 3 days after adoptive transfer. Without any further stimulation, cells were counted and immediately plated in triplicate in an anti-cytokine precoated PVDF- backed 96-well microplate (R&D Systems ELISpot mouse IFN-γ and IL-4 kits). Seven serial 2-fold dilutions of cells were made for each triplicate. Plates were incubated and developed according to the kit protocol, and spots were counted manually using a stereomicroscope.

Histology

Livers were removed from animals. Liver pieces were placed in 10% formalin in tissue cassettes. Liver pieces in formalin were embedded in paraffin, cut into 5-micron sections, and stained with hematoxylin and eosin according to standard protocol.

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