

The Proapoptotic Bcl-2 Family Member Bim Plays a Central Role during the Development of Virus-Induced Hepatitis

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The proapoptotic Bcl-2 homolog Bim was shown to control the apoptosis of both T cells and hepatocytes. This dual role of Bim might be particularly relevant for the development of viral hepatitis, in which both the sensitivity of hepatocytes to apoptosis stimuli and the persistence of cytotoxic T cells are essential factors for the outcome of the disease. The relevance of Bim in regulating survival of cytotoxic T cells or induction of hepatocyte death has only been investigated in separate systems, and their relative contributions to the pathogenesis of T cell-mediated hepatitis remain unclear. Using the highly dynamic model system of lymphocytic choriomeningitis virus-mediated hepatitis and bone marrow chimeras, we found that Bim has a dual role in the development of lymphocytic choriomeningitis virus-induced, T cell-mediated hepatitis. Although the absence of Bim in parenchymal cells led to markedly attenuated liver damage, loss of Bim in the lymphoid compartment moderately enhanced hepatitis. However, when both effects were combined in Bim^{-/-} mice, the effect of Bim deficiency in the lymphoid compartment was overcompensated for by the reduced sensitivity of Bim^{-/-} hepatocytes to T cell-induced apoptosis, resulting in the protection of Bim^{-/-} mice from hepatitis.

Millions of people worldwide suffer from liver diseases associated with viral infections, particularly hepatitis B and C virus. Despite the fact that hepatitis B and C virus are not cytopathic, persistent infections with these viruses lead to life-threatening liver injuries. Tissue damage is histopathologically characterized by liver fibrosis, fatty liver disease, occasionally evolving into a hepatocellular carcinoma. The clinical course of viral hepatitis critically depends on the activity of CTL. Activated CD8⁺ T cells are able to limit the spread of the virus and to protect against persistent viral infections by killing virus-infected hepatocytes in a perforin- and Fas ligand (FasL)-dependent manner, as well as by the concomitant production of proinflammatory cytokines, such as IFN- γ . Unfortunately, the same CTL also contribute to tissue injury by killing infected and uninfected tissue cells in an uncontrolled manner (1–5).

The respective roles of CTL in the elimination of virus-infected cells and the induction of tissue injury have been investigated for many years in mouse models of hepatitis, such as mice infected with the noncytopathic lymphocytic choriomeningitis virus (LCMV) strain WE (LCMV-WE) (6). The induction of liver injury in LCMV-WE-infected mice critically depends on several parameters, such as the immune status of the host, the route of infection, and the virus dose administered (6–10). As an example, infections with a low dose of LCMV-WE are usually cleared in

a CD8⁺ T cell-dependent manner (10). Under these circumstances, activated CTL are able to rapidly eliminate virus-infected cells and limit viral spreading without significant signs of immunopathology (6, 8). Nonetheless, when the mice are infected with high doses of the same virus, CTL are no longer able to control the propagation of the virus, and they contribute to tissue injury. Under these circumstances, the recovery from primary viral infections via CTL-mediated elimination of infected target cells and bystander killing of uninfected cells is accompanied by the development of liver disease (5, 6, 8). Hepatocytes represent one of the major targets of activated T cells during the development of hepatitis. As a consequence, regulation of hepatocyte apoptosis and T cell survival and death after viral infection represent an essential factor in the outcome of hepatitis.

The proapoptotic Bcl-2 homolog Bim plays a central role in both the regulation of lymphocyte homeostasis after viral infection and hepatocyte apoptosis (11–18). Bim^{-/-} mice accumulate increased numbers of lymphoid and myeloid cells, and old mice (on a mixed C57BL/6 \times 129SV background) develop a fatal systemic lupus erythematosus-like autoimmune disease (19). Lymphocytes from Bim^{-/-} mice are abnormally resistant to negative selection and to different apoptotic stimuli, such as cytokine deprivation and deregulated calcium flux (19, 20). Bim has been implicated in the regulation of immune homeostasis, and it represents an important factor in the termination of immune responses during virus infections (13–16).

In addition to being a key regulator of lymphocyte homeostasis, Bim is critically involved in the regulation of hepatocyte apoptosis. In contrast to most cell types in which Fas triggering directly induces the activation of caspases and, subsequently, apoptosis (type I cells), hepatocytes require the amplification of the signal via the mitochondria (type II cells) (21). In this process, the proapoptotic molecule Bid plays an essential role, because Bid^{-/-} mice are largely resistant to anti-Fas- and FasL-induced hepatocyte apoptosis, liver damage, and death (22, 23). However, our results suggested that the Fas-signaling pathway in this cell type is even more complex and involves additional apoptosis effector molecules. We demonstrated that Bim plays a central role in the modulation of Fas-induced hepatocyte cell death. Consequently,

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Abbreviations used in this article: ALT, alanine aminotransferase; FasL, Fas ligand; LCMV, lymphocytic choriomeningitis virus; LCMV-WE, lymphocytic choriomeningitis virus strain WE; WT, wild-type.

similar to *Bid*^{-/-} mice, *Bim*^{-/-} animals are also protected from anti-Fas-induced liver damage (17). However, *Bim*-mediated modulation of hepatocyte apoptosis is not limited to the Fas-signaling pathway; it extends to other apoptosis triggers involving the mitochondrial pathway, thus most probably representing a general regulatory mechanism of apoptosis in hepatocytes. This notion is supported by the findings that *Bim* plays an important role in hepatocyte apoptosis induced by a variety of death triggers, such as paracetamol and chemotherapeutic agents; the development of TNF- α -mediated liver damage; and free fatty acid-induced JNK-dependent hepatocyte lipoapoptosis (18, 24–26).

Based on the function of *Bim* in the regulation of hepatocyte apoptosis and in the regulation of adaptive-immune responses during virus infections, we aimed to analyze the respective roles of *Bim* expressed in the hepatocytes, as well as in the lymphoid compartment, during the development of T cell-mediated hepatitis in LCMV-infected mice. Using such a unique experimental system in which *Bim* is involved in the regulation of both the effector and target cell populations, we demonstrated that the absence of *Bim* expression in hepatocytes partially protects from T cell-mediated liver damage postinfection with LCMV, whereas a specific loss of *Bim* in the hematopoietic compartment has the potential to moderately increase the severity of the disease. Therewith, we were able to demonstrate that *Bim* carries out a dual role during the development of T cell-mediated liver damage, either protective or exacerbating, critically defined by the cell type of its expression.

Materials and Methods

Mice, viruses, and reagents

Bim^{-/-} mice were kindly provided by A. Strasser (Walter and Eliza Hall Institute, Melbourne, VIC, Australia). C57BL/6 wild-type (WT) and *Bim*^{-/-} mice were bred and kept in individually ventilated cages in the Central Animal Facility of the Department of Medicine, University of Bern. Animal experiments were performed in compliance with Swiss laws and guidelines and were approved by the animal experimentation committee of the State of Bern. LCMV-WE was originally obtained from A. Ochsenbein (University of Bern) and propagated on L929 cells. For the generation of bone marrow chimeras, mice were lethally irradiated (twice with 6.5 Gy at a 4-h interval) and reconstituted i.v. with 10⁷ bone marrow cells isolated from femurs and tibias of donor mice. After 7–8 wk, chimeras were infected i.v. with the indicated doses of LCMV-WE. The synthetic peptides GP33-41 (KAVYNFATC), NP396-404 (FQPQNGQFI), and Adn5 were purchased from Neosystem. All other reagents were purchased from Sigma-Aldrich, unless stated otherwise.

Preparation of liver lysates

Liver samples were immersed in 500 μ l lysis buffer (300 mM NaCl, 2 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitor mixture [Roche] in PBS) and disrupted for 60 s at 20 Hz using a Tissue Lyser System (Qiagen). Subsequently, 1% Triton X-100 was added, and samples were incubated at 4°C for 15 min. After centrifugation for 15 min at 16,000 \times g at 4°C, supernatant was collected and used for caspase-3-activity assays.

Caspase-3-activity assay

Twenty microliters of liver lysate was added to 180 μ l HEPES buffer (20 mM HEPES [pH 7.5], 10% glycerol, 2 mM DTT) containing 111 μ M Ac-DEVD-AFC (Alexis Biochemicals) and incubated for 1 h at 37°C. Enzymatic activity was analyzed on a Spectramax M2e (Molecular Devices) microplate reader (400 nm excitation, 505 nm emission).

Measurement of serum transaminases

Alanine aminotransferase (ALT) in the serum of the mice was determined using standard clinical chemistry reagents (Teco Diagnostics).

Focus-forming assay

LCMV-infected mice were sacrificed at the indicated time points, and pieces of liver and spleen were frozen in liquid nitrogen and stored at -80°C until the day of analysis. For the determination of the viral titers, organ samples

were transferred into 1.5 ml MEM containing 5% FCS and disrupted using a Tissue Lyser system (Qiagen). Focus-forming assays were performed, as described previously (27).

Isolation of intrahepatic lymphocytes

LCMV-infected mice were sacrificed at the indicated time points, and livers were perfused with PBS containing 2% horse serum and passed through a 70- μ m cell strainer (BD Biosciences). Cells were resuspended in 40% Percoll (GE Healthcare) and underlain with 60% Percoll. After centrifugation at 900 \times g for 20 min, intrahepatic lymphocytes were recovered from the interphase.

Flow cytometry

Single-cell suspensions of splenocytes or intrahepatic lymphocytes were incubated with 2.4G2 hybridoma supernatant for 10 min at 4°C to block unspecific Fc γ R binding. Then the cells were stained with anti-CD8, anti-CD45.1, anti-CD45.2 (all from BioLegend), or D^bGP33-41 MHC class I tetramers (Beckman Coulter) in PBS plus 2% FCS, 2 mM EDTA, and 2 mM NaN₃ for 30 min at 4°C. Stained cells were analyzed on an LSR II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Intracellular cytokine staining

Splenocytes or isolated intrahepatic lymphocytes were incubated with 100 nM the specific peptide in 200 μ l IMDM plus 10% FCS, 50 μ M 2-ME, and 50 μ g/ml gentamicin in the presence of 10 μ g/ml brefeldin A for 5 h at 37°C. Then cells were incubated with anti-CD8 and anti-CD45.1 or anti-CD45.2 Abs (all from BioLegend) in PBS plus 2% FCS, 2 mM EDTA, and 2 mM NaN₃ for 20 min at 4°C. Cells were fixed with 4% formaldehyde in PBS for 10 min at 4°C, followed by incubation with anti-IFN- γ and anti-TNF- α Ab (BioLegend) in permeabilization buffer (PBS, 2% FCS, 2 mM EDTA, 2 mM NaN₃, and 0.1% saponin) overnight at 4°C. Stained cells were analyzed by flow cytometry.

Cytotoxicity assay

LCMV (GP33-41)-specific CTL activity was measured by DNA-fragmentation assays, as described previously (28). In brief [³H]thymidine-labeled and GP33-41- or Adn5-loaded target cells (RMA) were incubated with isolated splenocytes or intrahepatic lymphocytes from LCMV-infected mice in round-bottom 96-well plates at different E:T ratios at 37°C. Effector cell numbers were normalized on the basis of a D^bGP33-41 MHC class I tetramer staining prior to incubation. After 5 h, cells were lysed, and unfragmented DNA was harvested on Unifilter-96 GF/C glass fiber plates and counted in a beta counter (Perkin-Elmer). DNA fragmentation was calculated as follows: % DNA fragmentation = 100 \times (1 - cpm experimental sample/cpm target only).

Statistical analysis

Statistical significance of differences was determined using the unpaired Student *t* test. Differences were considered significant at *p* < 0.05.

Results

*Attenuated T cell-mediated liver damage in LCMV-infected *Bim*^{-/-} mice*

To investigate the role of *Bim* in the development of LCMV-induced hepatitis, WT and *Bim*^{-/-} mice were infected with a high dose of LCMV-WE (1 \times 10⁶ PFU i.v.), and liver damage was analyzed at different time points postinfection. In agreement with previous publications (6), we observed strongly elevated ALT values in the serum of LCMV-infected WT mice between days 8 and 15, with the highest level around days 10–11 (Fig. 1A). In contrast to WT mice, *Bim*^{-/-} mice were partially protected from LCMV-induced liver damage, as indicated by significantly reduced serum transaminase levels at the peak of the disease (days 10–11). To define whether transaminase activity in the sera correlates with enhanced apoptosis in the liver, caspase-3 activity was measured by Ac-DEVD-AFC (fluorogenic caspase-3 substrate) cleavage in liver samples at the peak of the disease (i.e., peak of transaminase activity in the sera). Caspase-3 activity measured at day 10 postinfection correlated with the elevated ALT levels and

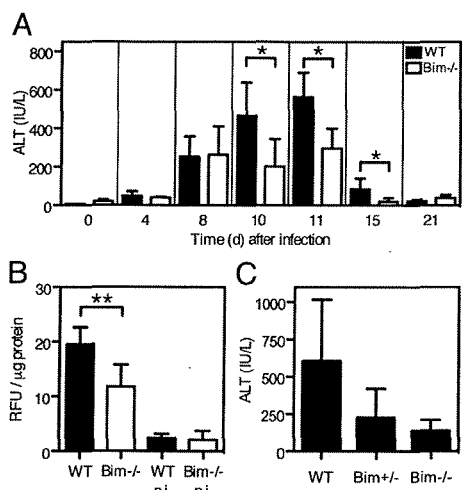


FIGURE 1. Attenuated liver damage in LCMV-infected $Bim^{-/-}$ mice. WT, $Bim^{-/-}$, and heterozygous ($Bim^{+/-}$) mice were infected with 1×10^6 PFU LCMV-WE i.v., and liver damage was analyzed at different time points postinfection. **A**, ALT levels in the serum of WT and $Bim^{-/-}$ mice. **B**, Caspase-3 activity (relative fluorescent units/ μ g protein) in liver lysates 10 d postinfection. **C**, ALT levels in the serum of WT, $Bim^{+/-}$, and $Bim^{-/-}$ mice 10 d postinfection. Mean \pm SD of three to five mice/group and time point are shown. The experiments were repeated three times, yielding similar results. * $p < 0.05$, ** $p < 0.01$, unpaired Student t test.

was significantly reduced in $Bim^{-/-}$ mice compared with WT mice (Fig. 1B). Hence, the attenuated serum ALT levels and caspase-3 activity in liver lysates of $Bim^{-/-}$ mice clearly demonstrated that the absence of Bim partially protects from T cell-mediated liver damage after LCMV infection. Attenuation of liver disease after LCMV infection was Bim dose dependent, because we observed an intermediate phenotype in $Bim^{+/-}$ mice compared with WT and $Bim^{-/-}$ mice (Fig. 1C).

Reduced liver damage in LCMV-infected $Bim^{-/-}$ mice is not caused by an impaired antiviral $CD8^+$ T cell response or by altered kinetics of virus propagation

Liver disease in LCMV-infected mice is predominantly caused by virus-specific cytotoxic T cells involved in the elimination of virus-infected hepatocytes and bystander killing of noninfected cells (6, 29). Consequently, the outcome of the disease critically depends on the numbers and activity of liver-infiltrating CTL, as well as the kinetics of virus expansion and clearance. Because normal T cell homeostasis and contraction of virus-specific T cells after viral clearance are impaired in $Bim^{-/-}$ mice (14, 19), we investigated whether the attenuated liver damage observed in $Bim^{-/-}$ mice is the consequence of major alterations in the virus-specific T cell response and/or clearance of the virus.

Thus, the number of $CD8^+$ T cells specific for the dominant T cell epitope GP33-41 was determined by FACS analysis of splenocytes and intrahepatic lymphocytes stained with D^b GP33-41 MHC class I tetramers. In agreement with a previous report (16) using other strains of LCMV, we found that the numbers of virus-specific (tetramer $^+$) CTL in spleen and liver of $Bim^{-/-}$ mice were significantly higher than in WT mice throughout the observation period of 15 d (Fig. 2A). However, tetramer staining does not provide any information about the activation status and effector functions mediated by these epitope-specific T cells. Because a previous study proposed that $Bim^{-/-}$ $CD4^+$ T lymphocytes show impaired activation and cytokine production in model systems of autoimmune inflammation (30), effector $CD8^+$ T cell

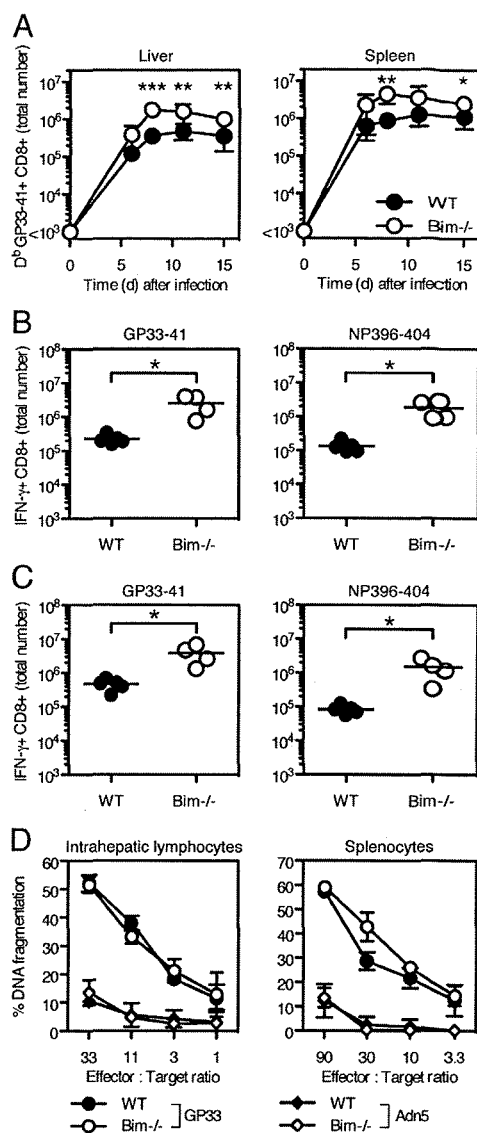


FIGURE 2. WT and $Bim^{-/-}$ mice were infected with 1×10^6 PFU LCMV-WE i.v. and sacrificed for analysis at the indicated time points. **A**, Total numbers of GP33-41-specific $CD8^+$ T cells in liver and spleen of the mice determined by staining with D^b GP33-41 MHC class I tetramers. Mean \pm SD of three to eight mice/group and time point are shown. Total numbers of functional $CD8^+$ T cells specific for the viral epitopes GP33-41 and NP396-404, as determined by intracellular IFN- γ staining of in vitro-stimulated T cells isolated from liver (**B**) and spleen (**C**) 11 d postinfection. **D**, Direct ex vivo cytolytic activity of virus-specific $CD8^+$ T cells isolated from liver and spleen 8 d postinfection, as determined in a 5-h DNA-fragmentation assay with GP33-41- or Adn5-labeled RMA target cells. Mean \pm SD of triplicate cultures is shown. The experiments were repeated three times, yielding similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired Student t test.

functions in LCMV-infected mice were analyzed in more detail. Intracellular IFN- γ staining of T cells isolated at day 11 postinfection and ex vivo restimulated with GP33-41 or NP396-404 peptide revealed significantly higher numbers of IFN- γ -producing GP33-41- and NP396-404-specific $CD8^+$ T cells in liver (Fig. 2B) and spleen (Fig. 2C) of the $Bim^{-/-}$ mice, thereby confirming the functionality of the $Bim^{-/-}$ $CD8^+$ T cells and the results obtained by MHC class I tetramer staining. These results were also confirmed by simultaneous intracellular IFN- γ and TNF- α stain-

ing of the *ex vivo* restimulated T cells (Supplemental Fig. 1). At the peak of the disease, the numbers of GP33-41- and NP396-404-specific CD8⁺ T cells producing both IFN- γ and TNF- α were significantly increased in the livers of the Bim^{-/-} mice compared with WT mice. Moreover, 10 d postinfection, lymphocytes were isolated from liver and spleen of Bim^{-/-} and WT mice and sorted for CD8⁺ T cells, and the expression of FasL, TNF- α , and TRAIL was measured at the mRNA level. As illustrated in Supplemental Fig. 1, Bim^{-/-} and WT CD8⁺ T cells expressed TNF- α at comparable levels, whereas Bim^{-/-} CD8⁺ T cells expressed increased amounts of FasL and TRAIL. Despite this difference in expression of FasL and TRAIL mRNA between WT and Bim^{-/-} CD8⁺ T cells, no difference in their cytotoxic activity was observed. This is demonstrated in Fig. 2D, where after normalizing the numbers of virus-specific T cells based on tetramer staining, the direct *ex vivo* cytotoxic activity of virus-specific CD8⁺ T cells isolated from liver and spleen was determined in a 5-h DNA-fragmentation assay with GP33-41- or Adn5-labeled RMA target cells. Taken together, these analyses demonstrated that fully functional virus-specific CTL are present at increased numbers in spleen and liver of LCMV-infected Bim^{-/-} mice and exclude the possibility that the reduced liver damage in these mice is caused by an impaired cytotoxic T cell response.

However, the outcome of liver disease in LCMV-infected mice strictly depends on the interplay between propagation of the virus in hepatocytes and activity of virus-specific T cells. Hence, we investigated whether the presence of increased numbers of activated T cells in Bim^{-/-} mice leads to an accelerated virus clearance compared with WT mice, which may account for the partial protection from LCMV-induced liver disease in Bim^{-/-} mice. Virus titers in spleen and liver were determined at different time points postinfection of Bim^{-/-} and WT mice with 1×10^6 PFU LCMV-WE. Despite the elevated numbers of T cells in the liver of Bim^{-/-} mice, virus titers showed only minor, not significant, differences around day 8 postinfection, whereas the initial expansion and the final clearance from the liver appeared to be identical to WT mice (Fig. 3). In the spleen, virus titers were completely indistinguishable between WT and Bim^{-/-} mice throughout the entire course of infection (Fig. 3). Based on these findings, it appears unlikely that the partial protection of Bim^{-/-} mice from LCMV-induced hepatitis is predominantly caused by an altered viral clearance in the liver of Bim^{-/-} mice.

Bim expression in hepatocytes plays a relevant role during the development of T cell-mediated liver damage

Although these experiments revealed an important role for Bim in the development of LCMV-induced hepatitis, they did not allow us to specifically define the respective contributions of Bim expressed

in the hepatocytes or in the hematopoietic compartment to the outcome of the disease. To reveal the specific contribution of Bim expression in hepatocytes to the development of T cell-induced liver disease, bone marrow from CD45.1⁺ WT mice was transferred into lethally irradiated CD45.2⁺ WT (WT \rightarrow WT) or Bim^{-/-} (WT \rightarrow Bim^{-/-}) recipients. Seven weeks after transfer, both types of chimeras were infected with 1×10^6 PFU LCMV-WE *i.v.*, and liver damage was analyzed at the peak of the disease (11 d postinfection). Measurement of serum transaminase activity revealed that liver damage in the WT \rightarrow Bim^{-/-} chimeras was substantially reduced compared with the WT \rightarrow WT mice (Fig. 4A). This finding was further confirmed by reduced levels of caspase-3 activity in the livers of the WT \rightarrow Bim^{-/-} chimeras (Fig. 4B). As expected, intracellular IFN- γ staining showed equal numbers of activated CD8⁺ T cells specific for the dominant LCMV epitopes GP33-41 and NP396-404 in liver (Fig. 5A) and spleen (Fig. 5B) of the WT \rightarrow WT and WT \rightarrow Bim^{-/-} chimeras. Because Bim^{-/-} T cells appear to be less sensitive to irradiation-induced cell death (19), the extent of chimerism was determined in the spleen and the liver of the chimeras. Tetramer staining of virus-specific T cells, together with staining for CD45.1⁺ (donor-derived) and CD45.2⁺ (recipient-derived) cells, confirmed a comparable expansion of CD8⁺ T cells specific for GP33-41 in liver and spleen of the chimeras (Fig. 5C). The equal numbers of virus-specific CD8⁺ T cells also correlated with comparable virus titers in liver and spleen of the WT \rightarrow WT and WT \rightarrow Bim^{-/-} mice (Fig. 5D). Taken together, these results demonstrated that Bim expression in the hepatocytes contributed to the observed cell death and the severity of LCMV-induced, T cell-mediated immunopathology in the liver and, thus, represents an important factor in regulating the susceptibility of hepatocytes to T cell-induced apoptosis.

Loss of Bim in the lymphoid compartment may have the potential to increase the severity of the disease

Because liver damage in LCMV-infected mice is primarily caused by CTL (6, 29), which are present in increased numbers in Bim^{-/-} mice during the course of infection (Fig. 2A), we investigated whether accumulation of activated T cells in Bim^{-/-} mice influences the outcome of liver disease. Therefore, lethally irradiated CD45.1⁺ WT mice were reconstituted with bone marrow from either CD45.2⁺ WT (WT \rightarrow WT) or Bim^{-/-} mice (Bim^{-/-} \rightarrow WT). Seven weeks after transfer, chimeras were infected with 1×10^6 PFU LCMV-WE *i.v.* and sacrificed for analysis 10 d postinfection. As previously shown in Bim^{-/-} mice (Fig. 2A–C), numbers of CD8⁺ T cells specific for GP33-41 and NP396-404 were increased in both the liver (Fig. 6A) and the spleen (Fig. 6B) of the

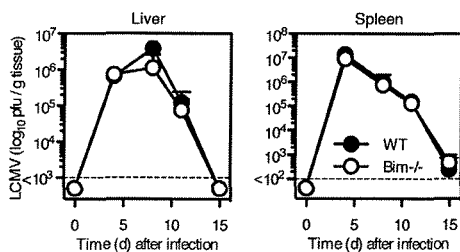


FIGURE 3. Similar kinetics of virus clearance in WT and Bim^{-/-} mice. WT and Bim^{-/-} mice were infected *i.v.* with 1×10^6 PFU LCMV-WE. At the indicated time points, virus titers (PFU/g tissue) were determined in liver and spleen of the mice. The dashed line indicates the detection limit. Mean \pm SD of three to five mice/group and time point are shown. The experiment was repeated three times, yielding similar results.

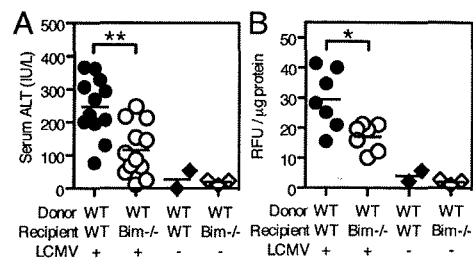


FIGURE 4. Crucial role of Bim expression in hepatocytes during the development of T cell-mediated liver damage. WT \rightarrow WT and WT \rightarrow Bim^{-/-} chimeras were infected with 1×10^6 PFU LCMV-WE *i.v.*, and liver damage was analyzed 11 d postinfection. *A*, ALT levels in the serum of the mice. *B*, Caspase-3 activity (relative fluorescent units/ μ g protein) in liver lysates. The experiment was repeated three times, yielding similar results. * $p < 0.05$, ** $p < 0.01$, unpaired Student *t* test.

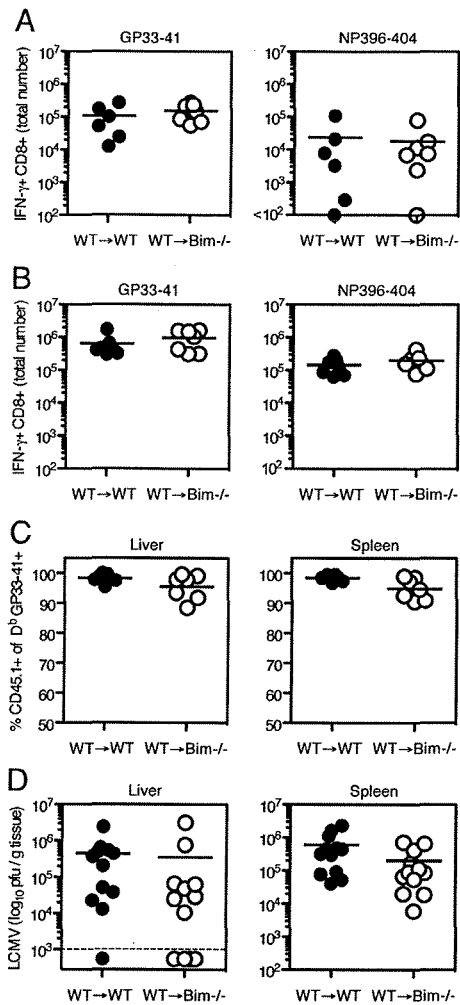


FIGURE 5. Comparable LCMV-specific CTL response and virus titers in WT→Bim^{-/-} and WT→WT chimeras. WT→WT and WT→Bim^{-/-} chimeras were infected with 1×10^6 PFU LCMV-WE i.v. and analyzed 11 d postinfection. Total numbers of functional CD8⁺ T cells specific for the viral epitopes GP33-41 and NP396-404, as determined by intracellular IFN- γ staining of in vitro-restimulated T cells isolated from liver (A) and spleen (B). C, Extent of chimerism of CD8⁺ T cells specific for the viral epitope GP33-41 isolated from liver and spleen of the mice, as determined by staining with D^bGP33-41 MHC class I tetramers. Values represent the percentage of donor-derived (CD45.1⁺) cells of D^bGP33-41⁺ CD8⁺ T cells. D, Virus titers (PFU/g tissue) in liver and spleen of the mice. The dashed line indicates the detection limit. The experiments were repeated three times, yielding similar results.

Bim^{-/-}→WT chimeras compared with the WT→WT mice. Furthermore, the analysis of the chimerism (Fig. 6C) confirmed that almost all of these virus-specific T cells in spleen and liver originated from the transferred bone marrow, with no differences between WT→WT and Bim^{-/-}→WT chimeras. Despite the increased virus-specific CTL response in Bim^{-/-}→WT mice, virus titers in liver and spleen of these mice did not differ from those in the WT→WT chimeras (Fig. 6D). To define whether persistence of activated T cells would affect the severity of liver pathology in Bim^{-/-}→WT chimeras, we analyzed serum transaminase activity (Fig. 7A) and caspase-3 activity in liver lysates (Fig. 7B). A trend, although statistically not significant, toward increased liver damage in Bim^{-/-}→WT chimeras compared with WT→WT chimeras was observed.

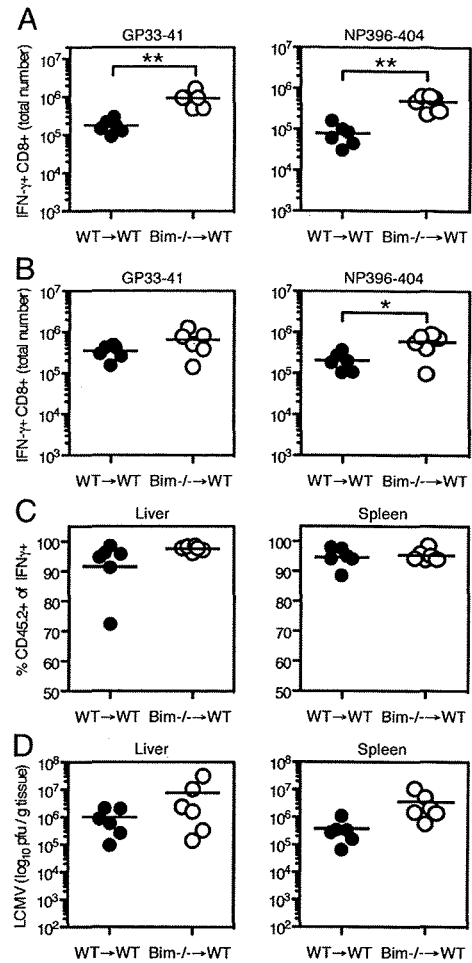


FIGURE 6. Increased LCMV-specific CTL response but normal virus titers in Bim^{-/-}→WT chimeras compared with WT→WT mice. WT→WT and Bim^{-/-}→WT chimeras were infected with 1×10^6 PFU LCMV-WE i.v. and analyzed 10 d postinfection. Total numbers of functional CD8⁺ T cells specific for the viral epitopes GP33-41 and NP396-404, as determined by intracellular IFN- γ staining of in vitro-restimulated T cells isolated from liver (A) and spleen (B). C, Extent of chimerism of CD8⁺ T cells specific for the viral epitope GP33-41 isolated from liver and spleen of the mice, as determined by intracellular IFN- γ staining of in vitro-restimulated T cells. Values represent the percentage of donor-derived (CD45.2⁺) cells of IFN- γ ⁺ CD8⁺ T cells. D, Virus titers (PFU/g tissue) in liver and spleen of the mice. The experiments were repeated four times, yielding similar results. * $p < 0.05$, ** $p < 0.01$, unpaired Student t test.

Taken together, these experiments indicated that a loss of Bim in the hematopoietic compartment might have the potential to partially exacerbate the severity of the disease. However, in the liver of LCMV-infected Bim^{-/-} mice, this effect was overcompensated for by the reduced sensitivity of Bim^{-/-} hepatocytes to T cell-induced apoptosis, resulting in attenuated liver damage in Bim^{-/-} mice upon LCMV infection.

Discussion

Bim has been shown to play a central role in T cell homeostasis during viral infection, as well as in the regulation of hepatocyte apoptosis (13–18). To date, the dual role of Bim in hepatocyte apoptosis and in the maintenance of the lymphoid homeostasis has been analyzed in a strictly separated manner.

To our knowledge, ours is the first study to analyze the role of Bim in a highly dynamic system of T cell-mediated liver disease

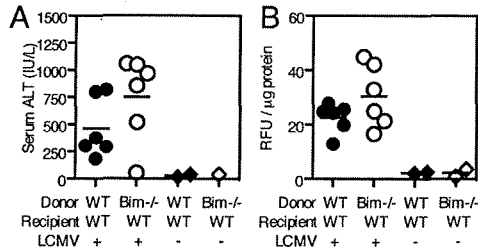


FIGURE 7. Loss of Bim in the lymphoid compartment has the potential to increase the severity of the disease. WT→WT and Bim^{-/-}→WT chimeras were infected with 1×10^6 PFU LCMV-WE i.v., and liver damage was analyzed 10 d postinfection. *A*, ALT levels in the serum of the mice. *B*, Caspase-3 activity (relative fluorescent units/ μ g protein) in liver lysates. The experiment was repeated four times, yielding similar results.

in which it is involved in the regulation of apoptosis in hepatocytes and lymphocytes. Liver disease in LCMV-infected mice is predominantly caused by virus-specific CTL, inducing hepatocyte cell death (6, 29). Hence, the outcome of the disease is the consequence of the sensitivity of hepatocytes to apoptosis-inducing triggers, and it critically depends on the numbers and activity of liver-infiltrating CTL, as well as the kinetics of viral spreading and clearance.

By performing bone marrow chimera experiments, we were able to specifically dissect the respective roles of Bim expressed in the lymphoid compartment versus hepatocytes during the pathogenesis of T cell-mediated liver disease in mice infected with LCMV. The analysis of the respective roles of Bim in both compartments revealed that it plays a dual role during the development of T cell-mediated liver damage. Although Bim deficiency in the hepatocytes led to markedly reduced liver damage upon infection with LCMV, loss of Bim in the lymphoid compartment had the potential to increase the severity of the disease. Interestingly, when both effects were combined in Bim^{-/-} mice, the protective effect of Bim deficiency in the hepatocytes was stronger than the deteriorating effect of Bim deficiency in the lymphoid compartment, leading to a partial protection of Bim^{-/-} mice from T cell-mediated hepatitis.

Our results demonstrated that, although Bim plays a critical role during the pathogenesis of T cell-mediated liver disease, its absence in hepatocytes did not affect the clearance of the virus, suggesting that the intracellular apoptotic mechanisms involved in the elimination of virus-infected cells differ from those involved in the induction of liver injury.

It was demonstrated that cytotoxic mechanisms implicated in the elimination of virus-infected cells do not completely overlap with those involved in the induction of liver pathology. Several reports demonstrated that mouse strains deficient in one or more components of the granules exocytosis pathway (i.e., perforin and granzymes A and B) are impaired in the recovery from primary viral infection. These observations, combined with the fact that Fas^{-/-} mice seem to recover from LCMV infection with similar kinetics as WT mice, suggested that viral clearance is solely dependent on the perforin-granzymes exocytosis pathway (7, 10). In contrast, liver disease was shown to be the consequence of the interplay of the cytotoxic pathways, the granule exocytosis pathway, and the death ligand pathway, in particular via FasL/Fas. The essential role of Fas during the development of CD8⁺ T cell-mediated liver damage was mainly demonstrated by adoptive transfer of transgenic CD8⁺ T cells (31, 32). In accordance with this, we also observed a profound reduction in liver damage in LCMV-infected *gld* mice compared with WT animals, whereas no

difference was observed in virus clearance between WT and *gld* mice (C. Lauer and N. Corazza, unpublished observations).

We previously showed that Bim is a key regulator of Fas-mediated apoptosis, resulting in an impressive protection of Bim^{-/-} mice from Fas-mediated liver damage upon treatment with an agonistic anti-Fas Ab (17). In contrast to normal cells, in which the activation of the Fas pathway directly activates the caspase cascade and, subsequently apoptosis, hepatocytes require the amplification of the signal via the mitochondria. In this respect, it was demonstrated that the proapoptotic molecule Bid plays an essential role, and Bid^{-/-} mice are largely resistant to FasL-induced hepatocyte apoptosis (22, 23). However, our results suggested that the Fas-signaling pathway in this cell type is even more complex and is critically controlled by TRAIL, Jun kinase, and Bim (17). This additional control of apoptosis may add a further safeguard for the protection of these cells from accidental death induction and liver damage.

Thus, the reduced liver damage in Bim^{-/-} mice is most probably a consequence of a reduced susceptibility of Bim^{-/-} hepatocytes to T cell-induced apoptosis via the FasL/Fas pathway, and the residual caspase-3 activity measured in Bim^{-/-} mice could be due, at least in part, to the activation of the caspase cascade via the granule exocytosis pathway (33). This notion is supported by the fact that Bid^{-/-} mice were also partially protected from LCMV-induced liver disease (data not shown) and the observation that liver damage was similarly reduced in the chimeras deficient for Bim solely in nonhematopoietic cells (WT→Bim^{-/-}), although the antiviral immune response was equal to that of the WT control chimeras (WT→WT), which exhibited a strong induction of hepatitis.

In line with previous work showing increased numbers of antiviral CTL in Bim^{-/-} mice during acute and chronic infections with the LCMV strains Armstrong and Clone 13 (16), we also observed increased numbers of virus-specific T cells and impaired retraction of activated T cells in Bim^{-/-} mice. Although Bim^{-/-} mice accumulated elevated numbers of virus-specific T cells in the liver, the severity of the disease was reduced compared with WT mice. This indicated that the number of activated T cells in Bim^{-/-} mice did not directly affect the development of liver disease. However, this could be due to an impaired capacity of Bim^{-/-} T cells to mount a proper immune response after virus infection. Although Ludwinski et al. (30) postulated that Bim^{-/-} CD4⁺ T cells have severe defects in activation and cytokine production, protecting these mice against experimental allergic encephalomyelitis and type 1 diabetes, our detailed analysis of the antiviral CD8⁺ T cell response in LCMV-infected WT and Bim^{-/-} mice did not reveal any signs of functional impairment of T cells from liver and spleen of Bim^{-/-} mice. Thus, we can exclude that Bim^{-/-} mice are protected from LCMV-induced hepatitis as the result of an impaired CTL response. This finding is further supported by the observation that chimeras specifically deficient for Bim in the hematopoietic compartment (Bim^{-/-}→WT) showed a clear tendency toward more pronounced liver damage than did WT controls (WT→WT) postinfection with LCMV.

Because the outcome of liver disease strictly depends on the interplay between propagation of the virus in hepatocytes and activation of virus-specific T cells, one could argue that the presence of increased numbers of activated T cells in Bim^{-/-} mice might lead to an accelerated virus clearance compared with WT mice, which then could account for the partial protection from LCMV-induced liver disease in Bim^{-/-} mice. Despite the increased numbers of virus-specific CTL in Bim^{-/-} mice, we were not able to detect significant differences in the kinetics of viral expansion and clearance between WT and Bim^{-/-} mice. This

observation is in accordance with previous work showing comparable viral kinetics in the livers of WT and Bim^{-/-} mice during chronic infections with LCMV Clone 13 (16). Thus, it is very unlikely that the reduction in the disease is predominantly caused by altered kinetics of viral expansion and clearance in the livers of Bim^{-/-} mice.

Although we showed that Bim^{-/-} mice are partially protected from T cell-mediated liver damage, most likely due to the lack of Bim in hepatocytes, our work also specifically analyzed the respective role of Bim expressed in the lymphoid compartment during the development of T cell-mediated hepatitis. Chimeras specifically deficient for Bim in the hematopoietic compartment (Bim^{-/-}→WT) showed a tendency toward increased disease severity compared with WT controls (WT→WT). Because CTL are the main effector cells responsible for the induction of liver damage in LCMV-infected mice (6, 29), the partial worsening of the disease in Bim^{-/-}→WT chimeras can be attributed to the significantly increased numbers of antiviral CTL in the liver. Nevertheless, the consequence of Bim deficiency in T cells for the course of the disease is minor compared with the effect of a lack of Bim in parenchymal cells. If Bim is missing in both effector and target cells, the effect of Bim in hepatocytes overcomes and masks the disadvantageous effects of the accumulation of activated antiviral lymphocytes.

Taken together, our findings may have important implications for the treatment of patients suffering from other forms of viral hepatitis, such as hepatitis B or C. Thus, a specific targeting of Bim in hepatocytes may represent an attractive therapeutic strategy for significantly reducing liver damage without negatively affecting the T cell response and clearance of the virus in these patients.

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Disclosures

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