Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling

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The liver is known to be a classical immunoprivileged site with a relatively high resistance against immune responses. Here we demonstrate that highly activated liver-specific effector CD8+ T cells alone were not sufficient to trigger immune destruction of the liver in mice. Only additional innate immune signals orchestrated by TLR3 provoked liver damage. While TLR3 activation did not directly alter liver-specific CD8+ T cell function, it induced IFN-α and TNF-α release. These cytokines generated expression of the chemokine CXCL9 in the liver, thereby enhancing CD8+ T cell infiltration and liver disease in mice. Thus, nonspecific activation of innate immunity can drastically enhance susceptibility to immune destruction of a solid organ.

Introduction

The liver represents a so-called immunoprivileged site according to its relative resistance against organ rejection after transplantation (1). This is in contrast to the rapid rejection of purified transplanted allogeneic hepatocytes in vivo (2). Also, autoimmune hepatitis due to attack by B and T cells is a relatively rare manifestation of autoimmune disease (3, 4). Interestingly, diagnostic markers of autoimmune hepatitis such as antimitochondrial antibodies are also found in healthy people (5). Together, these findings suggest that there exist mechanisms protecting immune attack against this solid organ. Some studies of immune reactivity against components of solid peripheral organs (such as pancreatic islet cells or salivary gland or thyroid antigens) indicate that self-reactive effector T or B cells alone may not be sufficient for disease induction without additional “inflammatory signals” being required for efficient induction of disease (6). Consistent with clinical observations, results of studies in animal models suggest that naive liver-reactive T cells ignore the liver antigen (7) or become tolerized within the liver (8, 9). Inflammation (e.g., infection with systemic bacteria) may upregulate costimulatory molecules in the liver and can break this tolerance (10, 11).

Besides priming of an adaptive immune response, viruses can promote inflammatory signals through their ability to activate the innate immune system via TLRs (12). Recently it was shown that activation of TLR3 or TLR7 (which recognize double-stranded and single-stranded RNA [dsRNA and ssRNA], respectively) promotes autoimmunity in mice exhibiting high frequencies of functional autoreactive CD8+ T cells. Disease onset/progression closely correlated with IFN-α production (13, 14), suggesting that TLR-induced production of proinflammatory cytokines such as IFN-α and TNF-α may influence the development of autoimmune.

Here we analyzed the requirements of autoimmune liver destruction in a mouse model where the lymphocytic choriomeningitis virus–glycoproteinα1–60 (LCMV-glycoproteinα1–60) is expressed in the liver as a transgene under the control of the mouse albumin promoter (Alb-1 mice) (7).

Results

Requirement of TLR3 signaling for destructive autoimmunity. We injected splenocytes from TCR-Tg 318 mice in which 50% of the CD8+ T cells express a TCR recognizing the LCMV-glycoproteinα13–41 epitope presented on H-2Db MHC class I molecules into Alb-1 mice or control C57BL/6 mice. Without further treatment, those T cells virtually ignored the antigen in the liver and did not further expand, with no signs of liver damage (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28349DS1) (7). Infection of such recipient mice with 200 PFU of the LCMV strain WE (LCMV-WE) caused massive expansion of gp33-specific CD8+ T cells, as detected by gp33 tetramer binding (7) (Figure 1B), and resulted in liver injury as determined by increased serum activity of alanine amino-transferase (ALT) and high bilirubin serum concentration (7) (Figure 1B). In this experimental model, LCMV induces both a strong adaptive T cell response against liver antigens and a strong innate immune response. To separately analyze the influence of this innate activation, we immunized mice with gp33 peptide together with the TLR9 ligand CpG (ODN-1826). This treatment resulted in a similar expansion of tetramer-positive T cells but only a small increase in ALT and undetectable bilirubin in the serum (Figure 1C). CpG (ODN-1826) is a ligand for TLR9, and its binding to TLR9 results in DC activation and upregulation of costimulatory molecules (15) but does not induce massive IFN-α production. In contrast, LCMV, CpG (ODN-2216), and the RNA repeat polyinosinic-polycytidylic acid [poly(I:C)] activate innate immunity and result in production of IFN-α (16, 17). We therefore tested whether poly(I:C) could mimic the ability of replicating LCMV in promoting liver disease. Mice adoptively transferred with autoreactive TCR-Tg 318 CD8+ T cells were immunized on days 0 and 4 with gp33 plus CpG (ODN-1826). Peptide priming without...
CpG (ODN-1826) also expanded TCR-Tg CD8+ T cells (data not shown); however, gp33 plus CpG (ODN-1826) yielded maximal T cell responses. After priming of TCR-Tg CD8+ T cells, 1 group of mice was additionally treated with poly(I:C) on day 7. Administration of poly(I:C) resulted in a rapid elevation of ALT and bilirubin in the serum, indicating hepatocyte destruction (Figure 1C). In separate experiments, peptide-treated mice were further treated with the IFN-α-inducing CpG (ODN-2216) on day 7. This activation of TLR9 resulted in a mild but significant increase in ALT, suggesting that other TLRs may also be able to trigger hepatitis (Supplemental Figure 2).

Induction of type I interferons can result in upregulation of MHC class I molecules on pancreatic islet cells, which correlates with onset of autoimmune diabetes (14, 18). In contrast, hepatocytes have been described as constitutively expressing abundant surface MHC class I (19), which was not further affected by interferon (Supplemental Figure 3). Thus, antigen presentation on MHC class I molecules did not explain the observed differences in immunologically induced liver destruction.

**TLR3-independent CD8+ T cell effector function.** Our findings might be explained by enhanced CD8+ T cell function as a consequence of TLR3 engagement. To investigate this possibility, we transfused naive C57BL/6 mice with 106 gp33-specific TCR-Tg CD8+ T cells and immunized recipient mice with gp33 plus CpG (ODN-1826) on days 0 and 4, with or without additional treatment with poly(I:C) on day 7. Thereafter, autoreactive CD8+ T cells were identified by tetramer binding and their expression of various activation markers analyzed by FACS analysis. First we analyzed the early activation markers CD25 and CD69. There was a weak expression of CD25 in both groups (Figure 2A). During treatment with poly(I:C), a subset of gp33-specific CD8 T cells upregulated CD69, known to be a target molecule of type I interferon (20). CD8+ T cells from either control or poly(I:C)-treated mice showed no difference in other activation markers (Figure 2B), nor were any differences observed in the ability of these cells to produce IFN-γ or to express granzyme B following in vitro restimulation with gp33 peptide (Figure 2C). To examine whether poly(I:C) affected effector function of CD8+ T cells, we performed in vitro and in vivo killing assays. Both mice primed with peptide and CpG (ODN-1826) alone and mice additionally treated with poly(I:C) generated effector CD8+ T cells capable of lysing gp33-labeled target cells in vivo and in vitro (Figure 2, D and E). Staining of the effector protein granzyme B directly ex vivo revealed no significant difference between tet-gp33+ T cells treated with and without poly(I:C) (Figure 2F). Recently it has been shown that CD8+ T cells can express FoxP3 and have regulatory function (21). Peptide priming might also induce a regulatory status in gp33-specific CD8 T cells. However, we did not observe expression of FoxP3 in gp33-specific cells (Supplemental Figure 4). We found that in both C57BL/6 and Alb-1 mice, CD4+CD25+ T cells were present in the liver after LCMV infection (Supplemental Figure 4). Therefore, we examined whether peptide treatment followed by poly(I:C) treatment had an effect on frequencies of Tregs. While peptide treatment did not result in any significant change in CD4+ Tregs, we have monitored a slight increase in CD25+FoxP3+CD4+ T cells after poly(I:C) treatment in the liver. We therefore concluded that TLR3 signals did not induce hepatitis by directly altering liver-specific CD8+ T cell function or by downregulation of Treg subsets.

**Effects of TLR3 ligation on liver gene expression.** To determine factors responsible for poly(I:C)-induced autoimmunity, we performed...
gene array experiments. Thirty-eight genes that were likely to influence autoimmune disease were selected for analysis. These genes were involved in cell survival and apoptosis, signaling, T cell migration, or antiviral activity. RNA analysis was performed on liver samples taken from naive or poly(I:C)-treated C57BL/6 mice as described in Methods. Only one gene out of the selected 38, CXC chemokine ligand 9 (Cxcl9), showed an increased expression of greater than 10-fold following poly(I:C) treatment (Table 1). The expression of several other genes was upregulated by a factor of 2- to 10-fold. These included the signaling molecule interferon regulatory factor 7 (Irf7), antiviral molecules Mx-1 and Usp18, adhesion molecules Vcam-1 and Icam-1, the inflammatory cytokine Tnf-α, the chemokine Cxcl10, and the antiinflammatory cytokine Il-10 (Table 1). In addition, we found more than 2-fold upregulation of Il-7, Fas-ligand, and Igfbp5.

Poly(I:C) induced upregulation of mRNA for Cxcl9, Vcam-1, and Icam-1 as determined by quantitative RT-PCR of CDNA generated from liver tissue (Figure 3A). Additional immunohistological analysis indicated that ICAM-1 was expressed constitutively in the liver in naive animals (data not shown). In contrast, expression of VCAM-1 or CXCL9 was limited in livers of naive mice. However, expression was detectable following poly(I:C) treatment (Figure 3B). The ability to upregulate CXCL9 expression was TLR3 dependent. This was confirmed by poly(I:C) treatment of Tlr3−/− mice, which exhibited reduced upregulation (Figure 3B). This effect was mediated through TLR3 ligation on bone marrow–derived cells but not on hepatocytes, as Tlr3−/− bone marrow transferred into irradiated TLR3-competent mice (Tlr3−/−→BALB/c) yielded a reduced Cxcl9 expression similar to that in control Tlr3−/− mice. TLR3-competent bone marrow transferred into irradiated Tlr3−/− mice (BALB/c→Tlr3−/−) resulted in Cxcl9 expression similar to that in wild-type control mice (Figure 3B). Loss of TLR3 did not completely abolish poly(I:C)-induced Cxcl9 expression, possibly due to the activation of other molecules, e.g., retinoic acid–inducible gene 1 (RIG-I), which can recognize dsRNA (22).

To analyze the role of TLR3-induced cytokines such as interferons and TNF-α in upregulation of Cxcl9 and Vcam-1, mice deficient in receptors for type I or type II interferons or TNF-α were employed. Both IFN-α and TNF-α were produced following poly(I:C) administration (Table 1 and Supplemental Figure 5), and the absence of receptors for either factor abolished the ability of poly(I:C) to induce Cxcl9 upregulation (Figure 3C). No role for type II interferons (IFN-γ) was found, since mice deficient in interferon type II receptors (Ifngr−/−) revealed responses comparable to those in wild-type mice following poly(I:C) treatment (Figure 3C). Vcam-1 expression appeared to be regulated solely by TNF-α, with no obvious role observed for either type I or type II interferons (Figure 3D). Taken together, the results suggest that TLR3 engagement on bone marrow–derived cells changed expression of T cell–attracting molecules.

Enhanced CD8+ T cell recruitment into the liver by TLR3 ligation. The upregulation of genes involved in T cell homing prompted us to investigate whether poly(I:C) treatment resulted in increased T cell migration into the liver in vivo. It was likely that CXCL9 could attract memory T cells, as most of the CD44+CD8+ T cells in C57BL/6 mice expressed its receptor CXCR3 (Figure 3E). Treatment with poly(I:C) diminished staining of CXCR3 on memory
Table 1
mRNA profile of livers 24 hours after treatment with poly(I:C)

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<th>Fold difference</th>
<th>Homing/ inflammation</th>
<th>T cell survival</th>
<th>Apoptotic</th>
<th>Antia apoptotic</th>
<th>Signaling</th>
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<td>2–10</td>
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<td>Il-7</td>
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<tr>
<td>0.1–0.5</td>
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Three of 6 naive C57BL/6 mice were treated with 500 μg poly(I:C). After 24 hours, livers were removed, and mRNA was isolated. For every individual liver, RT-PCR assay was performed to determine expression levels of genes involved in T cell homing, inflammation, T cell survival, apoptosis, signaling, and antiviral activity, which were normalized to the housekeeping gene 18S RNA (see Methods). Thirty-eight of 48 genes showed measurable gene expression. The fold difference between the median expression in 3 naive mice and the median expression in 3 poly(I:C)-treated mice was calculated. *Ccam-1 and Icam-1 were measured separately and were not included on a gene array (raw data are shown in Figure 3A). All other genes were analyzed in an RT-PCR array. Analyzed genes were sorted according to the expression difference between naive and poly(I:C)-treated mice and designated as: “highly increased” (10- to 100-fold); “slightly increased” (2- to 10-fold); “not affected” (0.5- to 2-fold); and “slightly decreased” (0.1- to 0.5-fold). Igfbp5, insulin-like growth factor binding protein 5; Ifn-7, interferon regulatory factor 7.

CD8+ T cells (Figure 3E), suggesting binding of its ligand CXCL9 and consequent downregulation of CXCR3 (23). To analyze homing of gp33-specific cells, we transferred 10^6 TCR-Tg 318 splenocytes into C57BL/6/2 recipients, followed by immunization with gp33 on days 0 and 4 together with CpG, with or without additional poly(I:C) treatment on day 7. Again, poly(I:C) treatment induced an upregulation of Cxcl9 and Vcam-1, suggesting that peptide treatment had no influence on CXCL9 and VCAM-1 upregulation in the liver (Figure 3, F and G). After peptide priming, most gp33-specific cells were found to express CXCR3, which was also downregulated following poly(I:C) treatment (Figure 3H). gp33-specific CD8+ T cells were recruited from the blood twelve hours after poly(I:C) treatment (Figure 3I) and were found within the increased numbers in Alb-1 mice (Figure 3J). Together these data suggest that an innate immune activation by poly(I:C) recruits CD8+ T cells into the liver, which subsequently leads to liver damage.

Role of innate signals in virus-induced hepatitis. To investigate the role of IFN-α in virus-induced hepatitis, we crossed the Alb-1 onto Ifnar–/– mice. A total of 10^7 TCR-Tg 318 splenocytes were transferred on day -1 into Alb-1 Ifnar–/– mice or littermate control mice (Alb-1 Ifnar–/– or Alb-1 Ifnar–/–), followed by infection with 200 PFU LCMV on day 0. Lack of IFN-α signaling resulted in an inability to clear the virus (Figure 4A), and as a consequence all mice died of generalized immune pathology by day 7 (data not shown). The transferred CD8+ T cells expressing type I interferon receptor could raise a functional T cell response (24) (Figure 4B). In littermate control mice, immunohistologically demonstrated T cell infiltration in the portal in liver areas correlated with increased serum bilirubin concentrations, reflecting liver damage. Despite high viral load in the liver, LCMV-infected Alb-1 Ifnar–/– mice demonstrated a reduced infiltration of T cells into portal liver areas (Figure 4A), correlating well with reduced Cxcl9 expression within the liver of Ifnar–/– mice (Figure 4D). The lack of T cell infiltration into the portal area of the liver also correlated with reduced serum bilirubin concentrations (Figure 4C).

We next tested whether T cell migration into the liver could be inhibited while preserving the beneficial antiviral function of IFN-α. For this purpose, we used pertussis toxin (PTX), which acts as a inhibitor of all G protein–coupled chemokine receptors (23). PTX-treated, LCMV-infected mice no longer displayed CD8+ T cell accumulation within the liver (Figure 4E), did not develop liver disease (Figure 4F), and showed limited virus antigen in the liver (Figure 4G). To rule out the possibility that PTX treatment affected CD8+ T cell priming, we investigated the ability of CD8+ T cells isolated from control or PTX-treated mice to lyse gp33-loaded target cells in vitro. PTX treatment did not alter the CTL activity of gp33-specific CD8+ T cells (Figure 4G). Analysis of gp33-specific CD8+ T cells indicated that PTX-treated Alb-1 mice contained more circulating cells expressing high levels of the IL-7 receptor α (IL-7Rα) chain (Figure 4H). Since IL-7Rα downregulation is indicative of recent antigenic stimulation (25), these data imply that gp33-specific CD8+ T cells in Alb-1 mice receiving PTX did not interact with their antigen, likely resulting from the lack of migration into the liver.

Discussion
The immunoprivileged status of the liver may be built of more than one mechanism. Immune destruction of the liver requires first the priming of liver-specific T cells. These activated T cells then have to migrate into the target organ, where autoimmunity finally occurs. T cell priming has been proposed to be controlled by costimulatory factors, innate immune signals, and Tregs (26, 27). The findings presented here strongly suggested that there exists a second line of immunoprivilege in the liver. Usually, the liver does not attract liver-specific T cells because chemokines are expressed at low levels in the liver. Thus, even though highly active liver-specific T cells were found at high frequencies in the blood, few migrated into the liver. Our present study further demonstrates that proinflammatory signals derived from ligation of TLR3 can lead to homing of CD8+ T cells to the liver with subsequent enhancement of liver disease. The mechanism by which TLR3 signaling promoted disease involved IFN-α- and TNF-α-dependent upregulation of genes and their products involved in T cell homing and migration. This pathogenic mechanism may explain earlier observations that autoimmunity, including hepatitis, was promoted by Listeria infection (10).
Our findings are in accordance with previous observations indicating that expression of CXCR3 on T cells is required for the onset of autoimmune diabetes (28) and implicate TLR-induced chemokine production as a mechanism by which inflammation can be generated, resulting in exacerbated hepatitis (29, 30).

The most dramatic effect of TLR ligation on liver cells was the upregulation of CXCL9, which was dependent on production of type I interferon and TNF-α. These findings suggest that treatment of chronic HCV infection with interferon (31) may enhance infiltration of T cells in the liver and that although this may help to reduce viral load, it could aggravate liver destruction and cirrhosis (32) or accelerate the course of recurrent disease after transplantation.

We found that PTX-mediated inhibition of chemokine signaling provoked infiltration of only a small number of T cells and strongly reduced liver enzyme release and serum bilirubin concentrations. Virus was limited but still detectable. This finding correlates with a genetic polymorphism characterized by reduced chemokine signaling and chronic HCV infection in humans (33). From this data we would suggest that the inhibition of chemokine signaling in combination with interferon therapy might be a possible strategy for the treatment of chronic infection with HBV or HCV or disease recurrence following liver transplantation.

Tregs have been proposed to play an important role in the prevention of autoimmune disease and inflammatory processes (34). Here we have demonstrated a rise in the number of CD25+FoxP3+CD4+ T cells after LCMV infection in Alb-1 mice (Supplemental Figure 4). Also, poly(I:C) treatment slightly increased the number of Tregs in the liver (Supplemental Figure 4). While the increase in Tregs...
cannot explain the induction of hepatitis, it might be required for the observed downregulation of disease after day 7.

TNF-α–induced CXCL9 may play a role in human hepatitis, as there is a direct link between a mutation in the TNF-α locus and autoimmune hepatitis (35). These clinical observations together with our data suggest that inhibition of chemokines may prove an interesting therapeutic approach against immune hepatitis in humans.

In conclusion, the presented experiments revealed that highly activated CD8+ T cells can “peacefully” coexist with hepatocytes expressing the relevant autoantigen without causing overt disease. Engagement of TLR3 was required to break this immunoprivileged state of the liver and converted autoreactivity into overt autoimmune disease and organ destruction. Taken together, our results have identified TLR3 as one important mechanism of solid organ immune privilege.

**Methods**

Mice and viruses. LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. Virus titers were measured using a plaque-forming assay as described previously (36). Mice were infected with 200 PFU LCMV-WE. Mice transgenic for a TCR recognizing LCMV-glycoprotein 33–41 (LCMV-gp33/H-2Db–specific TCR, 318 mice), Alb-1 mice (7), Myd88−/−, and Tnfr1−/− mice were maintained on the C57BL/6 genetic background, while Ifngr−/− mice were of the 129Sv background. Ifnar−/− mice were of the 129Sv or of the C57BL/6 background (for crossings to Alb-1). Tlr3−/− mice were on the BALB/c background. For generation of bone marrow chimera, recipient mice were irradiated with 9.50 Gy on day –1. On day 0, 10^7 bone marrow cells were transferred i.v., and mice were used for experiments 7 weeks later. All experiments were performed with animals housed in ventilated cages. Animal experiments were approved and authorized by...
cited literature references.