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Plasmodium berghei Infection in Mice Induces Liver Injury by an IL-12- and Toll-Like Receptor/Myeloid Differentiation Factor 88-Dependent Mechanism[1]

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Malaria, caused by infection with *Plasmodium* spp., is a life cycle-specific disease that includes liver injury at the erythrocyte stage of the parasite. In this study, we have investigated the mechanisms underlying *Plasmodium berghei*-induced liver injury, which is characterized by the presence of apoptotic and necrotic hepatocytes and dense infiltration of lymphocytes. Although both IL-12 and IL-18 serum levels were elevated after infection, IL-12-deficient, but not IL-18-deficient, mice were resistant to liver injury induced by *P. berghei*. Neither elevation of serum IL-12 levels nor liver injury was observed in mice deficient in myeloid differentiation factor 88 (MyD88), an adaptor molecule shared by Toll-like receptors (TLRs). These results demonstrated a requirement of the TLR-MyD88 pathway for induction of IL-12 production during *P. berghei* infection. Hepatic lymphocytes from *P. berghei*-infected wild-type mice lysed hepatocytes from both uninfected and infected mice. The hepatocytotoxic action of these cells was blocked by a perforin inhibitor but not by a neutralizing anti-Fas ligand Ab and was up-regulated by IL-12. Surprisingly, these cells killed hepatocytes in an MHC-unrestricted manner. However, CD1d-deficient mice that lack CD1d-restricted NK T cells, were susceptible to liver injury induced by *P. berghei*. Collectively, our results indicate that the liver injury induced by *P. berghei* infection of mice induces activation of the TLR-MyD88 signaling pathway which results in IL-12 production and activation of the perforin-dependent cytotoxic activities of MHC-unrestricted hepatic lymphocytes. *The Journal of Immunology,* 2001, 167: 5928–5934.

Malaria, caused by infection with pathogenic *Plasmodium* spp., is a life-threatening disease that kills three million people per year worldwide (1, 2). *Plasmodium* has a complicated life cycle in the intermediate host that includes mice and human. The protozoa induce stage-specific pathological changes, including asymptomatic changes during the liver stage and symptomatic changes during the erythrocyte stage (3). Indeed, human and mice suffer from liver injury after infection with the fatal strains *Plasmodium falciparum* and *Plasmodium berghei* (3), respectively (4, 5). Because erythrocyte-stage *P. berghei* (merozoites) do not parasitize liver parenchymal cells, the mechanism of *P. berghei*-induced liver injury must be distinct from that of liver injury that is caused by Ag-specific CTL following hepatitis B virus infection (6). Instead, the liver injury induced by *P. berghei* is most likely to be caused by the local production of cytokines that activate lymphocytes that reside in the liver.

IL-12 and IL-18 are potent proinflammatory cytokines (7, 8). IL-18 is produced as a biologically inactive precursor and, upon appropriate stimulation, becomes active by cleavage with caspase-1 or a caspase-1-like enzyme (8–11). In some infectious diseases, IL-12 and/or IL-18, although frequently critical for expelling of the microbes, are critically important for the development of immunopathological changes in host target organs (7, 8). For example, LPS, a constituent of Gram-negative bacterial cell walls, can cause liver injury and lethal shock in humans and mice with an important role for IL-12 and IL-18 (12, 13). A recent study revealed that administration of neutralizing anti-IL-12 Abs protects mice from *P. berghei*-induced liver injury without affecting clearance of the parasite (5).

The Toll-like receptor (TLR)[3] family represents a set of essential surface molecules for recognition of microbe-derived products to activate host innate immunity (14, 15). Following activation of TLRs by their corresponding ligands, the cytoplasmic domain of TLRs recruits a common adaptor molecule, myeloid differentiation factor 88 (MyD88), to activate a common signaling pathway, leading to production of cytokines including IL-12 (14–16). Although both bacterial and fungal components such as LPS and zymosan can activate this signaling pathway, it remains unclear whether microbial infection initiates activation of the TLR-MyD88 pathway in the host (15–19). Our recent study clearly demonstrated that *Listeria monocytogenes* infection of mice induces IL-12 production by a TLR-MyD88-dependent pathway (17). The role of the TLR-MyD88 signaling pathway for production of cytokines and liver injury caused by *P. berghei* is unknown.

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; DKO, double-knockout mice; Fas L, Fas ligand; mFas L, murine Fas ligand; CMA, concanamycin A; GPT, glutamic-pyruvic transaminase; WT, wild type.

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In this study, we have investigated the mechanism by which *P. berghei* causes liver injury in mice. Although both IL-12 and IL-18 serum levels were elevated after infection, only IL-12-deficient mice were resistant to liver injury. Furthermore, MyD88-deficient mice were resistant to liver injury with production of IL-18 but not IL-12. Therefore, we conclude that protozoa can activate the TLR-MyD88 signaling pathway to induce pathological changes in the host. In addition, hepatic lymphocytes from *P. berghei*-infected mice gained the capacity to kill normal hepatocytes in a perforin-dependent and MHC-unrestricted manner. These findings suggest the involvement of an unusual killer-cell mechanism in *P. berghei*-induced liver injury.

Materials and Methods

Mice

Female C57BL/6 mice (6–8 wk old), C57BL/6 *lpr/lpr* mice (6–8 wk old), C3H/HeJ, and C3H/HeN (6–8 wk old) were purchased from SLC (Shizuoka, Japan). Female SCID mice (6–8 wk old) and BALB/c mice (6–8 wk old) were purchased from CLEA Japan (Osaka, Japan). Female perforin-deficient mice on a C57BL/6 background (10 wk old) were kindly provided by Dr. H. Yagita (Juntendo University, Tokyo, Japan). IL-18-deficient mice were backcrossed onto the C57BL/6 background and F_8 (female, 6–8 wk old) animals were used in this study (20). IL-12-deficient mice on the C57BL/6 background were kindly provided by Dr. J. Magram (Hoffmann-La Roche, Nutley, NJ) and female mice (6–8 wk old) were used for this study (21). MyD88-deficient mice were backcrossed onto the BALB/c background and F_8 (female, 6–8 wk old) animals were used (22). TLR6-deficient mice (8–11 wk old) were backcrossed with C57BL/6 mice and F_2 animals were used (23). TLR2 and TLR4 double-knockout (DKO) mice onto the C57BL/6 129 background (9–11 wk old) were used for investigation. CD1d-deficient mice (6–8 wk old) have been described (24). All mice were kept under specific pathogen-free conditions.

Reagents

rIL-12 was a kind gift from Hayashibara (Okayama, Japan). Purified anti-Fas ligand (Fas L) mAb (MFL-1, hamster IgG) and murine Fas L-transfected cells (mFas L) were kindly provided by Dr. N. Kayagaki at Juntendo University (Tokyo, Japan) (25). Concanamycin A (CMA) was purchased from Wako (Osaka, Japan). The culture medium generally used was William's medium (ICN Pharmaceuticals, Aurora, OH) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM of 2-ME, and 2 mM L-glutamine.

P. berghei-*induced liver injury*

Mice were i.p. inoculated with 10^6 erythrocytes parasitized with *P. berghei* (NK65) which was purchased from American Type Culture Collection (Manassas, VA). At various time points, liver specimens and serum were sampled for histological analysis and for measurement of the liver enzyme glutamic-pyruvic transaminase (GPT) and cytokine levels, respectively. Liver specimens were fixed in 3.5% formaldehyde in PBS and the slides were stained with H&E. Parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. The percentage of parasitemia was calculated as follows: parasitemia (%) = [(number of infected erythrocytes)/(total number of erythrocytes counted)] $\times$ 100.

Assay for cytokines

IL-18 levels were measured by a commercially available ELISA kit (MBL, Nagoya, Japan). IL-12 p40 levels were also measured by a commercially available ELISA kit (Genzyme, Minneapolis, MN).

Preparation of hepatocytes and liver lymphocytes

Hepatocytes and liver lymphocytes were prepared from inoculated or noninoculated mice, as previously described (26, 27).

Ex vivo assay for hepatocytotoxicity

Hepatocytotoxicity of liver lymphocytes was determined by 4-h ^{51}Cr release assays as previously described (26) with some modifications. In some experiments, liver lymphocytes were precultured with various doses of IL-12 overnight, or with 10 μg/ml anti-Fas L mAb or 20 nM CMA for 1 h at 37°C. Percent cytotoxicity was calculated as previously described (27). Spontaneous release of ^{51}Cr by hepatocytes was <5% of the maximal release.

RT-PCR

Total RNA was extracted from hepatic lymphocytes isolated from inoculated or noninoculated mice. RT-PCR for IL-12Rβ1, IL-12Rβ2, and β-actin was performed as previously described (27).

Statistics

All data are shown as the mean value of triplicate samples. Significance between the control group and a treated group was examined with the unpaired Student's *t* test. Values of $p < 0.05$ were regarded as significant.

Results

IL-18-independent, but IL-12-dependent, liver injury in P. berghei-*infected mice*

Because both IL-12 and IL-18 are potent proinflammatory cytokines (7, 8, 27), we measured the serum concentration of IL-12 and IL-18 after infection of mice with *P. berghei*. IL-12 and IL-18 levels peaked at day 4 and at day 6 after inoculation, respectively (Fig. 1, *A* and *B*). IL-18 in the serum contained biologically active forms because it induced IFN-γ production by IL-18-sensitive cells (data not shown).

Next, we investigated the susceptibility of IL-12- and IL-18-deficient mice to *P. berghei*-induced liver injury. Infection of IL-18-deficient mice with *P. berghei* resulted in enhancement of GPT levels that were comparable to wild-type (WT) mice (Fig. 1*C*). Histological analysis of the liver specimens of WT and IL-18-deficient mice (Fig. 1*D*) revealed a mixture of focal necrosis of hepatocytes and scattered apoptotic hepatocytes characterized by

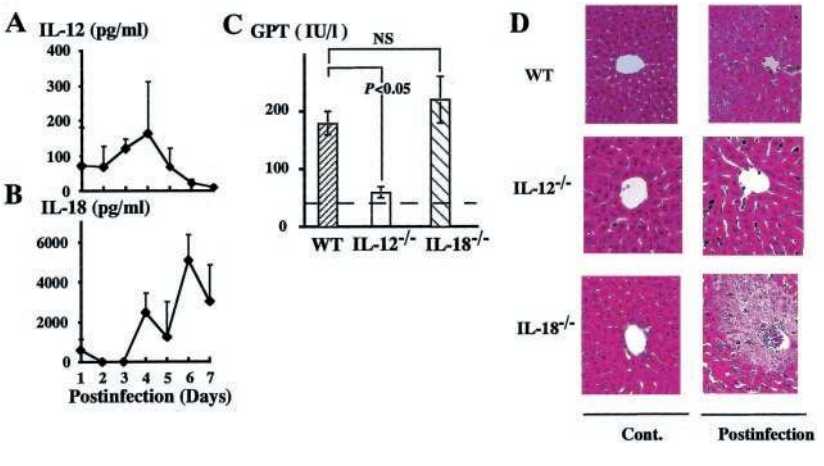

FIGURE 1. *P. berghei* causes liver injury in mice in an IL-12-dependent, but IL-18-independent, manner. *A* and *B*, Sera were sampled from infected C57BL/6 mice at various time points for measurement of IL-12 (*A*) or IL-18 (*B*) concentration. Data represent mean + SD of three mice in each group. *C* and *D*, IL-12-deficient mice (IL-12$^{-/-}$), IL-18-deficient mice (IL-18$^{-/-}$), and WT mice were inoculated with (postinfection) or without (control) parasitized erythrocytes. At day 7, sera and liver specimens were sampled for measurement of GPT (*C*) and histological analysis (H&E; original magnification, $\times$40; *D*). The horizontal dotted line in *C* indicates mean GPT serum levels of normal WT, IL-12-deficient, and IL-18-deficient mice. Serum GPT levels of the uninfected various genotype mice were all <50 IU/L. Data represent mean $\pm$ SD of three mice in each group. Similar results were obtained in three independent experiments.

condensed and fragmented nuclei, accompanied by dense infiltration of mononuclear cells including lymphocytes. In sharp contrast, GPT levels in *P. berghei*-infected IL-12-deficient mice were similar to those of untreated animals. Histological analysis demonstrated the absence of necrotic and apoptotic hepatocytes but the presence of infiltrating lymphocytes. There were no differences of GPT levels among uninfected WT, IL-12-deficient, and IL-18-deficient mice (data not shown). Parasitemias in WT (Table I), IL-12-deficient, and IL-18-deficient mice were comparable (data not shown). These results indicate that IL-12, but not IL-18, is essential for *P. berghei*-induced liver injury.

Critical roles of MyD88 for induction of IL-12 after P. berghei *infection*

To investigate whether the elevation of serum levels of IL-12 and/or IL-18 was mediated through the TLR-MyD88 signaling pathway, we inoculated MyD88-deficient mice with *P. berghei*-infected erythrocytes. The serum levels of IL-18 in MyD88-deficient mice were almost the same as in WT mice, whereas those of IL-12 were dramatically reduced (Fig. 2*A*). As expected from the failure of IL-12-deficient mice to show Th1-dominant immune response upon Bacille bilié de Calmette-Guérin infection, a potent Th1 polarizer (20), MyD88-deficient mice showed marked impairment in Th1 cell development (data not shown). Moreover, like IL-12-deficient mice (Fig. 1*D*), MyD88-deficient mice did not suffer from liver injury (Fig. 2, *B* and *C*) (22) but showed equivalent infiltration of lymphocytes in their livers compared with WT mice (Table I), indicating the important role of IL-12 in *P. berghei*-induced liver injury. Additionally, infected MyD88-deficient mice showed similar mortality and parasitemia to *P. berghei*-infected WT mice (Table I). Taken together, our findings indicate that MyD88 is essential for serum accumulation of IL-12 but not IL-18. Therefore, we conclude that *P. berghei* infection activates the TLR-MyD88 pathway to induce IL-12, which in turn results in liver injury. However, despite our efforts to identify the TLR involved, TLR(s)-deficient or mutant mice we tested showed production of comparable levels of IL-12 and liver injury (Fig. 2, *D–F*, and data not shown), suggesting the possible engagement of other TLRs and/or simultaneous engagement of multiple TLRs with the complex components of *P. berghei* protozoa.

Liver injury induced by P. berghei *requires perforin but not Fas/Fas L interactions*

Animals treated with IL-12 do not show fatal pathological changes (12). Therefore, we hypothesized that the liver injury observed after infection of mice with *P. berghei* is caused by the induction of effector molecules in response to IL-12 and other factors. Furthermore, histological findings led us to investigate whether infiltrating lymphocytes kill hepatocytes by the perforin/granzyme and/or Fas/Fas L pathways, which can be activated by IL-12 and IL-18, respectively (27–29). To address this possibility, we examined whether functional Fas-deficient *lpr/lpr* mice (30) were susceptible to liver injury induced by *P. berghei. lpr/lpr* mice inoculated with *P. berghei*-parasitized erythrocytes manifested almost the same levels of liver injury with dense infiltration as in WT mice (Fig. 3, *A* and *B*) suggesting that the Fas/Fas L pathway is not critical.

To investigate the involvement of perforin in this liver injury, we inoculated perforin-deficient mice with the parasitized erythrocytes. Perforin-deficient mice remained healthy after administration of the infected erythrocytes, although similar density of lymphocyte infiltration was observed in their livers as compared to WT mice (Fig. 3, *A* and *B*). Thus, liver injury induced by *P. berghei* infection occurs in a perforin-dependent Fas/Fas L-independent manner.

Unusual hepatocytotoxic lymphocytes accumulate in the liver of P. berghei*-infected mice*

To investigate the cellular and molecular mechanism of *P. berghei*-induced liver injury, we conducted ex vivo hepatocytotoxicity assays (26). Liver lymphocytes from uninfected mice failed to kill hepatocytes from either infected or uninfected mice (Fig. 4*A*). However, liver lymphocytes from *P. berghei*-infected mice showed cytotoxic activity against hepatocytes isolated from infected mice (Fig. 4*A*). Surprisingly, hepatic lymphocytes from the infected mice also attacked hepatocytes from uninfected mice (Fig. 4*A*). Furthermore, hepatic lymphocytes from the infected mice killed hepatocytes from MHC-mismatched BALB/c mice (our unpublished data), indicating that cytotoxicity occurs in an MHC-unrestricted manner.

Fas L has been implicated in the killing of self cells independently of MHC restriction (25). Moreover, many investigators have reported that normal hepatocytes are sensitive to Fas L (30, 31). However, our studies demonstrated that Fas-mutant *lpr/lpr* mice are sensitive to liver injury induced by *P. berghei* (Fig. 3). To provide further evidence that the Fas/Fas L pathway of cell-mediated cytotoxicity is not required for the liver injury induced by *P. berghei*, we performed neutralization experiments with anti-Fas L mAb that completely inhibit the killing action of Fas L-expressing cells against normal hepatocytes (Fig. 4*B*). Anti-Fas L treatment had little effect on the killing activity of hepatic lymphocytes from *P. berghei*-infected mice against hepatocytes from either uninfected or infected mice (Fig. 4*A*), indicating that the effector lymphocytes killed hepatocytes in a Fas/Fas L-independent manner. In separate experiments, we observed that the effector lymphocytes precultured with control hamster IgG showed no reduction in this cytotoxicity to both targets (data not shown). To confirm the perforin-dependence of hepatocytotoxicity, we incubated liver lymphocytes from the infected mice with CMA, an inhibitor for maturation of perforin (27), and tested their hepatocytotoxicity. Strong inhibitory effects of CMA for the cytotoxic activities of hepatic lymphocytes from *P. berghei*-infected mice were observed (Fig. 4*A*). CMA used in this study did not affect the hepatocytotoxic action of mFas L (Fig. 4*B*). We therefore conclude that *P. berghei* infection stimulates hepatic lymphocytes to kill both autologous and allogeneic normal hepatocytes in a perforin-dependent manner.

Freshly isolated hepatic lymphocytes from infected mice killed hepatocytes ex vivo (Fig. 4*A*) and required continuous stimulation with IL-12 in vitro to sustain their hepatocytotoxic activity (Fig.

Table I. *Similar systemic responses of MyD88-deficient mice to* Plasmodium berghei *infection to those of WT*[a]

	Survival Rate[b]	Parasitemia[c]	Increase of Hepatic Lymphocytes[d]
WT	9/12	26.6 ± 9.3%	>10-fold
MyD88$^{-/-}$	8/12	27.7 ± 5.0%	>10-fold

[a] Twelve WT mice and 12 MyD88-deficient mice (MyD88$^{-/-}$) were inoculated with parasitized erythrocytes.

[b] Survival rates at 10 days after inoculation are shown. No differences of the survival rate between WT and MyD88$^{-/-}$ were observed until all mice died by day 21.

[c] Parasitemia at 10 days after inoculation are shown. The difference of parasitemia between WT and MyD88$^{-/-}$ was statistically not significant.

[d] Mean increases of hepatic lymphocytes from three infected mice at day 7 after inoculation to those from three uninfected mice were shown. There were no significant differences in the two groups. Mean absolute number of hepatic lymphocytes from uninfected WT or MyD88$^{-/-}$ was about 1–2 × 10^6 cells per mouse.

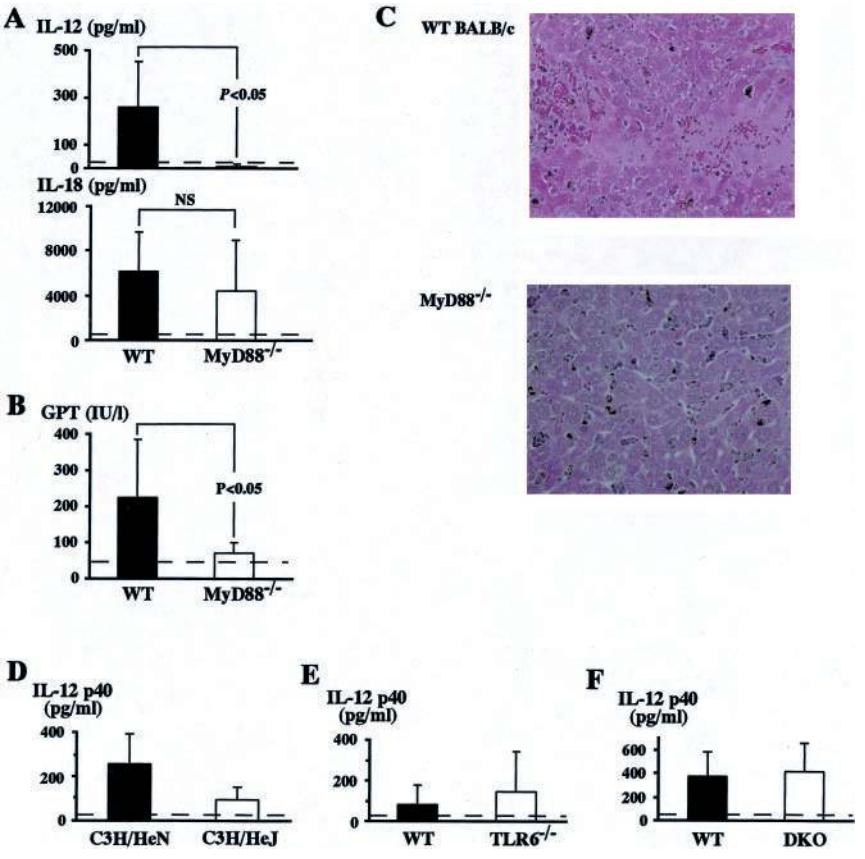

FIGURE 2. Critical role of MyD88 for induction of IL-12 production following *P. berghei* infection. MyD88-deficient mice (MyD88$^{-/-}$) on the BALB/c background and WT-control mice were inoculated with the parasitized erythrocytes. At day 7, sera were sampled for measurement of IL-18, IL-12 (*A*), and GPT (*B*) and liver specimens were examined by histology (H&E; magnification, ×20; *C*). Serum GPT levels of the uninfected WT and MyD88-deficient mice were <50 IU/L. Data represent mean + SD of eight WT and seven MyD88-deficient mice. C3H/HeJ mice (*D*, $n = 5$), TLR6-deficient mice (TLR6$^{-/-}$; *E;* $n = 5$), TLR2, TLR4 DKO mice (*F*, $n = 3$), and WT mice with the corresponding genetic background (*D*; $n = 5$; *E,* $n = 3$; *F,* $n = 5$) were inoculated and sera were sampled for measurement of IL-12 at day 7. Data represent mean + SD of WT and mutant mice. The horizontal dotted line indicates mean IL-12 serum levels of normal WT and mutant mice.

4*C*). However, IL-12-treated normal hepatic lymphocytes did not kill hepatocytes from either uninfected or infected mice (data not shown), suggesting that hepatic lymphocytes become sensitive to IL-12 during infection of *P. berghei*. To investigate this possibility, we evaluated mRNA expression of the β1 and β2 chains of IL-12R (7) in hepatic lymphocytes before and after infection. Hepatic lymphocytes from uninfected mice expressed only the β1 component, whereas those from infected mice expressed both the β1 and β2 components (Fig. 4*D*). Thus, hepatic lymphocytes increase their hepatocytotoxicity in response to IL-12 because they have acquired increased levels of IL-12R after infection.

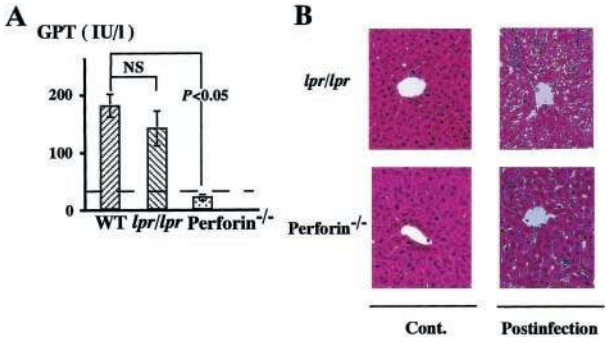

FIGURE 3. *P. berghei*-induced liver cytotoxicity is perforin-dependent, but Fas/Fas L-independent. *lpr/lpr* mice, perforin-deficient mice (perforin$^{-/-}$), and WT mice were inoculated with (postinfection) or without (Cont.) sampled parasitized erythrocytes. At day 7, sera and liver specimens were sampled for measurement of GPT (*A*) and histological analysis (H&E; original magnification, ×40; *B*). The horizontal dotted line indicates mean GPT serum levels of normal WT, *lpr/lpr*, and perforin-deficient mice. Serum GPT levels of the uninfected various genotype mice were all <50 IU/L. Data represent mean ± SD of three mice in each group. Similar results were obtained in three independent experiments.

CD1d-restricted NK T cells are not required for induction of liver injury by P. berghei

Finally, we analyzed characteristics of the unique hepatocyte-killing lymphocytes that accumulate in the liver of *P. berghei*-infected mice. SCID mice lacking T cells and B cells did not suffer from this liver injury, indicating the requirement of T cells in *P. berghei*-induced liver injury (Fig. 4*E*). Recently, it has been reported that CD1d-restricted NK T cells can induce liver injury (32–34). For example, administration of α-galactosylceramide, a selective activator of CD1d-restricted NK T cells (35), induced moderate liver injury in WT mice but not in CD1d-deficient mice. To investigate the possible involvement of CD1d-restricted NK T cells in the liver injury induced by *P. berghei* infection, we inoculated CD1d-deficient mice with parasitized erythrocytes. CD1d-deficient mice were sensitive to the liver injury with similar histological changes as WT mice (Fig. 4*E*) (data not shown). These results indicate that T cells other than CD1d-restricted NK T cells, such as conventional T cells and/or CD1d-unrestricted NK T cells, play an essential role in this liver injury. Because conventional T cells usually exert their cytotoxic action in an Ag-specific and MHC-restricted manner (6), CD1d-unrestricted NK T cells may be candidates for the effector cells in this liver injury.

Discussion

The cytokines IL-12 and IL-18 can play a critical role in the induction of liver injury following infection. In a previous study, we showed that sequential administration of heat-killed *Propionibacterium acnes*, a Gram-positive bacterium, and LPS induces acute liver injury in mice (13, 36). In this model, *P. acnes*-elicited

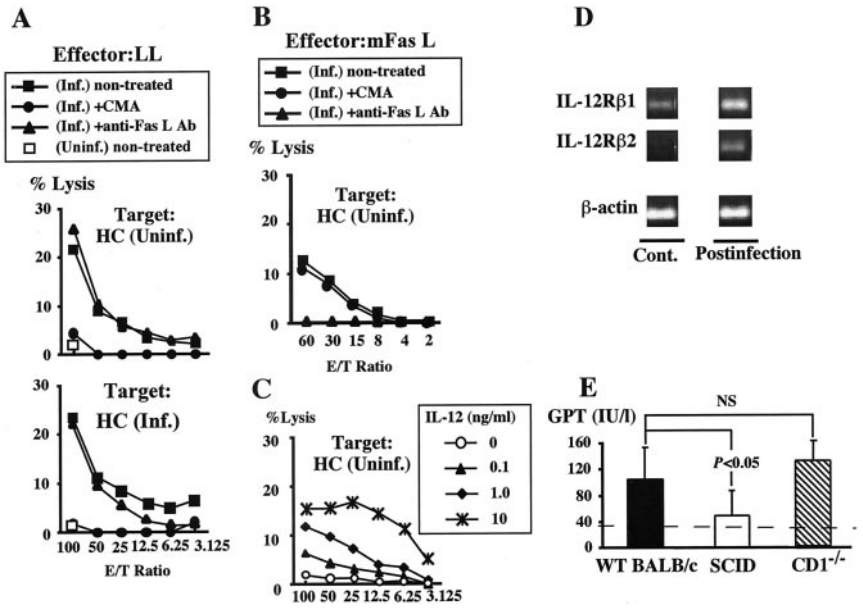

FIGURE 4. Hepatocyte-killing lymphocytes accumulate in the liver after *P. berghei* infection. *A*, Target hepatocytes (HC) were prepared from C57BL/6 mice at day 6 after inoculation (infected, *lower panel*) or from uninfected mice (uninfected, *upper panel*). Hepatic lymphocytes isolated from mice at day 6 post inoculation (infected, filled symbols) were precultured with CMA (●), anti-Fas L Ab (▲), or no substrate (■) for 1 h before mixing with targets. Hepatic lymphocytes from uninfected mice were precultured without any substrates (uninfected, □). In separate experiments, hepatic lymphocytes precultured with control Ab showed no inhibition of hepatocytotoxicity (data not shown). Similar results were obtained in three independent experiments. *B*, Labeled target hepatocytes were incubated with mFas L, which had been treated with or without CMA or anti-Fas L Ab as shown in *A*. *C* and *D*, Hepatic lymphocytes from the infected mice were incubated with various doses of IL-12 overnight and their hepatocytotoxicity was determined by using normal hepatocytes (*C*). Total RNA was extracted from hepatic lymphocytes from uninfected (control) or infected mice (postinfection) and their IL-12Rβ1, IL-12Rβ2, and β-actin mRNA expressions were determined by RT-PCR (*D*). Similar results were obtained in three independent experiments. (*E*) SCID mice, CD1d-deficient mice ($CD1d^{-/-}$), or WT mice were inoculated with the parasitized erythrocytes and day 6 sera were sampled for measurement of the liver enzyme GPT. The horizontal dotted line indicates mean GPT serum levels of normal WT, SCID, and CD1d-deficient mice. Serum GPT levels of the uninfected various genotype mice were all <50 IU/L. Data indicate mean + SD of five mice in each experimental group. Similar results were obtained in three independent experiments.

Kupffer cells produce IL-12 and IL-18 in response to LPS challenge which results in induction of hepatotoxic TNF-α and Fas L in the liver (28, 36). Liver injury in this model is prevented by the administration of neutralizing anti-IL-18 Abs (13) and is absent in IL-18-deficient mice (37), indicating that IL-18 is essential for *P. acnes* and LPS-induced liver injury. Furthermore, liver injury in this model was dependent on induction of Fas L expression but independent of perforin (11). Thus, IL-18 is required for the induction of some types of liver injury. In contrast, the studies described demonstrate that *P. berghei*-induced liver injury is independent of IL-18 but dependent on endogenous IL-12 (Fig. 1, *C* and *D*), although both IL-18 and IL-12 levels in the serum were augmented (Fig. 1, *A* and *B*). IL-18-deficient mice were sensitive to *P. berghei*-induced liver injury (Fig. 1, *C* and *D*). In addition, administration of IL-18 into *P. berghei*-infected mice resulted in no obvious changes in their liver disease (data not shown). In contrast, IL-12 (Fig. 4*C*), but not IL-18 treatment (data not shown), sustained or enhanced hepatocytotoxicity of hepatic lymphocytes from the infected mice. Taken together, these studies suggest that nonspecific liver injury can be caused by at least two different mechanisms: 1) an IL-18-induced, Fas L-dependent mechanism and 2) an IL-12-activated, perforin-dependent mechanism, as seen in *P. acnes*/LPS-induced liver injury and *P. berghei*-induced liver injury, respectively.

This is the first report demonstrating that a protozoan infection can activate the TLR-MyD88 signaling pathway. TLR family members are pattern-recognition receptors that are conserved among species from insects to humans (14). To date, nine different TLRs have been described (14, 15, 18). Recent studies have revealed that some TLRs are critically important for recognition of microbial pathogens by cells from innate immune systems (15, 18). In this study, we have shown that TLR-MyD88-mediated IL-12 production is responsible for perforin-dependent liver injury induced by *P. berghei* infection (Fig. 3, 4). In contrast, IL-18 was induced in a MyD88-independent manner after *P. berghei* infection, which was also observed after *L. monocytogenes* infection (17). However, as MyD88-deficient mice evaded *P. berghei*-induced liver injury, IL-18 is not involved as a causative factor for this liver injury.

Recently, it was reported that a protozoan glycosylphosphatidylinositol anchor has the capacity to activate TLR2-mediated signaling (38). Indeed, the glycosylphosphatidylinositol anchor in *P. falciparum* can activate host innate immunoresponses (39). The particular TLR(s) that is involved in IL-12 induction in *P. berghei* infection still remains unknown (Fig. 2, *D–F*). After *P. berghei* infection, not only WT mice, but also TLR6-deficient mice and TLR4-mutant C3H/HeJ strain mice, (19, 40, 41) showed obvious increases in IL-12 serum levels compared to uninfected mice (Fig. 2, *D* and *E*) and liver injury (data not shown), indicating that TLR6 or TLR4 is not solely responsible for the induction of the liver injury. Furthermore, TLR2 and TLR4 DKO mice also exhibited increases in IL-12 serum levels like WT mice (Fig. 2*F*) and liver injury (data not shown).

After infection with attenuated strains of malaria, host-derived IL-12 or exogenous IL-12 plays a critical role in its clearance (42,

43), suggesting that the TLR-MyD88 pathway is essential for host defense against malaria. However, after infection with the fatal strain *P. berghei*, IL-12 produced by the activation of the TLR-MyD88 pathway causes liver injury but fails to clear the protozoan parasite. Lymphocytes that can kill normal hepatocytes in a MHC-unrestricted manner accumulated in the liver after *P. berghei* infection. These lymphocytes expressed increased levels of IL-12R (Fig. 4*D*) and were highly responsive to IL-12 stimulation, which may explain their unusual cytotoxic activities (Fig. 4, *A* and *C*). It has been reported that IL-12 up-regulates IL-12R expression on lymphocytes (44) and that IL-12 up-regulates the cytotoxic activity of hepatic NK T cells that constitutively express both β1 and β2 components of IL-12R in vitro (28). Therefore, we investigated whether multiple administrations of IL-12 can induce liver injury. This treatment protocol failed to induce liver injury (45), suggesting that a factor(s) other than IL-12 and/or cells other than CD1d-restricted NK T cells are required to induce liver injury. Additional studies will be required to identify this factor(s) and/or effector cells. Splenocytes from the infected mice failed to kill hepatocytes from either uninfected or infected mice (data not shown), suggesting that these cells selectively accumulate in the liver of *P. berghei*-infected mice. Interestingly, CD1d-deficient mice were susceptible to *P. berghei*-induced liver injury (Fig. 4*D*), indicating that CD1d-restricted NK T cells are not required for liver injury. This raises the possibility that CD1d-unrestricted NK T cells accumulated in the liver become effector cells after stimulation with IL-12 and possible other factors. This unusual lymphocyte population may participate in infection-associated tissue injuries that are now categorized in autoimmune disorders. Further analysis of these cells will provide novel insights into effector mechanisms underlying inflammatory disorders of the liver.

In summary, *P. berghei* infection induces activation of TLR-MyD88 signaling to produce IL-12, leading to liver injury in a perforin/granzyme-dependent manner.

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