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Platelets mediate cytotoxic T lymphocyte–induced liver damage

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We found that platelet depletion reduces intrahepatic accumulation of virus-specific cytotoxic T lymphocytes (CTLs) and organ damage in mouse models of acute viral hepatitis. Transfusion of normal but not activation-blocked platelets in platelet-depleted mice restored accumulation of CTLs and severity of disease. In contrast, anticoagulant treatment that prevented intrahepatic fibrin deposition without reducing platelet counts did not avert liver injury. Thus, activated platelets contribute to CTL-mediated liver immunopathology independently of procoagulant function.

Hepatic damage resulting from infection by nonlytic viruses, such as hepatitis B virus (HBV) and replication-deficient adenoviruses, is a consequence of the antigen-specific CTL response[1,2] aimed at viral clearance. We show here that platelets, which participate with leukocytes in inflammatory reactions[3,4], are involved in CTL-induced immunopathology and antiviral activity.

We initially observed that HBV transgenic mice injected with HBV-specific CTLs and C57BL/6J mice infected with a replication-deficient adenovirus expressing *lacZ* (RAd35) showed platelet aggregates in the liver alongside apoptotic hepatocytes and inflammatory cells (**Supplementary Fig. 1** online). To verify whether platelets contribute to the development of these lesions, we injected mice with antibodies (α-PLT) against mouse glycoprotein (GP) Ibα (**Supplementary Methods** online) that caused a >97.5% reduction in platelet count within 30 min and for up to 6 d (**Supplementary Table 1** online). Platelet counts remained normal (8–10 × 10^5/μl of blood) in mice that received saline (NaCl) solution or an irrelevant antibody (α-Irr). We then evaluated liver disease severity in Thy-1.2$^+$ HBV transgenic mice injected with α-PLT or α-Irr before transfer of Thy-1.1$^+$ HBV-specific CTLs. In agreement with previous results[5–8], the liver of α-Irr–treated mice killed at the peak of serum alanine aminotransferase (sALT) elevation showed scattered necroinflammatory foci, in which loss of hepatocytes (hepatocellular dropout) was readily detectable (**Fig. 1a**). The degree of hepatocellular dropout as well as the size of necroinflammatory foci (mean size, 1,245 μm^2 versus 6,724 μm^2; **Fig. 1b**) was smaller in thrombocytopenic mice, which also showed 80% lower sALT levels (**Fig. 1c**) and ~40% ($P < 0.01$) and 30% ($P < 0.01$) fewer intrahepatic Thy-1.1$^+$ HBV-specific CTLs at days 1 and 2 after transfer, respectively (**Fig. 1d**). CTL function was unaffected by the low platelet count, as Thy-1.1$^+$ HBV-specific CTLs recovered from the liver of normal or thrombocytopenic mice had identical cytotoxic activity against target cells that express hepatitis B surface antigen (**Fig. 1e**) and identical interferon (IFN)-γ (encoded by *Ifng*) production *ex vivo* (data not shown). In thrombocytopenic mice, intrahepatic *Ifng* mRNA (a marker of antigen recognition by CTL[6,9]) was reduced (**Supplementary Fig. 2** online) in proportion to the number of CTLs in the liver (**Fig. 1d**), but probably yielded sufficient levels of this antiviral cytokine[6,9] to abolish viral replication (**Supplementary Fig. 2** online).

To investigate further the role of platelets in CTL-induced liver disease, we transfused thrombocytopenic mice with washed mouse platelets lacking endogenous but expressing human GP-Ibα (**Supplementary Methods** online). These platelets (TKK-PLT) are not recognized by α-PLT (**Supplementary Fig. 3** online) and function normally in mice[10]. Transfusion of 6 × 10^8 TKK-PLTs into C57BL/6J mice treated with α-PLT 1 d earlier caused platelet counts of ~40% (at 0.5, 3 and 24 h) and ~22% (at 48 h) of NaCl-injected mice, whereas the same number of C57BL/6J-derived platelets yielded counts <3% of control as early as 0.5 h after transfusion (**Supplementary Fig. 3** online). TKK-PLT transfusion restored severity of liver disease, intrahepatic accumulation of CTL and levels of *Ifng* mRNA (**Supplementary Fig. 4** online), unless TKK-PLTs were treated with the activation inhibitor prostaglandin (PG) E_1 before transfusion (**Fig. 1f** and data not shown). Unlike untreated TKK-PLTs, circulating PGE_1-treated TKK-PLTs isolated at autopsy did not aggregate in response to collagen stimulation *in vitro* (data not shown). Thus, activated platelets contribute to CTL-mediated immunopathological responses. Accordingly, we found that CTLs adhere to platelets activated on a model-reactive surface *ex vivo* (**Supplementary Video 1** online).

Livers from HBV transgenic mice that received HBV-specific CTLs after injection of α-Irr showed abundant fibrin deposition as compared to NaCl-injected mice; fibrin deposits were reduced in mice rendered thrombocytopenic before CTL injection, but restored in thrombocytopenic mice that received TKK-PLT transfusion before CTL injection (**Supplementary Fig. 5** online). This indicates that platelets are required for fibrin deposition. Treatment with the anticoagulant warfarin at a dose that caused a tenfold increase in whole-blood clotting time before CTL transfer also prevented liver fibrin deposition but had no impact on liver damage (**Fig. 1g**), CTL accumulation or intrahepatic *Ifng* mRNA levels (data not shown). Fibrin deposition, therefore, is a consequence and not a cause of CTL-induced liver injury.

To confirm our findings in a different model of acute viral hepatitis, we immunized C57BL/6J mice with a plasmid expressing *lacZ*

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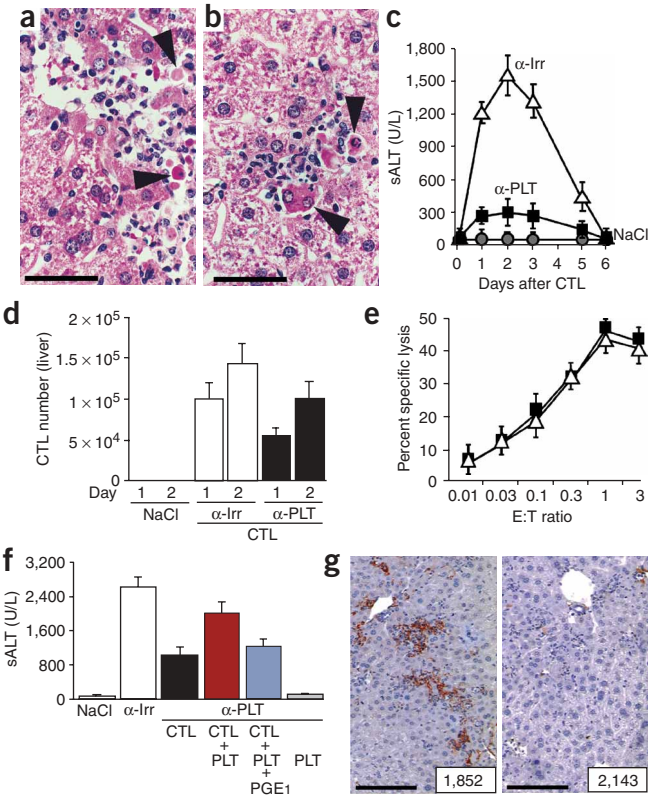

Figure 1 Activated platelets enhance intrahepatic accumulation of CTLs and severity of liver disease in HBV transgenic mice independently of fibrin deposition. Histological analysis of representative HBV transgenic mice injected with either irrelevant antibody (α-Irr; **a**) or antibody against mouse GPIbα (α-PLT; **b**) and killed 2 d after transfer of HBV-specific CTLs. Apoptotic hepatocytes are indicated by arrowheads. Scale bars, 50 μm. (**c**) Mean sALT activity (U/L ± s.d.) measured at the indicated days after CTL transfer in groups of mice ($n = 6$) that received α-Irr (open triangles), α-PLT (filled squares) or NaCl (gray circles). (**d**) Absolute number (mean ± s.d.) and (**e**) *in vitro* cytotoxicity (mean ± s.d. at different effector-to-target (E:T) ratios) of intrahepatic HBV-specific CTLs recovered from the same mice. (**f**) sALT values (mean ± s.d.) measured at the time of autopsy (day 1 after CTL transfer) in groups ($n = 4$) of HBV transgenic mice injected either with α-Irr (white bars) or α-PLT (black, red and blue bars) 24 h before injection of HBV-specific CTLs. Four hours after CTL transfer, one group (red bars) also received TKK-PLTs and one group (blue bars) received TKK-PLTs pretreated with PGE_1. Mice receiving NaCl (dark gray bars) or α-PLT and TKK-PLTs but no CTLs (light gray bars) served as controls. (**g**) Immunohistochemical analysis of fibrin in the liver of representative HBV transgenic mice treated with warfarin (right) or left untreated (left) and killed at day 1 after CTL transfer. Insets show mean sALT levels at the time of autopsy. Scale bars, 150 μm.

before infecting them with RAd35. This allowed us to measure the pathogenic *lacZ*-specific CTL response and define the role of platelets independently of CTL priming. Mice with normal platelet counts killed at the peak of sALT elevation showed an overwhelming disease resembling fulminant hepatitis in humans (**Fig. 2a**). Thrombocytopenia markedly improved this picture, with most hepatocytes retaining normal cytological appearance (**Fig. 2b**), paralleled by a ~90% reduction in sALT activity (**Fig. 2c**), and an 88% ($P < 0.01$) and 66% ($P < 0.01$) reduction in the intrahepatic accumulation of *lacZ*-specific CTLs at days 3 and 4 after infection, respectively (**Fig. 2d**). The ability of CTLs recovered from the liver of thrombocytopenic mice to express granzyme B or to produce IFN-γ *ex vivo* was not impaired (data not shown). Accordingly, intrahepatic *Ifng* mRNA levels in thrombocytopenic and control mice (**Fig. 2e**) paralleled the number of intrahepatic CTLs at the same time points (**Fig. 2d**). By day 5, thrombocytopenic mice showed a more abundant expression of *lacZ* RNA (**Fig. 2f**) and higher percentage of β-galactosidase–positive hepatocytes (**Fig. 2g**), an indication that RAd35 was not readily cleared. This suggests that, in the absence of platelets, the number of functionally normal *lacZ*-specific CTLs that had accumulated in the liver was not sufficient for viral clearance within the time frame of our experiments. As occurred in HBV transgenic mice, TKK-PLT transfusion in RAd35-infected thrombocytopenic mice restored severity of liver disease, intrahepatic accumulation of CTLs, levels of *Ifng* mRNA (**Supplementary Fig. 4** online) and deposition of fibrin (**Supplementary Fig. 5** online).

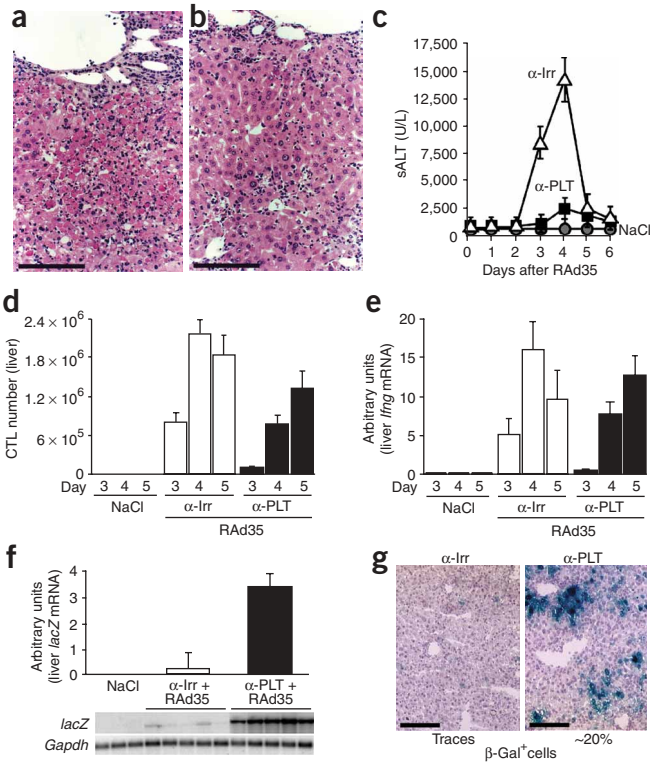

Figure 2 Platelet depletion ameliorates liver disease in adenovirus-infected mice and reduces the intrahepatic accumulation of virus-specific CTLs. Histological analysis of representative *lacZ*-immunized C57BL/6J mice injected with either α-Irr (**a**) or α-PLT (**b**) and killed 4 d after RAd35 infection. Scale bars, 150 μm. (**c**) Mean sALT activity (U/L ± s.d.) measured at the indicated days after RAd35 infection in mice that received α-Irr (open triangles), α-PLT (filled squares) or NaCl (gray circles). (**d**) Absolute number (mean ± s.d.) of intrahepatic *lacZ*-specific CTLs recovered from the same mice killed at days 3, 4 and 5 after RAd35 infection. (**e**) Total hepatic RNA derived from the same mice was analyzed for the expression of IFN-γ by RNAse protection assay. (**f**) The same total hepatic RNA was analyzed for *lacZ* RNA (day 5 after RAd35) by northern blot (lower part) and quantified by phosphorimaging analysis (upper part). (**g**) Fresh frozen liver sections from mice killed at day 5 after RAd35 infection were stained for β-galactosidase activity, and the mean percentage of *lacZ*$^+$ cells in each section is indicated. Scale bars, 300 μm.

In conclusion, our findings show that platelet activation is necessary to accumulate virus-specific CTLs at the site of inflammation, thus mediating immunopathogenic responses. Events of this kind may occur during liver infections caused by noncytopathic viruses (such as hepatitis B and C viruses), in which CTLs have a crucial role in the progression of organ damage[1,11,12]. The experiments described in this report were approved by the Animal Research Committee of The Scripps Research Institute.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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