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TNF-Induced Target Cell Killing by CTL Activated through Cross-Presentation

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SUMMARY

Viruses can escape cytotoxic T cell (CTL) immunity by avoiding presentation of viral components via endogenous MHC class I antigen presentation in infected cells. Cross-priming of viral antigens circumvents such immune escape by allowing noninfected dendritic cells to activate virus-specific CTLs, but they remain ineffective against infected cells in which immune escape is functional. Here, we show that cross-presentation of antigen released from adenovirus-infected hepatocytes by liver sinusoidal endothelial cells stimulated cross-primed effector CTLs to release tumor necrosis factor (TNF), which killed virus-infected hepatocytes through caspase activation. TNF receptor signaling specifically eliminated infected hepatocytes that showed impaired anti-apoptotic defense. Thus, CTL immune surveillance against infection relies on two similarly important but distinct effector functions that are both MHC restricted, requiring either direct antigen recognition on target cells and canonical CTL effector function or cross-presentation and a noncanonical effector function mediated by TNF.

INTRODUCTION

Viruses have developed strategies to escape virus-specific immunity. Among these, impairment of the classical endogenous MHC class I antigen-presentation pathway allows the virus to escape recognition by virus-specific effector CD8 T cells (CTLs) that normally would destroy infected cells (Reddehase, 2002). Antigen cross-presentation, which is a hallmark of professional antigen-presenting cells (APCs) such as dendritic cells (DCs), allows presentation of endocytosed antigen on MHC class I molecules to CTLs (Kurts et al., 2010) and is usually not affected by such viral escape strategies (Sigal et al., 1999). Hence, this viral escape mechanism is not operational in noninfected DCs, allowing them to cross-present antigens captured from infected cells to naive CTLs. Thereby, cross-priming DCs initiate virus-specific CTL immunity regardless of viral immune escape in infected cells (Allan et al., 2003). To this end, DCs must be licensed by CD4 helper T cells or by NKT cells to cross-prime CTLs efficiently (Schoenberger et al., 1998; Semmling et al., 2010). Also, type I interferon (IFN), which is expressed and released from virus-infected cells, has antiviral effects and can stimulate cross-presentation (Le Bon et al., 2003). Taken together, cross-priming of viral antigens by appropriately licensed DCs is an important step in antiviral immunity, which establishes large numbers of virus-specific CTLs to combat infection at peripheral sites (Kurts et al., 2010).

However, already cross-primed CTLs still have to recognize viral antigens on infected cells in peripheral organs in order to attack them, and viral escape from MHC class I presentation in virus-infected cells obviously impedes this critical step of T cell immunity (Holtappels et al., 2004). Moreover, infection of solid organs like the liver by viruses such as hepatitis B or C or lymphocytic choriomeningitis virus (LCMV) is believed to require direct interaction of virus-specific CTLs with virus-infected hepatocytes to control infection (Guidotti and Chisari, 2001; Rehermann and Nascimbeni, 2005), which may require additional time to recruit significant numbers of CTLs from the circulation into infected parenchymal tissue. It is unresolved how cross-primed CTLs can perform effector functions under conditions of viral immune escape in infected cells because it is believed that direct MHC class I-restricted antigen recognition on

virus-infected cells is required for CTL-mediated elimination. Here, we identify a noncanonical CTL effector mechanism that overcomes these limitations.

RESULTS

Cross-Presentation by Liver Endothelial Cells Triggers CTL Effector Function

We investigated the relevance of endogenous antigen presentation versus cross-presentation within a virus-infected organ for CTL effector function. We used an experimental viral hepatitis model where mice were infected with recombinant hepatotropic adenoviruses (AdOVA), which led to preferential infection and expression of Ovalbumin in hepatocytes (Figure S1A). Then, these mice received in vitro-activated OVA-specific H-2K^b-restricted CTLs (OT-I cells) by adoptive transfer, which readily exert effector function upon antigen recognition. As recipients, we used transgenic mice with cell-type-specific expression of MHC class I (H-2K^b) in order to identify the cell on which antigen must be recognized by specific CTLs to elicit immune surveillance.

Adoptive transfer of in vitro-activated OVA-specific CTLs into AdOVA-infected wild-type mice rapidly caused liver damage within 48 hr as measured by elevation in serum liver enzymes (serum ALT [sALT]) (Figure 1A), consistent with the key role of CTLs for viral hepatitis (Ando et al., 1993; Maini et al., 2000). To study the relevance of direct MHC I-restricted recognition of infected hepatocytes by CTLs, we used transgenic mice with hepatocyte-restricted MHC class I expression (CRP-K^b). In these mice we found unexpectedly reduced CTL-mediated hepatitis compared to mice with ubiquitous H-2K^b expression (Figure 1A), indicating that CTLs recognized not only infected hepatocytes but also other APCs. To further investigate the relevance of antigen cross-presentation by nonhepatocytes, we used a transgenic mouse where H-2K^b expression under tie2 promoter drives expression in endothelial cells and some bone marrow-derived immune cells (Limmer et al., 2005). Because liver sinusoidal endothelial cells (LSECs) are as competent in cross-presentation as DCs (Limmer et al., 2000; Schurich et al., 2009), we investigated their contribution by targeting these tissue-resident cells with transgenic tie2-K^b mice. CTL transfer into virus-infected tie2-K^b mice caused hepatitis similar to H-2^b mice with ubiquitous H-2K^b expression (Figure 1A), which is consistent with cross-presentation of hepatocyte-derived antigens by LSECs. The importance of cross-presenting LSECs for CTL-mediated viral hepatitis was substantiated by the prominent lymphocyte accumulation in tie2-K^b—similar to H-2^b mice (Figure 1B). We excluded a role for bone marrow-derived APCs by using [DBA/2- > tie2-K^b] chimeric mice where only organ-resident LSECs but not macrophages or DCs expressed H-2K^b, which sufficed for induction of viral hepatitis (Figure 1C). There was no contribution from bone marrow-derived immune cells to CTL-mediated viral hepatitis in [tie2-K^b- > DBA/2] chimeric mice (Figure 1C). A contribution from macrophages was further excluded because clodronate-induced depletion did not significantly alter CTL-mediated viral hepatitis (Figure S1B), which is distinct from the direct pathogenetic role of Kupffer cells in viral hepatitis when naive CTLs are transferred (Giannandrea et al., 2009). It is important to note that in tie2-K^b mice, virus-infected hepatocytes did not express H-2K^b and thus did not present antigen to CTLs at all. We conclude that local cross-presentation by LSECs contributed to CTL-induced viral hepatitis.

We confirmed the relevance of these findings by repeating the experiments with OVA-specific CTLs that were primed during a viral infection in vivo. Their transfer also elicited liver damage if they recognized their antigen on cross-presenting LSECs in vivo (Figure S1C). Antibody-mediated depletion studies revealed that NK cells were not involved in hepatitis (Figure S1D) and that no antiviral T cell immunity from the endogenous T cell repertoire was observed within the time frame of the experiments (Figure S1E).

We next validated LSEC cross-presentation of hepatocyte-derived viral antigens to CTLs. LSECs isolated from AdOVA-infected tie2-K^b mice but not from AdGFP-infected mice stimulated OVA-specific CTLs directly ex vivo (Figure 1D). We excluded transfer of peptide-loaded MHC class I molecules from hepatocytes to LSECs because LSECs isolated from CRP-K^b mice that did not express H-2K^b on endothelial cells (Figure S1F) also did not stimulate CTLs ex vivo (Figure 1D). Similarly, CTLs killed hepatocytes isolated from CRP-K^b but not from tie2-K^b mice in vitro (Figure S1G). LSECs cross-presented in vitro OVA that was released from AdOVA-infected HepG2 cells (Figure 1E). LSECs were not infected by AdOVA, excluding direct antigen presentation by this cell population (Figure 1E). Taken together, LSECs cross-presented hepatocyte-derived antigens to CTLs and thereby triggered viral hepatitis even in the absence of MHC class I-restricted CTL recognition of virus-infected hepatocytes, which may serve to counteract viral immune escape of MHC class I-restricted antigen presentation in infected hepatocytes.

Next, we examined the relevance of CTL recognition by cross-presenting cells for antiviral immunity. The ability of CTLs to eliminate virus-infected hepatocytes even in the absence of MHC I-restricted target cell recognition was shown by reduction in the numbers of virus-infected hepatocytes in tie2-K^b mice (Figure 1F). Furthermore, we employed a highly sensitive method for detection of antiviral CTL effector function in the liver, which is based on quantitative in vivo bioluminescence imaging of virus-encoded luciferase (Stabenow et al., 2010). Following adoptive transfer, CTLs significantly controlled viral gene expression in infected hepatocytes demonstrated by reduced bioluminescence in the livers of virus-infected tie2-K^b mice, whereas CTLs in CRP-K^b mice required 48 hr to achieve similar antiviral effects (Figure 1G). This indicated that local cross-presentation of hepatocyte-derived antigens to CTLs allowed for rapid control of viral infection and may synergize with direct antigen presentation by infected hepatocytes at later time points, as illustrated in Figure 1H.

CTL-Derived TNF Causes Liver Damage after Viral Infection

Given the independence of CTL-induced liver damage from MHC class I-restricted antigen recognition on virus-infected hepatocytes, we wondered what had triggered hepatocyte death after CTL activation by cross-presenting LSECs. Whereas cross-presentation by LSECs to naive CTLs results in

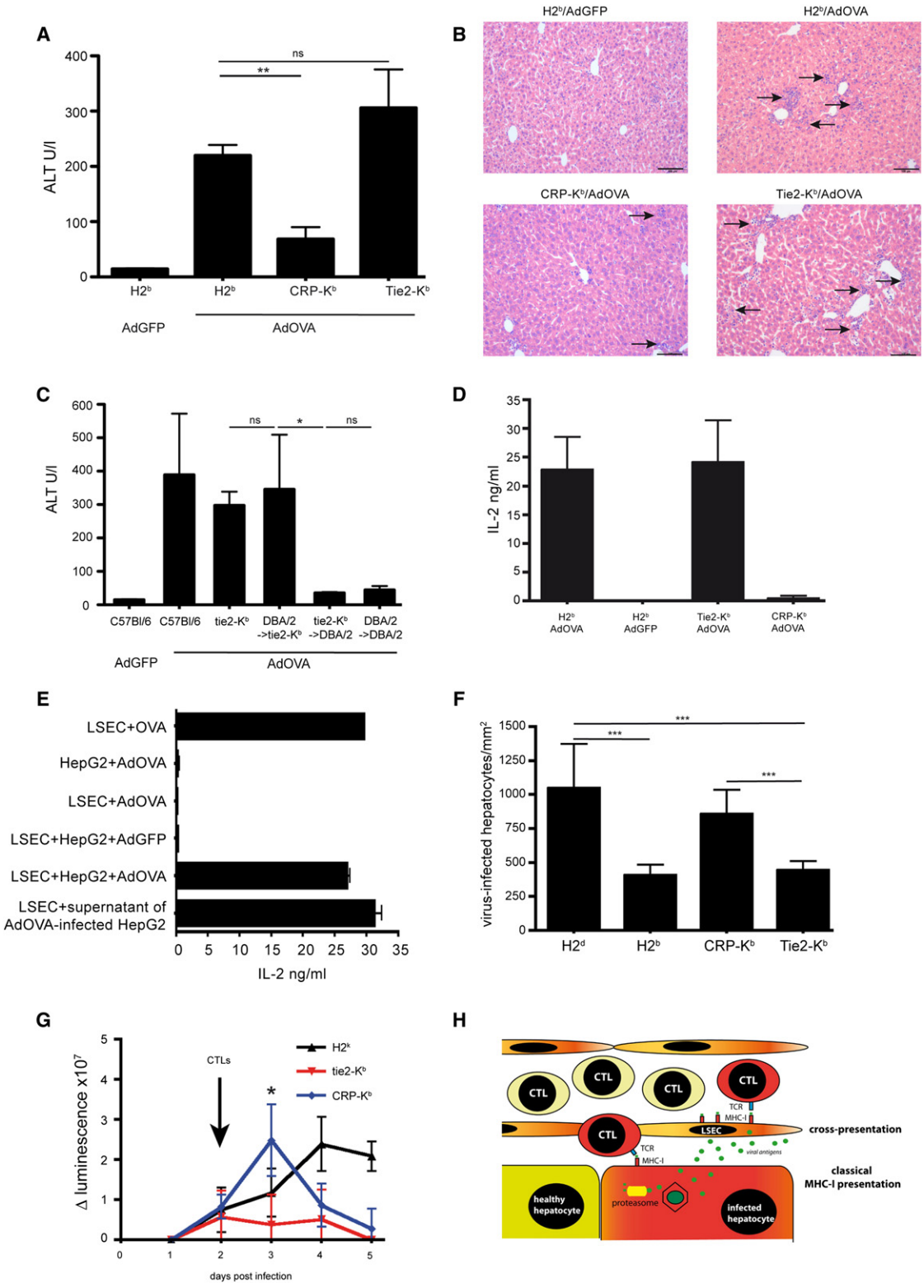

Figure 1. Cross-Presentation by LSECs Triggers a Noncanonical CTL Effector Function

(A) H-2^b or transgenic mice with hepatocyte (CRP-K^b) or endothelial cell (tie2-K^b)-restricted H-$2K^b$ expression were infected i.v. with recombinant AdGFP or AdOVA (2×10^8 pfu/mouse) and 2 days later received in vitro-activated OVA-specific OT-I effector CTLs by adoptive transfer (10^7/mouse). After further 2 days, sALT was determined (n = 4 mice per group; three separate experiments). ns, nonsignificant (p = 0.2979); **, significant (p = 0.0028).

development of T cell nonresponsiveness leaving LSECs intact (Diehl et al., 2008; Limmer et al., 2000), we observed killing of LSECs in vitro and in vivo after cross-presentation to CTLs that had been activated 4 days before by DCs (Figures 2A and S2A). This may damage the sinusoidal endothelial layer in the liver. Endothelial lesions are known to elicit platelet activation (Wu and Thiagarajan, 1996). Although we observed platelet accumulation at sites of viral infection in the liver (Figure 2B), platelet depletion by antibodies (Figure S2B) did not attenuate CTL-induced hepatitis (Figure 2C). This excluded platelet-mediated microvascular thrombosis as the cause of liver damage in our experimental setting. Notwithstanding, platelets become activated in the context of viral infection of the liver, alter the hepatic microcirculation, and can worsen viral hepatitis (Iannacone et al., 2005; Lang et al., 2008). How then could CTLs exert cytotoxicity in the absence of MHC class I-restricted antigen recognition on infected hepatocytes?

We reasoned that cytokines might play a role, and first examined IFNγ and IFNα/β, two obvious candidates with potent antiviral activity (Guidotti and Chisari, 2001). However, hepatitis was not attenuated in virus-infected IFN$\gamma R^{-/-}$ or IFN$\alpha/\beta R^{-/-}$ mice (Figure 2D). In contrast, CTL-mediated viral hepatitis was significantly reduced in $TNFR^{-/-}$ mice, revealing a crucial role for tumor necrosis factor (TNF) (Figure 2D), which is involved in noncytopathic antiviral defense (Guidotti and Chisari, 2001). By intracellular flow cytometry we found that antigen-presenting LSECs induced strong TNF expression in CTLs (Figures S2C and S2D), arguing for a role of CTL-derived TNF in viral hepatitis. Transfer of TNF-competent CTLs into virus-infected $TNF^{-/-}$ mice elicited liver damage, confirming that CTL-produced TNF sufficed for inducing viral hepatitis (Figure 2E). Moreover, transfer of $TNF^{-/-}$ CTLs into TNF-competent virus-infected mice led to a 40% reduction in liver damage, indicating that TNF accounted for a significant part of the CTL effector function against virus-infected hepatocytes (Figure 2F). Taken together, these results define a CTL effector function that depends on stimulation by cross-presenting cells, is independent of direct MHC class I-restricted target cell recognition and, therefore, of viral immune escape from antigen presentation, and relies on CTL-derived TNF as effector molecule.

Viral Infection Sensitizes Hepatocytes for TNF-Induced Cell Death

If CTLs employed TNF to kill hepatocytes, the question arises whether such killing was restricted to infected cells. We therefore studied the situation in which LSECs cross-presented OVA in the absence of viral infection of hepatocytes by injecting soluble OVA, which is rapidly and efficiently cross-presented by LSECs (Schurich et al., 2009), followed by CTL transfer. There was no hepatitis even after providing additional TNF by intravenous (i.v.) injection (Figure 3A), demonstrating that CTL activation and release of TNF are required for viral hepatitis but are not sufficient to induce liver damage in noninfected mice.

This suggested that additional factors associated with viral infection rendered hepatocytes susceptible for TNF-mediated cell death. In a reductionist's approach we show that TNF injection alone independent of other CTL-associated death-inducing molecules leads to hepatitis in virus-infected mice (Figure 3B). There was no difference in levels of TNF receptor (TNFR) expression in infected versus noninfected hepatocytes (Figure S3A), which excluded a direct effect of infection at the level of TNF sensing. TNF-induced hepatitis was independent of OVA expression and also occurred after infection with AdGFP or AdLacZ (data not shown), demonstrating that sensitivity of infected hepatocyte to TNF was not a specific feature of OVA expression. Injection of UV-inactivated adenovirus did not suffice to elicit liver damage by TNF (Figure 3B), suggesting that immune recognition of viral structural patterns did not cause hepatocyte sensitization. We directly characterized the role of innate receptors involved in recognition of adenoviral infection and liver damage (Zhu et al., 2007) by injecting animals with synthetic ligands to these receptors followed by TNF challenge. None of these ligands rendered hepatocytes susceptible to death by TNF (Figure 3C). There was also no role for inflammasome activation because the severity of hepatitis after viral infection and TNF challenge was similar in $ASC1^{-/-}$ mice compared to wild-type mice or after inhibition of caspase-1 (data not shown).

If cell-autonomous mechanisms predisposed infected hepatocytes to TNF killing, then the numbers of infected hepatocytes should directly correlate with liver damage. Indeed, higher infectious doses of adenoviruses led to increasing sALT levels in mice treated with the same concentration of TNF (Figure 3D). Following infection with high-dose adenovirus (10^9 pfu/mouse), liver histology and sALT elevation revealed development of severe liver damage peaking within 4 hr after challenge with TNF (Figures 3E and 3F). There was a decline of sALT 4 hr after TNF challenge (Figure S3B), indicating that a single TNF injection did not suffice to elicit long-lasting liver damage. The synergy of viral infection and TNF for hepatocyte death was not restricted to infection with replication-incompetent DNA virus but was also

(B) Mice were treated as in (A). Histopathological analysis (H&E) of liver sections taken 2 days after CTL transfer. Arrows indicate accumulation and infiltration of lymphocytes. Scale bars, 100 μm.
(C) Bone marrow chimeric mice were generated as indicated. Adenoviral infection and OT-I CTL transfer followed by determination of sALT were performed as described in (A). *, significant ($p = 0.0242$).
(D) Mice were infected as in (A), LSECs were isolated from H-2^b, CRP-K^b, or tie2-K^b mice 2 days later, and OVA cross-presentation was determined ex vivo by measuring IL-2 release from B3Z cells.
(E) LSECs and/or HepG2 cells were infected in vitro with AdOVA or AdGFP, and cross-presentation to B3Z cells was determined.
(F) Enumeration of virally infected hepatocytes by immunohistochemistry of mice treated as in (A). ***, $H2^d$ versus Tie2-K^b $p = 0.0002$; all others $p < 0.0001$.
(G) Bioluminescence from H-2^k (devoid of H-$2K^b$, as negative control), tie2-K^b, or CRP-K^b mice after infection with recombinant adenovirus expressing OVA and luciferase as fusion protein and CTL transfer (n = 4 mice per group). *$p = 0.0114$ for tie2-K^b versus CRP-K^b on day 3.
(H) Scheme for CTL-mediated liver damage in absence of direct MHC I-restricted target cell recognition.
(C and D) One out of three independent experiments is shown.
See also Figure S1.

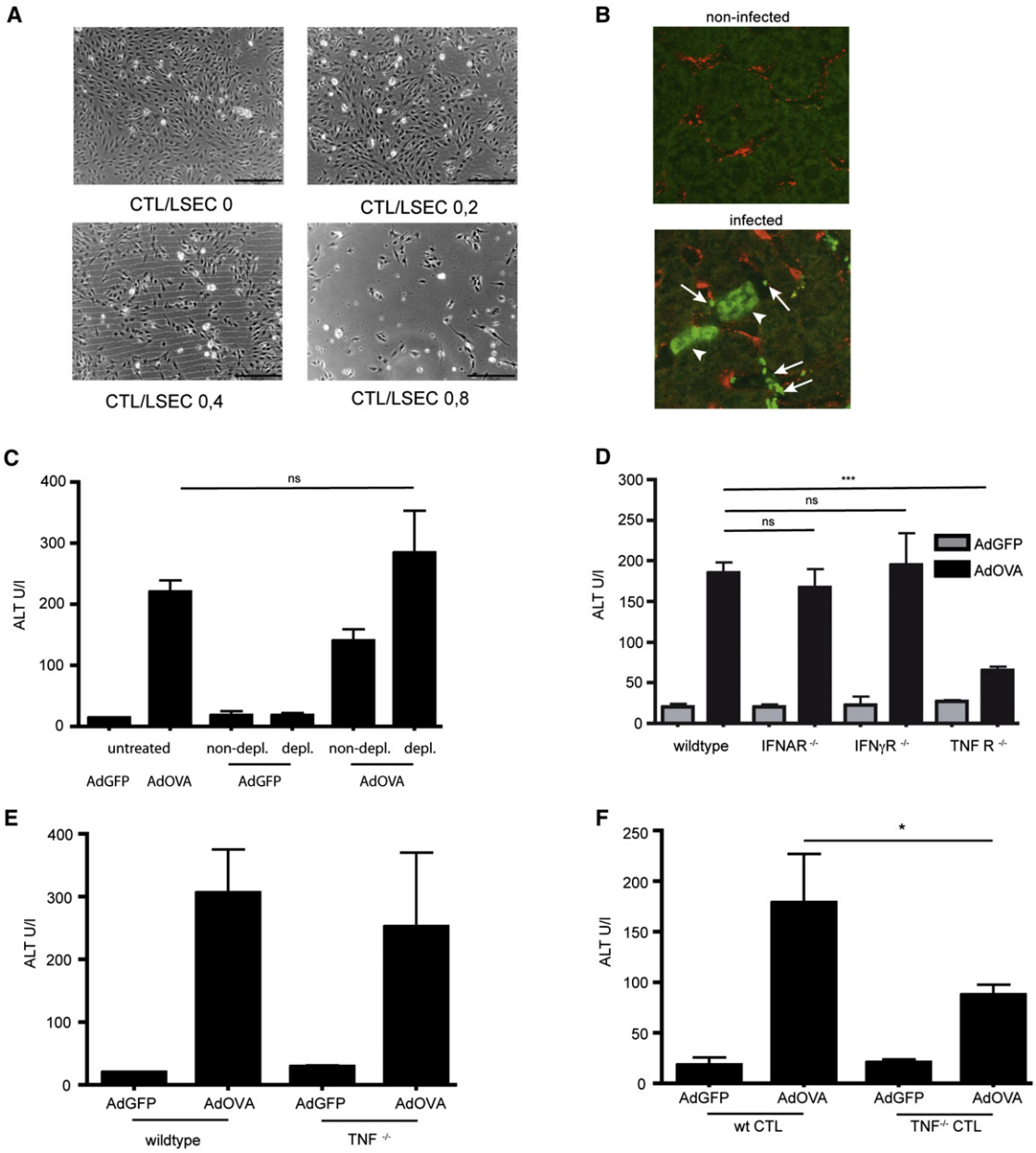

Figure 2. CTL-Derived TNF Causes Liver Damage

(A) Incubation of cross-presenting LSECs with increasing numbers of specific CTLs in vitro for 3 hr. Scale bar, 100 μm.

(B–F) Mice were infected with AdGFP or AdOVA (2×10^8 pfu/mouse), 2 days later adoptive CTL transfer (10^7/mouse). (B) Visualization of sinusoidal endothelium by uptake of Alexa 647-labeled BSA; platelet staining with DyLight 488-labeled αGPIb antibody (arrows) in the vicinity of AdGFP-infected hepatocytes (arrowheads). Confocal laser-scanning microscopy of liver sections 24 hr after CTL transfer. (C) Platelet depletion with αCD42b antibody (4 μg/kg) compared to isotype control antibody (nondepleted). sALT determination 2 days after CTL transfer (n = 4 mice per group; one of two representative experiments is shown). ns, nonsignificant ($p = 0.1306$). (D) sALT determination 2 days after adoptive CTL transfer into the indicated virus-infected knockout mice (n = 3 mice per group). Wild-type versus IFNγR$^{-/-}$ nonsignificant ($p = 0.8176$), wildtype versus IFNAR$^{-/-}$ nonsignificant ($p = 0.4766$), wildtype versus TNFR$^{-/-}$ ***, significant ($p = 0.0001$). (E and F) Adenoviral infection of wild-type or TNF$^{-/-}$ mice; sALT determination 2 days after transfer of TNF-competent or TNF$^{-/-}$ CTLs. *, significant ($p = 0.0407$). See also Figure S2.

observed after infection with the replication-competent RNA virus LCMV (Figure 3G). TNF application reduced viremia in LCMV-infected animals (Figure 3H), demonstrating that TNF contributed to control of viral infection. These results identified a nonredundant combinatorial effect of viral infection and TNF in killing of infected hepatocytes. We further wondered whether preexistent inflammation would alter the outcome of TNF-induced liver damage in virus-infected mice. PolyIC-induced

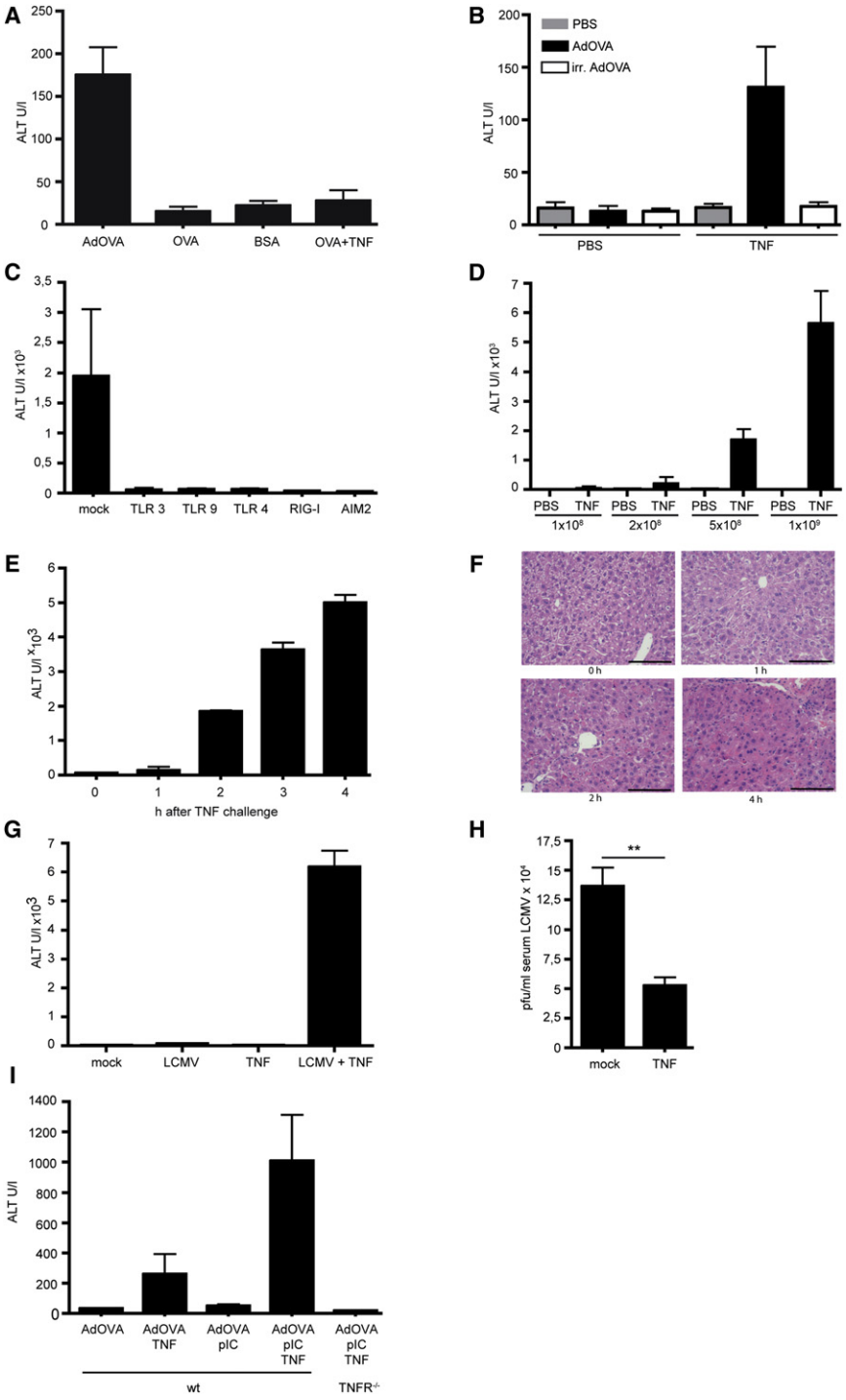

Figure 3. Viral Infection Predisposes to TNF-Induced Liver Damage

(A) Intravenous challenge of H-2^b mice with OVA or BSA (2 mg/mouse) ± TNF (1 mg/mouse) followed by CTL transfer (10^7/mouse); sALT was measured 1 day later.

(B) Infection with AdOVA (2 × 10^8 pfu/mouse) or UV-irradiated virus followed by TNF challenge (400 ng/mouse) 2 days later; sALT at 4 hr.

(C) Systemic application of TLR, RIG-I, and AIM2 ligands followed by i.v. TNF-challenge and sALT measurements at 4 hr. AdOVA-infected (5 × 10^8 pfu/mouse) and TNF-challenged mice served as positive controls.

(D) Infection with increasing virus dose; TNF-challenge and sALT determination as in (B).

(E an F) Viral infection (10^9 pfu/mouse) of mice; sALT (E) and liver histology (F) at indicated time points after TNF challenge. Scale bars, 200 μm.

(G and H) LCMV infection (10^4 pfu/mouse) followed by TNF challenge 2 days later and (G) sALT determination at 4 hr (n = 4 mice per group; one out of five experiments is shown) or (H) determination of viremia at day 1 (n = 3 mice per group; one out of two experiments is shown). **, significant (p = 0.0011).

(I) WT or TNFR knockout mice were infected with AdOVA as in Figure 1 and i.v. challenged 1 day later with pIC (100 μg/mouse) followed by TNF and sALT determination as above (n = 3 mice per group; one out of three experiments is shown).

In (B)–(G), n = 4–5 mice per group; one out of three representative experiments is shown.

See also Figure S3.

TNFR Signaling in Infected Hepatocytes Causes Cell Death

To test whether CTL-derived TNF acted directly on hepatocytes, we employed mice deficient in hepatocellular TNFR signaling by hepatocyte-specific ablation of FADD, an essential component of the TNFR-signaling pathway (Ermolaeva et al., 2008). These mice were protected from TNF-induced liver damage after viral infection (Figure 4A), which identified hepatocytes as direct targets of TNF and suggested selective TNF-induced death in infected hepatocytes. Accordingly, active caspase-3 was only detected in hepatocytes of mice infected with adenovirus or LCMV and challenged with TNF, but not in mock-treated infected livers or upon TNF challenge alone (Figure 4B). Importantly, cell death was observed exclusively in virus-infected hepatocytes (Figure 4C). We did not observe collateral cell death because TUNEL-positive hepatocytes were all infected with adenovirus, whereas no uninfected hepatocytes were TUNEL positive (Figure 4D). Because hepatocytes were the direct target of TNF, these findings indicated that the

TLR3 stimulation of infected mice amplified the ensuing TNF-mediated liver damage (Figure 3I). Clearly, liver damage depended on TNF even under these circumstances because no effect was seen if poly-IC was applied alone or when mice deficient for TNFR were employed (Figure 3I), indicating that low-grade inflammation accentuates the effector function of TNF in mediating viral hepatitis.

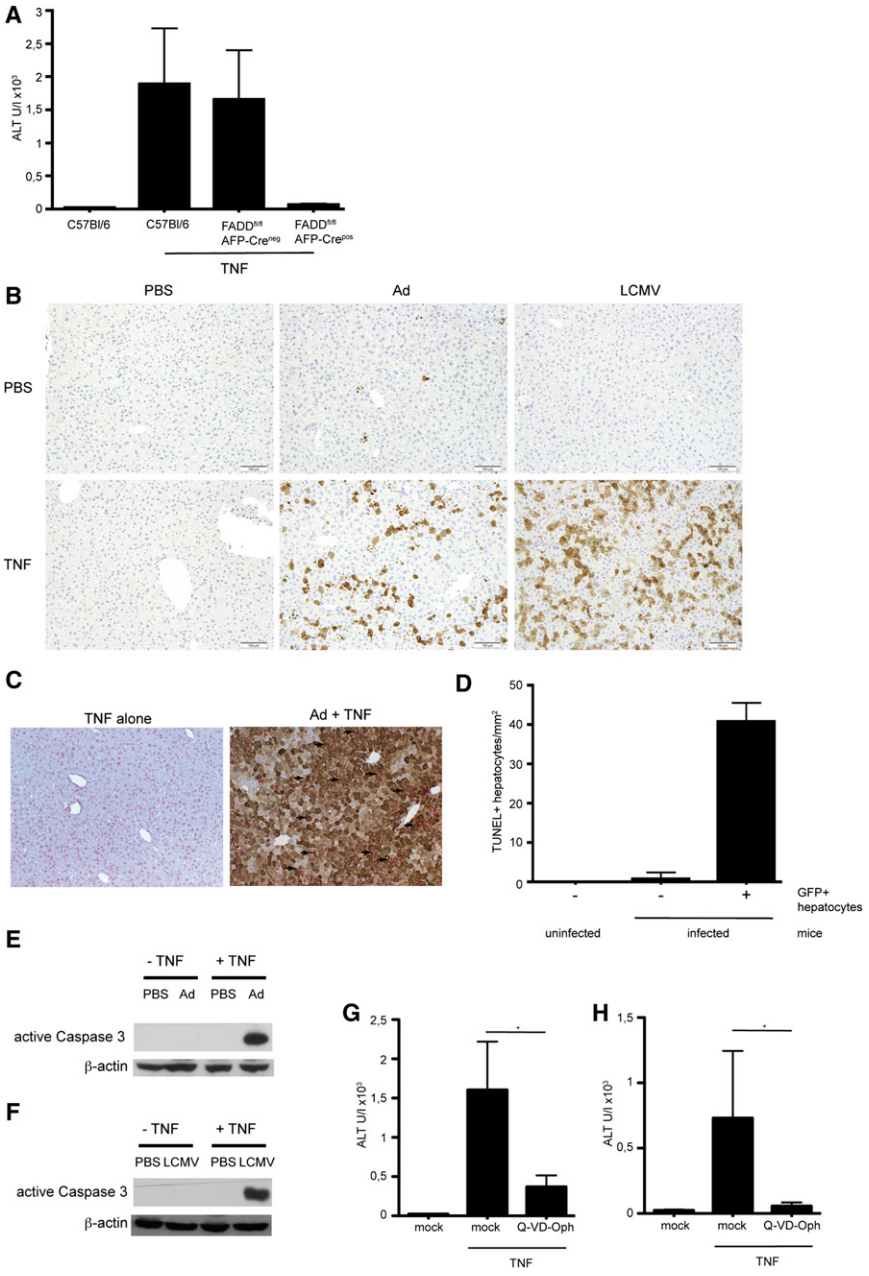

Figure 4. TNF Directly Targets Virus-Infected Hepatocytes

(A) Adenoviral infection of transgenic mice bearing a hepatocyte-specific FADD ablation ($FADD^{fl/fl}$ xAPF-Cre^{pos}; control: $FADD^{fl/fl}$ xAPF-Cre^{neg}), 2 days later TNF challenge, and sALT after 4 hr.

(B and C) Mice were infected with adenovirus (5×10^8 pfu/mouse) or LCMV (1×10^2 pfu/mouse); 2 days later TNF challenge and 1 hr after TNF-challenge liver immunohistochemistry for (B) cleaved active caspase-3 (brown) and for (C) GFP expression (brown) in combination with TUNEL staining (red and black arrowheads).

(D) Quantification of $TUNEL^+$ hepatocytes in uninfected and adenovirus-infected mice (5×10^8pfu/mouse) (n = 4 mice per group; one out of three experiments is shown).

(E and F) Immunoblot detecting caspase activation in hepatocytes isolated from livers of adenovirus-infected (E) or LCMV-infected (F) mice treated as indicated.

(G and H) Treatment of adenovirus-infected (G) or LCMV-infected mice (H) with a caspase inhibitor, sALT 4 hr after TNF challenge (n = 4 mice per group; one out of four independent experiments is shown). *, significant (p = 0.0153) in (G). *, significant (p = 0.0306) in (H).

DISCUSSION

Viruses have evolved various strategies to evade the immune response, which include escape from CTL recognition by downregulation of MHC I expression and prevention of classical MHC I-restricted antigen presentation as well as escape from innate immune response by interfering with IFN signaling and induction of apoptosis (Klenerman and Hill, 2005; Rehermann and Nascimbeni, 2005). Cross-priming by DCs solves this problem only partially by generating sufficient numbers of CTLs in lymphatic tissues (Kurts et al., 2010) because as long as viral immune escape is operational in infected cells, activated CTLs fail to recognize and kill their targets and remain ineffectual (Holtappels et al., 2004). Here, we report on the discovery of a noncanonical CTL effector mechanism that selectively kills virus-infected target cells in the absence of MHC I-restricted target cell recognition and thereby complements conventional CTL-mediated immune surveillance effected through CD95L, perforin, and granzyme B.

It is believed that the key mechanism of immune surveillance by CTLs is target cell killing upon recognition of peptide-loaded MHC I molecules directly on the target cell. However, this MHC I-restricted direct target cell recognition accounted for only about 50%–60% of total CTL effector function in an experimental model of viral hepatitis where only hepatocytes expressed MHC I molecules. This raised the question by which mechanism the sensitization in infected hepatocytes observed in our experiments resulted from cell-autonomous mechanisms. Because we observed increased numbers of TUNEL-positive and active caspase-3-positive hepatocytes in virus-infected livers, we wondered whether caspase activation was involved in TNF-induced death of infected hepatocytes. Western blotting confirmed this assumption, showing activated caspase-3 only in virus-infected hepatocytes of mice challenged with TNF (Figures 4E and 4F). Moreover, a pan-caspase inhibitor attenuated TNF-induced viral hepatitis (Figures 4G and 4H). These results demonstrated that CTLs activated by cross-presenting LSECs killed specifically virus-infected hepatocytes through TNF-induced apoptosis.

remaining CTL effector function was achieved. We found that cross-presentation of antigens derived from adenovirus-infected hepatocytes by organ-resident LSECs to blood-borne CTLs was sufficient to induce hepatitis even in the complete absence of MHC class I-restricted antigen recognition on infected hepatocytes. When we characterized the molecular mechanisms by which LSEC cross-presentation to effector CTLs led to hepatitis, we found a so far unrecognized CTL effector function that depends on TNF. This was surprising because we had previously shown that naive CTLs are rendered nonresponsive when stimulated by cross-presenting LSECs (Diehl et al., 2008; Limmer et al., 2000). Our present findings demonstrate that the tolerogenic effect of LSECs is restricted to stimulation of naive CTLs and does not compromise the execution of CTL effector function, which allows these cells to fulfill their function in peripheral immune surveillance. Our findings also reveal that CTL immune surveillance can occur independently from direct MHC-restricted target cell recognition, which allows CTLs to control viral infection in the infected liver more rapidly and efficiently.

Microvascular endothelial cells may contribute to CTL immune surveillance also in other organs because endothelial cells of the pancreas and the blood-brain barrier are capable of cross-presentation and contribute to CTL transmigration into those organs (Galea et al., 2007; Savinov et al., 2003). However, in most studies reported, endothelial cells did not mediate antigen-specific CTL activation but their attraction through increased chemokine expression, e.g., following CD4 T cell activation by antigen-presenting DCs within infected tissue that triggered local chemokine expression as shown in the genitourinary tract (Nakanishi et al., 2009) or the kidney (Heymann et al., 2009). Although we cannot exclude a role for chemokine-mediated attraction of CTLs in amplification of viral hepatitis, the antigen-specific activation of CTLs by cross-presenting LSECs clearly was the initiating event for development of hepatitis.

Given the independence of target cell killing from direct MHC class I recognition on target cells, we explored the pathogenic role of soluble mediators involved in antiviral immunity and liver damage during viral infection, such as TNF, type I and type II IFNs (Guidotti and Chisari, 2001; Lang et al., 2006). Type II IFN release from CTLs has been shown to contribute to control of viral infection in hepatocytes (Giannandrea et al., 2009; Jo et al., 2009). We identified TNF released from activated CTLs as the only essential and sufficient factor that mediates cell death selectively in virus-infected hepatocytes. However, TNF released from macrophages, neutrophils, NK/T cells, or $CD4^+$ T cells (Gao et al., 2009; Mosser and Edwards, 2008; Nathan, 2006) may further amplify the response initiated by CTLs that were locally reactivated through cross-presentation. Because the lack of TNF expression in CTLs caused a 40% reduction of liver damage in infected mice with ubiquitous MHC I expression, we conclude that the TNF-dependent effector function accounts for a substantial portion of total CTL effector function even in the presence of FAS-L, perforin, or granzyme B. This demonstrates that CTL immune surveillance against viral infection involves two distinct mechanisms, which both are MHC I restricted, but only one requires direct MHC-restricted antigen recognition on target cells, whereas the other relies on cross-presentation and TNF-mediated killing of target cells that does not require direct MHC I recognition.

A role of TNF in noncytolytic control of viral replication but not in mediation of hepatocyte death was observed in mice with transgenic expression of viral antigens in hepatocytes (Guidotti and Chisari, 2001). Our experiments clearly demonstrate that TNF does not inflict damage to a noninfected liver but that it causes hepatitis once viral infection sensitizes hepatocytes for the death-inducing effects of TNFR signaling. TNF acted directly on infected hepatocytes to induce cell death because mice with hepatocyte-selective knockout of FADD, which constitutes an important component of TNFR signaling (Ermolaeva et al., 2008), did not develop TNF-dependent viral hepatitis. Because FADD also contributes to FAS-mediated death (Peter and Krammer, 2003), we cannot formally exclude an additional role for FAS-L in noncanonical CTL effector function. However, liver damage inflicted through anti-FAS antibody develops in noninfected livers (Ogasawara et al., 1993), indicating that FAS death receptor signaling does not discriminate between healthy and infected hepatocytes. Death of virus-infected hepatocytes following TNFR signaling involved caspase activation because pharmacologic caspase inhibition alleviated hepatitis. Although the molecular mechanisms determining the increased susceptibility of infected hepatocytes to the proapoptotic effects of TNF still need to be determined, our findings establish a robust experimental system for future research on the differential outcome of TNFR signaling in vivo.

This noncanonical CTL effector function may bear advantages for antiviral immunity in situations such as (1) viral escape from CTL and NK cell immune surveillance through expression of MHC class I decoy molecules (Farrell et al., 1997), (2) viral interference with IFN signaling or prevention of IFN induction in the liver (Protzer et al., 2012; Rehermann and Nascimbeni, 2005), or (3) impaired sensing of viral infection by innate immune receptors or by danger-sensing inflammasomes that prohibits execution of programmed cell death in infected cells (Bowie and Unterholzner, 2008; Schroder and Tschopp, 2010). However, impaired generation of sufficient numbers of antiviral CTLs may compromise also the effect of the noncanonical effector function because large CTL numbers are required for efficient pathogen-specific immunity against infected hepatocytes (Protzer et al., 2012). A further implication of our findings is that therapies aiming at neutralizing TNF, for example in rheumatoid arthritis or colitis, suppress such antiviral defense, which is suggested by recent reports by Chung et al. (2009) and Li et al. (2009).

In summary, we report a noncanonical CTL effector function that may not only overcome viral immune escape in infected cells, which interferes with CTL recognition or innate immune sensing in infected cells directly causing apoptosis. This noncanonical CTL effector function may also accelerate and improve local antiviral immune surveillance by complementing conventional CTL-mediated elimination of virus-infected cells. It reveals a novel principle of how innate immune sensing in infected target cells supports cell death execution and attributes specificity to death-inducing effector functions from adaptive immunity. The identification of this noncanonical CTL effector function will aid in the design of strategies for overcoming dysfunctional immune responses in persistent viral infections.

EXPERIMENTAL PROCEDURES

Determination of Liver Damage

sALT levels were determined with Reflotron test strips (GPT) with a Reflotron analysis system from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Animal experiments were conducted after approval of experimental protocols by local authorities and kept under standardized SPF-conditions in the central animal facility of the Medical Faculty of the University of Bonn.

Viruses

Recombinant adenovirus expressing Ovalbumin, GFP, and Luciferase driven by a CMV promoter (AdOVA) and adenovirus expressing GFP driven by CMV promoters (AdGFP) were generated as described by Stabenow et al. (2010). Recombinant adenoviral stocks were grown and purified as described earlier by Sprinzl et al. (2001). Recombinant Ad-Virus was UV irradiated 50 mJ/cm^2 for 2.5 min. LCMV strain WE was kindly provided by K. Lang.

Flow Cytometry

A total of 5 × 10^5 to 1 × 10^6 cells were stained with saturating concentrations of antibodies plus 10 μg/ml Fc-block (clone 2.4G2) in FACS buffer (PBS/1% bovine serum albumin, 2 mM EDTA/0.02%NaAz). Data acquisition and analysis were conducted on a FACS CantoII (BD Bioscience) using FlowJo software (Tree Star, Ashland, OR, USA). To exclude dead cells, Hoechst 33258 (Sigma-Aldrich, Munich) was added at a final concentration of 1 μg/ml. TNF concentration was measured using FlowCytomix Basic kit (BenderMed Systems, Vienna) according to the manufacturer's instructions.

Statistical Analysis

Student's t test was used to determine statistical significance of results. Results are shown as mean ± SD for representative experiments. The p values <0.05 were considered significant: *<0.05, **<0.01, ***<0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.001.

LICENSING INFORMATION

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