RAPID COMMUNICATION

Bioluminescence Imaging Allows Measuring CD8 T Cell Function in the Liver

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In vivo **evaluation of CD8 T cell effector (cytotoxic T lymphocyte [CTL]) function in peripheral organs such as the liver is currently not possible but would greatly improve our understanding of local immune regulation, because simple determination of antigen-specific CTL numbers does not predict the outcome of immune responses. In particular, measurement of alanine aminotransferase serum levels is not sensitive enough to detect T cell immunity against low numbers of target hepatocytes. We developed a procedure that detects virus-specific effector function of CTLs in the liver after simultaneous adenoviral transfer of reporter and immune target genes into hepatocytes, followed by bioluminescence imaging of reporter genes. Bioluminescence imaging enabled detection of as few as 10,000 infected hepatocytes *in vivo*, and even more importantly, quantification of antiviral effector function of as few as 50,000 CTLs. *Conclusion:* Our results provide evidence that low numbers of antigen-specific CTLs are sufficient to control viral gene expression and eliminate viral infection from hepatocytes. The experimental system established here is a highly sensitive method to simultaneously detect viral infection of hepatocytes and to quantify antiviral CTL function in the liver *in vivo* and will help in characterizing principles of hepatic immune regulation.** (HEPATOLOGY 2010;51:1430-1437)

The function of CD8 T cells is essential to control viral infection and combat cancer in peripheral organs. Determination of numbers of virus-specific or cancer-specific CD8 T cells (cytotoxic T lymphocytes [CTLs]) by sophisticated tetramer/multimer technology or by ELISpot (enzyme-linked immunosorbent spot assay) revealed the presence of significant numbers of antigen-specific T cells circulating in the bloodstream,[1-3] but it did not reflect clinical outcome in persistent viral infection or in cancer. Rather, numbers of tissue-infiltrating CTLs correlated with the clinical course.[4,5] This indicated that measurement of CTL function in liver is mandatory for prediction of efficacy of immune treatment modalities. So far, antiviral CTL function in liver was determined by indirect measures: (1) quantification of hepatocyte-damage by serum alanine aminotransferase (ALT) levels; (2) quantification of CTL infiltration by histochemistry; or (3) reduction in serum levels of viral proteins or genes. Although these are well-recognized parameters for measuring antiviral immune responses, they are subject to low sensitivity (ALT measurements), sampling error (liver biopsy for histochemistry), or only reflect the effect of immune responses in the past (serum levels of viral proteins/DNA or RNA). Our understanding of antiviral T cell immunity in the liver is based on experiments involving genetically modified animals with hepatocyte-restricted transgene expression and human material where a dual role of noncytopathic mechanisms together with cytotoxic effector function of virus-specific CD8 T cells was reported.[6,7]

Abbreviations: AdLGO, recombinant adenovirus expression luciferase, green fluorescent protein, and ovalbumin; ALT, alanine aminotransferase; CFSE, carboxyfluorescein succinimidyl ester; CTL, cytotoxic CD8 T lymphocyte; EGFP, enhanced green fluorescent protein; IFA, incomplete Freund's adjuvant; IFN, interferon; OVA, ovalbumin; PCR, polymerase chain reaction.

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Both mechanisms cooperate in order to achieve viral clearance from infected hepatocytes.[8] However, virus-specific T cells infiltrating the liver have been found to be functionally impaired[9,10] or become deleted,[11,12] and thus simple enumeration of infiltrating T cells in the peripheral blood or even in the liver does not reflect local T cell function.[13] Here, we report that bioluminescence imaging of reporter gene expression in hepatocytes can be used to quantify CTL function in the liver *in vivo*.

Materials and Methods

Mice. C57BL/6 mice were obtained from Elevage Janvier (France). C2J (B6(Cg)-*Tyr*$^{c-2J}$/J) albino mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred under specific pathogen-free (SPF) conditions in the central animal facility of the University Hospital Bonn. *In vivo* experiments were approved by the Animal Care Commission of Nordrhein-Westfalia.

Recombinant Adenovirus. E1-deleted and E3-deleted adenoviral vectors (recombinant adenovirus expression luciferase, green fluorescent protein, and ovalbumin [AdLGO]) expressing fusion proteins of the enhanced green fluorescent protein (EGFP) (Promega), the H2-K^b-binding peptide epitope OVA$_{aa257-264}$ SIINFEKL (OVAp) and the click-beetle luciferase (Promega, Mannheim, Germany) were generated through Cre-lox recombination as described.[14] AdLGO was propagated on human embryonic kidney (HEK 293) cells, purified by cesium chloride density-gradient centrifugation according to standard protocols.[15]

Isolation and Generation of CD8 T Effector Cells. CD8 T cells isolated from spleen and lymph nodes of OT-1 T cell receptor transgenic mice recognizing the H2-K^b-restricted peptide SIINFEKL derived from ovalbumin (OVA) were stimulated with peptide-pulsed splenic dendritic cells *in vitro*. After 4 days, cells were purified by gradient centrifugation and were injected into the tail vein.

Bioluminescence Measurement. Measurement of bioluminescence was performed with an IVIS 200 system (Caliper LifeSciences, Hopkinton, MA) 5 minutes after intraperitoneal injection of Luciferin (50 mM) (Caliper LifeSciences) in 200 μL of phosphate-buffered saline. Acquisition time for bioluminescence measurement was in the range between 10 and 60 seconds, depending on the charge-coupled device camera saturation. Data analysis was performed with Living Image 2.50.1 software (Caliper LifeSciences). For data analysis, a region of interest was set around the maximum of light emission and kept constant during all measurements within an experiment.

In Vivo *Cytotoxicity.* The assay was performed as described.[16,17] Briefly, splenocytes were loaded with SIINFEKL peptide and labeled CFSE^{high} (carboxyfluorescein succinimidyl ester; 2 μM) whereas control cells were left unloaded and labeled CFSE^{low} (0.2 μM). Cells were mixed at a 1:1 ratio, and specific killing of 1×10^5 target cells in spleen and liver was determined after 5 hours by flow cytometry. Specific kill was calculated as follows: % specific kill $= 100 - (100^*[\text{CFSE}^{high}/\text{CFSE}^{low}]_{sample}/[\text{CFSE}^{high}/\text{CFSE}^{low}]_{control})$.

Real-Time Polymerase Chain Reaction. For quantitative real-time polymerase chain reaction (PCR), DNA was extracted from 9-12 mg of liver tissue with the viral$^+$DNA-OLS kit (Omni Life Science, Hamburg, Germany). Serial dilutions of pAd-Track plasmids were used as a standard. For each sample, 4 μL LightCycler FS DNA Master Plus SYBR Green I (Roche, Mannheim, Germany), 12 μL double distilled H_2O, forward (5′ TAAGCGACGGATGTGG 3′) and reverse (5′ CCACGTAAACGGTCAAAG 3′) primers (20 μM) each 1 μL, and 2 μL DNA were mixed in a capillary and used for measurement by means of a LightCycler (Roche, Mannheim, Germany).

Statistical Analysis. The Student *t* test was used for *in vitro* and *in vivo* experiments. Results are shown as mean ± standard error. *P* values < 0.05 were considered significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

In Vivo *Bioluminescence Imaging Detects 10,000 Hepatocytes Infected with Recombinant Adenovirus Expressing Luciferase.* The lack of a reliable and sensitive method to directly assess T cell function in the liver prompted us to develop a procedure that can be easily implemented to determine CTL function in the liver. Advances in the sensitivity of *in vivo* bioluminescence imaging of gene expression[18] provided the basis for discovering novel principles of infection by pathogens using recombinant luciferase-expressing microorganisms,[19] for detection of tumor growth of luciferase-expressing tumor cells,[20] and for tracking the *in vivo* migration pattern of immune cells.[21] We used a recombinant adenoviral vector bearing the genes for firefly luciferase, green fluorescent protein, and ovalbumin as a fusion protein under cytomegalovirus promoters (AdLGO) that allows for simultaneous

detection of both virus-infected hepatocytes by marker genes and CTL function against infected hepatocytes expressing OVA as target gene. Intravenous injection of adenovirus vectors led predominantly to infection of the liver visualized by *in vivo* detection of luciferase expression and within the liver to preferential infection of hepatocytes detected by GFP expression with confocal laser scanning microscopy (Fig. 1A,B), as previously reported.[15,22] Measurement of luciferase expression by *in vivo* bioluminescence imaging allowed quantification of hepatocellular infection with recombinant AdLGO with much higher sensitivity than detection of GFP expression in liver tissue by confocal laser scanning microscopy (Fig. 1B-D). Hepatic bioluminescence was directly proportional to the numbers of infectious virions injected up to 3 days after infection (Fig. 1C). Assuming that more than 90% of intravenously injected adenoviruses infected hepatocytes,[15] bioluminescence imaging enabled sensitive detection of as little as 10,000 to 100,000 infected hepatocytes *in vivo* (Fig. 1D). To further increase sensitivity, we used C2J-H2^b-albino mice that show less nonspecific photon adsorption. In these mice, we were able to augment sensitivity of bioluminescence detection of virally encoded luciferase expression in hepatocytes by a factor of five (Fig. 1E). It was astonishing that little variation in infection efficiency existed between animals even when low numbers of infectious viruses were used (Fig. 1C,D), indicating that determination of hepatocellular infection by bioluminescence imaging was very sensitive yet also robust.

Sensitive* In Vivo *Detection of CD8 T Cell Function in the Liver. Next, we determined the ability of activated OVA-specific H2-K^b-restricted CTLs to exert antigen-specific effector function by recognizing OVA-derived SIINFEKL peptide that is presented on liver cells of AdLGO-infected mice. After adoptive transfer of 5×10^6 *in vitro*-activated CD69$^+$CD44$^+$CD25$^+$ CTLs, we observed increased ALT serum levels only in AdLGO-infected but not in AdLG-infected animals, which confirms antigen-specific execution of CTL function against infected hepatocytes *in vivo* (Fig. 2A). Under these conditions, maximally 2×10^8 AdLGO-infected hepatocytes served as targets for 5×10^6 CTLs, leading to immune-mediated liver damage detected as an increase in ALT level. However, after reducing the numbers of infected hepatocytes to approximately 10^6 cells, we failed to detect CTL-mediated liver damage as determined by ALT serum levels (Fig. 2B). This revealed the low sensitivity of ALT as a measure to detect effector CTL function in the liver. It is of interest to note that hepatocyte damage in combination with CTL infiltration into liver tissue was only observed in mice infected with 2×10^8 plaque-forming units (pfu) of AdLGO, i.e., high numbers of infected hepatocytes. We observed no liver damage, but scattered infiltrating CTLs were observed after adoptive transfer of 5×10^5 to 5×10^6 CTLs into mice infected with 10^6 pfu AdLGO (Fig. 2C). These findings suggested that infiltrating CTLs may exert effector functions against infected hepatocytes even in situations where only low numbers of infected hepatocytes are present and no ALT serum levels can be detected.

When bioluminescence imaging was used for detection of virus-encoded luciferase expression, thus providing a direct measure of infected hepatocytes, we observed that adoptive transfer of CTLs reduced detectable luciferase expression (Fig. 2D), indicating execution of CTL function in the liver under the conditions of the experiment. Importantly, titration of the number of adoptively transferred CTLs showed that bioluminescence imaging allowed detection of antiviral effector function of as little as 10^4 to 5×10^5 CTLs. These results demonstrate the superior sensitivity of bioluminescence imaging over ALT measurements. Time course experiments further revealed that adoptive transfer of 10^6 CTLs eliminated established luciferase activity *in vivo*, whereas adoptive transfer of 10^5 CTLs only controlled viral gene expression in infected hepatocytes (not shown). To exclude noncytolytic control of viral infection by release of interferon-gamma (IFNγ) from activated CTLs, we used IFNγR$-/-$ animals for infection and as recipients of CTLs. Clearly, reduction in bioluminescence was maintained under the conditions of the experiment (Fig. 2E), indicating that elimination of virus-infected hepatocytes rather than noncytolytic control of viral infection by IFNγ was operative in CTL-mediated antiviral immunity. Our results establish that bioluminescence imaging is more sensitive than ALT serum levels for detection of local effector CTL function against virus-infected hepatocytes.

In Vivo *Detection of Antiviral CTL Function in the Liver After Immunization.* We next aimed to demonstrate the usefulness of our assay system for monitoring CTL function in the liver after vaccination. C2J mice bearing 5×10^4 OVA-specific naïve CD8 T cells were immunized with OVA in incomplete Freund's adjuvant (IFA), which is known to elicit only a weak immune stimulatory reaction, or were left untreated. Four days after vaccination, animals were challenged with AdLGO (10^5 pfu, thus infecting approximately 0.01% of hepatocytes) and determined

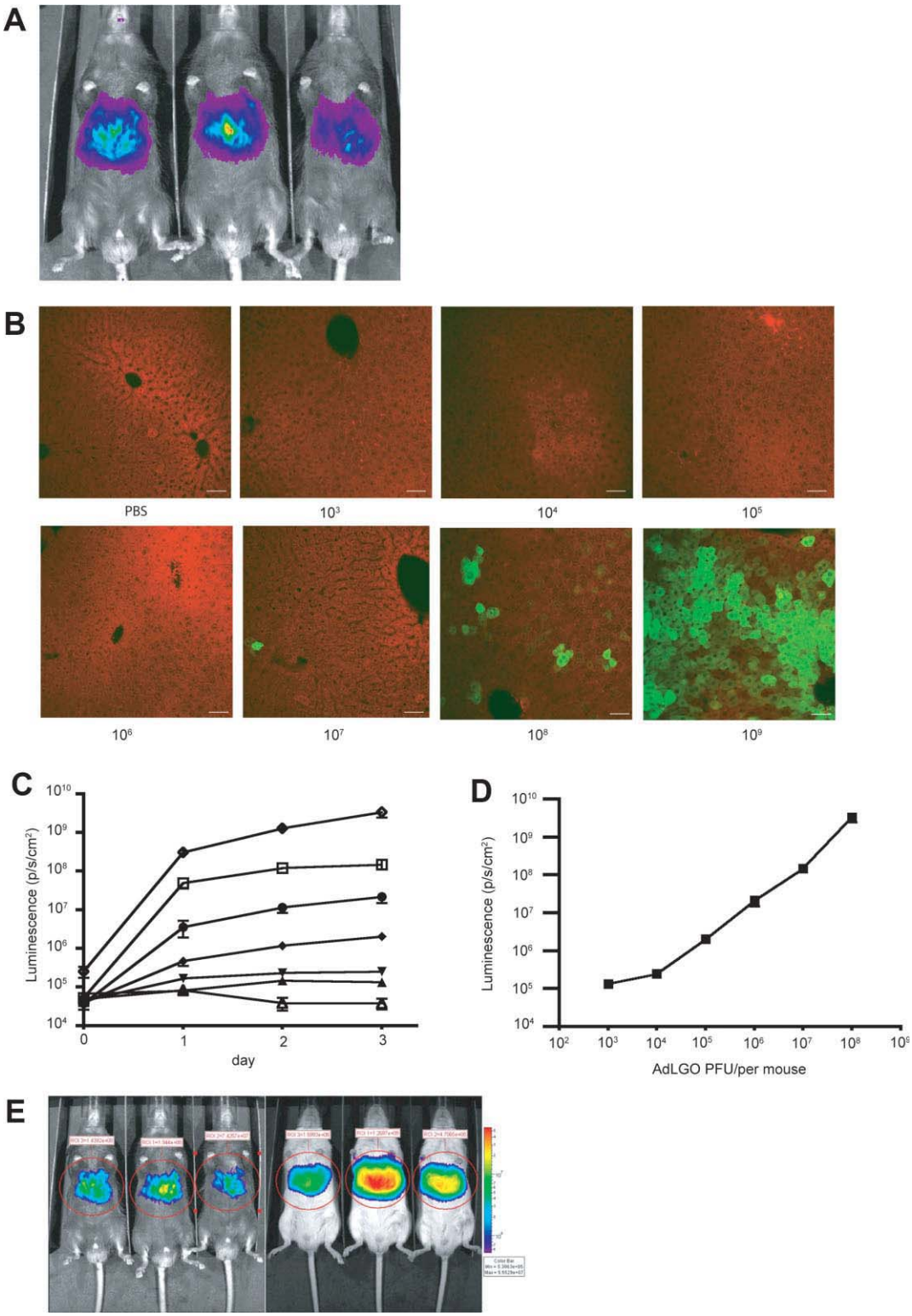

Fig. 1. Sensitive detection of hepatocellular virus infection by in vivo bioluminescence measurement. (A) Bioluminescence image of $H2^b$ mice at day 3 after intravenous infection with AdLGO. (B) Detection of GFP+ hepatocytes by confocal laser scanning microscopy in AdLGO-infected mice. Numbers denote pfu AdLGO/mouse; bar = 100 μm. (C) Time course of in vivo bioluminescence determination in AdLGO-infected (σ, 1×10^3, τ, 1×10^4, ◆, 1×10^5, ●, 1×10^6, □, 1×10^7, ◇, 1×10^8 pfu/mouse) or mock-treated (△) mice. (D) Bioluminescence values from (C) at day 3. (E) Comparison of bioluminescence obtained from C57Bl/6 mice (left) versus C2J (right) after intravenous infection with equal amounts of AdLGO (10^7 pfu/mouse).

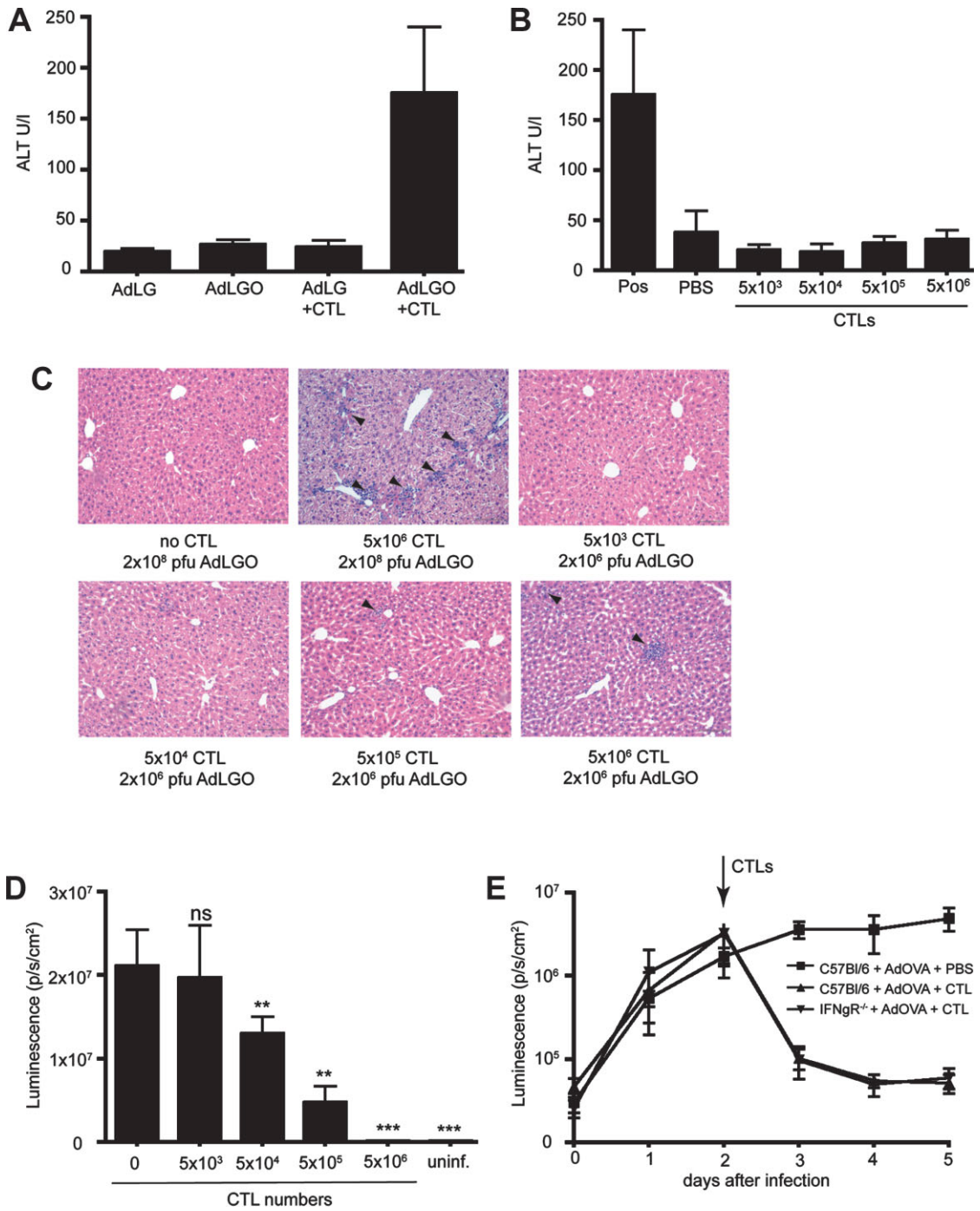

Fig. 2. Sensitive detection of antiviral CD8 T cell function in the liver using in vivo bioluminescence imaging. (A) Two days after intravenous infection of $H2^b$-mice with 2×10^8 pfu/mouse of AdLGO or AdLG, in vitro-activated OVA-specific CTLs (5×10^6) were adoptively transferred and serum ALT levels were determined 2 days later. (B) Mice were injected with AdLGO as indicated, and 2 days later different numbers of OVA-specific CTLs were adoptively transferred followed by serum ALT measurements 2 days later. (C) Hematoxylin/eosin staining of liver sections from mice treated as in (B). Bar = 100 μm. Arrowheads indicate lymphocytic infiltration. (D) Two days after intravenous infection with 10^6 pfu AdLGO/mouse, different numbers of OVA-specific CTLs were adoptively transferred followed by in vivo bioluminescence measurement 2 days later. (E) Wild-type and IFN$\gamma R^{-/-}$ mice were adoptively transferred with OVA-specific CTLs (10^6/mouse) 2 days after AdLGO infection (10^6 pfu/mouse); time couse of bioluminescence is as indicated.

bioluminescence on the subsequent days. Figure 3A clearly shows that luciferase expression in the liver was controlled only in animals subjected to OVA/IFA immunization but not in mock-treated animals. Longitudinal measurement of bioluminescence demonstrated that hepatocellular luciferase expression after adenoviral infection was controlled in vaccinated animals right from the start, whereas mock-treated animals

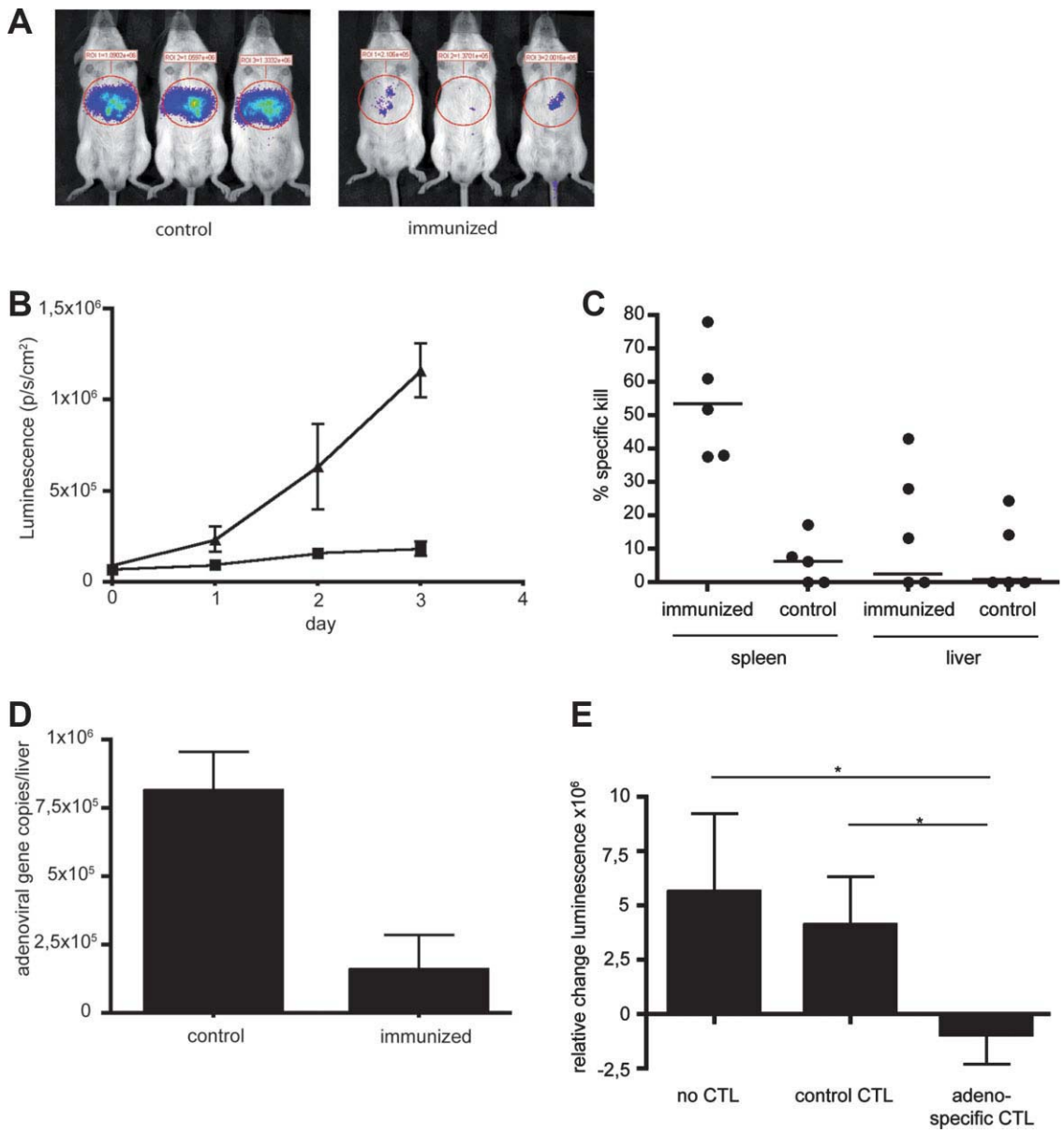

Fig. 3. Monitoring antiviral CD8 T cell function in the liver by bioluminescence imaging after immunization. (A,B) Naïve OT-1 T cells (5×10^4 cells) were transferred into mice that were subsequently immunized with OVA/IFA or were mock-treated. AdLGO infection (10^5 pfu/mouse) 4 days after immunization. (A) Bioluminescence images of immunized or mock-treated mice at day 3 after infection. (B) Time course of luciferase activity over a period of 3 days. (C) Mice received the same number of naïve OT-1 T cells and were immunized with OFA/IFA or mock-treated, as in (A). Four days later, Ova-specific in vivo CTL cytotoxicity was determined. (D) Mice were treated as in (A); real-time PCR quantitation of adenoviral DNA from liver extracts on day 3 after infection. (E) CTLs (5×10^6) obtained from mice infected 8 days previously with AdCre (10^6 pfu/mouse) or mock-treated mice were adoptively transferred into mice that were infected 2 days previously with AdLGO (10^6 pfu/mouse). Bioluminescence was measured 24 hours after adoptive transfer.

experienced a failure to control infection and subsequent viral marker gene expression (Fig. 3B). We evaluated whether measurement of CTL activity by *in vivo* cytotoxicity assay was similar in sensitivity as bioluminescence. Using low numbers of target cells and low numbers of antigen-specific CTLs, sensitive detection of specific cytotoxicity was only possible in the spleen, whereas no specific activity was detected in the liver (Fig. 3C), demonstrating that antiviral CTL activity in the liver fails to be detected by determination of intravascular CTL function.

In order to determine whether effector CTLs generated by immunization contributed not only to the control of luciferase expression but also to clearance of adenovirus from infected hepatocytes, we investigated hepatocellular persistence of adenoviral DNA by real-

time PCR. Only immunized but not mock-treated animals experienced a clearance of adenoviral DNA from the liver at day 3 after infection (Fig. 3D), thus indicating that control of *in vivo* bioluminescence in AdLGO-infected animals correlated with viral clearance from infected hepatocytes.

To provide more compelling evidence for specific CTL activity against viral determinants in our system, we adoptively transferred CTLs from mice that raised an immune response against adenoviral infection. In comparison to CTLs obtained from nonimmune mice, adenovirus-specific CTLs efficiently controlled luciferase expression in AdLGO-infected animals (Fig. 3E).

Discussion

Antigen-specific T cell immunity controls viral infection of the liver. Persistent infection with hepatotropic viruses, however, has been linked to viral immune escape strategies and the unique tolerogenic immune function of the liver.[12,23] Often, transgenic animal models were used to study antiviral immunity in the liver.[7] In these models, all hepatocytes expressed the viral antigen as a transgene and large numbers of virus-specific T cells were adoptively transferred to study the course of antiviral T cell immunity, which was determined by an increase in ALT or decline of viral antigens in serum. This does not reflect the real situation of hepatocyte infection *in vivo*, where low numbers of CD8 T cells have to interact with low numbers of virus-infected hepatocytes. The direct evaluation of CTL function *in vivo* under these conditions, however, is difficult and has not been established yet. Methods to determine CTL function, such as the *in vivo* cytotoxicity assay, mainly determine intravascular immune responses,[24] but ignore local control of CTL function by liver-resident cells and microenvironmental immune-regulatory cues.[25,26] Thus, no methods are available to directly determine with high sensitivity CTL function in the liver in situations when only few infected hepatocytes serve as targets and only low numbers of antigen-specific CTLs are present.

Here, we report a new assay system based on bioluminescence imaging that allows very sensitive evaluation of antigen-specific CTL function in the liver and is more sensitive in detection of liver-specific CTL function than conventional *in vivo* cytotoxicity assays. Previously, bioluminescence imaging was successfully used to unravel important pathophysiological mechanisms of infection and persistence of various microorganisms.[18,19] Here, we employ bioluminescence detection of luciferase expression in adenovirus-infected hepatocytes to measure antigen-specific CTL responses directed against these infected hepatocytes that express a fusion protein of luciferase and OVA. First, bioluminescence imaging allows for the most sensitive detection of up to 10,000 infected hepatocytes expressing luciferase. Second, our assay system is several orders of magnitude more sensitive in detection of local antiviral CTL effector function in the liver than determination of serum ALT, and our assay allows for longitudinal measurements of T cell function because bioluminescence imaging can be repeatedly performed in anesthetized animals. Importantly, our assay system allows quantification of the activity of low numbers of circulating CTLs (10^4) against a low number of infected hepatocytes (10^5), thus reflecting the natural situation during viral infection. Because the numbers of liver-infiltrating CTLs are considerably lower than the numbers of circulating CTLs (data not shown), we assume that we still underestimate the true sensitivity of the assay. Importantly, direct *in vivo* determination of CTL function provides a new tool to characterize the principles underlying antiviral immunity in the liver. Our data demonstrate that low numbers of CTLs were sufficient to control viral gene expression in infected hepatocytes *in vivo*. Given its high sensitivity, robustness, convenience, and speed, we anticipate that this assay system will become a powerful tool for anyone studying T cell immunity in the liver.

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