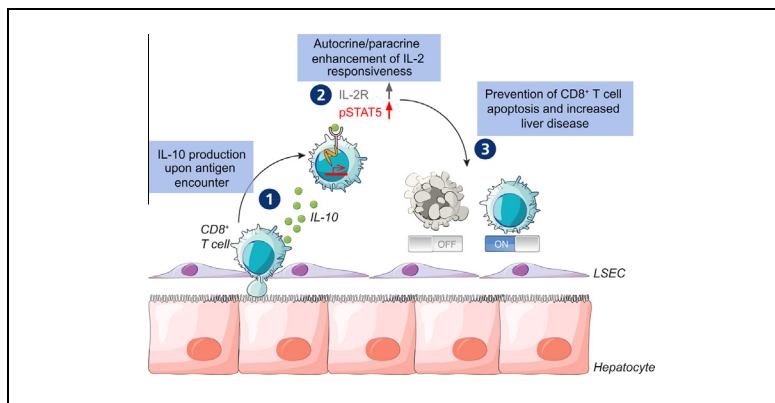


Effector CD8⁺ T cell-derived interleukin-10 enhances acute liver immunopathology

Graphical Abstract



Highlights

- Effector CD8⁺ T cells produce IL-10 upon hepatocellular antigen encounter.
- IL-10 enhances IL-2 responsiveness.
- IL-10 inhibits antigen-induced effector CD8⁺ T cell apoptosis.
- CD8⁺ T cell-derived IL-10 supports liver immunopathology.

Authors

Jessica Fioravanti, Pietro Di Lucia, Diletta Magini, ..., Francis V. Chisari, Luca G. Guidotti, Matteo Iannacone

Correspondence

guidotti.luca@hsr.it
(L.G. Guidotti), iannacone.matteo@hsr.it
(M. Iannacone)

Lay summary

Interleukin-10 is mostly regarded as an immunosuppressive cytokine. We show here that HBV-specific CD8⁺ T cells produce IL-10 upon antigen recognition and that this cytokine enhances CD8⁺ T cell survival. As such, IL-10 paradoxically promotes rather than suppresses liver disease.

Effector CD8⁺ T cell-derived interleukin-10 enhances acute liver immunopathology

Jessica Fioravanti¹, Pietro Di Lucia¹, Diletta Magini¹, Federica Moalli^{1,3}, Carolina Boni², Alexandre Pierre Benechet¹, Valeria Fumagalli¹, Donato Inverso^{1,3}, Andrea Vecchi², Amleto Fiocchi¹, Stefan Wieland⁴, Robert Purcell⁵, Carlo Ferrari², Francis V. Chisari⁴, Luca G. Guidotti^{1,*}, Matteo Iannacone^{1,3,6,*}

¹Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy;

²Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, 43100 Parma, Italy; ³Vita-Salute San Raffaele University, 20132 Milan, Italy; ⁴Department of Immunology and Microbial Sciences, The Scripps Research Institute, La Jolla, CA 92037, USA;

⁵Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA; ⁶Experimental Imaging Center, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy

Background & Aims: Besides secreting pro-inflammatory cytokines, chemokines and effector molecules, effector CD8⁺ T cells that arise upon acute infection with certain viruses have been shown to produce the regulatory cytokine interleukin (IL)-10 and, therefore, contain immunopathology. Whether the same occurs during acute hepatitis B virus (HBV) infection and role that IL-10 might play in liver disease is currently unknown.

Methods: Mouse models of acute HBV pathogenesis, as well as chimpanzees and patients acutely infected with HBV, were used to analyse the role of CD8⁺ T cell-derived IL-10 in liver immunopathology.

Results: Mouse HBV-specific effector CD8⁺ T cells produce significant amounts of IL-10 upon *in vivo* antigen encounter. This is corroborated by longitudinal data in a chimpanzee acutely infected with HBV, where serum IL-10 was readily detectable and correlated with intrahepatic CD8⁺ T cell infiltration and liver disease severity. Unexpectedly, mouse and human CD8⁺ T cell-derived IL-10 was found to act in an autocrine/paracrine fashion to enhance IL-2 responsiveness, thus preventing antigen-induced HBV-specific effector CD8⁺ T cell apoptosis. Accordingly, the use of mouse models of HBV pathogenesis revealed that the IL-10 produced by effector CD8⁺ T cells promoted their own intrahepatic survival and, thus supported, rather than suppressed liver immunopathology.

Conclusion: Effector CD8⁺ T cell-derived IL-10 enhances acute liver immunopathology. Altogether, these results extend our understanding of the cell- and tissue-specific role that IL-10 exerts in immune regulation.

Lay summary: Interleukin-10 is mostly regarded as an immunosuppressive cytokine. We show here that HBV-specific CD8⁺ T cells produce IL-10 upon antigen recognition and that this cytokine enhances CD8⁺ T cell survival. As such, IL-10 paradoxically promotes rather than suppresses liver disease.

© 2017 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Introduction

It is widely recognized that hepatitis B virus (HBV) replicates non-cytopathically in the hepatocyte. Most of the clinical complications related to this infection is reflected in the adaptive immune response, particularly the virus-specific effector CD8⁺ T cell response.^{1–5} Indeed, by killing infected cells and secreting antiviral cytokines, effector CD8⁺ T cells (CD8 T_E) reaching the infection sites are major contributors to viral clearance as well as tissue immunopathology.^{1–5}

Paradoxically, during some acute viral infections (e.g. those caused by influenza virus, respiratory syncytial virus, coronavirus and vaccinia virus), CD8 T_E have been shown to produce the regulatory cytokine interleukin (IL)-10,^{6–11} in addition to pro-inflammatory cytokines, chemokines and effector molecules. CD8 T_E-derived IL-10 is generally thought to limit tissue damage,^{6,7,11} however the net effect induced by this cytokine might depend on the pathophysiological context.¹² Whether HBV-specific CD8 T_E produce IL-10 upon hepatocellular antigen (Ag) recognition and the role that this cytokine plays in liver immunopathology are currently unknown.

We show here that serum IL-10 is readily detectable in an acutely HBV-infected adult chimpanzee in a manner that coincides with intrahepatic CD8⁺ T cell infiltration. Using mouse models of acute HBV pathogenesis, we confirmed that IL-10 is indeed produced by HBV-specific CD8 T_E upon hepatocellular Ag recognition *in vivo*. To our surprise, IL-10 blockade ameliorated rather

Keywords: CD8⁺ T cells; IL-10; Liver immunopathology; Hepatitis B virus.

Received 13 September 2016; received in revised form 27 March 2017; accepted 25 April 2017; available online 5 May 2017

* Corresponding author. Address: Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Via Olgettina 58, Milan 20132, Italy. Tel.: +39 (02) 2643 6359; fax: +39 (02) 2643 6822.

E-mail addresses: guidotti.luca@hsr.it (L.G. Guidotti), iannacone.matteo@hsr.it (M. Iannacone).



Research Article

than worsened liver disease. *Ex vivo* analyses of HBV-specific CD8 T_E isolated from the livers of HBV replication-competent transgenic mice or from the blood of acutely infected patients revealed that IL-10 acts in an autocrine/paracrine fashion to increase IL-2 responsiveness and rescue CD8 T_E from Ag-induced apoptosis.

Materials and methods

Chimpanzees

Chimpanzee A0A006 has already been described.¹³ The animal was handled according to humane use and care guidelines specified by Animal Research Committees at the National Institutes of Health, The Scripps Research Institute, and Bioqual Laboratories. The chimpanzee was individually housed at Bioqual Laboratories (Rockville, MD), an American Association for Accreditation of Laboratory Animal Care International-accredited institution under contract to the National Institute of Allergy and Infectious Diseases. Chimpanzee A0A006 was inoculated with 10¹⁰ genome equivalents of HBV obtained from an HBV-positive serum of chimpanzee 5835 that was previously inoculated with a monoclonal HBV isolate (genotype D, ayw subtype; GenBank accession no. V01460)²⁵ contained in HBV transgenic mouse serum,¹⁴ as described. Blood was obtained by venipuncture and analyzed for serum IL-10 (see below).

Mice

C57BL/6, CD45.1 (inbred C57BL/6), and Balb/c mice were purchased from Charles River. *IL-10*^{-/-} mice (B6.129P2-*Il10*^{tm1Cgn/J}) were purchased from The Jackson Laboratory. HBV replication-competent transgenic mice (lineage 1.3.32, inbred C57BL/6, H-2^b), that express all of the HBV antigens and replicate HBV in the liver at high levels without any evidence of cytopathology, were previously described.^{13,14} In indicated experiments, these mice were used as C57BL/6 x Balb/c H-2^{bxd} F1 hybrids. HBV nucleocapsid (Cor)-specific (referred to as Cor93 cells) T cell receptor (TCR) transgenic mice (lineage BC10.3, inbred CD45.1), in which >98% of the splenic CD8⁺ T cells recognize a K^b-restricted epitope located between residues 93–100 in the HBV Core protein (MGLKFRQL), were previously described.¹⁷ Env28 (envelope) TCR transgenic mice (lineage 6C2.36, inbred Balb/c), in which ~83% of the splenic CD8⁺ T cells recognize a L^d-restricted epitope located between residues 28–39 of HBsAg (IPQSLDSWWTSL), were previously described.¹⁷ In indicated experiments, lineage BC10.3 or lineage 1.3.32 were crossed with *IL-10*^{-/-} mice. Mice were housed under specific pathogen-free conditions and used at 8–10 weeks of age. In all experiments, mice were matched for age, sex and (for the 1.3.32 animals) serum HBeAg levels before experimental manipulations. All experimental animal procedures were approved by the Institutional Animal Committee of San Raffaele Scientific Institute.

Patients

Five patients with acute self-limited HBV infection were enrolled at the Unit of Infectious Diseases and Hepatology in Parma, Italy. Patients had clinical, biochemical, and virological evidence of acute HBV infection (aminotransferase levels at least 10 times the upper normal limit and detection of HBsAg and IgM anti-HBcAg Ab in the serum). Patients were negative for anti-HCV, anti-delta virus, anti-HIV-1 and anti-HIV-2 Ab and for other markers of viral or autoimmune hepatitis. T cell response was tested one month from the time of acute illness.

The study was approved by the Ethical Committee of the Azienda Ospedaliero-Universitaria of Parma, and all subjects gave written informed consent.

Generation of CD8 T_E and adoptive transfer

In vitro generation of CD8 T_E was performed as described.^{16,26} Briefly, splenocytes from Cor93 or Env28 TCR transgenic mice were incubated with 10 µg/ml of Cor93-100 (K^b; MGLKFRQL) or Env28-39 (L^d; IPQSLDSWWTSL) peptides (Primm), respectively, at 37 °C for 1 h, washed, and cultured in complete RPMI 1640 (10% FBS, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, HEPES 10 mM, non essential amino acid 100 µM and penicillin plus streptomycin). Two days later, cells were cultured in fresh medium supplemented with 2.5% EL-4 supernatant. Media sup-

plemented with cytokines were replaced every 2 days. After 8 or 9 days of culture, cells were tested for the expression of CD8, CD69, CD25, CD44, CD62L, CCR7, IFN-γ and granzyme B by FACS prior to subsequent use, as described.¹⁶ 10⁷ cells of each cell type were injected intravenously into recipient animals.

RNA analyses

Total RNA was isolated from cultured cells or frozen livers (left lobe) with RNeasy Lysis Miniprep system (Promega) following the manufacturer's instructions. For quantitative RT-PCR, 1 µg of total RNA was reverse transcribed prior to qPCR analysis for mouse *il10* and *ifng* (TaqMan Mm01288386 and Mm01168134 probes, Applied Biosystems) in an ABI 7900HT fast real-time PCR system (Applied Biosystems). All experiments were performed in triplicate and normalized to the reference gene *GAPDH*.

Enzyme-linked immunosorbent assays

IL-10 and IFN-γ in mouse sera or cell supernatants were measured by ELISA (Biolegend and R&D, respectively), according to the manufacturer's instructions. HBeAg in mouse sera was measured by ELISA (Diapron) following the manufacturer's instructions. IL-10 in chimpanzee sera was measured using a human IL-10 ELISA (R&D Systems) according to the manufacturer's instructions.

Biochemical analyses

The extent of hepatocellular injury was monitored by measuring serum alanine aminotransferase (ALT) activity at multiple time points after treatment, as previously described.¹⁶

In vivo IL-10R-specific antibody treatment

Mice were injected i.v. with 250 µg of anti-IL-10Rα blocking Ab (clone 1B1.3 A, BioXCell) 2 h prior to CD8 T_E transfer.

Isolation of primary hepatocytes

Primary hepatocytes were isolated from wild-type or *IL-10*^{-/-} HBV replication-competent transgenic mice (inbred C57BL/6) exactly as described.¹⁶ Hepatocyte purity (assessed by flow cytometry-based parameters of size) and viability (assessed by light microscopy-based morphology and Trypan blue dye exclusion) were routinely greater than 70% and 80%, respectively. Hepatocytes (10⁶ cells/ml) were incubated at a 1:2 ratio with Cor93 CD8 T_E for 4 h in the presence of 10 µg/ml brefeldin A (BFA, Sigma) prior to intracellular IFN-γ staining.

Cell isolation and flow cytometry

Single-cell suspensions of livers were generated as described.¹⁸ For analysis of *ex vivo* intracellular cytokine production, cell suspensions of livers were obtained as described above except that 1 µg/ml of BFA (Sigma) was included in the digestion buffer. All flow cytometry stainings of surface-expressed and intracellular molecules were performed as described.²⁷ Antibodies (Abs) used included PB- and PE-conjugated anti-CD8α (53-6.7), Alexa Fluor 488-, PerCP-, and APC-Cy7-conjugated anti-CD45.1 (A20), Alexa Fluor 488-, and Alexa Fluor 647-conjugated anti-IFN-γ (XMG1.2), PE- and PB-conjugated anti-CD25 (PC61), APC-conjugated Annexin V (EOS9.1), 7AAD (BD Pharmingen). All Abs were purchased from BioLegend, unless otherwise indicated. For phosphorylated STAT5 analysis, cells were fixed with 4% paraformaldehyde, permeabilized with absolute methanol and stained with PE-Cy7-conjugated anti-phosphoSTAT5 (47/Stat5pY694, eBioscience). All flow cytometry analyses were performed in FACS buffer containing PBS with 2 mM EDTA and 2% FBS on a FACS CANTO (BD Pharmingen) and analyzed with FlowJo software (Treestar).

Histochemistry

For Haematoxylin and Eosin staining, livers were perfused with PBS, harvested in Zn-formalin and transferred into 70% ethanol 24 h later. Tissue was then processed, embedded in paraffin and stained as previously described.¹⁶ Bright-field images were acquired through an Aperio Scanscope System CS2 microscope and an ImageScope program (Leica Biosystem) following the manufacturer's instructions. The extent of hepatocellular injury was monitored by histopathological

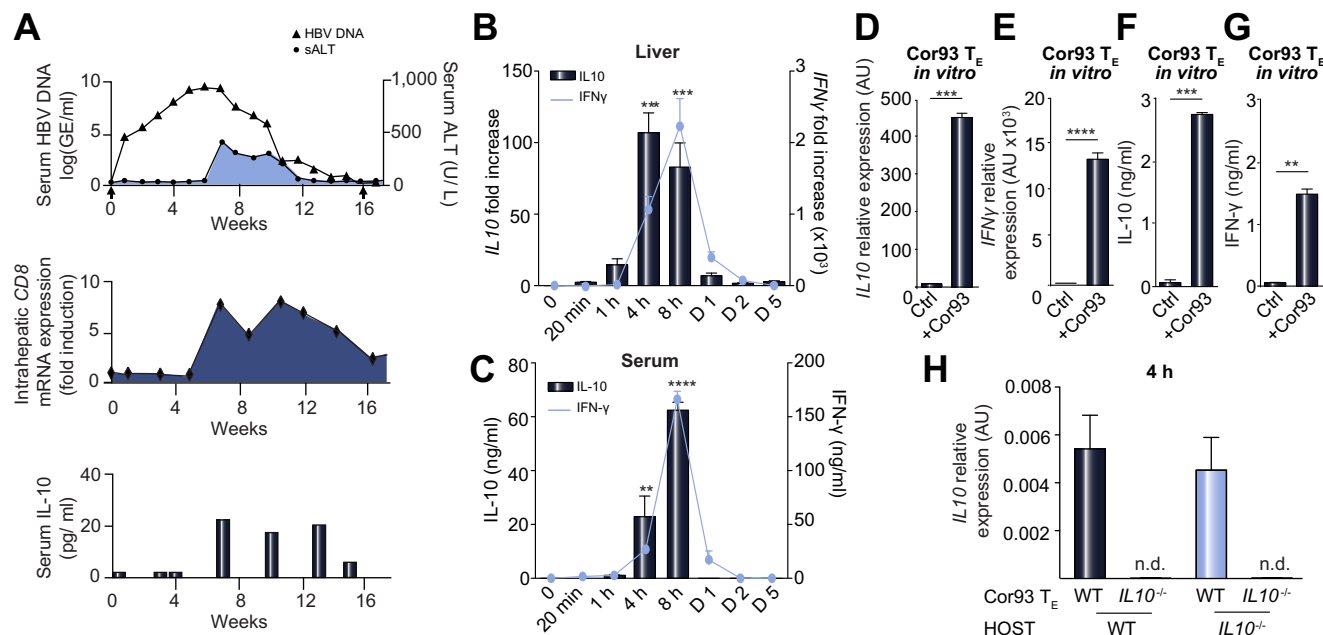


Fig. 1. CD8 T_E produce IL-10 upon hepatocellular Ag recognition. (A) Determination of serum IL-10 in chimpanzee A0A006 after experimental intravenous inoculation of 10¹⁰ of HBV.¹³ (Top panel) Serum HBV DNA levels were determined by quantitative real-time PCR and are shown as GE/ml (left axis). Serum ALT activity (light blue shaded area) is shown as U/L (right axis); (middle panel) intrahepatic CD8 mRNA expression is shown as a shaded blue area (fold induction compared to two pre-inoculation time points); (bottom panel) Serum IL-10 is expressed in pg/ml. (B) Quantification of hepatic IL-10 (dark blue bars) and IFN-γ (light blue line) mRNA (fold increase over PBS-injected controls) at the indicated time points upon adoptive transfer of Cor93 CD8 T_E into HBV replication-competent transgenic mice. n ≥ 3/time point; results are representative of 3 independent experiments. (C) Quantification of IL-10 (dark blue bars) and IFN-γ (light blue line) in the serum of the same mice described in B. (D-G) Cor93 T_E cells were incubated *in vitro* with PBS (Ctrl) or Cor93 peptide for 4 h prior to quantifying IL-10 (D) or IFN-γ (E) mRNA in cell lysates (expression relative to the mouse reference gene GAPDH), and IL10 (F) or IFN-γ (G) proteins in the supernatant. n = 3; results are representative of 3 independent experiments. (H) Quantification of hepatic IL-10 mRNA (expression relative to the mouse reference gene GAPDH) 4 h after adoptive transfer of 10⁷ IL-10^{+/+} or IL-10^{-/-} Cor93 CD8 T_E into IL-10^{+/+} or IL-10^{-/-} HBV replication-competent transgenic mice. n = 3; results are representative of 2 independent experiments. Results are expressed as mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Means between two groups were compared with two-tailed t test. Means among three or more groups were compared with one-way or two-way analysis of variance with Bonferroni's post-hoc test.

and quantitative morphometric analyses as described.¹⁸ The number of injured hepatocytes (identified as either apoptotic or necrotic based on standard cytopathological criteria) and intrahepatic inflammatory cells (mononuclear and polymorphonuclear) were counted in at least 50 high power fields of liver tissue (corresponding to about 2 mm²). Results are expressed as number of cells per mm².

In vitro cell culture assays with murine CD8 T_E

To test the expression and production of IL-10 and IFN-γ by Cor93 T_E cells were incubated in complete RPMI 1640 media with 2 μg/ml of rhIL-2 (Roche) at 5 × 10⁶ cells/ml for 4 h at 37 °C in the presence or absence of the Cor93 peptide. To assess the effect of IL-10 on Ag-induced apoptosis, Cor93 CD8 T_E (10⁷ cells/ml) were incubated for 1 h at 37 °C with 18 μg/ml of anti-IL-10R Ab (BioXCell), 400 ng/ml of recombinant mIL-10 (BioLegend) or left untreated prior to the addition of Cor93 peptide (1 mg/ml) and human IL-2 (1 mg/ml). For the assessment of cell viability, cells were harvested 8 h later and stained with Annexin V and 7AAD, as described above. For the assessment of CD25 expression, cells were harvested 24 h after peptide stimulation. For STAT5 phosphorylation, Cor93 CD8 T_E were cultured overnight in serum-free RPMI complete medium (Gibco, Life Technologies), prior to treating them as described above. Cells were harvested 15 min after peptide stimulation.

In vitro cell culture assays with human CD8 T_E

To measure the effect of IL-10 on Ag-induced apoptosis, peripheral blood mononuclear cells (PBMC) from five HLA-A201⁺ patients with acute self-limited hepatitis B (concentration of 2 × 10⁶/ml) were incubated for 1 h at 37 °C with 20 μg/ml of anti-IL-10R Ab (BD Pharmingen), 200 ng/ml of recombinant human IL-10 (BioLegend) or left untreated prior to the addition of Core 18-27 peptide

(1 μg/ml) and human IL-2 (100 IU/ml). After a 5 h incubation, cells were extensively washed, stained with Core 18-27 dextramer, anti-CD8 and anti-CD3 mouse Abs for 15 min in the dark, then stained with Annexin V and 7AAD (BD Pharmingen), according to the Annexin V staining protocol (BD Pharmingen). The cells were acquired immediately on a FACSCANTO II multicolor flow cytometer and were analyzed with the DIVA software (BD Biosciences, Immunocytometry Systems, CA, USA).

Tissue DNA analyses

Total DNA was isolated from frozen livers (left lobe) for Southern blot analysis, as previously described.¹⁸

Statistical analyses

Results are expressed as mean + SEM. All statistical analyses were performed in Prism 5 (GraphPad Software). Means between two groups were compared with two-tailed t test. Means among three or more groups were compared with one-way or two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. Some data were analyzed using Fisher's Least Significant Difference (LSD) test (stated in figure legend). Statistical analysis was performed on single independent experiments utilizing individual results from each animal. The n indicated in the figure legends always reflects the number of biological replicates (e.g. mice) that were included in the single experiment that is shown in the figures and on which statistical analysis was performed. When we indicated in the figure legends that the results are representative of x independent experiments, it means that we have completed x experiments and they all showed similar statistical significance. Those additional experiments are however not shown nor included in the statistical analysis.

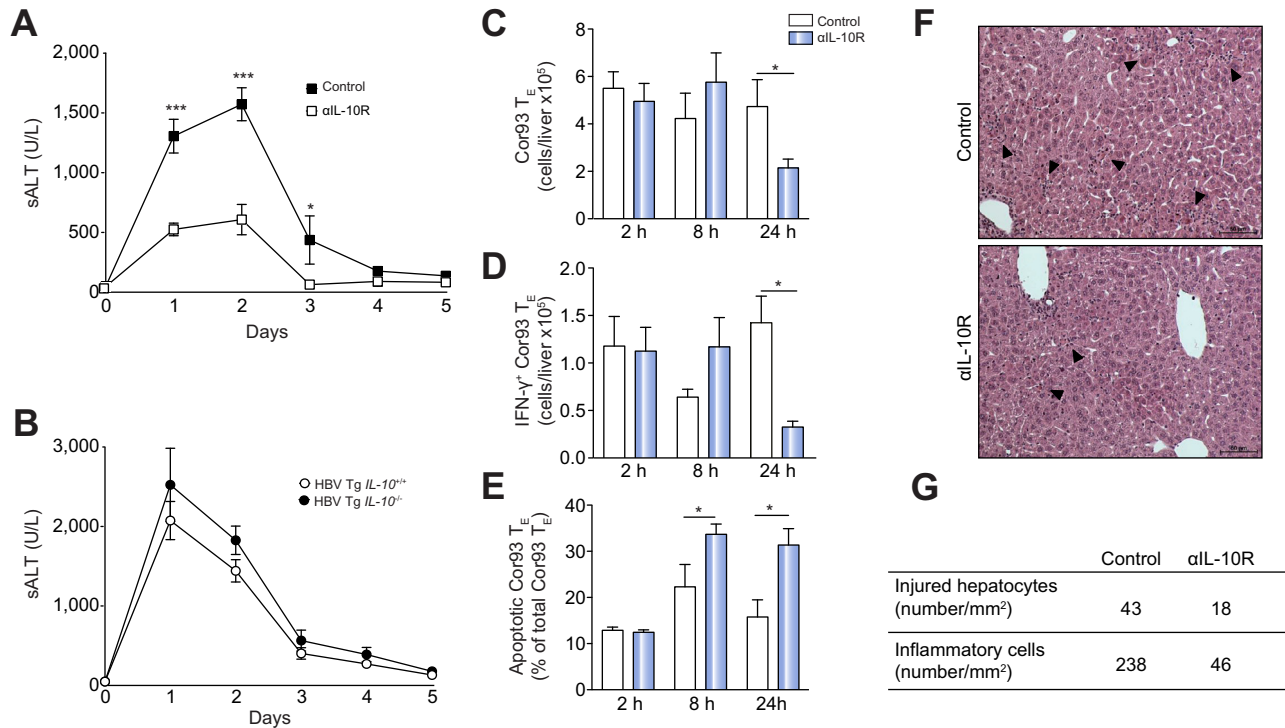


Fig. 2. IL-10 promotes liver immunopathology by preventing CD8 T_E apoptosis. (A) ALT activity measured in the serum (sALT) of HBV replication-competent transgenic mice treated or not with anti-IL10Rα 2 h before the injection of 10⁷ Cor93 CD8 T_E. n = 5; results are representative of 3 independent experiments. (B) ALT activity measured in the serum of IL-10^{+/+} or IL-10^{-/-} HBV replication-competent transgenic mice upon 10⁷ Cor93 CD8 T_E injection. n = 10; results are representative of 2 independent experiments. (C–E) Quantification of the absolute number (C), IFN-γ⁺ (D), and apoptotic (E) Cor93 CD8 T_E recovered at the indicated time points from the livers of HBV replication-competent transgenic mice that were treated or not with anti-IL-10Rα Abs prior to Cor93 CD8 T_E injection. n = 5; results are representative of 2 independent experiments. (F) Histological analysis of representative HBV replication-competent transgenic mice treated or not with anti-IL10Rα 2 and sacrificed 2 days after the injection of 10⁷ Cor93 CD8 T_E. Arrowheads denote necroinflammatory foci. Scale bars represent 50 μm. (G) Quantitative morphometry of dead and degenerating (injured) hepatocytes and intrahepatic mononuclear and polymorphonuclear (inflammatory) cells in the same mice described in F. A minimum of 50 high power (400×) fields representing 2 mm² of liver tissue were examined. The results represent the mean of five observations (five mice). Results are expressed as mean + SEM. *p < 0.05, ***p < 0.001. Means between two groups were compared with two-tailed t test. Means among three or more groups were compared with one-way or two-way analysis of variance with Bonferroni's post-hoc test.

For further details regarding the materials used, please refer to the [Supplementary material](#) and the [CTAT table](#).

Results and discussion

CD8 T_E produce IL-10 upon hepatocellular Ag recognition

We first assessed whether IL-10 is produced upon acute HBV infection. We longitudinally analyzed the sera of a chimpanzee (A0A006) that had been inoculated with a monoclonal HBV inoculum of 10¹⁰ GE of HBV DNA, as described.¹³ Serum IL-10 was not detectable until CD8⁺ T cells accumulated in the liver and its appearance coincided with the onset of a necroinflammatory liver disease (Fig. 1A), suggesting that this cytokine might have been produced by virus-specific CD8 T_E upon hepatocellular Ag recognition. To test this hypothesis and to assess the role of IL-10 in liver immunopathology, we employed a well-established model of acute HBV pathogenesis, *i.e.* the adoptive transfer of HBV-specific CD8⁺ T_E into HBV replication-competent transgenic mice.^{14–16} Naïve CD8⁺ T cells from HBV nucleocapsid (Cor)-specific TCR transgenic mice¹⁷ (referred to as Cor93 cells) were differentiated *in vitro* into *bona fide* CD8 T_E. Upon intravenous injection of 10⁷ Cor93 CD8 T_E into HBV replication-competent transgenic mice, the hepatic mRNA expression of the *il10* gene

increased sharply, reaching peak levels at 4–8 h after T cell transfer and mirroring the kinetics of the prototypical CD8 T_E-derived pro-inflammatory and antiviral cytokine gene *ifng* (Fig. 1B). Accordingly, IL-10 was also detected in the serum of these mice between 4 and 8 h after T cell transfer, again echoing the IFN-γ kinetics (Fig. 1C). Consistent with the hypothesis that IL-10 is produced by CD8 T_E upon hepatocellular antigen recognition, we found that Cor93 T_E cells stimulated for 4 h *in vitro* with the cognate Cor93 peptide produced both cytokines (Fig. 1D–G; Fig. S1). Finally, to unambiguously identify the cellular source of the IL-10 detected in the liver of HBV replication-competent transgenic mice upon adoptive transfer of Cor93 CD8 T_E, we genetically deleted the *il10* gene in HBV replication-competent recipient mice, in Cor93 CD8 T_E or in both. By using this approach, we could demonstrate that the transferred CD8 T_E cells are the unique source of IL-10 in this experimental setting, as *il10* expression was only detected when the transferred T cells were IL10-competent, regardless of the recipient genotype (Fig. 1H).

IL-10 promotes liver immunopathology by preventing CD8 T_E apoptosis

To gain insight into the role of CD8 T_E-derived IL-10 expression in liver immunopathology, we selectively blocked IL-10 receptor

signaling by injecting HBV replication-competent transgenic mice with an anti-IL-10R α Ab 2 h prior to Cor93 T_E transfer. To our surprise, we found that IL-10 blockade decreased, rather than increased, liver damage by about 3-fold at the peak of the disease (Fig. 2A). Of note, anti-IL-10R α Ab injection did not affect the total number of circulating Cor93 T_E cells (data not shown), indicating that this treatment did not deplete the transferred T cells. Also, anti-IL-10R α Ab treatment significantly reduced liver disease when HBV envelope-specific TCR transgenic^{16,17} or polyclonal CD8 T_E¹⁸ were injected into HBV replication-competent transgenic mice instead of Cor93 T_E (data not shown). Moreover, consistent with IL-10 being produced exclusively by the transferred CD8 T_E in this model, deletion of the *il10* gene in HBV replication-competent recipient mice – which did not alter the Ag presentation capacity of hepatocytes (Fig. S2) – did not affect the severity of liver disease induced by Cor93 T_E transfer (Fig. 2B). To explore the mechanisms underlying this IL-10-mediated increase in liver immunopathology, we quantified the number, function and viability of intrahepatic Cor93 CD8 T_E 2, 8, and 24 h after adoptive transfer into HBV replication-competent transgenic mice that were or were not subjected to anti-IL-10R α Ab treatment. IL-10R blockade significantly decreased the number of total and IFN- γ ⁺ intrahepatic Cor93 CD8 T_E cells recovered 24 h after injection (Fig. 2C and D). We next addressed if this reflected an increase in cell death. Indeed, the percentage of

intrahepatic Cor93 CD8 T_E that underwent apoptosis at 8 and 24 h after transfer was significantly increased by IL-10R blockade (Fig. 2E; Fig. S3), and this was reflected by a lower disease severity (Fig. 2F and G). Although the number of intrahepatic Cor93 T_E was reduced upon IL-10R blockade, these cells probably produced enough IFN- γ to abolish viral replication (Fig. S4).

IL-10 rescues CD8 T_E from Ag-induced apoptosis by increasing IL-2 responsiveness

To explore if CD8 T_E-derived IL-10 directly decreased Ag-induced apoptosis, we exposed purified Cor93 CD8 T_E to Ag in the presence or absence of IL-10R blockade or exogenous IL-10. As shown in Fig. 3A, Ag exposure for 8 h triggered Cor93 CD8 T_E apoptosis. Notably, IL-10R blockade increased the percentage of Cor93 CD8 T_E that became apoptotic, whereas the addition of exogenous recombinant IL-10 decreased Cor93 CD8 T_E apoptosis (Fig. 3A and Fig. S5A). We next set out to determine the mechanism whereby IL-10 prevents CD8 T_E from dying. Since IL-10 has been proposed to enhance the growth of activated CD8⁺ T cells in the presence of IL-2,^{19–21} we reasoned that CD8 T_E-derived IL-10 might increase IL-2 responsiveness by, for instance, modulating IL-2 receptor expression. Indeed, Ag-induced IL-2R α (CD25) upregulation on CD8 T_E was lower upon IL-10R blockade, whereas the addition of exogenous recombinant IL-10 increased CD25 upregulation (Fig. 3B and Fig. S5B). Accordingly, blocking IL-10R signaling decreased the capacity of CD8 T_E to respond to IL-2 (as assessed by STAT5 phosphorylation), and IL-10 treatment increased IL-2 sensitivity (Fig. 3C; Fig. S5C). Finally, we explored whether the capacity of CD8 T_E-derived IL-10 to act in an autocrine/paracrine fashion to rescue CD8 T_E from Ag-triggered apoptosis was restricted to murine CD8 T_E or it extended to HBV-specific CD8 T_E isolated from acutely infected patients. To this end, PBMCs from 5 HLA-A201⁺ patients with acute hepatitis B, in whom HBV-specific CD8⁺ T cells can be specifically visualized by HBV-specific dextramers²² (Fig. S6), were stimulated with the cognate Cor18 peptide in the presence or absence of anti-IL-10R α Abs or exogenous IL-10. The results mirrored those obtained with murine CD8 T_E, in that IL-10R blockade increased Ag-induced apoptosis, whereas the addition of recombinant IL-10 partially rescued CD8 T_E from cell death (Fig. 3D).

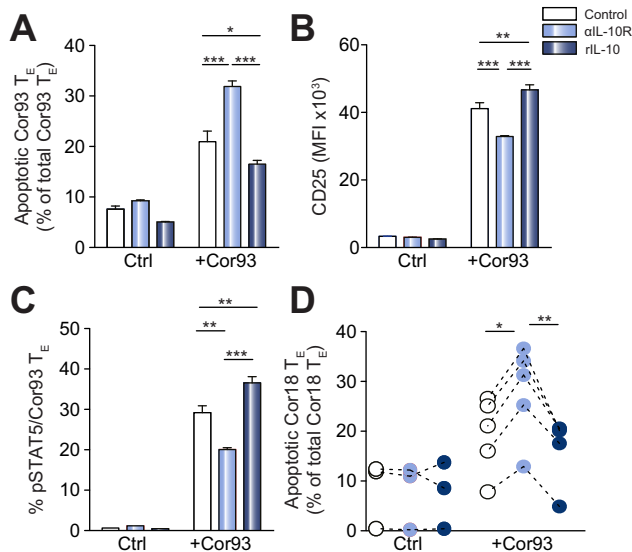


Fig. 3. IL-10 rescues CD8 T_E from Ag-induced apoptosis by increasing IL-2 responsiveness. (A–C) Cor93 T cells were incubated with medium (white), anti-IL10R α (18 μ g/ml, light blue) or rIL-10 (400 ng/ml, dark blue). After 1 h Cor93 peptide (1 mg/ml) was added (+ Cor93) or not (Ctrl). The percentage of Annexin V⁺ (apoptotic) cells (A), the CD25 expression (B) and the percentage of phospho-STAT5⁺ cells (C) was determined on Cor93 CD8 T_E 8 h, 24 h and 15 min later, respectively. n = 3; results are representative of 3 independent experiments. (D) PBMCs from 5 HLA-A201⁺ patients acutely infected with HBV were incubated with medium (white), anti-IL-10R α (20 μ g/ml, light blue), or rIL-10 (200 ng/ml, dark blue). After 1 h Cor18-27 peptide (1 μ g/ml) was added (+Cor18) or not (Ctrl). The percentage of Annexin V⁺ (apoptotic) cells was determined on Core 18-27 dextramer⁺ cells 5 h later. Results are representative of 2 independent experiments. Results are expressed as mean + SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Means between two groups were compared with two-tailed *t* test. Means among three or more groups were compared with one-way or two-way analysis of variance with Bonferroni’s post-hoc test. Data in Fig. 3D were analyzed using Fisher’s Least Significant Difference (LSD) test.

Conclusion

In conclusion, our results indicate that CD8 T_E-derived IL-10 acts in an autocrine/paracrine fashion to increase IL-2 responsiveness, rescuing CD8 T_E from Ag-induced apoptosis. Although the net contribution that IL-10 plays during a natural HBV infection – where this cytokine may be produced by additional cell types^{23,24} – remains to be determined, the results described herein suggest that IL-10 may promote rather than suppress liver immunopathology.

Financial support

This work was supported by ERC grants 281648 (to M.I.) and 250219 (L.G.G), Italian Association for Cancer Research (AIRC) grants 9965 and 15350 (to M.I.), Italian Ministry of Health grant GR-2011-02347925 (to M.I.) and RF-2013-02355209 (to L.G.G),

Research Article

Fondazione Regionale per la Ricerca Biomedica grant 2015-0010 (to M.I.), European Molecular Biology Organization (EMBO) Young Investigator Program (to M.I.), EMBO Long-Term Fellowships ALTF 694-2016 (to A.B.) and ALTF 1018-2016 (to D.I.), a Marie Curie Intra-European Fellowship (IEF) for Career Development 327336 (to J.F.) and a Career Development Award from the Giovanni Armenise-Harvard Foundation (to M.I.).

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Please refer to the accompanying [ICMJE disclosure](#) forms for further details.

Authors' contributions

J.F., D.M., P.D.L., C.B., A.P.B., F.M., V.F., D.I., A.V., A.F., S.W. performed experiments; J.F., D.M., P.D.L. and M.I. analyzed data; J.F., D.M. and F.M. prepared the figures; R.P., C.F., F.V.C. and L.G.G. provided conceptual advice; F.V.C. and L.G.G. revised the manuscript; M.I. and L.G.G. provided funding; M.I. designed and coordinated the study and wrote the manuscript.

Acknowledgements

We thank L. Giustini, M. Mainetti and M. Raso for technical support; M. Silva for secretarial assistance and the members of the Iannacone laboratory for helpful discussions. Flow cytometry was carried out at FRACTAL, a flow cytometry resource and advanced cytometry technical applications laboratory established by the San Raffaele Scientific Institute.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2017.04.020>.

References

- [1] Guidotti LG, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006;1:23–61.
- [2] Iannacone M, Guidotti LG. Mouse models of hepatitis B virus pathogenesis. *Cold Spring Harbor Perspect Med* 2015;5.
- [3] Wong YC, Tay SS, McCaughan GW, Bowen DG, Bertolino P. Immune outcomes in the liver: Is CD8 T cell fate determined by the environment? *J Hepatol* 2015;63:1005–1014.
- [4] Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 2003;77:68–76.
- [5] Phillips S, Chokshi S, Riva A, Evans A, Williams R, Naoumov NV. CD8(+) T cell control of hepatitis B virus replication: direct comparison between cytolytic and noncytolytic functions. *J Immunol* 2010;184:287–295.
- [6] Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat Med* 2009;15:277–284.
- [7] Sun J, Cardani A, Sharma AK, Laubach VE, Jack RS, Müller W, et al. Autocrine regulation of pulmonary inflammation by effector T-cell derived IL-10 during infection with respiratory syncytial virus. *PLoS Pathog* 2011;7:e1002173.
- [8] Puntambekar SS, Bergmann CC, Savarin C, Karp CL, Phares TW, Parra GI, et al. Shifting hierarchies of interleukin-10-producing T cell populations in the central nervous system during acute and persistent viral encephalomyelitis. *J Virol* 2011;85:6702–6713.
- [9] Cush SS, Reynoso GV, Kamenyeva O, Bennink JR, Yewdell JW, Hickman HD. Locally produced IL-10 limits cutaneous vaccinia virus spread. *PLoS Pathog* 2016;12:e1005493.
- [10] Zou Q, Wu B, Xue J, Fan X, Feng C, Geng S, et al. CD8+ Treg cells suppress CD8+ T cell-responses by IL-10-dependent mechanism during H5N1 influenza virus infection. *Eur J Immunol* 2014;44:103–114.
- [11] Trandem K, Zhao J, Fleming E, Perlman S. Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis. *J Immunol* 2011;186:3642–3652.
- [12] Gao B. Hepatoprotective and anti-inflammatory cytokines in alcoholic liver disease. *J Gastroenterol Hepatol* 2012;27:89–93.
- [13] Asabe S, Wieland SF, Chattopadhyay PK, Roederer M, Engle RE, Purcell RH, et al. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. *J Virol* 2009;83:9652–9662.
- [14] Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus infection in transgenic mice. *J Virol* 1995;69:6158–6169.
- [15] Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity* 1996;4:25–36.
- [16] Guidotti LG, Inverso D, Sironi L, Di Lucia P, Fioravanti J, Ganzer L, et al. Immunosurveillance of the liver by intravascular effector CD8(+) T cells. *Cell* 2015;161:486–500.
- [17] Isogawa M, Chung J, Murata Y, Kakimi K, Chisari FV. CD40 activation rescues antiviral CD8+ T cells from PD-1-mediated exhaustion. *PLoS Pathog* 2013;9:e1003490.
- [18] Iannacone M, Sitia G, Isogawa M, Marchese P, Castro MG, Lowenstein PR, et al. Platelets mediate cytotoxic T lymphocyte-induced liver damage. *Nat Med* 2005;11:1167–1169.
- [19] Chen WF, Zlotnik A. IL-10: a novel cytotoxic T cell differentiation factor. *J Immunol* 1991;147:528–534.
- [20] Groux H, Bigler M, de Vries JE, Roncarolo MG. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J Immunol* 1998;160:3188–3193.
- [21] Geginat J, Larghi P, Paroni M, Nizzoli G, Penatti A, Pagani M, et al. The light and the dark sides of Interleukin-10 in immune-mediated diseases and cancer. *Cytokine Growth Factor Rev* 2016. <http://dx.doi.org/10.1016/j.cytogfr.2016.02.003>.
- [22] Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, et al. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007;81:4215–4225.
- [23] Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683–765.
- [24] Gabryšová L, Howes A, Saraiva M, O'Garra A. The regulation of IL-10 expression. *Curr Top Microbiol Immunol* 2014;380:157–190.
- [25] Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* 1979;281:646–650.
- [26] Manjunath N, Shankar P, Wan J, Weninger W, Crowley MA, Hieshima K, et al. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J Clin Invest* 2001;108:871–878.
- [27] Tonti E, Jiménez de Oya N, Galliverti G, Moseman EA, Di Lucia P, Amabile A, et al. Bisphosphonates target B cells to enhance humoral immune responses. *Cell Reports* 2013;5:323–330.