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.

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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.
**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 increase IL-2 responsiveness, thus preventing antigen-induced IL-10 was found to act in an autocrine/paracrine fashion to increase IL-2 responsiveness, thus preventing antigen-induced 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. Altogether, these results extend our understanding of the cell- and tissue-specific role that IL-10 exerts in immune regulation.

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

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

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Materials and methods

Chimpanzees

Chimpanzee A04006 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 Bioal Laboratories. The chimpanzee was individually housed at Bioal 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 A04006 was inoculated with 1010 genome equivalents of HBV obtained from an HBV-positive serum of chimpanzee S835 that was previously inoculated with a monoclonal HBV isolate (genotype D, ayw subtype; GenBank accession no. V01460)16 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-Il10tm1Gve) were purchased from The Jackson Laboratory. HBV replication-competent transgenic mice (lineage BC10.3, inbred CD45.1), in which >98% of the splenic CD8+ T cells recognize a K5-restricted epitope located between residues 93–100 in the HBV Core protein (MGLKFRQL), were previously described.17 In indicated experiments, these mice were used as C57BL/6 x Balb/c H-2b x 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 K5-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 L6-restricted epitope located between residues 28–39 of HBVAg (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 weeks of age. In all experiments, mice were matched for age, sex, and (for the 1.3.32 animals) serum HBsAg 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 Tc and adoptive transfer

In vitro generation of CD8 Tc was performed as described.18,20 Briefly, splenocytes from Cor93 or Env28 TCR transgenic mice were incubated with 10 µg/ml of Cor93-100 (Kb; MGLKFRQL) or Env28-39 (Ld; 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 supplemented 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.18 107 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 ReliaPrep RNA 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. HBsAg in mouse sera was measured by ELISA (Diapro) 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-10R antibody (clone 1B1.3 A, BioXCell) 2 h prior to CD8 Tc 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 Tc 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.16 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.17 Antibodies (Abs) used included PE- and PE-conjugated anti-CD8α (53-67.7), 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 (EOS3.1), 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 Haemotoxylin 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.19 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 and quantitative morphometric analyses as described.19 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 Tc

To test the expression and production of IL-10 and IFN-γ by Cor93 Tc cells, cells were incubated in complete RPMI 1640 media with 2 µg/ml of rhIL-2 (Roche) at
CD8 Te 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 $10^{10}$ GE of HBV DNA, as described. Serum IL-10 was not detectable until CD8+ T cells accumulated in the liver. The production of IL-10 was accompanied by an increase in liver IFN-γ production. The results suggest that IL-10 may play a role in the development of acute liver immunopathology.

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.

Results and discussion

CD8 Te produce IL-10 upon hepatocellular Ag recognition

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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-IL-10R Ab 2 h before the injection of $10^7$ Cor93 CD8 T E. n = 10; results are representative of 2 independent experiments. (B) ALT activity measured in the serum of IL-10-/- or IL-10+/+ HBV replication-competent transgenic mice upon $10^7$ Cor93 CD8 T E injection. n = 5; results are representative of 3 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-10R 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-IL-10R Ab. Arrowheads denote necroinflammatory foci. Scale bars represent 50 μm. (G) Quantitative morphology 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 x) 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.

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 $10^7$ 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 CD8+ T 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 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 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 Ab treatment significantly reduced liver disease when HBV envelope-specific TCR transgenic16,17 or polyclonal CD8+ T E 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 CD8 T<sub>e</sub> 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<sub>e</sub> 2, 8, and 24 h after adoptive transfer into HBV replication-competent transgenic mice that were or were not subjected to anti-IL-10RA Ab treatment. IL-10R blockade significantly decreased the number of total and IFN-γ<sup>+</sup> intrahepatic Cor93 CD8 T<sub>e</sub> 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<sub>e</sub> 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<sub>e</sub> was reduced upon IL-10R blockade, these cells probably produced enough IFN-γ to abolish viral replication (Fig. S4).

**IL-10 rescues CD8 T<sub>e</sub> from Ag-induced apoptosis by increasing IL-2 responsiveness**

To explore if CD8 T<sub>e</sub>-derived IL-10 directly decreased Ag-induced apoptosis, we exposed purified Cor93 CD8 T<sub>e</sub> 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<sub>e</sub> apoptosis.

![Image](https://example.com/image.png)

**Fig. 3. IL-10 rescues CD8 T<sub>e</sub> from Ag-induced apoptosis by increasing IL-2 responsiveness.** (A-C) Cor93 T<sub>e</sub> cells were incubated with medium (white), anti-IL-10Rα (18 μg/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<sup>+</sup> (apoptotic) cells (A), the CD25 expression (B) and the percentage of phospho-STAT5<sup>+</sup> cells (C) were determined on Cor93 CD8 T<sub>e</sub> 8 h, 24 h and 15 min later, respectively. n = 3; results are representative of 3 independent experiments. (D) PBMCs from 5 HLA-A201<sup>-</sup> patients acutely infected with HBV were incubated with medium (white), anti-IL-10Rα (20 μg/ml, light blue), or rIL-10 (200 ng/ml, dark blue). After 1 h Cor18-27 peptide (1 μg/ml) was added (+Cor18) or not (Ctrl). The percentage of Annexin V<sup>+</sup> (apoptotic) cells were determined on Core 18-27 dextramers<sup>+</sup> 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. 3A were analyzed using Fisher’s Least Significant Difference (LSD) test.

Notably, IL-10R blockade increased the percentage of Cor93 CD8 T<sub>e</sub> that became apoptotic, whereas the addition of exogenous recombinant IL-10 decreased Cor93 CD8 T<sub>e</sub> apoptosis (Fig. 3A and Fig. S5A). We next set out to determine the mechanism whereby IL-10 prevents CD8 T<sub>e</sub> from dying. Since IL-10 has been proposed to enhance the growth of activated CD8<sup>+</sup> T cells in the presence of IL-2, we reasoned that CD8 T<sub>e</sub>-derived IL-10 might increase IL-2 responsiveness by, for instance, modulating IL-2 receptor expression. Indeed, Ag-induced IL-2Rα (CD25) upregulation on CD8 T<sub>e</sub> 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<sub>e</sub> 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<sub>e</sub>-derived IL-10 to act in an autocrine/paracrine fashion to rescue CD8 T<sub>e</sub> from Ag-triggered apoptosis was restricted to murine CD8 T<sub>e</sub> or it extended to HBV-specific CD8 T<sub>e</sub> isolated from acutely infected patients. To this end, PBMCs from 5 HLA-A201<sup>-</sup> patients with acute hepatitis B, in whom HBV-specific CD8<sup>+</sup> T cells can be specifically visualized by HBV-specific dextramers<sup>22</sup> (Fig. S6), were stimulated with the cognate Cor18 peptide in the presence or absence of anti-IL-10RA Abs or exogenous IL-10. The results mirrored those obtained with murine CD8 T<sub>e</sub> in that IL-10R blockade increased Ag-induced apoptosis, whereas the addition of recombinant IL-10 partially rescued CD8 T<sub>e</sub> from cell death (Fig. 3D).

**Conclusion**

In conclusion, our results indicate that CD8 T<sub>e</sub>-derived IL-10 acts in an autocrine/paracrine fashion to increase IL-2 responsiveness, rescuing CD8 T<sub>e</sub> 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<sup>23,24</sup> – remains to be determined, the results described herein suggest that IL-10 may promote rather than suppress liver immunopathology.

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**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.
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Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions


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Supplementary data

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