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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.

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

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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.
than worsened liver disease. Ex vivo analyses of HBV-specific CD8 Tc cells 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 Tc cells from Ag-induced apoptosis.

Materials and methods

Chimpanzees

Chimpanzee A0A006 has already been described. 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 10^9 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) contained in HBV transgenic mouse serum, 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-H2^Mdg^tm1Kob/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. In indicated experiments, these mice were crossed with C57BL/6 x Balb/c H-2^b-/- F1 hybrids. HBV nucleoprotein (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 Ld-restricted epitope located between residues 93–100 in the HBV Core protein (MGLKFRQQL), were previously described. Env28 (envelope) TCR transgenic mice (lineage 6C2.36, inbred Balb/c) were purchased from Primm, transgenic mouse serum, as described. Blood was obtained by venipuncture and analyzed for serum IL-10 (see below).

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.

Materials and methods

Generation of CD8 Tc and adoptive transfer

In vitro generation of CD8 Tc cells was performed as described. Briefly, splenocytes from Cor93 or Env28 TCR transgenic mice were incubated with 10 µg/ml of Cor93-100 (Kb; MGLKFRQQL) or Env28-39 (Ld; IQSYSDDSVWVTSL) 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, HEPE 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 (BD Pharmingen) and analyzed with FlowJo software (Treestar).

Materials and methods

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 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. 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 (10^6 cells/ml) were incubated at a 1:2 ratio with Cor93 C57BL/6 Tc cells for 4 h in the presence of 10 µg/ml brefedin A (BFA, Sigma) prior to intracellular IFN-γ staining.

Cell isolation and flow cytometry

Single-cell suspensions of livers were generated as described. 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. Antibodies (Abs) used included PE- and PE-conjugated anti-CD8A (53-6.7), Alexa Fluor 488-, PerCP-, and APC-Cy7-conjugated anti-CD45 (120), Alexa Fluor 488-, and Alexa Fluor 647-conjugated anti-IFN-γ (XMG1.2), PE- and PB-conjugated anti-CD25 (PC61), APC-conjugated Annexin V (E059.1), 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-phospho-STAT5 (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 10% for 2 h, washed, and cultured in complete RPMI 1640 (10% FBS, 2 mM L-glutamine, HEPE 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 (BD Pharmingen) and analyzed with FlowJo software (Treestar).
and quantitative morphometric analyses as described.\textsuperscript{15} 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 mm\textsuperscript{2}). Results are expressed as number of cells per mm\textsuperscript{2}.

\textit{In vitro cell culture assays with murine CD8 T cells.}\n
To test the expression and production of IL-10 and IFN-γ by Cor93 T\_\_ cells, cells were incubated in complete RPMI 1640 media with 2 \mu\text{g/ml} of rIL-2 (2 \text{Roche}) at 5 \times 10^6 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\_\_ cells (10^6 cells/ml) were incubated for 1 h at 37 °C with 18 \mu\text{g/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 24 h after peptide stimulation. For STAT5 phosphorylation, Cor93 CD8 T\_\_ cells 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.

\textit{In vitro cell culture assays with human CD8 T cells.}\n
To measure the effect of IL-10 on Ag-induced apoptosis, peripheral blood mononuclear cells (PBMC) from five HLA-A20\_1 patients with acute self-limited hepatitis B (concentration of 2 \times 10^6/ml) were incubated for 1 h at 37 °C with 20 \mu\text{g/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 Cor 18-27 peptide (1 \mu\text{g/ml}) and human IL-2 (100 IU/ml). After a 5 h incubation, cells were extensively washed, stained with Cose 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 FACS\textsuperscript{Canto II} multicolor flow cytometer and were analyzed with the DIVA software (BD Biosciences, Immunocytometry Systems, CA, USA).

\textbf{Tissue DNA analyses}\n
Total DNA was isolated from frozen livers (left lobe) for Southern blot analysis, as previously described.\textsuperscript{15}

\textbf{Statistical analyses}\n
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 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 10\(^{10}\) GE of HBV DNA, as described. Serum IL-10 was undetectable 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. Naive CD8\(^+\) T cells from HBV nucleocapsid (Cor)-specific TCR transgenic mice (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 sera of these mice between 4 and 8 h after T cell transfer, again echoing the IFN-\(\gamma\) 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 ifng 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 selected blocking IL-10 receptor...
signaling by injecting HBV replication-competent transgenic mice with an anti-IL-10Rα Ab 2 h prior to Cor93 T 푇 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-10Rα Ab injection did not affect the total number of circulating Cor93 T 푇 cells (data not shown), indicating that this treatment did not deplete the transferred T cells. Also, anti-IL-10Rα Ab treatment significantly reduced liver disease when HBV envelope-specific TCR transgenic or polyclonal CD8 푇 푇 were injected into HBV replication-competent transgenic mice instead of Cor93 T 푇 (data not shown). Moreover, consistent with IL-10 being produced exclusively by the transferred CD8 푇 푇 in this model, deletion of the ii10 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 푇 transfer (Fig. 2B).

To explore the mechanisms underlying this IL-10-mediated decrease in liver immunopathology, we quantified the number, function and viability of intrahepatic Cor93 CD8 푇 푇 2, 8, and 24 h after adoptive transfer into HBV replication-competent transgenic mice that were or were not subjected to anti-IL-10Rα Ab treatment. IL-10R blockade significantly decreased the number of total and IFN-γ 푑 intrahepatic Cor93 CD8 푇 푇 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 푇 푇 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 푇 was reduced upon IL-10R blockade, these cells probably produced enough IFN-γ to abolish viral replication (Fig. S4).

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

To explore if CD8 푇 푇-derived IL-10 directly decreased Ag-induced apoptosis, we exposed purified Cor93 CD8 푇 푇 to Ag in the presence or absence of IL-10R blockade or exogenous IL-10. Notably, IL-10R blockade increased the percentage of Cor93 CD8 푇 푇 that became apoptotic, whereas the addition of exogenous recombinant IL-10 decreased Cor93 CD8 푇 푇 apoptosis (Fig. 3A and Fig. S5A). We next set out to determine the mechanism whereby IL-10 prevents CD8 푇 푇 from dying. Since IL-10 has been proposed to enhance the growth of activated CD8 푇 푇 cells in the presence of 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 푇 푇-derived IL-10 to act in an autocrine/paracrine fashion to rescue CD8 푇 푇 from Ag-triggered apoptosis was restricted to murine CD8 푇 푇 or it extended to HBV-specific CD8 푇 푇 isolated from acutely infected patients. To this end, PBMCs from 5 HLA-A201 푇 patients with acute hepatitis B, in whom HBV-specific CD8 푇 푇 cells can be specifically visualized by HBV-specific dextramers27 (Fig. S6), were stimulated with the cognate Cor18 peptide in the presence or absence of anti-IL-10Rα Abs or exogenous IL-10. The results mirrored those obtained with murine CD8 푇 푇 in that IL-10R blockade increased Ag-induced apoptosis, whereas the addition of recombinant IL-10 partially rescued CD8 푇 푇 from cell death (Fig. 3D).

**Conclusion**

In conclusion, our results indicate that CD8 푇 푇-derived IL-10 acts in an autocrine/paracrine fashion to increase IL-2 responsiveness, rescuing CD8 푇 푇 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 types23,24 – 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.

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.

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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.

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