Archivum Immunologiae et Therapiae Experimentalis (2022) 70:25
https://doi.org/10.1007/s00005-022-00663-8

ORIGINAL ARTICLE

Interleukin-35 has a Protective Role in Infectious Mononucleosis-Induced Liver Inflammation Probably by Inhibiting $CD8^+$ T Cell Function

Ying Gao[1] · Lan Li[1] · Xingxing Hu[1] · Weihua Zhang[1] · Yu Li[2]

Received: 2 June 2022 / Accepted: 15 September 2022 / Published online: 11 October 2022

Abstract

Interleukin (IL)-35 plays an immunosuppressive role in infectious diseases, autoimmune disorders, and cancers. However, IL-35 expression and its regulation of $CD8^+$ T cells in infectious mononucleosis (IM) are not fully understood. In this study, three groups of participants were compared, including twenty-three patients of IM without liver inflammation, twenty-eight patients of IM with liver inflammation, and twenty-one controls. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated. $CD8^+$ T cells were purified. Plasma IL-35 was measured by ELISA. PBMCs and $CD8^+$ T cells were stimulated with recombinant human IL-35 in vitro. Perforin and granzyme B secretion was assessed by ELISPOT. Immune checkpoint molecule expression was investigated by flow cytometry. $CD8^+$ T cells were co-cultured with HepG2 cells in direct contact and indirect contact manner. The cytotoxicity of $CD8^+$ T cells was calculated by measuring lactate dehydrogenase release and proinflammatory cytokine expression. There was no significant difference in plasma IL-35 levels between patients with IM without liver inflammation and the controls, but the IL-35 level was notably increased in patients with IM who presented with liver inflammation and negatively correlated with aminotransferase. $CD8^+$ T cells in patients with IM with liver inflammation showed stronger cytotoxicity. IL-35 stimulation inhibited $CD8^+$ T cell-induced target cell death in patients with IM, mainly through suppression of IFN-γ/TNF-α secretion and elevation of immune checkpoint molecule expression, but did not affect perforin or granzyme B secretion. The current data indicated that IL-35 dampened the cytotoxicity of $CD8^+$ T cells in patients with IM probably via repression of cytokine secretion. Elevated IL-35 may protect against $CD8^+$ T cell-induced liver inflammation in patients with IM.

Keywords Interleukin-35 · $CD8^+$ T lymphocytes · Infectious mononucleosis · Liver inflammation · Immunoregulation

Abbreviations

ALT	Alanine aminotransferase
AST	Asparatate aminotransferase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FBS	Fetal bovine serum
HBV	Hepatitis B virus
IFN-γ	Interferon-γ
IL	Interleukin
IM	Infectious mononucleosis
LAG-3	Lymphocyte activation gene-3
LDH	Lactate dehydrogenase
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed death-1
SFC	Spot-forming cells
T-BIL	Total bilirubin
TIM-3	T cell immunoglobulin and mucin domain 3
TNF-α	Tumor necrosis factor-α
Tregs	Regulatory T cells
VCA	Viral capsid antigen

✉ Yu Li
drlee2810@126.com

[1] Department of Hematology, Shaanxi Provincial People's Hospital, The Affiliated Hospital of Xi'an Medical University, Xi'an 710068, Shaanxi Province, China

[2] Department of Infectious Diseases, Shaanxi Provincial People's Hospital, The Affiliated Hospital of Xi'an Medical University, 256 West Youyi Rd, Xi'an 710068, Shaanxi Province, China

Introduction

Epstein-Barr virus (EBV) is a γ-herpesvirus that infects the majority of the population worldwide (Henle et al. 1979). 85.3% of individuals were EBV seropositive between 0 and 25 years old in the United Kingdom (Kuri et al. 2020), while 97.3% of men aged 18–70 years were seropositive for EBV in Brazil, Mexico, and United States (Rahman et al. 2021). EBV infection is often asymptomatic, but some adolescents and young adults present with a clinical syndrome of infectious mononucleosis (IM) (Niederman et al. 1976). IM is clinically characterized by at least two of the classical symptoms of fever, pharyngitis, and lymphadenopathy (Hoagland 1952). Furthermore, 40–80% of patients with EBV-induced IM have liver inflammation, which mainly manifests as jaundice or acute hepatitis (Vine et al. 2012; Zhang et al. 2021a). Although IM-related liver inflammation is self-limited, in rare cases, severe and potentially fatal cases of hepatitis have been reported (Hu et al. 2018; Kamal and Haboubi 2021; Mellinger et al. 2014). The risk factors for a poor prognosis are older age (> 60 years old), co-infection with other hepatitis viruses, and afflicted splenomegaly (Hu et al. 2018; Zhang et al. 2021a). However, the mechanisms of liver inflammation in patients with IM are still not completely understood.

The progression of IM is closely associated with altered expression of inflammatory cytokines and disturbed T-cell homeostasis (Panikkar et al. 2015). Cytokine-mediated immune dysfunction contributes to dysregulated homeostasis during acute IM (Panikkar et al. 2015). Interleukin (IL)-35 is a new member of the IL-12 cytokine family and mainly plays an immunosuppressive role in infectious diseases (Teymouri et al. 2018). Chronic hepatitis B virus (HBV) and hepatitis C virus infection induced elevation of peripheral and liver-resident IL-35 (Liu et al. 2017; Shao et al. 2017; Taghinejad et al. 2020). Increased IL-35 expression mediated immune tolerance and promoted viral persistence through suppression of cytotoxicity of $CD8^+$ T cells (Li et al. 2015; Shao et al. 2017), inhibition of Th9 and Th17 cell activity (Yang et al. 2019a; Zhang et al. 2021b), and enhancement of $CD4^+CD25^+CD127^{dim/-}$ regulatory T cell (Treg) function (Liu et al. 2017; Shao et al. 2017; Yang et al. 2019a). However, circulating IL-35 and IL-35-producing Tregs were decreased in patients with severe enterovirus 71-induced hand, foot, and mouth disease, which was positively correlated with the Tregs-to-Th17 cell ratio (Huang et al. 2017; Zhang et al. 2020). Limited studies have focused on IL-35 expression in patients with IM.

Asymptomatic EBV infection induces a strong EBV-specific $CD8^+$ T cell response, which plays a vital role in controlling latent EBV infection (Dunne et al. 2002). In contrast, EBV replication is poorly controlled in the acute phase in patients with IM despite the massive expansion of EBV lytic antigen-specific $CD8^+$ T cells (Angelini et al. 2013; Deng et al. 2021). IL-35 suppressed non-specific and antigen-specific $CD8^+$ T cells in HBV-related diseases (Shao et al. 2017; Yang et al. 2019b, 2020), and elevation of IL-35 suppressed acute HBV infection-induced liver inflammation and protected against hepatocyte damage (Teng et al. 2019). Thus, we hypothesized that IL-35 regulated $CD8^+$ T cells in patients with IM. To test this possibility, we investigated the IL-35 expression profile and $CD8^+$ T cell activity in patients with IM with and without liver inflammation. The cytotoxicity of $CD8^+$ T cells from patients with IM was assessed in response to recombinant human IL-35 stimulation in vitro.

Materials and Methods

Institutional Review Board Approval

The current study protocol was in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Shaanxi Provincial People's Hospital (No. 2017019). Written consent was obtained from each enrolled subject.

Enrolled Subjects

Fifty-one adult patients with IM were enrolled in this study between July 2017 and July 2021. The inclusion criteria: (1) Age > 18 years old when admitted. (2) Meeting the IM diagnostic criteria: clinical indicators (three or more): fever, pharyngeal tonsillitis, cervical lymphadenopathy, splenomegaly, hepatomegaly, and eyelid edema (Naughton et al. 2021). Laboratory indicators (meeting one or more): (a) positive for EBV DNA in the peripheral blood; (b) positive for anti-EBV-viral capsid antigen (VCA)-IgM and anti-EBV-VCA-IgG and negative for anti-EBV-nuclear antigen-IgG; (c) Serum anti-EBV-VCA-IgG titer was more than four-fold elevated in the convalescence phase compared with the acute phase; (d) atypical lymphocyte ratio in peripheral blood ≥ 0.10 and/or lymphocytosis ≥ 5.0×10^9/L (Ebell et al. 2016). (3) Treatment-naïve for non-steroidal anti-inflammatory drugs or glucocorticoids. The exclusion criteria were as follows: (1) afflicted with hepatitis virus or human immunodeficiency virus (HIV) infection; (2) afflicted with autoimmune diseases; (3) afflicted with cancers; (4) afflicted with severe systemic or organic disorders such as liver failure and impaired renal function. The diagnosis for IM-related liver inflammation was as follows: (1) abnormal liver function, including more than two times elevation of alanine aminotransferase (ALT), aspartate aminotransferase

(AST), and/or total bilirubin (T-BIL); (2) exclusion of drug-induced liver injury or hereditary metabolic disease. Meanwhile, twenty-one healthy individuals with a matched sex ratio and average age, who received physical examination in our hospital, were also enrolled as controls. The clinical characteristics of the enrolled subjects are shown in Table 1.

Plasma and Peripheral Blood Mononuclear Cells Isolation

Twenty milliliters of EDTA anti-coagulant peripheral blood was collected from all enrolled subjects. Plasma was obtained by centrifugation of whole blood at 1000×*g* for 10 min. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Premium (GE Healthcare Bio-Sciences AB, Bjokgatan, Uppsala, Sweden) as previously described (Teng et al. 2019). PBMCs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 conditions and were stimulated with recombinant human IL-35 (1 ng/ml; Peprotech, Rocky Hill, NJ, USA) (Teng et al. 2019) for 24 h.

$CD8^+$ T Cell Purification and Culture

$CD8^+$ T cells were purified using a $CD8^+$ Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) as previously described (Jin et al. 2019). The purity of enriched $CD8^+$ T cells was > 95% by flow cytometry determination. Enriched $CD8^+$ T cells were seeded into 24-well plates at a concentration of 10^5/ml, and were cultured in RPMI 1640 supplemented with 10% FBS at 37 °C under 5% CO_2 conditions. $CD8^+$ T cells were stimulated with anti-CD3/CD28 (1 mg/ml) in the presence or absence of IL-35 (1 ng/ml) (Teng et al. 2019) for 24 h. A total of 10^5 of IL-35-stimulated $CD8^+$ T cells from HLA-A*02 restricted individuals were co-cultured with 5×10^5 HepG2 cells, which are also HLA-A*02 restricted (Boegel et al. 2014), in either direct contact or indirect contact conditions (Phillips et al. 2010). HepG2 is an immortalized cell line consisting of human liver carcinoma cells derived from the liver tissue of a 15-year-old Caucasian male who had well-differentiated hepatocellular carcinoma. Briefly, in the direct contact co-culture condition, $CD8^+$ T cells and HepG2 cells were directly mixed and cultured in the presence of anti-CD3/CD28. In the indirect contact co-culture condition, a transwell plate (Corning, Corning, NY, USA) was used. $CD8^+$ T cells were added to the upper chamber with anti-CD3/CD28 stimulation. HepG2 cells were added to the lower well. Cells and supernatants were harvested for further analyses 48 h post co-culture.

Enzyme-Linked Immunosorbent Assay

Cytokine levels were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (ColofulGene, Wuhan, Hubei Province, China).

Enzyme-Linked Immunospot Assay

The secretion of perforin and granzyme B by PBMCs was analyzed using a Human Perforin enzyme-linked immunospot assay (ELISPOT) Kit (with precoated plates) (Abcam, Cambridge, MA, USA) and Human Granzyme B ELISPOT Kit (with precoated plates) (Abcam), respectively. Briefly, 10^5 PBMCs were added to antibody-precoated polyvinylidene difluoride membrane 96-well plates and stimulated with phytophemagglutinin (10 μg/ml) for 12 h. Perforin or granzyme B secretion is shown as spot-forming cells (SFCs)/10^6 PBMCs.

Cytotoxic Assay

Target HepG2 cell death was determined by measuring lactate dehydrogenase (LDH) levels in the cultured supernatants using an LDH cytotoxicity kit (Beyotime, Wuhan, Hubei Province, China). The LDH level in the supernatants of cultured HepG2 cells was defined as the low-level control,

Table 1 Clinical characteristics of enrolled subjects

	Control	IM without liver inflammation	IM with liver inflammation
Cases (*n*)	21	23	28
Gender (male/female)	11/10	13/10	15/13
Age (years)	30.14 ± 8.54	27.22 ± 6.42	28.14 ± 8.19
Lymphocytes ($\times 10^9$/L)	1.73 ± 0.64	5.01 ± 1.08	4.32 ± 1.17
Atypical lymophocyte ratio	Not available	0.12 ± 0.02	0.11 ± 0.02
ALT (IU/L)	19(14, 28)	45(24, 52)	162(123, 263)
AST (IU/L)	26(19, 33)	38(28, 47)	93(59, 152)
T-BIL (μmol/L)	13.82(12.22, 17.96)	21.82(18.18, 28.18)	75.91(51.59, 131.4)
EBV DNA positive (*n*, %)	Not available	14 (60.87%)	16 (57.14%)

IM infectious mononucleosis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *T-BIL* total bilirubin

while the LDH level in the supernatants of Triton X-100-treated HepG2 cells was defined as the high-level control. The percentage of HepG2 cell death was calculated using the following formula: (experimental LDH − low-level control)/(high-level control − low-level control) × 100% (Jin et al. 2019; Phillips et al. 2010).

Flow Cytometry

PBMCs were stained with PE-Cy7 Mouse Anti-Human CD3 (Clone UCHT1; BD Pharmingen, San Jose, CA, USA), FITC Mouse Anti-Human CD8 (Clone RPA-T8; BD Pharmingen), PE Mouse Anti-Human CD279 (programmed death-1, PD-1) (Clone EH12.1; BD Pharmingen), APC Mouse Anti-Human CD152 (cytotoxic T-lymphocyte-associated protein 4, CTLA-4) (Clone BNI3; BD Pharmingen), PerCP-Cy5.5 Mouse Anti-Human T cell immunoglobulin and mucin domain 3 (TIM-3) (CD366) (Clone 7D3; BD Pharmingen) and APC-R700 Mouse Anti-Human lymphocyte activation gene-3 (LAG-3) (CD233) (Clone T47-530; BD Pharmingen). Cells were analyzed using a FACS Aria II flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

All data were analyzed using SPSS version 23.0 for Windows (Chicago, IL, USA). The Shapiro–Wilk test was used for the normal distribution assay. Variables following a normal distribution are presented as the mean ± standard deviation, and statistical significance was determined by Student's *t* test, paired *t* test, one-way ANOVA, or Tukey test. Variables following a skewed distribution are presented as the median (Q1, Q3), and statistical significance was determined by the Mann–Whitney test, Wilcoxon paired test, Kruskal–Wallis test, or Dunn's multiple comparison test. Pearson or Spearman correlation analysis was performed for correlation analysis. *P* values of less than 0.05 were considered to indicate significant differences.

Results

Plasma IL-35 Levels were Elevated in Patients with IM with Liver Inflammation

The IL-35 level in the plasma was screened in all enrolled subjects. There was no significant difference in plasma IL-35 levels between controls and patients with IM [73.30(50.06, 99.36) pg/ml vs. 71.08(49.53, 125.0) pg/ml; $P = 0.824$, Mann–Whitney test; Fig. 1a]. Patients with IM with liver inflammation had notably elevated plasma IL-35 levels compared with those without liver inflammation [59.43(43.70, 89.82) pg/ml vs 84.59(62.28, 188.8) pg/ml; $P = 0.025$, Mann–Whitney test; Fig. 1b]. In patients with IM with liver

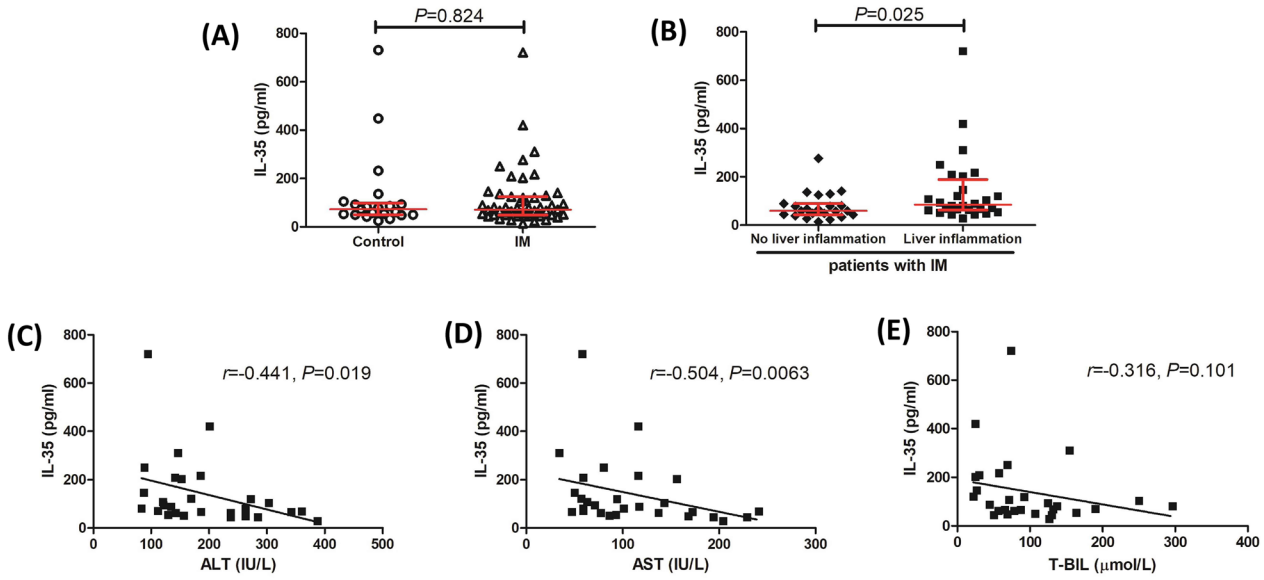

Fig. 1 Plasma IL-35 levels in controls ($n = 21$) and patients with IM ($n = 51$). The IL-35 level in the plasma was measured by ELISA. **a** There was no significant difference in plasma IL-35 levels between controls ($n = 21$) and patients with IM ($n = 51$). **b** IL-35 expression in the plasma was remarkably elevated in patients with IM with liver inflammation ($n = 28$) when compared with patients with IM without liver inflammation ($n = 23$). The Mann–Whitney test was used for comparison. **c** Plasma IL-35 level was negatively correlated with ALT in patients with IM with liver inflammation. **d** Plasma IL-35 levels were negatively correlated with AST in patients with IM with liver inflammation. **e** There was no statistical correlation between plasma IL-35 and T-BIL in patients with IM with liver inflammation. Spearman correlation analysis was used for correlation analysis. The individual level for each subject is shown

inflammation, IL-35 levels in the plasma were negatively correlated with ALT ($r=-0.441$, $P=0.019$, Spearman correlation analysis; Fig. 1c) and AST ($r=-0.504$, $P=0.0063$, Spearman correlation analysis; Fig. 1d). However, the IL-35 level did not correlate with the T-BIL level ($r=-0.316$, $P=0.101$, Spearman correlation analysis; Fig. 1e). There was no statistical correlation between IL-35 expression and other clinical indices (such as lymphocyte count, T-BIL level, etc., data not shown, $P>0.05$). Furthermore, there were no significant differences in IL-35 receptor subunits in $CD8^+$ T cells among controls, patients with IM without liver inflammation, and patients without liver inflammation ($P>0.05$; Fig. S1).

$CD8^+$ T Cells in Patients with IM with Liver Inflammation Presented Stronger Cytotoxicity

Perforin and granzyme B secretion by PBMCs was measured in all enrolled subjects. Perforin secretion was significantly elevated in patients with IM with liver inflammation (320.6 ± 62.94 SFC/10^6 PBMCs) compared with patients with IM without liver inflammation (247.1 ± 65.63 SFC/10^6 PBMCs, $P=0.0018$, Tukey test; Fig. 2a) and controls (258.1 ± 68.42 SFC/10^6 PBMCs, $P=0.0002$, Tukey test; Fig. 2a). Granzyme B secretion was also increased in patients with IM with liver inflammation [123.6(77.12, 207.8) SFC/10^6 PBMCs] compared with controls [60.81(47.27, 103.9) SFC/10^6 PBMCs] ($P=0.0047$, Dunn's multiple comparison test; Fig. 2b). However, the IL-35 level did not correlate with either perforin ($r=-0.108$, $P=0.583$, Spearman correlation analysis, Fig. 2c) or granzyme B ($r=0.136$, $P=0.489$, Spearman correlation analysis; Fig. 2d) in patients with IM with liver inflammation. Similarly, plasma IL-35 levels also did not correlate with either perforin or granzyme B in patients with IM without liver inflammation (data not shown; $P>0.05$).

A total of 10^5 of purified $CD8^+$ T cells from seven HLA-A*02 patients with IM without liver inflammation and from six HLA-A*02 patients with IM with liver inflammation were co-cultured with 5×10^5 HepG2 cells under both direct contact and indirect contact conditions. $CD8^+$ T cells from patients with IM with liver inflammation induced increased HepG2 cell death in both direct contact and indirect contact co-culture systems ($P<0.05$, Tukey test; Fig. 3a). Similarly, interferon (IFN)-γ and tumor necrosis factor (TNF)-α production in cultured

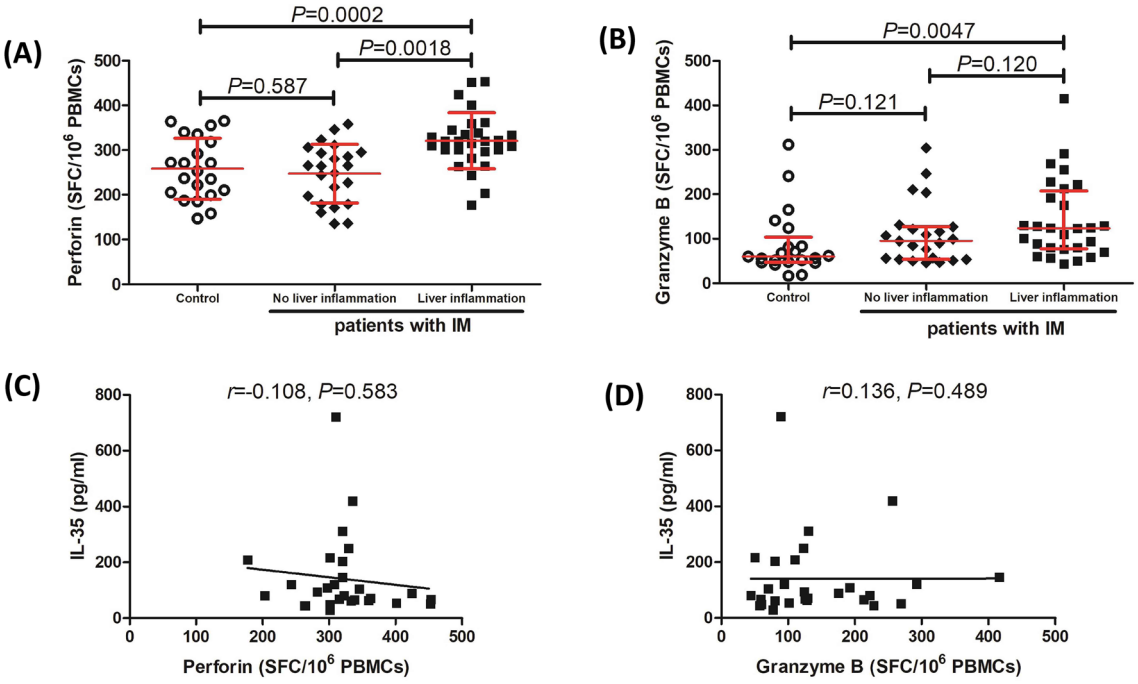

Fig. 2 Perforin and granzyme B secretion by PBMCs in controls ($n=21$) and patients with IM ($n=51$). Perforin and granzyme B secretion by PBMCs was measured by ELISPOT. **a** Perforin secretion was significantly elevated in patients with IM with liver inflammation compared with patients with IM without liver inflammation and controls. One-way ANOVA and Tukey tests were used for comparisons. **b** Granzyme B secretion was markedly increased in patients with IM with liver inflammation compared with controls. The Kruskal–Wallis test and Dunn's multiple comparison test were used for comparisons. **c** There was no statistical correlation between plasma IL-35 and perforin secretion in patients with IM with liver inflammation. **d** There was no statistical correlation between plasma IL-35 and granzyme B secretion in patients with IM with liver inflammation. Spearman correlation analysis was used for correlation analysis. The individual level for each subject is shown

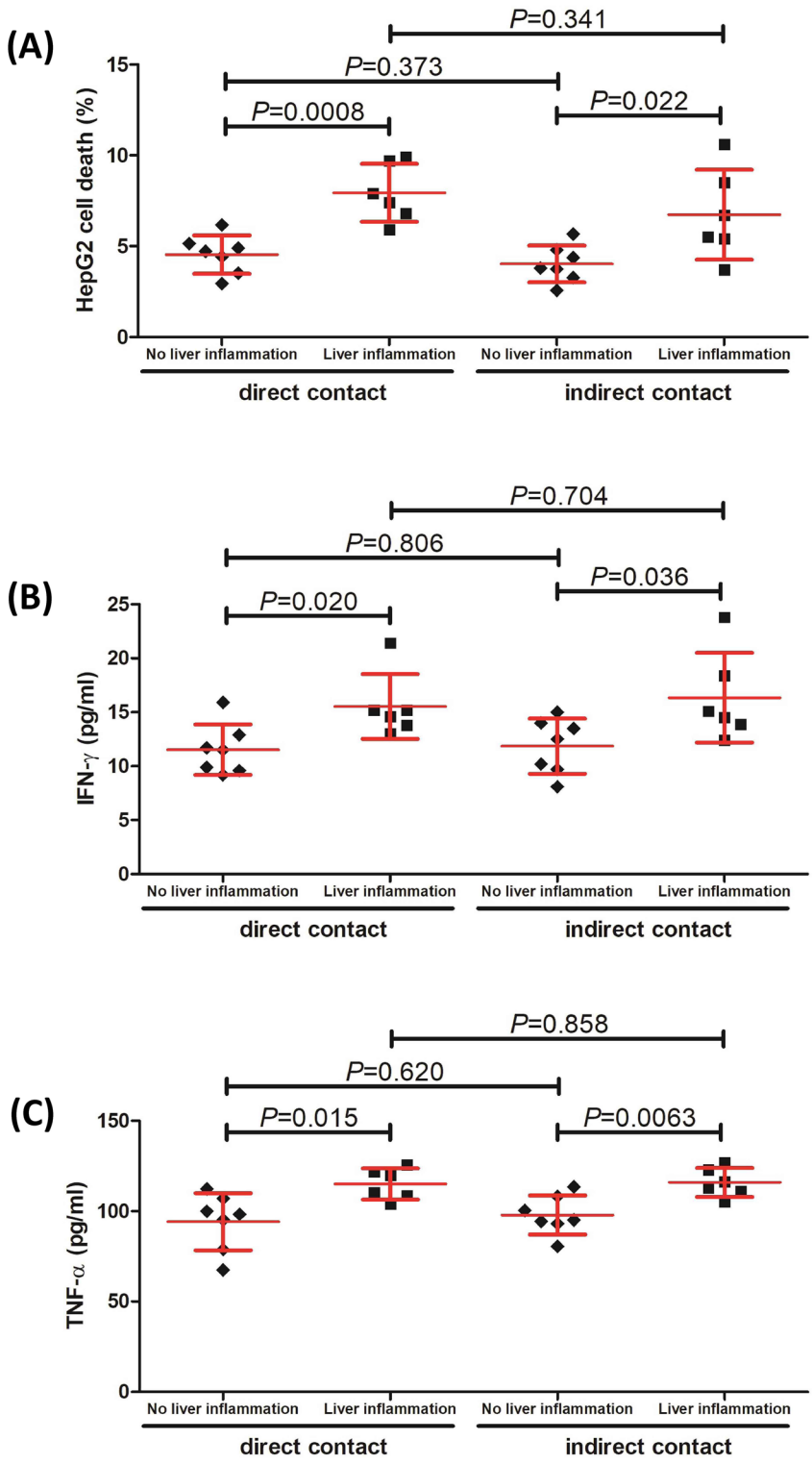

Fig. 3 Cytotoxicity of $CD8^+$ T cells in patients with IM. A total of 10^5 of purified $CD8^+$ T cells from HLA-A*02 patients with IM without liver inflammation ($n=7$) and from HLA-A*02 patients with IM with liver inflammation ($n=6$) were co-cultured with 5×10^5 HepG2 cells in both direct contact and indirect contact conditions. **a** The percentage of HepG2 cell death was calculated by measuring LDH release and was compared among groups. **b** IFN-γ and **c** TNF-α secretion was measured by ELISA and was compared among groups. The One-way ANOVA and Tukey test were used for comparisons. The individual level for each subject is shown

supernatants was also notably elevated in $CD8^+$ T cells from patients with IM with liver inflammation compared with those without liver inflammation ($P<0.05$, Tukey test; Fig. 3b, c). However, there were no significant differences in target cell death or IFN-γ/TNF-α levels between the direct contact and indirect contact co-culture systems in either patients with IM with liver inflammation or those without liver inflammation ($P>0.05$, Tukey test; Fig. 3a–c), suggesting that IFN-γ or TNF-α secretion may be responsible for EBV-associated liver inflammation.

In Vitro IL-35 Stimulation Suppressed the Cytokine-Induced Cytotoxicity of CD8$^+$ T Cells in Patients with IM

PBMCs were convenience-sampled from seventeen patients with IM without liver inflammation and from fifteen patients with IM with liver inflammation and were stimulated with recombinant human IL-35 for 24 h. Perforin and granzyme B secretion was then measured. In vitro IL-35 stimulation only slightly dampened perforin secretion in patients with IM with liver inflammation (302.0 ± 70.97 SFC/10^6 PBMCs vs. 314.4 ± 70.22 SFC/10^6 PBMCs, $P = 0.047$, paired t test; Fig. 4a), but not in patients with IM without liver inflammation (251.8 ± 68.88 SFC/10^6 PBMCs vs. 259.1 ± 66.24 SFC/10^6 PBMCs, $P = 0.190$, paired t test; Fig. 4a). IL-35 stimulation did not affect granzyme B secretion in either patients with IM without or with liver inflammation ($P > 0.05$, Wilcoxon paired test; Fig. 4b).

Purified CD8$^+$ T cells from seven HLA-A*02 patients with IM without liver inflammation and from six HLA-A*02 patients with IM with liver inflammation were stimulated with recombinant human IL-35 for 24 h, and 10^5 stimulated CD8$^+$ T cells were co-cultured with 5×10^5 of HepG2 cells in both direct contact and indirect contact conditions. In vitro IL-35 stimulation suppressed CD8$^+$ T cell-induced HepG2 cell death in patients with IM with and without liver inflammation in both direct contact and indirect contact co-culture systems ($P < 0.05$, paired t test; Fig. 5a). Similarly, IFN-γ and TNF-α secretion in cultured supernatants were also reduced in CD8$^+$ T cells in the presence of IL-35 stimulation ($P < 0.05$, Tukey test; Fig. 5b, c). However, there were no significant differences in target cell death or IFN-γ/TNF-α levels between the direct contact and indirect contact co-culture systems in either patients with IM with liver inflammation or those without liver inflammation in response to IL-35 stimulation ($P > 0.05$, Tukey test; Fig. 5a–c).

In Vitro IL-35 Stimulation Elevated Immune Checkpoint Molecules in CD8$^+$ T Cells in Patients with IM

PBMCs were convenience-sampled from nineteen patients with IM without liver inflammation and from thirteen patients with IM with liver inflammation and were stimulated with recombinant human IL-35 for 24 h. Immune checkpoint molecules, including PD-1, CTLA-4, TIM-3, and LAG-3, in CD8$^+$ T cells were assessed. Representative flow dots for PD-1$^+$, CTLA-4$^+$, TIM-3$^+$, and LAG-3$^+$ in CD3$^+$CD8$^+$ T cells are shown in Fig. S2. PD-1, CTLA-4, and TIM-3 were detected in all CD8$^+$ T cells. However, there was very limited LAG-3 expression in CD8$^+$ T cells with or without IL-35 stimulation. In vitro IL-35 stimulation strongly enhanced PD-1, CTLA-4, and TIM-3 expression in CD3$^+$CD8$^+$ T cells in both patients with IM without liver inflammation and with liver inflammation ($P < 0.05$, paired t test; Fig. 6a–c). However, there were no significant differences in PD-1$^+$, CTLA-4$^+$, or LAG-3$^+$ cell percentages between patients with IM without liver inflammation and with liver inflammation ($P > 0.05$, Tukey test; Fig. 6a–c).

Discussion

To our knowledge, this is the first report regarding IL-35 expression and its regulatory function in CD8$^+$ T cells in adults with EBV-associated IM with liver inflammation. Although acute EBV infection did not induce peripheral IL-35 elevation in all patients with IM, those who had liver

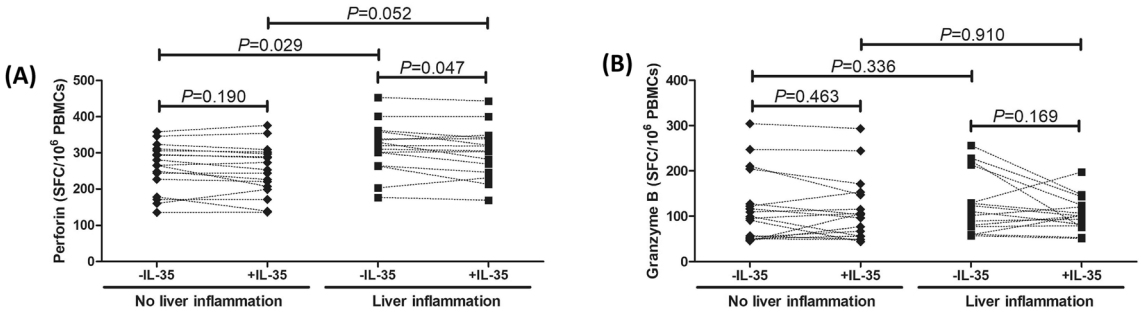

Fig. 4 Perforin and granzyme B secretion by PBMCs in patients with IM without liver inflammation ($n = 17$) and patients with IM with liver inflammation ($n = 15$) in response to IL-35 stimulation in vitro. A total of 10^5 of PBMCs were stimulated with recombinant human IL-35 (1 ng/ml) for 24 h. Perforin and granzyme B secretion by PBMCs was measured by ELISPOT. **a** IL-35 stimulation slightly dampened perforin secretion in patients with IM with liver inflammation but not in patients with IM without liver inflammation. The One-way ANOVA, Tukey test, and paired t test were used for comparisons. **b** IL-35 stimulation did not affect granzyme B secretion in either patients with IM without or with liver inflammation. The Kruskal–Wallis test, Dunn's multiple comparison test, and Wilcoxon paired test were used for comparisons. The individual level for each subject is shown

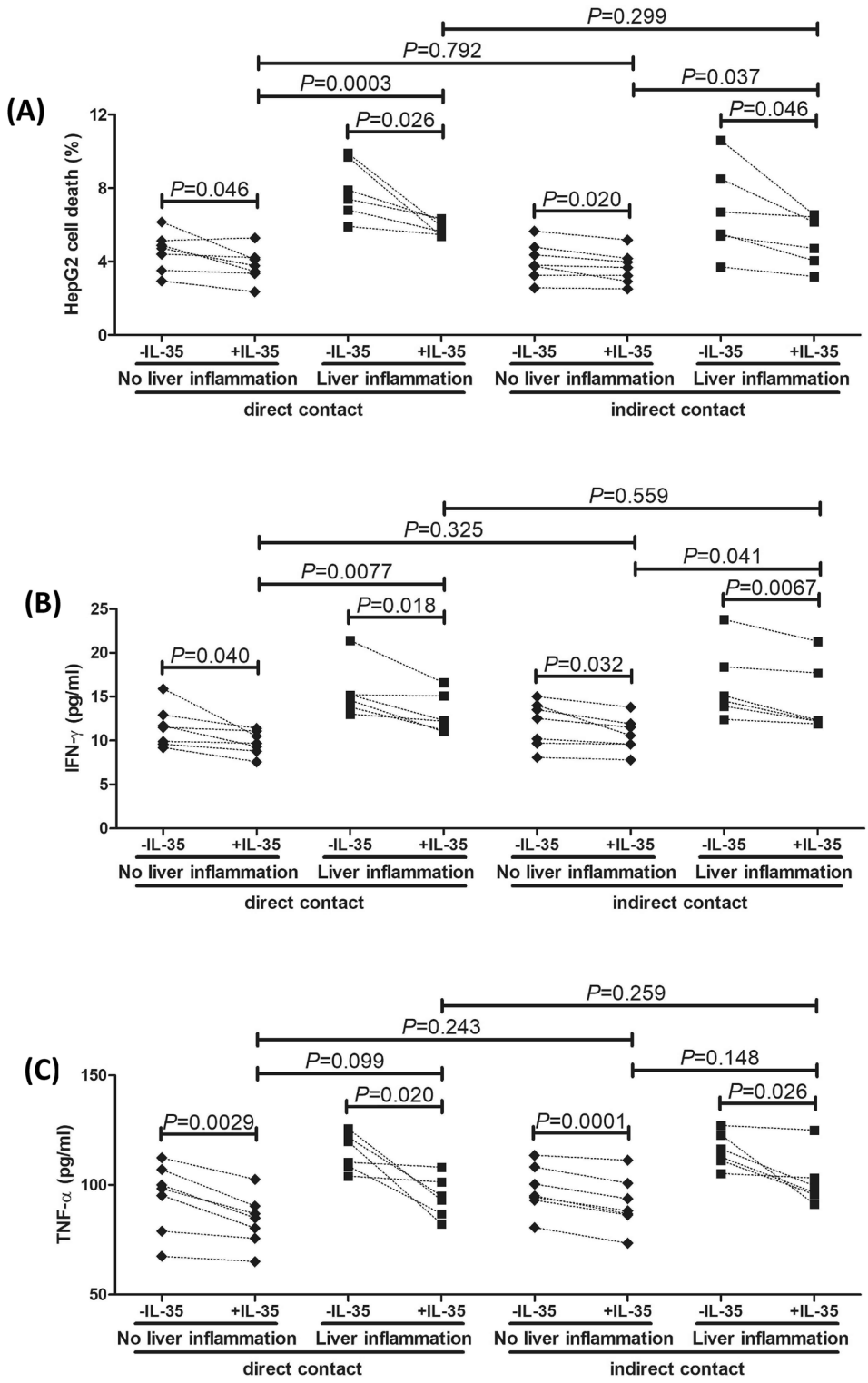

Fig. 5 Cytotoxicity of $CD8^+$ T cells in patients with IM in response to IL-35 stimulation in vitro. Purified $CD8^+$ T cells from HLA-A*02 patients with IM without liver inflammation ($n=7$) and from HLA-A*02 patients with IM with liver inflammation ($n=6$) were stimulated with recombinant human IL-35 (1 ng/ml) for 24 h, and 10^5 of stimulated $CD8^+$ T cells were co-cultured with 5×10^5 HepG2 cells in both direct contact and indirect contact conditions. **a** The percentage of HepG2 cell death was calculated by measuring LDH release and was compared among groups. **b** IFN-γ and **c** TNF-α secretion was measured by ELISA and was compared among groups. One-way ANOVA, The Tukey test, and paired *t* test were used for comparisons. The individual level for each subject is shown

inflammation revealed higher IL-35 expression in the plasma than those without liver inflammation, which negatively correlated with the severity of liver inflammation. $CD8^+$ T cells from patients with IM with liver inflammation demonstrated stronger cytotoxicity. Importantly, IL-35 dampened the percentage of $CD8^+$ T cell-induced target cell death, and this process was accompanied by an elevation of immune checkpoint molecule expression and inhibition of proinflammatory cytokine secretion in $CD8^+$ T cells. Our findings indicate that overexpression of IL-35 in patients with IM with liver inflammation might be one of the important regulators of $CD8^+$ T cell function.

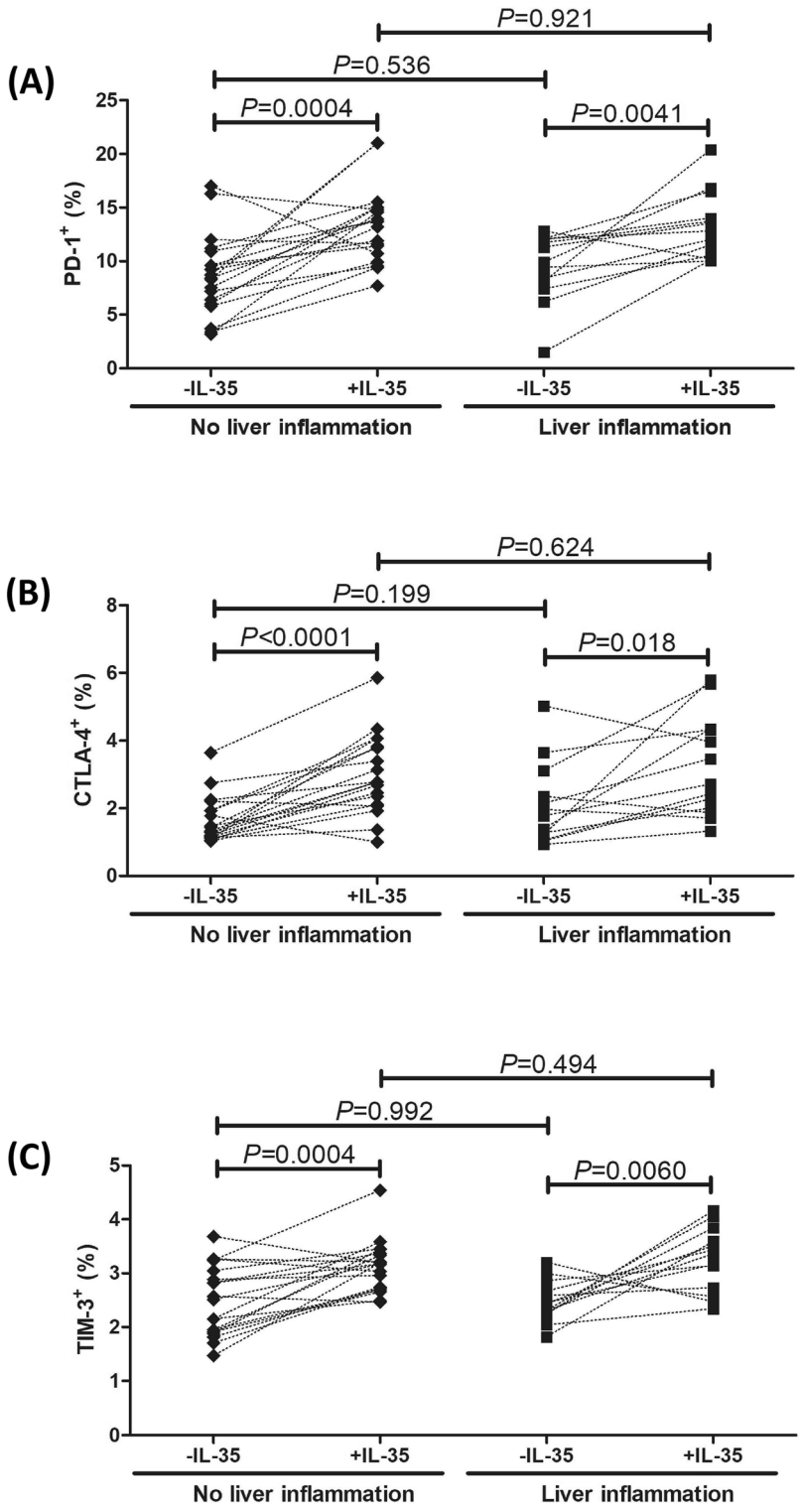

Fig. 6 Immune checkpoint molecule expression in $CD8^+$ T cells in patients with IM in response to IL-35 stimulation. PBMCs from patients with IM without liver inflammation ($n=19$) and from patients with IM with liver inflammation ($n=13$) were stimulated with recombinant human IL-35 for 24 h. PBMCs were stained with PE-Cy7 Mouse Anti-Human CD3, FITC Mouse Anti-Human CD8, PE Mouse Anti-Human PD-1, APC Mouse Anti-Human CTLA-4, PerCP-Cy5.5 Mouse Anti-Human TIM-3, and APC-R700 Mouse Anti-Human LAG-3. Cells were analyzed by flow cytometry. **a** $PD\text{-}1^+$, **b** $CTLA\text{-}4^+$, **c** $TIM\text{-}3^+$ cell percentage within $CD3^+CD8^+$ T cells was compared among groups. One-way ANOVA, The Tukey test, and paired *t* test were used for comparisons. The individual level for each subject is shown

IL-35 plays a dual function during infection. Sepsis is closely related to the enhancement of IL-35 release, and IL-35 is likely to facilitate bacterial dissemination in clinical and experimental abdominal sepsis (Cao et al. 2015). In contrast, recombinant IL-35 reduced inflammatory damage through suppression of inflammatory cytokine/chemokine secretion and inhibition of T cell and local neutrophil recruitment. The immunosuppressive property of IL-35 may be essential for improving the prognosis of a septic rat model (Wu et al. 2022). IL-35 was significantly reduced in peripheral blood in viral myocarditis patients (Ouyang et al. 2017) and severe enterovirus 71 infection (Huang et al. 2017).

However, few studies have focused on the IL-35 expression profile during EBV infection. IL-35 is highly expressed in nasopharyngeal carcinoma, which is the most common EBV-associated epithelial cancer. Overexpression of IL-35 was associated with lymph node metastases and advanced tumor stage and was an independent prognostic factor for nasopharyngeal carcinoma (Zhang et al. 2015). For the first time, we investigated IL-35 expression in the peripheral blood of patients with EBV-associated IM. There was no significant difference in plasma IL-35 levels between patients with IM without liver inflammation and the controls, but the IL-35 level was notably increased in patients with IM who presented with liver inflammation. Furthermore, IL-35 levels were negatively correlated with ALT and AST levels, which are the most important criterion and basis for liver inflammation. This result indicated that IL-35 might protect against EBV-induced liver damage in patients with IM, but the mechanism underlying the phenomenon still needs further elucidation.

EBV infection affects the percentage of functionally distinct T cell subsets in children with IM (Sulik et al. 2014). The $CD4^+/CD8^+$ ratio was decreased, and the memory $CD8^+$ T cell number was also rapidly down-regulated in patients with IM (Sulik et al. 2014). Herein, we analyzed the cytotoxicity of $CD8^+$ T cells in patients with IM. $CD8^+$ T cells presented cytolytic activity mainly through two different mechanisms. On the one hand, $CD8^+$ T cells produce cytotoxic molecules, such as perforin and granzyme B, which can directly induce apoptosis. This process requires direct cell-to-cell contact (Johnson et al. 2003). On the other hand, $CD8^+$ T cells secrete proinflammatory cytokines (e.g. IFN-γ and TNF-α) to mediate target cell death without the requirement for direct cell-to-cell contact (Rodrigues et al. 2021). Phillips et al. (2010) set up a direct contact and indirect contact co-culture system between $CD8^+$ T cells and target cells to separate these two mechanisms in vitro. The current results showed that $CD8^+$ T cells from patients with IM with liver inflammation induced a higher percentage of target cell death than those from patients with IM without liver inflammation, indicating the stronger cytotoxicity of $CD8^+$ T cells in patients with IM with liver inflammation. This process was accompanied by increased production of IFN-γ and TNF-α. Although perforin secretion by $CD8^+$ T cells was found to be increased in patients with IM with liver inflammation, there was no significant difference in $CD8^+$ T cell cytotoxicity between the direct contact and indirect contact co-culture systems, revealing that cytotoxicity was mainly related to secretory factors such as IFN-γ and TNF-α. Furthermore, a new terminal effector $CD8^+$ T cell subset, which was defined by a high level of IKAROS family zinc finger 2 and low expression of IL-7 receptor, expressed CD16 and mediated potent HIV-specific antibody-dependent cellular cytotoxicity (ADCC) (Naluyima et al. 2019). However, no $CD16^+CD8^+$ T cells were detected in patients with IM based on flow cytometry analysis (data not shown). Thus, ADCC function may not contribute to $CD8^+$ T cell function in patients with IM.

Cytokine-mediated immune cell dysfunction plays an important role during the acute phase of EBV-associated IM. IL-10 stimulation led to the loss of plasmacytoid dendritic cells in the acute phase in patients with IM (Panikkar et al. 2015). IL-35 mainly suppresses $CD8^+$ T cell cytotoxicity in hepatocellular carcinoma and acute-on-chronic liver failure through various mechanisms, including suppressing cellular proliferation, inhibiting cytotoxic molecule secretion, dampening cytokine production, and promoting immune checkpoint molecule expression (Yang et al. 2019b, 2020). We found that IL-35-stimulated $CD8^+$ T cells presented weaker cytotoxicity to target cells in patients with IM, but $CD8^+$ T cells from patients with IM with liver inflammation also induced elevated target cell death than those from patients with IM without liver inflammation in response to IL-35 stimulation. Consistent with the findings in Kawasaki disease (Sun and Xing 2021), we found that there was no significant difference in IL-35 receptor levels among controls, patients with IM without liver inflammation, and patients with liver inflammation. This suggested that IL-35 signaling might be comparable between patients with IM and controls, and the elevation of plasma IL-35 might contribute to $CD8^+$ T cell function in patients with IM with liver inflammation. Although granzyme B secretion by $CD8^+$ T cells was not significantly affected by IL-35 stimulation, IFN-γ and TNF-α production was down-regulated in response to IL-35 in patients with IM with liver inflammation. Moreover, PD-1, CTLA-4, and TIM-3, which are important immune checkpoint molecules, were increasingly expressed with IL-35 stimulation in $CD8^+$ T cells in patients with IM. The induction of immune checkpoint molecule expression by IL-35 was also found in hepatocellular carcinoma (Liu et al. 2021; Yang et al. 2019b) and acute-on-chronic liver failure (Yang et al. 2020). Although the exact mechanisms are not completely understood, it is possible that IL-35 regulates Janus kinase/signal transducers and activators of the transcription pathway and further affects the promoters of immune checkpoint molecules (Dong et al. 2020). Thus, IL-35 may suppress $CD8^+$ T cell cytotoxicity via inhibition of proinflammatory cytokine secretion and elevation of immune checkpoint molecule expression in patients with IM with liver inflammation. Elevated IL-35 may have a protective function by dampening cytokine-mediated $CD8^+$ T cell cytotoxicity in patients with IM with liver inflammation.

The strength of the study was using the PBMCs from patients with IM to investigate the regulatory function of IL-35 to immune cells. There were also several limitations of the study. On the one hand, the participants were hospitalized adults and thus not typical patients with IM. On the

other hand, further in vivo animal experiments should be performed to confirm the in vitro findings.

In summary, elevated IL-35 expression in patients with IM with liver inflammation might dampen the cytotoxicity of $CD8^+$ T cells probably through repression of IFN-γ/TNF-α secretion and elevation of immune checkpoint molecule expression. IL-35 may protect against cytokine-induced liver damage and may be considered a potential therapeutic target for patients with IM with liver inflammation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00005-022-00663-8.

Acknowledgements This work was supported by a grant from the SPPH Incubator Fund for Development of Science and Technology (2021YJY-19), SPPH Foundation for Development of Science and Technology (2021BJ-26), Xi'an Foundation for Development of Science and Technology [20YXYJ0009(11)], International Science and Technology Cooperation Projects of Shaanxi Province (2022KW-14), and Scientific and Technological Innovation Team of Shaanxi Province (2021TD-40).

Author contributions YG, LL, XH, and WZ performed the study. YG, WZ, and YL enrolled the patients. YG, LL, XH, WZ, and YL analyzed the data, and prepared the manuscript. YL designed and supervised the study. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Declarations

Conflicts of interest All authors declare no conflicts of interests.

Ethical approval The current study protocol was in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Shaanxi Provincial People's Hospital (No. 2017019).

Informed consent Written consent was obtained from each enrolled subject.

References

Angelini DF, Serafini B, Piras E et al (2013) Increased CD8+ T cell response to Epstein-Barr virus lytic antigens in the active phase of multiple sclerosis. PLoS Pathog 9:e1003220. https://doi.org/10.1371/journal.ppat.1003220

Boegel S, Lower M, Bukur T et al (2014) A catalog of HLA type, HLA expression, and neo-epitope candidates in human cancer cell lines. Oncoimmunology 3:e954893. https://doi.org/10.4161/21624011.2014.954893

Cao J, Xu F, Lin S et al (2015) IL-35 is elevated in clinical and experimental sepsis and mediates inflammation. Clin Immunol 161:89–95. https://doi.org/10.1016/j.clim.2015.08.016

Deng Y, Chatterjee B, Zens K et al (2021) CD27 is required for protective lytic EBV antigen-specific $CD8^+$ T-cell expansion. Blood 137:3225–3236. https://doi.org/10.1182/blood.2020009482

Dong Y, Li X, Yu Y et al (2020) JAK/STAT signaling is involved in IL-35-induced inhibition of hepatitis B virus antigen-specific cytotoxic T cell exhaustion in chronic hepatitis B. Life Sci 252:117663. https://doi.org/10.1016/j.lfs.2020.117663

Dunne PJ, Faint JM, Gudgeon NH et al (2002) Epstein-Barr virus-specific CD8(+) T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. Blood 100:933–940. https://doi.org/10.1182/blood-2002-01-0160

Ebell MH, Call M, Shinholser J et al (2016) Does this patient have infectious mononucleosis? The rational clinical examination systematic review. JAMA 315:1502–1509. https://doi.org/10.1001/jama.2016.2111

Henle W, Henle G, Lennette ET (1979) The Epstein-Barr virus. Sci Am 241:48–59. https://doi.org/10.1038/scientificamerican0779-48

Hoagland RJ (1952) Infectious mononucleosis. Am J Med 13:158–171. https://doi.org/10.1016/0002-9343(52)90154-x

Hu J, Zhao H, Lou D et al (2018) Human cytomegalovirus and Epstein-Barr virus infections, risk factors, and their influence on the liver function of patients with acute-on-chronic liver failure. BMC Infect Dis 18:577. https://doi.org/10.1186/s12879-018-3488-8

Huang Q, Wang Y, Si C et al (2017) Interleukin-35 modulates the imbalance between regulatory T cells and T helper 17 cells in enterovirus 71-induced hand, foot, and mouth disease. J Interferon Cytokine Res 37:522–530. https://doi.org/10.1089/jir.2017.0080

Jin B, Liang Y, Liu Y et al (2019) Notch signaling pathway regulates T cell dysfunction in septic patients. Int Immunopharmacol 76:105907. https://doi.org/10.1016/j.intimp.2019.105907

Johnson BJ, Costelloe EO, Fitzpatrick DR et al (2003) Single-cell perforin and granzyme expression reveals the anatomical localization of effector $CD8^+$ T cells in influenza virus-infected mice. Proc Natl Acad Sci USA 100:2657–2662. https://doi.org/10.1073/pnas.0538056100

Kamal T, Haboubi N (2021) Epstein-Barr virus and acute liver failure: an exceedingly rare amalgamation. J R Coll Physicians Edinb 51:46–48. https://doi.org/10.4997/JRCPE.2021.111

Kuri A, Jacobs BM, Vickaryous N et al (2020) Epidemiology of Epstein-Barr virus infection and infectious mononucleosis in the United Kingdom. BMC Public Health 20:912. https://doi.org/10.1186/s12889-020-09049-x

Li X, Tian L, Dong Y et al (2015) IL-35 inhibits HBV antigen-specific IFN-gamma-producing CTLs in vitro. Clin Sci 129:395–404. https://doi.org/10.1042/CS20140511

Liu S, Zhang Q, Shao X et al (2017) An immunosuppressive function of interleukin-35 in chronic hepatitis C virus infection. Int Immunopharmacol 50:87–94. https://doi.org/10.1016/j.intimp.2017.06.015

Liu X, Ren H, Guo H et al (2021) Interleukin-35 has a tumor-promoting role in hepatocellular carcinoma. Clin Exp Immunol 203:219–229. https://doi.org/10.1111/cei.13535

Mellinger JL, Rossaro L, Naugler WE et al (2014) Epstein-Barr virus (EBV) related acute liver failure: a case series from the US Acute Liver Failure Study Group. Dig Dis Sci 59:1630–1637. https://doi.org/10.1007/s10620-014-3029-2

Naluyima P, Lal KG, Costanzo MC et al (2019) Terminal effector CD8 T cells defined by an IKZF2(+)IL-7R(−) transcriptional signature express FcgammaRIIIA, expand in HIV infection, and mediate potent HIV-specific antibody-dependent cellular cytotoxicity. J Immunol 203:2210–2221. https://doi.org/10.4049/jimmunol.1900422

Naughton P, Healy M, Enright F et al (2021) Infectious mononucleosis: diagnosis and clinical interpretation. Br J Biomed Sci 78:107–116. https://doi.org/10.1080/09674845.2021.1903683

Niederman JC, Miller G, Pearson HA et al (1976) Infectious mononucleosis. Epstein-Barr-virus shedding in saliva and the oropharynx. N Engl J Med 294:1355–1359. https://doi.org/10.1056/NEJM197606172942501

Ouyang H, Xiang L, Chen J et al (2017) Significant reduction of peripheral blood interleukin-35 and CD4(+)EBI3(+) T cells, which are negatively correlated with an increase in the plasma IL-17 and cTnI level, in viral myocarditis patients. Cent Eur J Immunol 42:91–96. https://doi.org/10.5114/ceji.2016.65892

Panikkar A, Smith C, Hislop A et al (2015) Cytokine-mediated loss of blood dendritic cells during Epstein-Barr virus-associated acute infectious mononucleosis: implication for immune dysregulation. J Infect Dis 212:957–1961. https://doi.org/10.1093/infdis/jiv340

Phillips S, Chokshi S, Riva A et al (2010) CD8(+) T cell control of hepatitis B virus replication: direct comparison between cytolytic and noncytolytic functions. J Immunol 184:287–295. https://doi.org/10.4049/jimmunol.0902761

Rahman S, Wathington D, Waterboer T et al (2021) Seroprevalence of Chlamydia trachomatis, herpes simplex 2, Epstein-Barr virus, hepatitis C and associated factors among a cohort of men ages 18–70 years from three countries. PLoS ONE 16:e0253005. https://doi.org/10.1371/journal.pone.0253005

Rodrigues LS, Barreto AS, Bomfim LGS et al (2021) Multifunctional, TNF-alpha and IFN-gamma-Secreting CD4 and CD8 T cells and CD8(high) T cells are associated with the cure of human visceral Leishmaniasis. Front Immunol 12:773983. https://doi.org/10.3389/fimmu.2021.773983

Shao X, Ma J, Jia S et al (2017) Interleukin-35 suppresses antiviral immune response in chronic hepatitis B virus infection. Front Cell Infect Microbiol 7:472. https://doi.org/10.3389/fcimb.2017.00472

Sulik A, Oldak E, Kroten A et al (2014) Epstein-Barr virus effect on frequency of functionally distinct T cell subsets in children with infectious mononucleosis. Adv Med Sci 59:227–231. https://doi.org/10.1016/j.advms.2014.04.003

Sun M, Xing H (2021) Interleukin-35 regulates peripheral T cell activity in patients with Kawasaki disease. Int Immunopharmacol 96:107642. https://doi.org/10.1016/j.intimp.2021.107642

Taghinejad A, Barani S, Gholijani N et al (2020) Variations in IL-22, IL-27 and IL-35 serum levels in untreated and treated hepatitis C patients. Eur Cytokine Netw 31:134–139. https://doi.org/10.1684/ecn.2020.0455

Teng DK, Liu Y, Lv YF et al (2019) Elevated interleukin-35 suppresses liver inflammation by regulation of T helper 17 cells in acute hepatitis B virus infection. Int Immunopharmacol 70:252–259. https://doi.org/10.1016/j.intimp.2019.02.048

Teymouri M, Pirro M, Fallarino F et al (2018) IL-35, a hallmark of immune-regulation in cancer progression, chronic infections and inflammatory diseases. Int J Cancer 143:2105–2115. https://doi.org/10.1002/ijc.31382

Vine LJ, Shepherd K, Hunter JG et al (2012) Characteristics of Epstein-Barr virus hepatitis among patients with jaundice or acute hepatitis. Aliment Pharmacol Ther 36:16–21. https://doi.org/10.1111/j.1365-2036.2012.05122.x

Wu D, Wang L, Hong D et al (2022) Interleukin 35 contributes to immunosuppression by regulating inflammatory cytokines and T cell populations in the acute phase of sepsis. Clin Immunol 235:108915. https://doi.org/10.1016/j.clim.2021.108915

Yang L, Jia S, Shao X et al (2019a) Interleukin-35 modulates the balance between viral specific CD4(+)CD25(+)CD127(dim/-) regulatory T cells and T helper 17 cells in chronic hepatitis B virus infection. Virol J 16:48. https://doi.org/10.1186/s12985-019-1158-0

Yang L, Shao X, Jia S et al (2019b) Interleukin-35 dampens CD8(+) T cells activity in patients with non-viral hepatitis-related hepatocellular carcinoma. Front Immunol 10:1032. https://doi.org/10.3389/fimmu.2019.01032

Yang L, Zhang Q, Song J et al (2020) Interleukin-35 suppresses CD8(+) T cell activity in patients with viral hepatitis-induced acute-on-chronic liver failure. Dig Dis Sci 65:3614–3623. https://doi.org/10.1007/s10620-020-06077-w

Zhang Y, Sun H, Wu H et al (2015) Interleukin 35 is an independent prognostic factor and a therapeutic target for nasopharyngeal carcinoma. Contemp Oncol 19:120–124. https://doi.org/10.5114/wo.2014.44754

Zhang W, Huang Z, Huang M et al (2020) Predicting severe enterovirus 71-infected hand, foot, and mouth disease: cytokines and chemokines. Mediators Inflamm 2020:9273241. https://doi.org/10.1155/2020/9273241

Zhang C, Cui S, Mao G et al (2021a) Clinical characteristics and the risk factors of hepatic injury in 221 children with infectious mononucleosis. Front Pediatr 9:809005. https://doi.org/10.3389/fped.2021.809005

Zhang Q, Yang L, Liu S et al (2021b) Interleukin-35 suppresses interleukin-9-secreting CD4(+) T cell activity in patients with hepatitis B-related hepatocellular carcinoma. Front Immunol 12:645835. https://doi.org/10.3389/fimmu.2021.645835