

Original paper

Hepatic expression of programmed death-1 (PD-1) and its ligand, PD-L1, in children with autoimmune hepatitis: relation to treatment response

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Abstract

Aim of the study: Autoimmune hepatitis (AIH) is characterized histologically by aggressive inflammation with interface hepatitis and prominent lymphoplasmacytic infiltration. Programmed death-1 (PD-1) is expressed on activated lymphocytes. Engagement of PD-1 by its ligand PD-L1 leads to cell apoptosis and death. We aimed to evaluate the immunohistochemical expression of PD-1 and PD-L1 in children with AIH, and its relation to treatment outcome.

Material and methods: Pre-treatment liver biopsies of 31 children with AIH were compared to 30 children with chronic hepatitis C virus (HCV) infection as a control group. PD-1 was evaluated in lymphocytes, while PD-L1 was evaluated in lymphocytes, hepatocytes, biliary epithelial cells, sinusoidal endothelial cells and Kupffer cells. All AIH patients received the standard treatment.

Results: The mean PD-1 was significantly higher in AIH than HCV patients ($29.19 \pm 18.5\%$ vs. $15.2 \pm 10.1\%$; $p = 0.002$) while there was no statistically significant difference as regards PD-L1 on lymphocytes ($p = 0.853$). Neither PD-1 nor PD-L1 correlated with either liver fibrosis or the inflammatory activity ($p > 0.05$ for all). PD-1/PD-L1 ratio was significantly higher in AIH compared to HCV patients and in non-responder AIH patients compared to responders (46.9 vs. 6.58). PD-1 expression was comparable in both responders and non-responders ($p = 0.813$), while PD-L1 was significantly upregulated in responders ($4.17 \pm 3.15\%$ vs. $0.63 \pm 1.3\%$; $p = 0.046$). PD-L1 expression on hepatocytes, biliary epithelial cells, sinusoidal endothelial cells and Kupffer cells was comparable in AIH and HCV groups.

Conclusions: PD-1/PD-L1 ratio, which reflects immune aggression, was significantly higher in AIH compared to HCV patients and in non-responder AIH patients compared to responders.

Key words: autoimmune hepatitis, immunohistochemistry, hepatitis C virus, programmed death-1, programmed death ligand-1.

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Introduction

Autoimmune hepatitis (AIH) is a chronic progressive inflammatory liver disease characterized histologically by interface hepatitis, lymphoplasmacytic infiltrate, presence of circulating liver-related autoantibodies in the serum and hypergammaglobulinemia [1]. AIH is particularly aggressive in children and pro-

gresses rapidly unless immunosuppressive treatment is promptly started [2]. It affects mainly young girls, from infancy to late adolescence [3]. AIH can be classified into two types according to the autoantibody panel detected at diagnosis. AIH type 1 is the more common type and is characterized by the presence of anti-smooth muscle antibody (ASMA) and/or antinuclear antibody (ANA) while AIH type 2 is character-

ized by anti-liver-kidney microsomal antibody type 1 (anti-LKM1) [4].

AIH is a multifactorial disease of unknown etiology. Environmental factors act as a trigger with self-perpetuating liver inflammation in predisposed individuals who carry a complex genetic background [5]. Although an autoimmune reaction against self hepatocytes by autoreactive T cells is thought to participate in the pathogenesis of AIH, the details of the intrahepatic immunological reaction remain unclear [6].

Liver-infiltrating T cells play an essential role in the immunopathogenesis of autoimmune liver disease [7]. The activation of naïve T cells requires not only engagement of T-cell receptor by antigen/major histocompatibility complexes, but also engagement of co-stimulatory molecules, which can deliver either positive or negative co-stimulatory signals [8]. Many previous reports indicated that co-stimulatory molecules and their ligands, such as CD28/cytotoxic T-lymphocyte-associated antigen-4 and CD80/CD86, played an important role in the pathogenesis of autoimmune diseases [4]. Programmed death-1 (PD-1) is a new member of the CD28-B7 family. It is expressed on activated T, B and myeloid cells. The ligands for PD-1 are B7-H1/PD-L1 and B7-DC/PD-L2 [9, 10].

PD-1 is a key cell-surface receptor of the CD28 superfamily that triggers inhibitory pathways to attenuate T-cell responses and promote T-cell tolerance by binding to its ligand, PD-L1 [11]. The expression of PD-1, PD-L1, and PD-L2 has been investigated in several autoimmune diseases, such as rheumatoid arthritis, Sjögren syndrome, and inflammatory bowel disease, and in cancer immunity, including glioblastoma, lung, gastric, and pancreatic cancers [12, 13].

The aim of this study was to investigate hepatic expression of PD-1 and its ligand (PD-L1) in children with AIH and its relation to disease severity and treatment outcomes.

Material and methods

Study population

This retrospective study included pre-treatment liver biopsies of 31 children with AIH compared with 30 liver biopsies of children with chronic hepatitis C virus (HCV) as a disease control group. Ten liver biopsies of adults with normal liver tissue (accepted as living donor for liver transplantation) were also enrolled as healthy controls. AIH was diagnosed according to the criteria proposed by the International AIH groups [14]. In the HCV group all patients were positive for HCV-RNA by polymerase chain reaction for a period of at least 6 months. None of the AIH patients received corticoste-

roids and none of the HCV patients received interferon therapy as liver tissue samples were taken at the time of initial diagnosis. All AIH patients received immunosuppressive treatment in the form of low-dose prednisolone combined with azathioprine. According to the treatment response, AIH cases were divided into responders ($n = 24$ cases) and non-responders ($n = 4$). Response to treatment was defined by normalization of serum aminotransferases, bilirubin and γ globulin levels. Treatment non-response was defined by worsening clinical, laboratory, and histological features despite compliance with therapy [15]. Being a retrospective study, written informed consent was not needed from the parents or the legal guardians of the patients. The study was approved by the Research Ethics Committees of both the National Liver Institute (NLI), Menoufia University and the Faculty of Medicine, Benha University, Egypt. Patients' relevant demographic and serological (alanine aminotransferase [ALT], aspartate aminotransferase [AST], prothrombin time [PT] and complete blood count [CBC]) data as well as treatment and response to treatment were retrieved from the medical records of the Pediatric Hepatology, Gastroenterology and Nutrition Department, NLI, Menoufia University.

Histopathological studies

Liver biopsies were performed by ultrasonography-guided Tru-Cut needle for all the patients and controls (GTA, Quistello, MN, Italy). Paraffin-embedded liver biopsy specimens of the relevant patients and controls were retrieved from the Pathology Department archives, NLI, Menoufia University, and stained by hematoxylin and eosin (H&E), Masson's trichrome, Periodic acid Schiff, Orcein and Perls' stains for routine histopathological evaluation. Three four-micron thick sections were prepared for immunohistochemical staining.

Liver sections from all studied cases were re-evaluated by histopathologists who were unaware of their final diagnosis. The Ishak-modified Knodell system for scoring chronic hepatitis was applied for the histology activity index (HAI). Hematoxylin and eosin stained slides were evaluated for necroinflammatory activity, and trichrome stained slides were evaluated for fibrosis stage in biopsies from patients with AIH and HCV. According to the intensity of liver inflammation in the biopsy specimens, the grade of necroinflammatory activity was determined on a scale of 0-4; 0 (no inflammation), 1 (minimal inflammation, HAI score 1-3), 2 (mild inflammation, HAI score 4-8), 3 (moderate inflammation, HAI score 9-12), and 4 (marked inflammation, HAI score 13-18) [16]. Furthermore, fibro-

Table 1. Clinico-demographic findings of studied patients

Parameter	AIH	HCV	P-value
	(n = 31)	(n = 30)	
ALT (U/l)	399.55 ±343.25	48.9 ±51.96	< 0.0001
AST (U/l)	351.58 ±343.49	45.43 ±36.12	< 0.0001
PT (s)	14.56 ±1.97	12.78 ±1.53	0.001
Hemoglobin (gm/dl)	11.31 ±1.19	12.69 ±1.44	0.0003
WBCs (× 10 ³ /mm ³)	7.85 ±3.47	5.44 ±1.98	0.003
Platelets (× 10 ³ /mm ³)	260.84 ±114.87	262.97 ±97.53	0.829
Age (years)	7.91 ±4.19	11.6 ±4.1	0.001
Sex (male)	13 (41.9%)	19 (63.3%)	0.094

ALT – alanine aminotransferase, AST – aspartate aminotransferase, PT – prothrombin time, WBCs – white blood cells

Table 2. Histopathological findings

Parameter	AIH	HCV	P-value
	(n = 31)	(n = 30)	
Fibrosis (stage)			
F0	0 (0.0)	2 (6.7)	< 0.0001
F1	8 (25.8)	25 (83.3)	
F2	9 (29)	3 (10)	
F3	13 (41.9)	0 (0.0)	
F4	1 (3.2)	0 (0.0)	
Activity (grade)			
Minimal	0 (0.0)	18 (60)	< 0.0001
Mild	14 (45.2)	12 (40)	
Moderate	17 (54.8)	0 (0.0)	

sis staging was evaluated according to the METAVIR scoring system for fibrosis [17]. All biopsies were scored as follows; F0: no fibrosis; F1: portal fibrosis; F2: periportal or portal septa; F3: architectural distortion but no obvious cirrhosis; F4: definite cirrhosis.

PD-1 and PD-L1 immunohistochemistry

For immunohistochemical staining of PD-1 and PD-L1, 4 µm sections were cut from paraffin blocks and placed on positive charged slides. The primary antibody used were rabbit polyclonal PD-1 antibody (NBP1-77276; Novus Biologicals, Littleton, CO 80120, USA) at a dilution of 1 : 200 or rabbit polyclonal PD-L1 antibody (NBP2-15792; Novus Biologicals, Littleton, CO 80120, USA) at a dilution of 1 : 200. The detection kit was the Ultravision detection system (Cat #, TP-015-HD, Lab Vision, USA). The color development was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Negative (cold phosphate-buffered saline) and positive controls (brain tissue for PD-1 and colonic carcinoma for PD-L1) were enclosed in each run.

Interpretation of results

For PD-1 and PD-L1 liver-infiltrating lymphocyte, sections were screened on low power and areas of increased PD-1 were determined, usually centered on portal tracts and the periportal area or extending to hepatic parenchyma. Ten non-overlapping fields were examined using a 40× objective lens and a semi-quantitative estimation of proportions (percentages) of lymphocytes expressing PD-1 and PD-L1 was made. Then, the mean of the counted fields was considered for each liver section [7]. PD-L1 expression in Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), hepatocytes and biliary epithelial cells (BECs) was evaluated as either 0 = negative or 1 = positive [7, 18] using an Olympus Light Microscope (Tokyo, Japan).

Statistical analysis

Categorical data were presented as number and percentages while quantitative data were expressed as mean ± standard deviation (SD). The chi-square test (χ^2), or Fisher's exact test was used to analyze categorical variables. Quantitative data were tested for normality using the Shapiro-Wilk test, assuming normality at $p > 0.05$, using Student's *t* test if normally distributed, or the Mann-Whitney *U* test and Kruskal-Wallis test if not normally distributed for analyzing the difference. Spearman's test was used for correlation. Differences were considered significant at a calculated *p* value of < 0.05. Statistical analysis was performed using SPSS version 16 (SPSS Inc, Chicago, IL, USA).

Results

Demographic and clinical parameters

Children in the HCV group were older than those in the AIH group (11.6 ±4.1 vs. 7.91 ±4.19 years). The majority were male in the HCV group (63.3%) and were female in the AIH group (58.1%). AST, ALT and PT were significantly higher in the AIH group than those in the HCV group. Hemoglobin was significantly lower in the AIH group (Table 1).

Histopathological results

The majority of AIH patients (70.9%) were METAVIR F2 and F3 while the majority of HCV patients (83.3%) were METAVIR F1 ($p < 0.0001$). Similarly, necroinflammation was moderate in the majority of AIH patients (54.8%), while all HCV patients (100%) had either minimal or mild necroinflammation ($p < 0.0001$) (Table 2).

PD-1 and PD-L1 on liver-infiltrating lymphocytes in the studied groups

In AIH cases, PD-1 positive cells were abundantly observed on lymphocytes within liver tissue in AIH and HCV cases (Fig. 1A, B), while in healthy controls a few scattered PD-1 positive cells were observed (Fig. 1C). Mean PD-1 expression was significantly higher in the AIH group than in the HCV group (29.19 ± 18.5 vs. 15.2 ± 10.8 respectively; $p = 0.002$) (Fig. 2A). Comparing both AIH and HCV patients with mild activity only, PD-1 expression was significantly higher in AIH compared to that in HCV (33.57 ± 15.12 vs. 20.17 ± 10.57 respectively; $p = 0.015$) (Table 3). In addition, PD-L1 was expressed on some of lymphocytes in AIH and HCV with a mean percentage of 3.7 ± 3.22 and 3.6 ± 3.5 in AIH and HCV groups respectively with no statistically significant difference (Fig. 1D, E, 2B), while in healthy controls PD-L1 was negative (Fig. 1F). There was no correlation between the degree of PD-1 positive lymphocytes and activity grade, fibrosis stage, AST, ALT, PT or age in either AIH or HCV groups (Table 4).

Expression of PD-L1 on KC, LSEC, hepatocytes and BECs in the studied groups

PD-L1 expression on Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), hepatocytes and biliary epithelial cells (BECs) was comparable in AIH and HCV groups ($p > 0.05$ for all). In healthy controls, no PD-L1 positivity was detected. PD-L1 was expressed on KC in 19/31 (61.3%) AIH patients. Similarly, some KC expressed PD-L1 in 14/30 (46.7%) HCV patients. PD-L1 was also expressed on some LSEC in 18/31 (51.8%) patients with AIH, whereas some LSEC expressed PD-L1 in 17/30 (56.7%) patients with HCV. PD-L1 was also expressed on hepatocytes in 10/31 (32.3%) patients with AIH and in 5/30 (16.7%) HCV cases. In three AIH cases PD-L1 was expressed on BECs (Fig. 1 and Table 5).

PD-1 and PD-L1 lymphocytic expression in AIH according to treatment response

Of AIH patients, 27 (87%) were responders to immunosuppressive therapy while 4 (13%) were non-responders. PD-1 expression was comparable in responders and non-responders ($29.14 \pm 18.85\%$ vs. $29.58 \pm 18.77\%$; $p = 0.813$) (Fig. 3A). Interestingly, PD-L1 was significantly upregulated in responders compared to non-responders ($4.17 \pm 3.15\%$ vs. $0.63 \pm 1.3\%$; $p = 0.046$) (Fig. 3B). In addition, PD-1/PD-L1 ratio was much higher in non-responders compared to respond-

ers (PD-1/PD-L1; $[29.6/0.63] 46.9$ vs. $[29.1/4.17] 6.58$ respectively).

PD-L1 on non-lymphocytic cells in AIH according to treatment response

PD-L1 expression on hepatocytes, BECs, LSEC and KC was comparable in responder and non-responder AIH patients ($p > 0.05$ for all) (Table 6).

Discussion

PD-1 interaction with PD-L1 is critical to terminating immune responses. Elimination of either can result in the breakdown of tolerance and the development of autoimmunity. The PD-1 : PD-L pathway can impede self-reactive T cells and protect against autoimmunity in many ways [19]. The current study revealed upregulation of PD-1 expression in HCV and AIH groups while there were very few scattered PD-1 positive lymphocytes in the healthy control group. The same results were reported by Oikawa *et al.* [7]. The examination of synovial fluid from rheumatoid arthritis patients showed increased expression of PD-1 on lymphocytes [20] and previous reports have shown a regulatory function of PD-1 in organ-specific immune responses, such as experimental autoimmune encephalomyelitis [21], and autoimmune diabetes [11]. The results of the current study suggest that the upregulation of PD-1 was not only induced by autoimmune phenomena but also by viral infection, as reported in previous studies [22]. In addition, the mean PD-1 expression was significantly higher in AIH compared to that of the HCV group ($p = 0.002$). These findings are consistent with those in adults with chronic viral hepatitis and autoimmune liver diseases [23].

There was no significant correlation between the mean PD-1 expression and the stage of fibrosis ($p = 0.538$) or grade of inflammation ($p = 0.147$) in both AIH and HCV groups. These findings are consistent with those in adults with chronic autoimmune liver diseases [7]. The lack of correlation of PD-1 and PD-L1 with inflammatory activity can be explained by other factors affecting the inflammatory process such as cytokines, possible PD-1 and PD-L1 autoantibody formation as a part of autoimmunity [24], possible temporal changes in PD-1/PD-L1 expression [25] and the number of PD-1 and PD-L1 molecules per cell, which may reach up to 10^5 molecules per single cell [26]. Furthermore, PD-1 expression had no significant correlation with the laboratory findings of ALT or AST in either the AIH or HCV group. These results matched those of Kassel *et al.* [23] and Oikawa *et al.* [7].

In the current study, AIH and HCV groups up-regulated PD-L1 expression on lymphocytes infiltrating

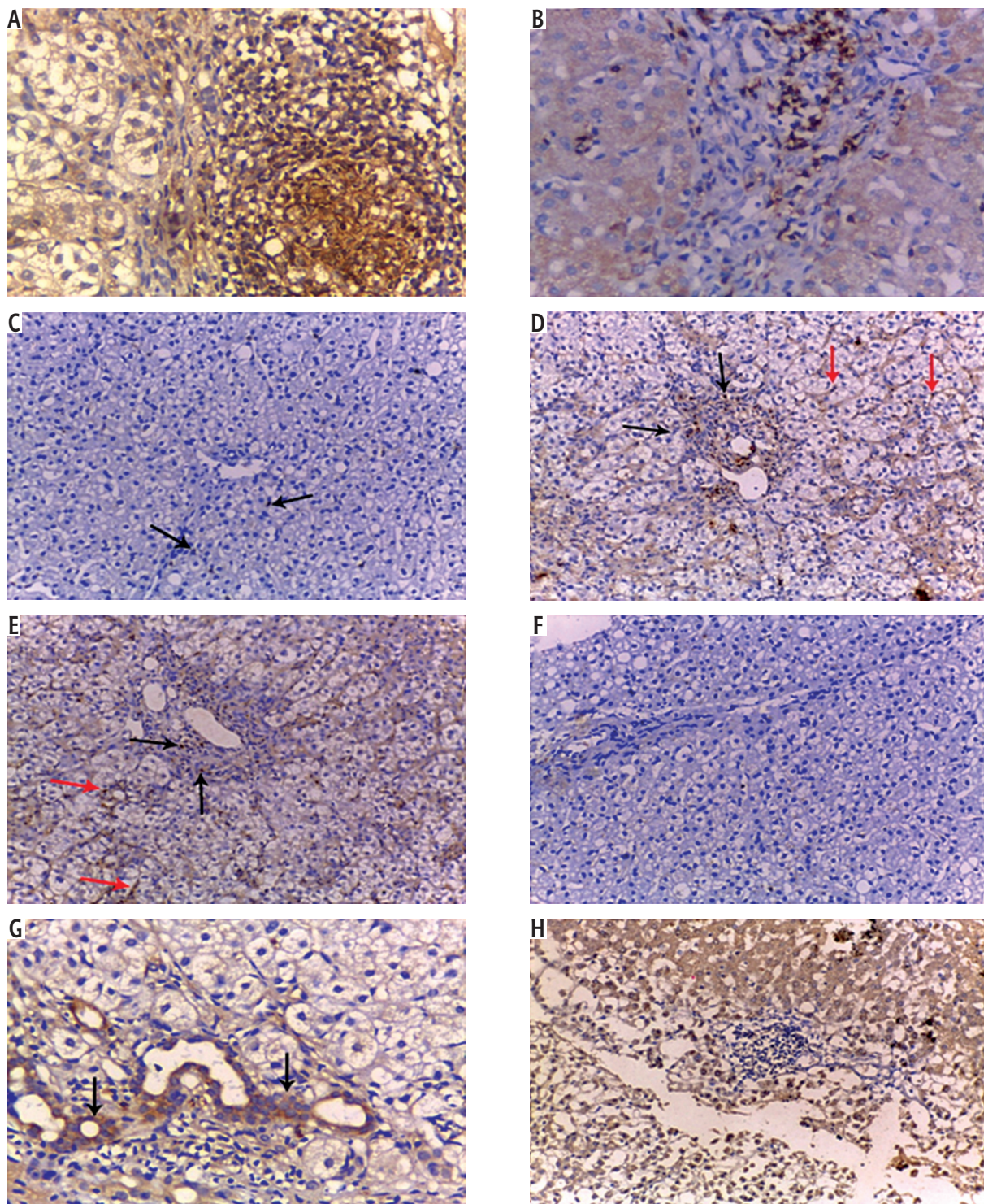


Fig. 1. PD-1 and PD-L1 immunohistochemical expression in the studied groups. **A)** Immunohistochemical staining of PD-1 on liver tissue from a child with AIH showing PD-1 positive brown cytoplasmic and membranous staining of lymphocytes with mean percentage (70%) (IHC 400×). **B)** Immunohistochemical staining of PD-1 on liver tissue from a child with HCV, showing PD-1 positive brown cytoplasmic and membranous staining of lymphocytes with mean percentage (20%) (IHC 400×). **C)** Immunohistochemical staining of PD-1 on healthy control liver tissue, showing few scattered PD-1 positive lymphocytes (arrows) (IHC 200×). **D)** Immunohistochemical staining of PD-L1 on liver tissue from a child with AIH, showing PD-L1 positive lymphocytes (black arrows), liver sinusoidal endothelial cells and Kupffer cells (red arrows) (IHC 200×). **E)** Immunohistochemical staining of PD-L1 on liver tissue from a child with HCV showing PD-L1 positive lymphocytes (black arrow) and liver sinusoidal endothelial cells and Kupffer cells (red arrow) (IHC 200×). **F)** Immunohistochemical staining of PD-L1 on healthy control liver tissue showing negative PD-L1 immune reaction (IHC 200×). **G)** Immunohistochemical staining of PD-L1 on liver tissue from a child with AIH, showing PD-L1 positive brown cytoplasmic staining of biliary epithelial cells (arrows) (IHC 400×). **H)** Immunohistochemical staining of PD-L1 on liver tissue from a child with AIH showing PD-L1 positive brown cytoplasmic staining of hepatocytes (IHC 200×)

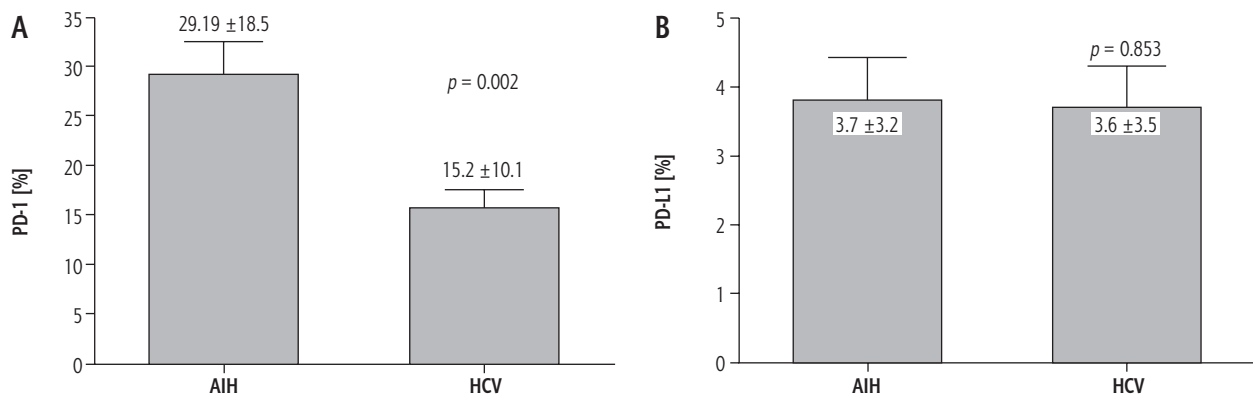


Fig. 2. Mean PD-1 and PD-L1 expression on lymphocytes in AIH and HCV groups. **A)** PD-1, **B)** PD-L1

liver tissue, LSEC, KC, hepatocytes as well as BECs, while PD-L1 was found in none of the healthy controls. The up-regulation of PD-L1 on lymphocytes, KC and LSEC was comparable in both AIH and HCV groups with no statistically significant difference. This is in agreement with other studies in adults with chronic autoimmune liver diseases and HCV [7, 23, 27].

KC together with LSEC are the first barrier for pathogens that enter the liver via the portal vein. This is extremely important, since venous portal blood is rich in pathogen-derived products, such as lipopolysaccharide, and pathogens from the gut, which need to be eliminated from the circulation to avoid systemic immune activation [28]. Intrahepatic PD-L1 expression on non-parenchymal cells such as KC and LSECs increases significantly during immune clearance episodes. When effector/memory T cells are attracted to the inflamed liver, they first contact with KC and LSECs [29]. The interaction between PD-1 on effector lymphocytes and PD-L1 on non-parenchymal cells can limit the degree of the immune response at the sites of the inflamed liver by increasing the negative regulatory signals [30].

It has been shown that activated lymphocytes express PD-L1 in autoimmune disease such as rheuma-

Table 3. Comparison between HCV and AIH with mild activity only

Parameter	AIH with mild activity n = 14		HCV with mild activity n = 12		P-value
Total PD-1	33.57	15.12	20.17	10.57	0.015
Total PD-L1	3.82	3.16	3.5	3.93	0.811

toid arthritis and inflammatory bowel disease [13, 31, 32]. Specific B-cell subsets can regulate T-cell immune responses, and are termed regulatory B (Breg) cells. The majority of Breg cells described in mouse and man have been identified by interleukin 10 (IL-10) production and are known to suppress allergy and autoimmunity [33]. However, Breg cell-mediated immune suppression, independent of IL-10, also occurs. Khan *et al.* [34] hypothesized that Breg cells play a critical role in regulating humoral immunity mediated by CD4/PD-1 positive follicular helper T cells, and can suppress inflammation in autoimmune disease through elevated expression of PD-L1. In addition, PD-L1 has been confirmed to play a critical role in the development and functional maintenance of Tregs. Francisco *et al.* [35] demonstrated that PD-L1 can inhibit T cell responses by converting naive CD4 T cells to iTreg cells, indicating that PD-L1

Table 4. Correlation of PD-1 and PD-L1 with transaminases and histopathological findings

Parameter	AIH (n = 31)				HCV (n = 30)			
	PD-1		PD-L1		PD-1		PD-L1	
	r	p	r	p	r	p	r	p
Age (years)	0.304	0.097	0.254	0.168	0.09	0.638	-0.177	0.348
Prothrombin time	-0.016	0.931	0.294	0.108	-0.281	0.133	0.065	0.733
ALT (U/l)	-0.112	0.55	0.093	0.617	0.11	0.564	0.028	0.885
AST (U/l)	0.103	0.582	0.189	0.308	0.181	0.339	-0.059	0.617
Fibrosis	-0.039	0.538	0.334	0.066	0.282	0.13	-0.314	0.091
HAI	-0.266	0.147	-0.034	0.855	0.321	0.083	-0.037	0.847

ALT – alanine aminotransferase, AST – aspartate aminotransferase, HAI – histological activity index

Table 5. PD-L1 in non-lymphocytic cells

Parameter	AIH (n = 31)	HCV (n = 30)	P-value
	n (%)	n (%)	
Kupffer cells	19 (61.3)	14 (46.7)	0.252
Sinusoidal endothelial cells	18 (58.1)	17 (56.7)	0.912
Hepatocytes	10 (32.3)	5 (16.7)	0.079
Biliary epithelial cells	2 (6.5)	0.0	0.492

AIH – autoimmune hepatitis, HCV – hepatitis C virus, PD-L1 – programmed death ligand 1

Table 6. PD-L1 in non-lymphocytic cells in AIH according to treatment outcome

Localization	Responders (n = 27)	Non-responders (n = 4)	P-value
Kupffer cells	17 (63)	2 (50)	0.63
Sinusoidal endothelial cells	17 (63)	1 (25)	0.284
Hepatocytes	9 (33.3)	1 (25)	1.0
Biliary epithelial cells	2 (7.4)	0.0	1.0

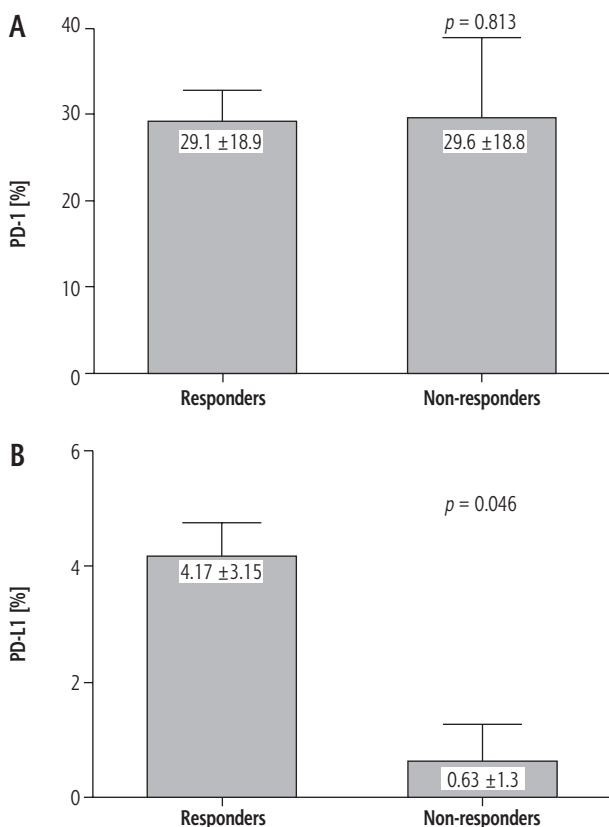


Fig. 3. Mean PD-1 and PD-L1 expression on lymphocytes in responder and non-responder AIH patients. **A)** PD-1, **B)** PD-L1

has a pivotal role in regulating induced Treg (iTreg) cell development and sustaining iTreg cell function.

The current study has shown that PD-L1 is expressed on some of the lymphocytes in AIH and HCV groups. It is possible that the PD-L1 expressing lymphocytes

in AIH might be of the regulatory type and associated with T-cell-mediated regulation [7]. PD-L1 expression in hepatocytes is of a major concern in our study, as hepatocytes are the primary site of infection for HCV and are recipients of the misdirected immune response that produce AIH. In this study, PD-L1 was found on hepatocytes in 32.3% of AIH cases, and in 13.3% of HCV cases with no statistically significant difference between the two groups ($p = 0.079$). This is in agreement with the results reported by Kassel *et al.* and Muhlbauer *et al.* [23, 36].

The positive PD-L1 BECs in AIH cases may be associated with unrevealed biliary pathology. This biliary pathology may be primary sclerosing cholangitis, which is known as autoimmune sclerosing cholangitis overlap syndrome. a Talwalkar *et al.* [37] and Knight *et al.* [38] reported that overlap syndrome appears to be more common in children. Schrupf *et al.* [39] reported that BECs are the target for several human immune mediated liver diseases, including primary biliary cholangitis, and stated that BEC-mediated T-cell inactivation occurs partially via PD-L1 in a cell contact dependent manner. They concluded that the regulatory activities of BECs are important for the maintenance of peripheral immune tolerance. Taken together, previous reports and the present results suggest that the interaction between PD-1 positive lymphocytes and increased expression of PD-L1 positive KC and LSEC might be involved in the downregulation of autoreactive lymphocytes and result in the regulation of pathogenesis in AIH. Additionally, hepatocytes, BECs as well as lymphocytes which also expressed PD-L1 could limit lymphocytes' effector function.

Although PD-L1 was expressed on KC, LSEC and hepatocytes, there was no significant association with clinicopathological features. These results matched those of Oikawa *et al.* [7] and Matakai *et al.* [40] but not those of Kassel *et al.* [23]. These can be attributed to multifactorial causes, but the most important aspects are probably the use of non-standardized immunohistochemical staining methods, besides using additional techniques rather than immunohistochemical methods such as PCR in assessment of PD-L1 upregulation.

In the current study, the PD-1/PD-L1 ratio in both AIH and HCV were calculated. Interestingly, the PD-1/PD-L1 ratio was higher in the AIH group; mean PD-1 increased by 6.68 times the increase of mean PD-L1 in the AIH group, while the mean PD-1 increased by 4.3 times the increase of mean PD-L1 in the HCV group. Furthermore, the ratio was significantly lower in non-responder AIH patients compared to responders (PD-1/PD-L1 ratio; 46.9 vs. 6.6 respectively), meaning that the expression of PD-1 greatly outweighed the

expression of the counteracting force of PD-L1. Similarly, Aarslev *et al.* [41] investigated PD-1 levels in serum, but not in liver tissue, of adult patients with AIH. They found that soluble PD-1 was significantly elevated in AIH patients with active disease and in incomplete responders to standard immunosuppressive therapy compared with responders and healthy controls. To the best of our knowledge, the current study is the first to investigate liver tissue expression of PD-1 and PDL-1 in pediatric AIH and its relation with treatment response.

Taken together, these results suggest a dysfunction of the PD-1/PD-L1 pathway in AIH. A possible reason is the genetic variability such as a single-nucleotide polymorphism (SNP), which was observed in the gene for cytotoxic T-lymphocyte associated antigen-4 in autoimmune liver disease [42]. Several studies have reported that SNP in PD-L1 was associated with the development of systemic lupus erythematosus [43], increased risk of type 1 diabetes [11], and rheumatoid arthritis [44].

It is likely that loss of control in regulation of the PD-1 pathway in the liver may contribute to inflammation and subsequently AIH. Manipulation of the PD-1/PD-L1 pathway may be a candidate target for the treatment of AIH in order to re-establish immunological tolerance [7]. Further, modulation of BEC function may be used for therapeutic modulation in overlap syndrome cases [39]. PD-1 immunotherapy was used recently in clinical trials. In a stage I clinical trial, anti-PD-1 monoclonal antibody therapy demonstrated a lack of significant side effects [45], and has been approved for the treatment of several cancers [46, 47]. On the other hand, blocking of PD-1 may be detrimental during AIH. Rather, PD-1 agonist may prove beneficial. It was reported that transforming growth factor- β plays a key role in activation of the PD-1 pathway [48]. In addition, glucocorticoids and hydrocortisone could enhance PD-1 expression in a dose-dependent manner. The effect was completely inhibited by a glucocorticoid receptor antagonist, indicating that the effect of glucocorticoid on PD-1 is mediated through the glucocorticoid receptor. Glucocorticoid could suppress T cell function via inhibition of cytokine production, or by induction of apoptosis through enhancing the expression of PD-1 [49]. In addition, epidermal growth factor receptor [29] and interferon γ [50] were reported to induce expression of the PD-L1 molecule.

In conclusion, expression of PD-1 and its ligand, PD-L1, is dysregulated in AIH with lower expression of PD-L1 compared to HCV and in treatment non-responders compared to responders in AIH patients. Future studies regarding PD-L1 induction as a therapeutic target in children with AIH are warranted.

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Disclosure

The authors report no conflict of interest.

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