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Original Article

Characteristic expression of PD-1 and its ligands mRNAs in patients with noninfectious uveitis

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Abstract: Purpose: To evaluate the characteristic expression of PD-1 and its ligands in patients with noninfectious uveitis. Methods: Thirty-four patients with noninfectious uveitis were examined. Fluorescent quantitative real-time polymerase chain reaction was used to analyze PD-1, PD-L1, and PD-L2 mRNA expression in peripheral blood lymphocytes. Their expression levels were compared according to uveitis stage, systemic or ocular manifestations, disease duration, visual acuity, and medication. Results: The expression of PD-1, PD-L1, and PD-L2 mRNAs was reduced in patients with uveitis, and most reduced in patients with active uveitis. Lower levels of PD-L2 mRNA were observed in patients with isolated ocular inflammation and in patients treated with topical steroid alone or combined with oral prednisone and immunomodulatory agents. There was no linear correlation between the expression of PD-1 or its ligands and visual acuity or disease duration. Conclusion: PD-1 and its ligands, especially PD-L2, may be involved in the development, manifestation, and treatment of noninfectious uveitis.

Keywords: Noninfectious uveitis, PD-1, PD-L1, PD-L2, mRNA

Introduction

Noninfectious uveitis is a group of sight-threatening inflammatory diseases associated with an exacerbated immunological response to ocular proteins [1]. It can be limited to the eye or be part of a generalized systemic syndrome [2]. The inflammation present in noninfectious uveitis can be triggered by an imbalance between the ocular immune privilege and an autoimmune response [1]. The induction of an antigen-specific T-cell response has been shown to mediate experimental autoimmune uveitis (EAU) in animals, and T cells are also considered to be central to the pathogenesis of human uveitis [2]. To fully activate a naïve T cell, not only must the T-cell receptor recognize the peptide/MHC complex on an antigen-presenting cell (APC), but co-stimulatory signals are also required.

The balance between positive and negative co-stimulatory signaling pathways dictates the fate of individual T cells and the immune response. As a member of the CD28/B7 family and a co-stimulatory molecule, programmed death 1 (PD-1) and its ligands (PD-Ls), PD-L1 and PD-L2, have emerged as a critical inhibitory signaling pathway that regulates the T-cell response and maintains peripheral tolerance. PD-1 contains an immunoreceptor tyrosine-based inhibiting motif and an immunoreceptor tyrosine-based switch motif [3]. It is upregulated on T cells, B cells, and some myeloid cells upon activation [4]. PD-L1 is constitutively expressed on T cells, B cells, macrophages, and dendritic cells (DCs), as well as on several nonhematopoietic cell types, whereas PD-L2 is mainly expressed on DCs and macrophages [4]. The engagement of PD-1 with its ligands inhibits the T-cell-receptor-mediated proliferation and cytokine production of previously activated T cells through a negative signal, by the recruitment of Src homology 2 (SH2)-domain-containing phosphatase-2 (SHP-2) to a phos-

phorylated tyrosine residue in the cytoplasmic region [5]. The interaction between PD-1 and PD-L has been shown to play an important role in autoimmune diseases, organ transplant rejection, microorganismal infections, tumor immune evasion, and so on [6, 7]. However, little is known about PD-1 and its ligands in patients with noninfectious uveitis.

Based on the importance of the PD-1-PD-L pathway in the immune response, we hypothesized that PD-1 and its ligands are involved in the regulation of noninfectious uveitis. In this study, the expression of PD-1, PD-L1, and PD-L2 was investigated in patients with noninfectious uveitis and the correlation between the PD-1-PD-L pathway and the clinical disease characteristics was determined.

Material and methods

Clinical samples

Thirty-four patients with noninfectious uveitis (23 males and 11 females, with an average age of 38.7±10.0 years) were included in this study. Twenty age- and sex-matched healthy volunteers (13 males and 7 females, with an average age of 40.3±11.2 years) acted as controls. All the subjects were recruited from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and the Guangdong General Hospital (Guangzhou, China). The noninfectious uveitis observed in this study was classified as Vogt-Koyanagi-Harada (VKH) syndrome (n = 12), panuveitis in Behcet's disease (n = 8), uveitis associated with ankylosing spondylitis (AS) (n = 5), and idiopathic anterior uveitis (n = 9). The ophthalmological diagnoses were based on a detailed clinical history and thorough ocular and systemic examinations. The diagnosis of VKH syndrome fulfilled the First International Workshop criteria for VKH disease [8]. The diagnosis of Behcet's disease was based on the criteria of the International Study Group for Behcet's disease [9]. Uveitis associated with AS was diagnosed according to the modified New York criteria for AS and the Standardization of Uveitis Nomenclature (SUN) [10, 11]. The SUN was also used to determine the uveitic clinical characteristics and the degree of inflammation. Exclusion criteria included a history of infection or chronic disease which could possibly influence the immune system (e.g. chronic hepatitis, diabetes, coronary heart disease, tumors), or any uncontrolled ocular disease other than noninfectious uveitis.

The data gathered from the medical records of each patient included demographic information (age, sex, race), systemic versus isolated ocular inflammatory disease, disease duration, the presence or absence of disease activity, visual acuity in decimal, and the use of steroid or immunomodulatory therapy (IMT) agents.

The local institutional ethics committee approved the study and written informed consent was obtained from all the study subjects. All research conformed to the Association for Research in Vision and Ophthalmology statement on human research and the Declaration of Helsinki.

Fluorescent quantitative real-time polymerase chain reaction (PCR) analysis of PD-1, PD-L1, and PD-L2

To determine the expression of PD-1, PD-L1, and PD-L2, fresh peripheral blood (2 ml) was collected in tubes containing EDTA from the patients with uveitis and the healthy controls. Lymphocytes were isolated with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Total RNA was extracted from the lymphocytes using TRIzol Reagent (Molecular Research Center, Cincinnati, USA) and quantified from the absorbance at 260 nm. cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Fluorescent quantitative real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) on the MJ Research Opticon 2 real-time PCR unit (MJ Research, California, USA), according to the manufacturer's instructions. The following primer pairs were used: Hu-PD-1: 5'-CCCTGGTGGTTGGTGTCGT-3', 5'-GCCTGGCTCCTATTGTCCCTC-3'; Hu-PD-L1: 5'-TGGTGTAGCACTGACATTCA-3', 5'-TCCAATGCTGGATTACGTCT-3'; Hu-PD-L2: 5'-TTCATAGCCACAGTGATAGC-3', 5'-GGTTCAGATAGCACTGTTCA-3'; and Hu-ra-b-actin: 5'-GCCAACACAGTGCTGTCTG-3', 5'-TACTCCTGCTTGCTGATCCA-3'. Each primer (Invitrogen, Shanghai, USA) was pretested and confirmed. The optimized protocol included an initial denaturation step at 95°C for 120 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Data analysis was based on the $2^{-\Delta\Delta Ct}$ method, with normalization of the raw data to the b-actin housekeeping gene. All PCR assays were performed in duplicate.

Table 1. Baseline characteristics of uveitis patients

Characteristic	Patients or eyes
Total patients enrolled	34 (68 eyes)
Mean age, yrs (range)	38.7±10.0 (16-55)
Male, number of patients (%)	23 (67.6)
Female, number of patients (%)	11 (32.4)
Uveitis diagnosis, number of patients (%)	
VKH syndrome	12 (35.3)
Panuveitis in Behcet's disease	8 (23.5)
Uveitis associated with AS	5 (14.7)
Idiopathic anterior uveitis	9 (26.5)
Uveitis phase, number of patients (%)	
Active	21 (61.8)
Inactive	13 (38.2)
Uveitis duration	
Mean, yrs (range)	5.1±6.4 (12 days to 28 years)
< 5 years, number of patients (%)	19 (55.9)
≥ 5 years, number of patients (%)	15 (44.1)
BCVA, number of eyes (%)	
≥ 0.3	14 (20.6)
0.05-0.3	28 (41.2)
< 0.05	26 (38.2)
Medication, number of patients (%)	
Topical steroid eye drops	9 (26.5)
Oral prednisone	18 (52.9)
Oral prednisone combined with IMT agents	7 (20.6)

VKH syndrome: Vogt-Koyanagi-Harada syndrome; AS: ankylosing spondylitis; BCVA: best corrected visual acuity; IMT agents: immunomodulatory agents.

Statistical analysis

The Wilcoxon two-sample rank sum test (Mann-Whitney U test) and Kruskal-Wallis test were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Data are expressed as medians and interquartile ranges (IQRs). A linear regression analysis was used to assess the relationship between visual acuity or disease duration and the expression of PD-1, PD-L1, and PD-L2. A value of $P < 0.05$ was considered to indicate a significant difference.

Results

The clinical characteristics of the enrolled patients with noninfectious uveitis are summarized in **Table 1**. All patients were young or middle-aged, and approximately two-thirds of the patients were male. All patients had bilateral disease. If one eye was in active phase and the contralateral eye was in remission, the subject was recorded as patient with active uveitis. Only two patients had early-onset disease, with durations of 12 days and 17 days. Nearly 80% of eyes (54 eyes) had a best corrected visual acuity of less than 0.3 decimal. All patients underwent the local or combined with systemic treatment for uveitis. Nine patients were given topical steroid eye drops alone at the time they visited us. Among them, seven patients had never used any systemic corticosteroids or IMT agents, and two patients had previously received low dose of prednisone or IMT agents but had stopped at least 2 weeks prior to blood sampling. Eighteen patients were treated with prednisone (20-60 mg/day) before visiting us. Other seven patients were treated with prednisone (10-20 mg/day) combined with IMT agents. Five of them were combined with cyclosporine A (CsA) (75-150 mg/day) treatment and the other two patients were combined with CsA and cyclophosphamide (50 mg/day).

Expression of PD-1, PD-L1, and PD-L2 in uveitis patients and healthy controls

PD-1, PD-L1, and PD-L2 mRNAs were detectable in all samples, but were significantly lower in the uveitis patients than in the healthy controls (**Table 2**).

Expression of PD-1, PD-L1, and PD-L2 in patients with active uveitis or inactive uveitis

The expression of PD-1 and PD-L2 mRNAs was lower in patients with active uveitis than in patients with inactive uveitis. PD-L1 mRNA was also reduced in active uveitis but was not significantly different from that in patients whose disease was in remission (**Table 3**).

Table 2. mRNA expressions of PD-1, PD-L1, and PD-L2 in uveitis patients and healthy controls

	Patients	PD-1 (×10E-4)			PD-L1 (×10E-4)			PD-L2 (×10E-4)		
		Median	Q_L	Q_U	Median	Q_L	Q_U	Median	Q_L	Q_U
Uveitis	34	1.56	1.05	2.49	2.05	1.53	3.23	1.63	1.32	6.86
Health controls	20	6.48	3.39	10.75	4.61	2.68	8.73	13.66	4.16	29.76
Z			-4.354			-3.003			-3.980	
P			<0.001			0.003			<0.001	

Q_L: lower quartile; Q_U: upper quartile.

Table 3. mRNA expression of PD-1, PD-L1, and PD-L2 in patients with active or inactive uveitis

	Patients	PD-1 (×10E-4)			PD-L1 (×10E-4)			PD-L2 (×10E-4)		
		Median	Q_L	Q_U	Median	Q_L	Q_U	Median	Q_L	Q_U
Active	21	1.21	0.92	1.59	2.04	1.03	2.80	1.49	1.13	1.65
Inactive	13	3.07	2.23	5.04	2.74	1.93	7.79	7.97	2.71	17.08
Z			-3.884			-1.774			-3.164	
P			<0.001			0.080			0.001	

Q_L: lower quartile; Q_U: upper quartile.

Expression of PD-1, PD-L1, and PD-L2 in patients with an isolated ocular diagnosis or systemic autoimmune disease

Although no statistically significant differences in the expression of PD-1 and PD-L1 mRNAs were observed between patients with isolated ocular diagnoses and those with known systemic autoimmune disease, PD-L2 expression was markedly lower in patients with ocular inflammation only (**Table 4**).

Expression of PD-1, PD-L1, and PD-L2 in patients treated with topical steroid, oral prednisone or oral prednisone combined with IMT agents

The analysis for the expression of PD-1 and its ligands in patients treated with topical steroid alone, combined with oral prednisone or combined with oral prednisone and IMT agents showed that there were significant differences of PD-1 and PD-L2 mRNAs in these groups, respectively (**Table 5**). Expressions of PD-1 and PD-L2 mRNAs in topical steroid group were significantly decreased compared with oral prednisone group (PD-1: $Z = -2.477$, $P = 0.012$; PD-L2: $Z = -2.973$, $P = 0.001$), but were similar to oral prednisone and IMT agents group. PD-L2 mRNA was lower in oral prednisone and IMT agents group than in oral prednisone group ($Z = -2.436$, $P = 0.013$).

Correlation between expression of PD-1, PD-L1, and PD-L2 and clinical features

Neither visual acuity nor disease duration correlated with the expression of PD-1, PD-L1, or PD-L2.

Discussion

Dysregulation of the immune response is considered one of the pathogenic mechanisms associated with the development of noninfectious uveitis. Idiopathic anterior uveitis, Behcet's disease, and VKH syndrome are the most common forms of uveitis in China [12]. PD-1 and its ligands have been implicated in the pathogenesis of noninfectious uveitis. In this study, changes in the expression of PD-1, PD-L1, and PD-L2 mRNAs were observed in the group of patients with noninfectious uveitis. The expression of PD-1 and its ligands was reduced in patients with uveitis, especially in those with active uveitis. The expression of PD-L2 mRNA was lower in patients with isolated ocular involvement. There was significant difference of PD-L2 mRNA in patients treated with topical steroid, oral prednisone or oral prednisone combined with IMT agents.

PD-1 has been implicated in a critical tolerance pathway since the discovery of spontaneous autoimmune disease in PD-1-knockout mice in the NOD mouse model of spontaneous type 1

Table 4. mRNA expression of PD-1, PD-L1, and PD-L2 in patients with an isolated ocular diagnoses or systemic autoimmune disease

	Patients	PD-1 (×10E-4)			PD-L1 (×10E-4)			PD-L2 (×10E-4)		
		Median	Q_L	Q_U	Median	Q_L	Q_U	Median	Q_L	Q_U
Ocular	9	1.10	0.68	1.73	1.53	0.99	2.10	1.08	0.98	1.44
Systemic	25	1.70	1.15	3.32	2.47	1.74	3.43	2.10	1.43	8.33
Z			-1.685			-1.748			-2.559	
P			0.099			0.086			0.008	

Q_L: lower quartile; Q_U: upper quartile.

Table 5. mRNA expression of PD-1, PD-L1, and PD-L2 in patients treated with topical steroid, oral predisone or oral predisone combined with IMT agents

	Patients	PD-1 (×10E-4)			PD-L1 (×10E-4)			PD-L2 (×10E-4)		
		Median	Q_L	Q_U	Median	Q_L	Q_U	Median	Q_L	Q_U
Topical steroid	9	1.14	0.96	1.27	1.61	1.13	2.13	1.21	0.96	1.67
Combined with oral prednisone	18	3.20	1.49	4.70	3.23	1.87	8.02	8.07	2.49	10.86
Combined with oral prednisone and IMT agents	7	1.29	0.53	3.05	2.05	1.40	2.55	1.32	1.09	2.17
H			6.691			5.360			11.236	
P			0.032			0.065			0.001	

Q_L: lower quartile; Q_U: upper quartile; IMT agents: immunomodulatory agents.

diabetes [13]. PD-1 deficiency in different genetic backgrounds results in the development of a lupus-like disease or dilated cardiomyopathy [14, 15]. Using an experimental autoimmune encephalomyelitis (EAE) mouse model of human multiple sclerosis, several studies have demonstrated that PD-1, PD-L1, and PD-L2 play influential roles in the pathogenesis of EAE [16, 17]. Our results show that the levels of PD-1, PD-L1, and PD-L2 mRNAs were lower in the peripheral blood of patients with noninfectious uveitis than in the healthy controls. Using the EAU mouse model, Chen et al. found that PD-1 and its ligands are expressed at sites of active inflammation in the posterior segment of the eye [18]. These results suggest that the PD-1-PD-L pathway is involved in the pathological process of noninfectious uveitis.

PD-L1 is expressed more broadly than PD-L2, which supports the different roles of these ligands in the immune response. PD-1-PD-L1 interactions have been shown to play a substantial role in regulating autoreactive T cells, which are specific for tissue-restricted self-antigens, whereas PD-L2 is critically involved in regulating immune responses to environmental antigens [4]. However, in the present study, the expression of PD-L2 mRNA, but not PD-L1 mRNA, changed significantly with the different phases of uveitis, its clinical manifestations, and its treatment. This result implies that PD-L2 is more closely associated with certain clinical characteristics of uveitis and some therapeutic interventions. However, the treatment for uveitis is susceptible to the dose, the time of drugs exposition, the inflammatory nature and severity. Thus, the effect of corticosteroids and immunosuppressive agents on the expression of PD-L and its ligands may be influenced by many factors, and still requires further research. As co-stimulatory molecules, the protein expression of PD-1 and its ligands on cell surface are important to mediate immune response, so an in-depth investigation about the frequencies of PD-1 on lymphocytes and PD-L1 and PD-L2 on APCs will help to further clarify the potential role of PD-1 pathway in noninfectious uveitis.

A major role of the PD-1-PD-L pathway is in the inhibition of T-cell function when PD-L1 or PD-L2 on APCs engages the PD-1 receptor on T cells [19]. PD-1 signaling has also been implicated in the reversal of the “stop signal” that is mediated by T-cell receptor signaling [20]. This means that in the presence of PD-1, T cells have a shortened dwell time in their interactions with APCs, which can reduce T-cell activation and may also favor the induction of regula-

tory T cells (Tregs) [19]. Treg cell populations are critical for the maintenance of peripheral tolerance, are potent inhibitors of many immune responses, and are effective in the prevention of autoimmune diseases [21, 22]. The PD-1-PD-L pathway has been shown to play a critical role in the generation of $Foxp3^+$ Tregs [23-25]. A reduction in $CD4^+FoxP3^+$ T cells has been detected in patients with active uveitis. The loss of Tregs in uveitis may be a salient feature in certain patients, and the treatment of autoimmune uveitis results in an increase in Tregs and the restoration of their functional state [26, 27]. A subpopulation of regulatory allospecific $CD8^+$ T cells expressing PD-1, which is induced by the ICOS-B7h blockade, is also important in immune regulation [28]. We found that PD-1, PD-L1, and PD-L2 mRNAs and proteins are markedly upregulated in the anterior-chamber-associated immune deviation mouse, and that $CD4^+PD\text{-}1^+$ T cells exhibit antigen-specific suppressive activity [29]. Therefore, further research is required to determine whether the PD-1-PD-L pathway is involved in the pathological processes of noninfectious uveitis by mediating the functions of Tregs.

There are some limitations in this study. Due to the restrictions of blood and eye tissue samples from each subject, the subset of cells, such as $CD4^+$ T cells, $Foxp3^+$ Tregs and macrophages, in blood and eye tissue were not evaluated. The present study also did not follow the patients longitudinally to see how levels change of PD-1 and its ligands with disease activity or treatment because some of the patients were lost to follow-up. Moreover, we should compare the expression levels to another immune mediated disease such as rheumatoid arthritis or multiple sclerosis.

In conclusion, the expression of PD-1 and its ligands is downregulated in noninfectious uveitis. PD-L2 may play a more important role in the development, manifestations, and treatment of uveitis than PD-1 or PD-L1.

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Disclosure of conflict interest

None.

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