Over-Expression of Immunosuppressive Molecules, PD-L1 and PD-L2, in Ulcerative Colitis Patients

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ABSTRACT

Background: Ulcerative colitis (UC) and Crohn's disease (CD) are the two forms of inflammatory bowel disease (IBD). Adaptive immune responses involving helper T cells play an important role in developing IBDs. Programmed death (PD)-1 and its ligands namely PD-L1 and PD-L2 are negative costimulatory molecules that control T cell motility and formation of an immune synapse between T cells and antigen-presenting cells (APCs). **Objective:** To investigate the role of PD-L1 and PD-L2 in patients with UC to clarify the mechanism of IBD development. **Methods:** Biopsy specimens were obtained from 50 UC patients and 45 sex- and age-matched control subjects. Total RNA was extracted from all samples and applied for cDNA synthesis. Relative expression of PD-L1 and PD-L2 mRNA was determined using Taqman qRT-PCR. **Results:** Relative gene expression levels of both PD-L1 and PD-L2 were higher in UC patients than the control groups ($p<0.05$ and $p<0.01$, respectively). Furthermore, both PD-L1 and PD-L2 expressions were positively correlated in all study subjects ($r=0.339$, $p<0.001$). However, among the groups with disease severity, the relative gene expression levels of PD-L1 and PD-L2 showed no significant difference. **Conclusions:** During IBD, the occurrence of PD-L1 and PD-L2 up-regulation may modulate the chronic inflammation of colonic mucosa.

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Keywords: PD-L1, PD-L2, Ulcerative Colitis

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory situation of the gastrointestinal tract that is manifested as ulcerative colitis (UC) or Crohn's disease (CD) (1). UC is a chronic remitting inflammation of the colonic mucosa (1). The etiology of IBDs has been poorly understood; it has been presumed that genetic susceptibility causes a prolonged abnormal activation of the mucosal immune system against regional normal flora in the intestine, which leads to inflammation of intestinal mucosa and developing IBD (2). Several studies have suggested the role of different subsets of helper T cells including type-1 and -2 CD4+ helper T cells (i.e., Th1 and Th2), Interleukin-17 (IL-17) producing CD4+ helper T cells (Th17), and regulatory T cells (Treg) in developing IBD (3). It has been postulated that IBD is caused by inadequate Treg mediated suppression of immune response to commensal organisms; however, the data have been somewhat conflicting (4). Dendritic cells (DCs), as the main antigen-presenting cells (APCs), are located in peripheral and mucosal lymphoid tissues, where they present antigens derived from bacteria and other pathogenic organisms to T cells. In the absence of danger signals, however, DCs may inhibit inflammatory T helper cell responses by inducing Treg production. Therefore, DCs play an important role in T cell-dependent immune homeostasis (5).

A variety of inhibitory molecules expressed on T-cell or DC surfaces may play a role in the maintenance of self-tolerance and prevention of autoimmune diseases. Cytotoxic T lymphocyte antigen-4 (CTLA-4) on the surface of T cells, for instance, mainly controls the initiation of T cell activation in lymphoid organs (6). In this regard, programmed death 1 (PD-1) is a co-inhibitory receptor expressed on B cells, T cells, monocytes, and natural killer cells (7). PD-1 is a member of the B7-CD28 family and has two ligands, programmed death ligand-1 and -2 (PD-L1 and -L2) (7). Newly, one of inhibitory B7 ligands PD-L1 (B7- H1, CD274) and PD-L2 (B7-DC, CD273) and their putative T cell counter-receptor PD-1 (CD279) have been described and a prominent role for PD-L1 and PD-L2 molecules in peripheral tolerance has been proposed (3-6, 8). PD-L1 is expressed on DCs and other APCs, activated T cells, and a variety of tissues (9), while PD-L2 is mainly expressed on DCs and other APCs (10). PD-L1 and -L2 may have distinct functions in the modulation of Th1-Th2 cell responses (8). These molecules also play different roles in the regulation of Th1-Th2 cell responses (8). While PD-L1-knocked-out mice show increased IFN-γ producing CD4+ and CD8+ T cells, suggesting that PD-L1 inhibits Th1 responses (9), PD-L2-knock-out mice indicate different results because of modulating Th2 responses (10).

Despite a large amount of information on the physiological role of B7 family negative co-stimulators, the exact role of PD-L1 and -L2 in the regulation of immune responses in intestinal mucosa has remained unclear (8, 10). Therefore, the aim of the present study is to determine the relative gene expression levels of PD-L1 and PD-L2 in patients with UC.

MATERIALS AND METHODS

Sample collection and processing. The participant of this study included patients with clinical symptoms of colitis who underwent a colonoscopy at Imam Hospital or Tooba Outpatient Clinic affiliated with Mazandaran University of Medical Sciences, Sari, Iran.

All procedures were performed between January 2016 and December 2017. None of the subjects received cyclosporine, none-steroidal anti-inflammatory drugs (NSAIDs), or any antibiotics during the past four weeks. Patients who showed signs of inflammatory bowel disease, such as edematous mucosa, submucosal bleeding, or ulcers confirmed by a pathologist, were considered as UC and those without the mentioned signs were considered as controls. Based on global colonoscopies appearance, clinical signs, and disease activity, patients with UC were further divided into three subgroups including mild, moderate, and severe UC patients (11). The biopsy specimens were collected and divided into two parts. One part was fixed and processed for routine histopathological examinations and the other part was stored for RNA extraction. This study was approved by the Ethics Committee of Mazandaran University of Medical Sciences and informed consent was obtained from all participants.

RNA isolation and cDNA synthesis. At room temperature, tissue specimens were homogenized. Total RNA was extracted from the dissected tissues using a RNA extraction kits (RNeasy Minikit, Qiagen, Germany), according to the manufacturer's instructions. The quality and quantity of extracted RNAs were assessed by agarose gel electrophoresis, and a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, the USA), respectively. RNA (1μg) was reverse-transcribed into complementary DNA (cDNA) using the RevertAidTM First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Massachusetts, USA) primed with random hexamer primer as per the manufacturer's instructions.

Quantitative Real time PCR (qRT-PCR). The sequences of PD-L1 and PD-L2 along with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a reference gene, were obtained from the GenBank (Table 1). Primers and TaqMan probes for amplification of PD-L1, PD-L2, and GAPDH were designed using the Beacon designer 7 software and synthesized by TIBmol (Germany) (Table 1). PCR amplifications were performed using TaqMan probes. Each reaction was performed in a volume of 25 μl using 2X reaction buffer (qPCR ProbesMaster with ROX, Jena Bioscience), 1 μl of each primer, 1 μl of the probe, 1.5 μl of cDNA, and 8 μl of PCR grade water (Jena Bioscience). All probes were labeled with the fluorophore FAM on the 5′ end with a black hole quencher BHQ-1 on the 3′ end. The reactions were carried out in a 96-well plate (Bio-Rad Laboratories Inc., Hercules, the USA) using an iQ5 real-time thermal cycler (Bio-Rad Laboratories Inc). The cycling conditions were as follows: 4 minutes at 95℃ for initial denaturation, then 40 repeating cycles of denaturation at 95℃ for 15 s, and annealing and extension at 57℃ for 1 minute. The reactions for each sample were performed in duplicate. An internal control (pooled cDNA of the samples), a non-template control, and a non-reverse transcribed control were applied in each run for optimization. Technique sensitivity was assayed by serial dilution of a sample of pooled cDNAs. Fluorescent data, collected during cycling, were analyzed with the FAM threshold set at 0.2 and the BHQ-1 threshold set at 0.04 on an iQ5 real-time thermal cycler (Bio-Rad Laboratories Inc.). A mean cycle of threshold (Ct) value for each duplicate measurement was calculated. Expression levels of PD-L1 and -L2 mRNA were determined by $2^{-\Delta Ct}$ value. The relative quantification system was used for data analysis based on the relative expression of PD-L1 and -L2 mRNA levels to GAPDH mRNA level as a housekeeping gene.

Table 1. Primers and probes sequences are shown for PD-L1, PD-L2, and GAPDH.

Gene	Gen Bank Accession Number	Primers and Probes (5'-3')*	Product Size (bps)
PD-L1	NM_014143	F: CCTGAGGAAAACCATACA R: ACACCAAGGCATAATAAGA P: AACTACCTCTGGCACATCCTCC	113
PD-L2	NM_025239	F: GGCAAGTCCTCATATCAA R: CTTCCAGTGTCAAAGTTG P: TCACATTGCTGCCATGCTCTATTATG	170
GAPDH	NM_001289746	F: GCTGCTTTTAACTCTGGTA R: CCATGTAGTTGAGGTCAA P: TGGATATTGTTGCCATCAATGACC	70

* F, Forward primer; R, Reverse primer; P, Probe.

Statistical Analysis. Statistical analyses were performed with GraphPad Prism 6 (San Diego, CA, USA) and SPSS 20 (North Castle, NY, the USA) software. First, the normality distribution of the obtained data was determined by the Kolmogorov-Smirnov test. Then, independent-samples t-test was used to calculate the mean difference between two groups, and more than 2 groups using ANOVA. Pearson's rank correlation analysis was applied to calculate the correlation coefficients. Findings were considered significant when P-values were <0.05. The results were presented in text and tables as mean ± standard deviation (SD).

RESULTS

Up-regulation of PD-L1 and PD-L2 mRNA in the UC patients. A total of 50 patients with UC and 45 non-UC subjects, as control groups, were enrolled in the present study (Table 2).

Table 2. Characteristics of the UC patients and control groups.

	Age (mean ± SD)	Severity of disease	Male	Female
UC patients (n=50)	34.3 ± 17.8	Mild (n=8)	4	4
		Moderate (n=29)	14	15
		Sever (n=13)	9	4
Controls (n=45)	27.5 ± 16.6		24	21

UC: Ulcerative Colitis

According to Mayo scoring system, 8 (16%) UC patients showed mild activity, 29 (58%) moderate activity, and 13 (26%) severe activity. All subjects in the control group showed normal activity (Table 2). The relative expression of PD-L1 and PD-L2 mRNA

was evaluated in all samples by a qRT-PCR method using GAPDH as an internal control. Our results demonstrated that PD-L1 and -L2 were significantly increased in UC patients compared to the control groups (p=0.02 and p=0.003, respectively, Figure 1). Indeed, PD-L1 mRNA level measured in UC patients was nearly two-fold higher than that of controls, but PD-L2 mRNA level in UC patients was nearly three-fold of its value in the controls (Figure 1).

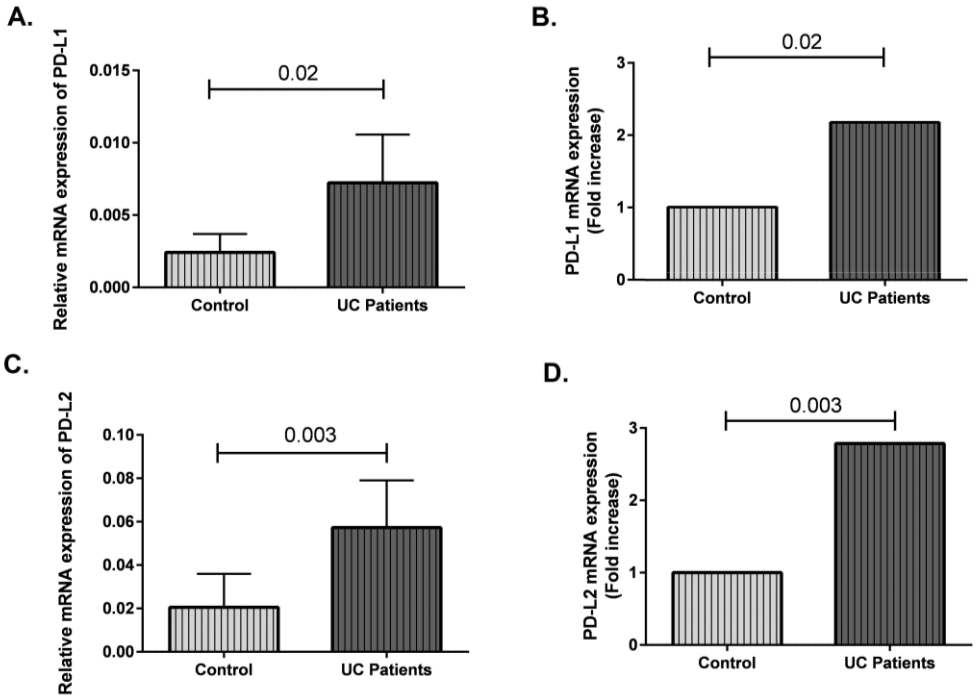

Figure 1. PD-L1 and PD-L2 mRNA expression in UC patients and controls. Total RNA was extracted from all subjects and single-strand cDNA was synthesized. Real-Time PCR was performed with specific primers for PD-L1, PD-L2, and GAPDH. (A) Relative mRNA transcript levels of PD-L1 in UC patients and controls; (B) Fold increase of PD-L1 mRNA expression in UC patients compared to controls; (C) Relative mRNA transcript levels of PD-L2 in UC patients and controls; (D) Fold increase of PD-L2 mRNA expression in UC patients compared to controls. Gene expression results were evaluated by independent-samples t-test and represented as mean ± SD of $2^{-\Delta Ct}$ after normalization with GAPDH as an internal control.

The difference between PD-L1 and PD-L2 mRNA in disease severity of UC patients. In the current study, we analyzed the correlation of PD-L1 and -L2 expression with disease severity (i.e., mild, moderate, and severe) of the UC patients. As shown in Figure 2, the relative gene expression levels of PD-L1 and -L2 showed no significant difference in the disease severity of the studied groups.

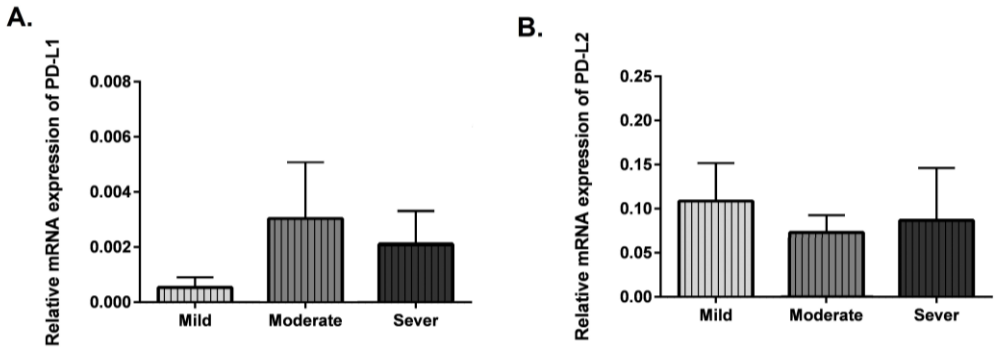

Figure 2. Comparison of the levels of PD-L1 and PD-L2 expression among patients with mild, moderate, and severe Ulcerative Colitis. Total RNA was extracted from all subjects and single-strand cDNA was synthesized. Real-Time PCR was performed with specific primers for PD-L1, PD-L2, and GAPDH. (A) Relative mRNA transcript levels of PD-L1 among the groups of the severity of UC patients; (B) Relative mRNA transcript levels of PD-L2 among the groups of the severity of UC patients. Gene expression results were evaluated by ANOVA and represented as mean ± SD of $2^{-\Delta Ct}$ after normalization with GAPDH as an internal control.

PD-L1 mRNA expression was positively correlated with PD-L2 expression in UC patients. To find any correlations between PD-L1 and PD-L2 expressions in the UC patients, we analyzed Pearson's rank correlation test. As shown in Figure. 3, PD-L1 mRNA expression showed a positive association with the PD-L2 mRNA expression, which was statistically significant (r=0.339, p<0.001).

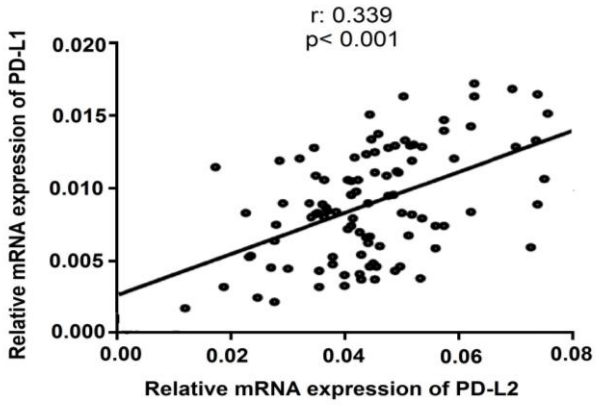

Figure 3. PD-L1 mRNA expression was significantly associated with PD-L2 (r=0.339, p<0.001). Statistical comparisons were done by Pearson's rank correlation test.

DISCUSSION

The present study was designed to investigate whether PD-L1 and -L2 gene expression are associated with UC progression in human. For this purpose, we evaluated the mRNA expression levels of PD-L1 and -L2 in patients with UC and compared them with the same measures from control group. We observed a significantly higher PD-L1 and -L2 expression in UC patients in comparison to the control group. Prolonged T-cell responses in chronic inflammations and infections could lead to up-regulation of inhibitory receptors, PD-1, CTLA-4, T cell immunoglobulin and mucin-domain-containing-3 (Tim-3), Lymphocyte activation gene-3 (LAG-3), 2B4, and CD160 (12, 13). PD-1/PD-L1,2 *axis* is responsible for inhibitory T cell signaling, mediating the mechanisms of tolerance, and providing immune homeostasis (14,15).

The interaction between the B7 family members and their receptors provides serious co-inhibition and co-stimulation that modulate T cell responses. Furthermore, the well-recognized pathway of B7-1/B7-2-CD28/CTLA-4, the interaction between PD-L1, a member of B7 family and its receptor PD-1, and a member of the CD28 family have a main role in inhibiting T cell functions and in inducing T cell exhaustion during viral infections and various tumors (13,16,17).

The role of the PD-1/PD-L1 axis in T cell-mediated intestinal inflammation and IBD remains unknown. A few studies have indicated that both PD-1 and its ligands were significantly increased in inflamed mucosa from IBD patients and experimental colitis mice. More importantly, it was shown that the blockade of PD-L1 by monoclonal antibodies (mAbs) enhanced the development of experimental colitis (18,19). These results suggested that PD-L1 and -L2 play a vital role in the development of colitis. However, another study presented that the loss of PD-1/PD-L1,2 signaling could lead to an expansion of gut antigen-specific CD8+ T cells (20) and the PD-1 inhibition in SIV-infected monkeys increases repair of gut-associated junctions (21). Our findings are consistent with a recent study reporting a significantly elevated expression of PD-1 and PD-L1 in lamina propria and peripheral mononuclear cells in patients with IBD and that anti-PD-L1 mAb, but not anti-PD-L2 mAb, prevented experimental colitis (18). However, Nakazawa *et al.* revealed an unchanged level of PD-L1 and -L2 mRNA in intestinal epithelial cells of patients with IBD (22). Nonetheless, they observed significantly higher protein expression of PD-L1 in patients with IBD especially in CD (22).

Also, in the present study, we evaluated the difference between PD-L1 and -L2 mRNA expressions in UC patients with different severity levels of UC (i.e., mild, moderate, and severe). We found no significant difference in the expressions of these molecules among three severity groups in patients with UC. The data from the animal study suggest that PD-L1 and -L2 control intestinal inflammation (16). Although these molecules did not affect tissue repair processes after an injury and insult, they led to apoptosis and necrosis of intestinal epithelial cells during gut inflammation (16,23). So, we can conclude elevated level of PD-L1 and -L2 expression in a more severe form of the disease is due to more production inflammatory cytokines that may prevent tissue and cell damage in intestinal tissue. We also evaluated the correlation between the expression of PD-L1 and -L2 in UC patients and control groups and found that PD-L1 expression was positively correlated with PD-L2 expression. As mentioned earlier, these two molecules play a role in controlling immune function mostly in peripheral tissue, but not in lymphoid tissue (23). Also, both PD-L1 and -L2 have the same receptor, i.e., PD-1, and the major difference between them is the expression pattern in tissues and immune cells (23). Therefore, according to the similarity of these two

molecules, function, and receptor, they could be indicative of the presence of different kind of cells in the intestinal mucosa, lymphoid, and non-lymphoid tissues that can express both molecules. We can conclude that the expression of PD-L1 and -L2 increases simultaneously in response to inflammatory cytokine and chemokine in mucosal tissue. PD-1 and its ligands have been recognized as the most essential targets in the development of effective immunotherapy in tumors, inflammatory, and autoimmune disease (24-26). In this context, a number of mAbs and biological inhibitors, including mAbs to PD-1 and PD-L1, as well as a PD-L2 fusion protein, have been developed. The anti-PD-L1 mAb might block the ligation of PD-L1 by PD-1 and thus prevent suppression of active T cell against tumor cells. According to the protective effect of PD-L1-Fc, Song *et al.* suggested that PD-1-mediated inhibitory signals have a crucial role in limiting the expansion of colonic inflammation. This result indicates that PD-L1-Fc may provide a novel therapeutic method to treat IBD (27). These facts suggest new approaches for the treatment of chronic inflammation of colonic mucosa in the future.

In conclusion, we noticed that chronic inflammation of colonic mucosa may lead to up-regulation of PD-L1 and -L2 expression in patients with UC, which may result in regulation of immune responses against this chronic inflammation and then prevent progressive and acute inflammation of the disease.

Also, the expression of PD-L1 was positively correlated with the expression of PD-L2 in UC patients. Therefore, these immunosuppressive molecules may be a novel target for clinical interventions in patients with UC. However, further studies on specific functions of these molecules are warranted to better understand their specific roles in the maintenance of intestinal peripheral tolerance.

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