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CORRESPONDENCE

Dysregulation of the PD-1/PD-L1 pathway contributes to the pathogenesis of celiac disease

Candelaria Ponce de León[1], Miguel Angel López-Casado[2], Pedro Lorite[1], Teresa Palomeque[1] and María Isabel Torres[1]

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In this study, we focus on the alteration of the programmed cell death 1 (PD-1)/PD-L1 pathway in celiac disease. We discuss the diverse roles of the PD-1 pathway in regulating immune responses and how this knowledge can improve celiac disease (CD) autoimmunity. The PD-1 and PD-L1 levels in the serum and in intestinal biopsies of CD patients may be relevant to the determination of a possible correlation between markers of the autoimmune response, inflammation, and disease activity. In previous work, we assessed the function of the IL-33/ST2 axis in the pathogenesis of CD.[1] These data point to a possible important interregulation of IFN-γ and IL-33 in CD. IFN-γ can induce IL-33 expression and may contribute to IL-33 release via its proapoptotic effect in the intestinal mucosa.[1] When IL-33 is released from cells, it can act on T cells to produce IFN-γ and can establish the ideal conditions for blocking the immune response through the PDL/PD-1 axis. Therefore, CD4+T cells lacking PD-1 have a significantly reduced likelihood of becoming regulatory cells.

CD is a chronic inflammatory disorder with autoimmune disease features that results from a loss of gluten tolerance.[2,3] Intestinal damage is caused by CD4+T cells, which recognize deamidated gluten peptides presented in complex with HLA-DQ2.5, HLA-DQ2.2, and/or HLA-DQ8.[4,5] Upon activation, these cells secrete high levels of IFN-γ, support B cell-mediated production of antibodies to tTG, modify gluten peptides, and enhance the lysis of stressed epithelial cells by CD8+T cells.[5,6] The active state of CD has been associated with impairments in regulatory T-cell (Treg) function.[7] Although the effector T-cell response in CD patients is well characterized, the role of Treg cells in the loss of tolerance to gluten remains poorly understood. These findings emphasize the hypothesis that innate defects in the Treg cell population eventually trigger inefficient immune regulation.

PD-1 is a member of the CD28 family, which modulates T-cell function and is primarily upregulated on the surface of CD4 and CD8 T cells upon activation.[8] PD-1 interacts with its ligands PD-L1 or PD-L2, and this engagement induces tyrosine phosphorylation of the cytoplasmic domain of PD-1.[9] Although PD-1 expression by antigen-specific CD8 T cells is associated with an exhausted phenotype, PD-1 is an activation marker of CD4 and CD8 T cells, similar to CTLA-4, and may be upregulated to potentially prime negative regulatory feedback mechanisms to limit inflammation.[10,11] PD-1 is induced by antigen-specific and nonspecific stimulation of T cells. PD-1 is expressed on all conventional CD4+T cells and CD8+T cells during acute T-cell activation and by some subsets of memory T cells and tolerant T cells.[11] Considering the diverse roles of PD-1 in these cell types, it is relevant to elucidate the contribution of PD-1 to CD.

A total of 24 patients with features typical of active CD and nine control patients in whom CD was ruled out were included in this study. The diagnosis of CD was established by means of serologic screening tests using tissue transglutaminase antibodies, and CD-specific HLA typing accompanied by biopsy of the small intestine following gastrointestinal endoscopy. This study was performed after approval of the Ethics Committee.

Formalin-fixed paraffin-embedded biopsies from patients with active CD and control subjects were analyzed by immunohistochemistry. Five-micrometer-thick sections were cut, deparaffinized, and rehydrated. Sections were microwaved in 10 mM citrate buffer (pH 6.0) for antigen retrieval. We used anti-human PD-1 antibody and anti-human PD-L1 antibody (Abcam, Cambridge, UK). Immunohistochemical staining was performed using the UltraTech HRP Streptavidin–Biotin Universal Detection System (Immunotech, France). Negative control experiments were performed by incubating sections with isotype-matched IgG1. Peripheral blood mononuclear cells of patients with CD were isolated from fresh blood using a standard Ficol-Hypaque gradient (Sigma-Aldrich, Spain). Briefly, 150 µl PBMC aliquots and 1 million isolated cells were incubated with a predetermined optimal concentration of the following antibodies: CD57–VioBlue, CD279 (PD-1)–APC, CD4–VioBright-FITC, and CD8–APC–Vio770, all from Miltenyi Biotec GmbH.

We assessed the levels of soluble PD-1 and PD-L1 in the serum of active celiac and nonceliac patients using commercial ELISA kits in accordance with the manufacturer's instructions (Sigma-Aldrich, Madrid, Spain and Invitrogen, Thermo Fisher Scientific, Madrid, Spain, respectively). The sensitivity of the assay was <2 pg/mL. The concentrations of soluble PD-1 (sPD-1) and sPD-L1 were determined from the value of the optical density according to the standard curves.

The expression of PD-1 and PD-L1 antigens were analyzed in the biopsies of active celiac patients. Our results provide the first evidence of high PD-L1 expression levels in celiac patients. Indeed, all tested patients showed positive expression of PD-L1 with different grades of immunoreaction, as demonstrated by immunohistochemistry. PD-L1 is highly expressed on the surface of intestinal epithelial cells and lamina propria cells of patients with active CD (Fig. 1b-d), suggesting an important role for the PD-L1: PD-1 pathway in regulating mucosal tolerance in situ. Interestingly, our results demonstrated negative expression of PD-1 in intestinal samples of patients with active CD (Fig. 1a). Without PD-1, excessive immune-mediated tissue damage can lead to devastating consequences. PD-1 plays crucial roles in central and

[1]Department of Experimental Biology, University of Jaén, Jaén, Spain and [2]Department of Pediatrics Gastroenterology, Hospital Virgen de las Nieves, Granada, Spain
Correspondence: María Isabel Torres (mitorres@ujaen.es)

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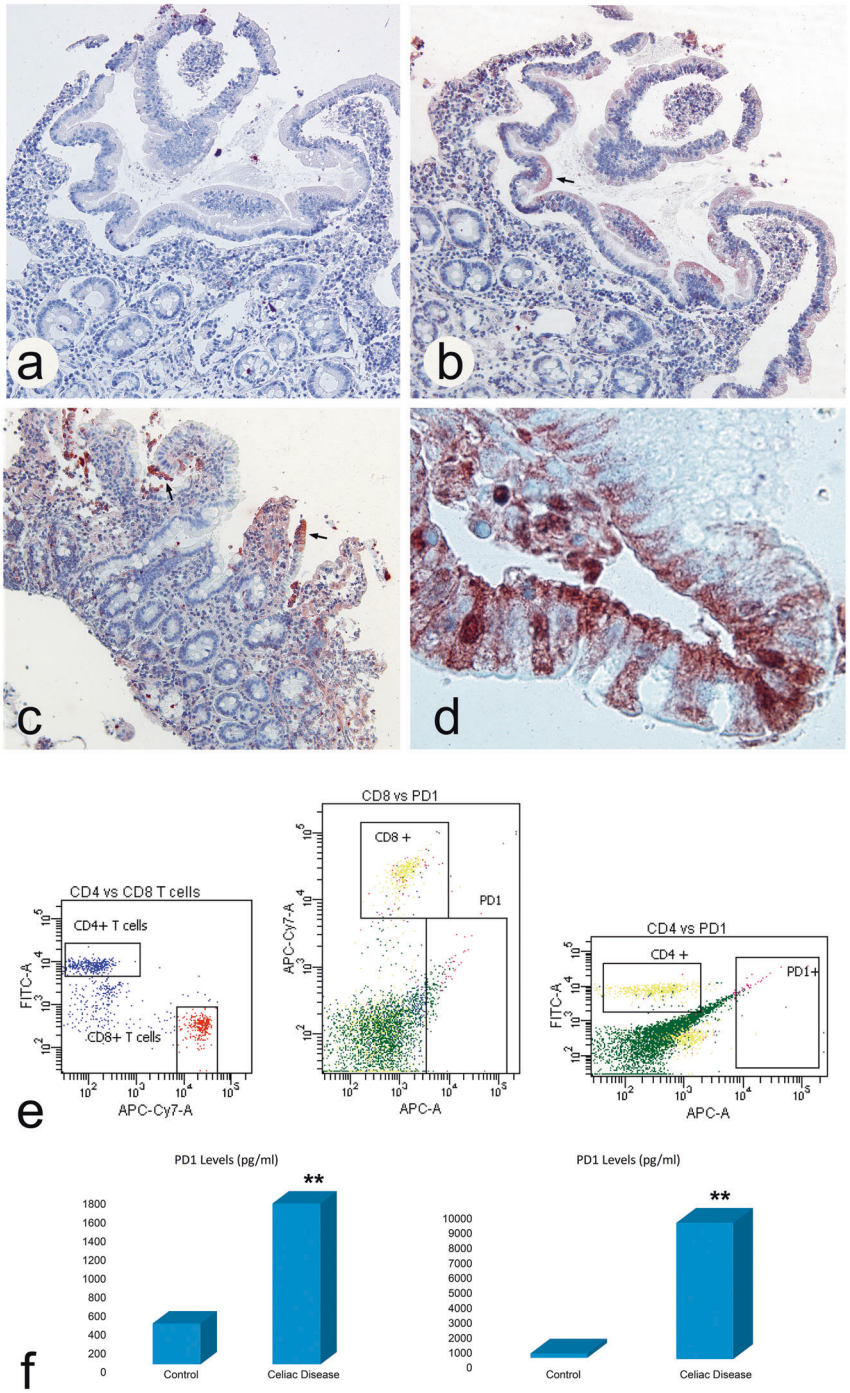

Fig. 1 PD-1/PD-L1 expression: **a**) immunohistochemical labeling of PD-1 in biopsy samples of celiac patients presenting negative immunoreactions. **b**–**d**) Immunohistochemical labeling of PD-L1 in biopsy samples of celiac patients showing expression in epithelial and lamina propria cells. Original magnification: **a**–**c** ×200. **d** ×1000. Arrows: cells with immunoreaction. **e**) Flow cytometry analysis of PD-1 expression in CD4+T cells and CD8+T cells in celiac patients. **f**) PD-1/PD-L1 expression in the serum of celiac disease patients and healthy controls. PD-1 and PD-L1 are highly expressed in serum from celiac disease patients ($n = 24$) in comparison with that from healthy controls ($n = 9$). Significantly different at $^*p < 0.05$ are shown

peripheral T-cell tolerance, aiding in the protection of self-tissues from autoimmune responses.

Using flow cytometric analysis, we assessed the frequencies of PD-1-positive T cells in patients with CD. We observed a negative result by flow cytometry in the frequencies of CD8+/PD-1+T cells. The frequencies of CD4+/PD-1+T cells in the CD group were very low (Fig. 1e). In view of the above results, we determined the levels of serum sPD-1 and sPD-L1 by ELISA in patients with active CD. We found that the levels of sPD-1 and sPD-L1 were considerably higher in the serum of patients with CD compared in that of the healthy controls (Fig. 1f). In addition to the membrane-bound form, PD-1 has a soluble form, sPD-1. sPD-1 is encoded by the alternative splice variant PD-1Deltaex3, which has a soluble extracellular domain but lacks the transmembrane domain of the PD-1 molecule.[12] sPD-1 may functionally block the regulatory effect of

membrane-bound PD-1 on T cells and can lead to alterations in T-cell proliferation and regulation.[12]

In patients with CD, excessive sPD-1 could serve as an "antibody" to block the PD-1/PD-Ls pathway and lead to aberrant T-cell proliferation. PD-1 is expressed by all T cells during activation and acts as a natural brake to temper the overactivation of T-cell responses. Without PD-1, excessive immune-mediated tissue damage can lead to devastating consequences.[11] In addition, PD-1 plays crucial roles in central and peripheral T-cell tolerance, aiding in the protection of self-tissues from autoimmune responses. It is important to consider that the crucial function of the PD-1 pathway is to limit immunopathological responses in host tissues by promoting the resolution of inflammation and restoration of immune homeostasis. If, for example, CD8+T-cell responses are not adequately controlled, severe immunopathology can result from the production of proinflammatory cytokines, such as IFN-γ and TNF-α.[11] These cytokines are potent stimulators of PD-L1 expression in T cells, B cells, endothelial cells, and epithelial cells. Reports show that sPD-1 can promote T-cell responses through blocking the PD-1/PD-Ls pathway. IFN-γ is crucial for this process but also contributes to the upregulation of PD-L1,[13] which indicates that sPD-1 plays a crucial role not only during the phase of T-cell exhaustion but also during primary T-cell activation and that sPD-1 can be used as an adjuvant to increase T-cell immunity[11]

These findings suggest that at the time of clinical diagnosis of CD, T cells can exhibit features of immune exhaustion. Our observation in CD patients with downregulated PD-1 expression in T cells is intriguing. It is not yet known what factor(s) contribute to the dysregulated PD-1 expression and may have increased susceptibility to the autoimmune complications of CD. Thus, PD-1 expression may be under epigenetic regulation, but further studies are needed to investigate how epigenetic modifications control PD-1 expression. Understanding how or why PD-1 is not upregulated in T cells from children with CD may help in the development of new approaches in specific T cells in the context of CD treatment. These possibilities should be investigated with an increased sample size and follow-up period.

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ADDITIONAL INFORMATION

Competing interests: The authors declare no conflict of interest.

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