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Decrease in CD226 expression on $CD4^+$ T cells in patients with endometriosis

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SUMMARY Endometriosis is a chronic inflammatory disease. The immune-checkpoint molecules CD226 and TIGIT play an important role in regulating T cells' function. However, little is known about the proportion and function of CD226 and TIGIT on $CD4^+$ T cells in endometriosis. The current study found no significant differences in the TIGIT percentage on peripheral $CD4^+$ T cells between patients with endometriosis and the control group. However, CD226 was lower in patients with endometriosis than that in the control group ($P < 0.01$). The cytokines TNF-α, IL10, and IFN-γ were significantly elevated in $TIGIT^+$ $CD4^+$ T cells compared to TIGIT- $CD4^+$ T cells. $HLA\text{-}DR^+$ cells were more numerous among $TIGIT^+$ $CD4^+$ T cells than among the TIGIT- subset ($P <0.001$). Similarly, the cytokines TNF-α, IL10, and IFN-γ were significantly elevated in $CD226^+$ $CD4^+$ T cells compared to levels in CD226- $CD4^+$ T cells. The proportion of $HLA\text{-}DR^+$ $CD4^+$ T cells among $CD226^+$ $CD4^+$ T cells was also significantly higher than that among the CD226- subset ($P < 0.001$). After TIGIT was blocked, the level of IL-10 in $TIGIT^+$ $CD4^+$ T cells was higher than that in cells with unblocked TIGIT. There were no differences in TNF-α and IFN-γ. After CD226 was blocked, TNF-α and IFN-γ were lower while IL-10 was higher. In conclusion, there is a diminution of CD226 in $CD4^+$ T cells in patients with endometriosis. This is correlated with the effector function of $CD4^+$ T cells, and blocking CD226 can suppress this function.

Keywords TIGIT, CD226, endometriosis, $CD4^+$ T cells

Endometriosis is a chronic inflammatory disease in which proinflammatory factors and chemokines in the local immune microenvironment are involved in normal and pathologic immune regulation (*1*), and this abnormal immune status is also reflected in peripheral blood (*2*). The T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) is an immunosuppressive molecule discovered in 2005 (*3*) that is associated with T-cell activation and suppression (*4*). This receptor molecule mediates inhibitory signals by interacting with the ligands CD155 and CD112. TIGIT has been proven to exist mainly on activated T cells (*5*) and to play a role in suppressing immune effects in tumors, immune-related physiology, or in various diseases (*6*). CD226 (DNAM-1) is a co-stimulatory molecule that interacts with CD155 to activate TCR, that promotes Th1-related signal transduction, that enhances the effector function of NK cells, and that promotes $CD4^+$T cells to produce the proinflammatory cytokine interferon γ (IFN-γ) (*7*).

Although abnormal expression of TIGIT/CD226 in some immune-related diseases has been reported, there are few existing reports concerning both molecules regarding their expression and possible role(s) in endometriosis. Thus, the current study evaluated the molecular expression, state of activation, and cytokine production of the two immune-checkpoint molecules on $CD4^+$ T cells in endometriosis in order to provide data to elucidate the development and progression of endometriosis.

Subjects were patients and female controls who met the following inclusion criteria and exclusion criteria. Inclusion criteria: The patients were diagnosed by a gynecologist, through clinical manifestations, ultrasound

or some other imaging exam, laparoscopy, or the like. To reduce the influence of age, subjects between the ages of 20 and 45 were selected. Exclusion criteria: Patients with diseases that might cause serious changes in the immune system were excluded. Patients with chromosomal diseases were excluded. Patients with unavailable data were also excluded. The control group: Healthy women seen at this hospital for a pre-pregnancy checkup were included as healthy controls; these women were age-matched to patients, had no chromosomal or immune system-related diseases, and consented to participate in this study.

Venous blood was anticoagulated with EDTA, and peripheral blood mononuclear cells were extracted using Ficoll-Paque lymphocyte separation medium (Huajing, Shanghai, China) and frozen in a −80°C freezer. When a particular number of patients was selected, the frozen cells were rewarmed and suspended in RPMI 1640 for subsequent use.

Antibodies were purchased from BD BioLegend (San Diego, California, USA) and included CD3 APC-CY7, CD4 BV510, CD25 PE, TIGIT FITC, CD226-BV785, HLA-DR APC, TNF-a PE-CY7, IFN-γ-PE-Cy5.5, and IL10 BV421. Intracellular cytokines were stimulated before testing. Leukocyte Activation Cocktail (BD Biosciences, Franklin Lakes, NJ, USA) was used to stimulate them in accordance with the manufacturer's instructions. Then, cytokine antibodies TNF-α PE-CY7, IFN-γ-PE-Cy5.5, and IL10 BV421 were added. Before stimulation, the cells were blocked or not blocked with anti-TIGIT or anti-CD226 antibodies.

The software GraphPad Prism Version 5.0 (GraphPad, San Diego, CA, USA) and SPSS 19 were used for statistical analysis and graphs. Variables with a normal distribution were expressed as the mean±standard deviation (X ± SD), and variables with an abnormal distribution were expressed as a median and range. Differences between the 2 groups were analyzed using a *t*-test or chi-square test. The Mann–Whitney *U* test was used for abnormally distributed variables.

As shown in Table 1, there were 23 patients with endometriosis in the endometriosis group and 24 healthy women in the control group. Their clinical and demographic data are shown in Table 1. To compare TIGIT/CD226 markers between the two groups and to identify clinical associations with CD226 in patients with endometriosis, the expression of TIGIT and CD226 was first determined without any stimulation or activation. There were no significant differences in the TIGIT percentage on $CD4^+$ T cells between the 2 groups. However, CD226 was significantly lower in patients with endometriosis compared to that in the control group ($P < 0.01$, Figure 1A, 1B). When the CD226 proportion on

Table 1. Clinical data on subjects

Parameters	Control group ($n = 24$)	Patients with endometriosis ($n = 23$)
Age (years)	36 ± 4.47	35 ± 5.04
CA125 (U/mL)	27.92 (4.89 - 63.22)	27.43 (3.64 - 73.19)
HE4 (pmol/L)	39.11 (15.01 - 137.00)	54.83 (22.02 - 116.75)
CRP (mg/L)	3.40 (0.8 - 8.72)	1.84 (0.8 - 13.64)
SAA (mg/L)	6.34 (2.65 - 22.75)	7.00 (2.45 - 23.76)
WBCs ($\times 10^9$/L)	6.51 ± 1.56	6.20 ± 1.95
Neutrophils ($\times 10^9$/L)	3.36 (2.22 - 5.93)	4.00 (1.81 - 9.94)
Lymphocytes ($\times 10^9$/L)	2.35 (1.11 - 3.49)	1.42 (0.62 - 2.76) *
PLTs ($\times 10^9$/L)	245.13 ± 46.24	233.48 ± 66.44
PDW (fL)	11.97 ± 1.45	12.53 ± 1.79
Endometrial antibody	6/24	6/23
NLR	1.67 (0.70 - 3.44)	2.52 (1.10 - 16.03) *
D dimer (mg/L)	0.28 (0.03 - 1.02)	0.33 (0.0 - 1.14)

Data are exhibited as means ± SD for continuous variables, or medians and ranges for non-normally distributed data. * represents $P < 0.05$.

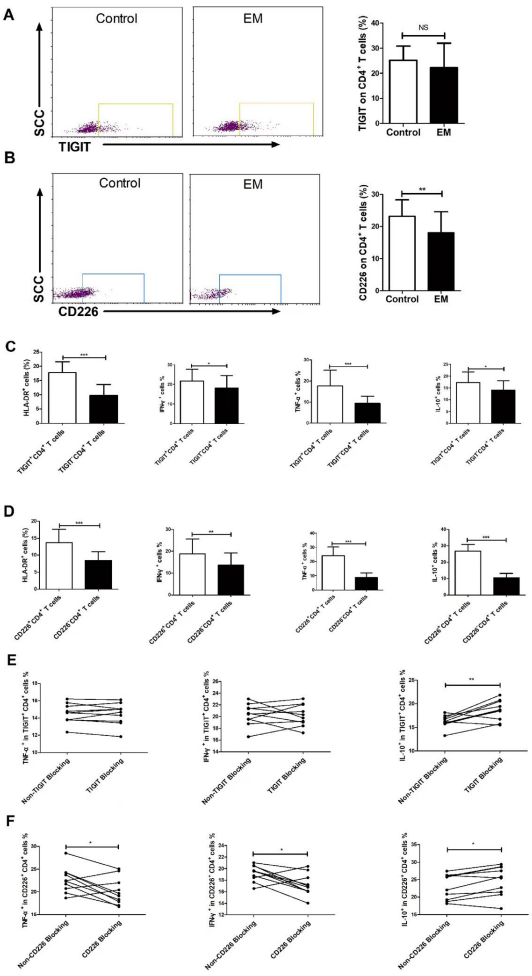

Figure 1. Characteristics of CD226 and TIGIT expressed on $CD4^+$ T cells in patients with endometriosis and healthy controls. (A, B) Samples were examined using flow cytometry, and CD4-positive lymphocytes were gated for CD226 and TIGIT on the T cell surface. Data are expressed as the mean±SD, and a t test was used to compare patients with endometriosis (n = 23) and the control group (n = 24). **(C)** HLA-DR^+ $CD4^+$ T cells were more prevalent among $TIGIT^+$ $CD4^+$ T cells than the TIGIT- subset. TNF-α, IFN-γ, and IL10 were significantly higher in $TIGIT^+$ $CD4^+$ T cells than in the TIGIT- subset. **(D)** HLA-DR^+ $CD4^+$ T cells were more numerous among $CD226^+$ $CD4^+$ T than the CD226- subset. **(E)** After TIGIT was blocked, there were no differences in TNF-α and IFN-γ in cells compared to cells with treated TIGIT and the IL-10 level was higher in $TIGIT^+$ $CD4^+$ T cells. **(F)** After CD226 was blocked, the levels of TNF-α, IFN-γ, and IL-10 were higher in $TIGIT^+$ $CD4^+$ T cells. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

$CD4^+$ T cells and the clinical data were analyzed, results revealed that the percentage of cells expressing CD226 was correlated with NLR ($r = 0.536$, $P = 0.008$). To evaluate the activation status and cytokine production of $CD4^+$ T cells in endometriosis, their characteristics were analyzed using flow cytometry. After activation for 4 hours, the cytokines TNF-α, IFN-γ, and IL10 were significantly elevated in $TIGIT^+$ $CD4^+$ T cells compared to the TIGIT- subset. $HLA\text{-}DR^+$ $CD4^+$ T cells were more numerous among $TIGIT^+$ $CD4^+$ T cells than among the TIGIT- subset ($P < 0.001$). Similarly, the cytokines TNF-α, IL10, and IFN-γ were significantly elevated in $CD226^+$ $CD4^+$ T cells compared to levels in the CD226- subset. The proportion of $HLA\text{-}DR^+$ $CD4^+$ T cells among $CD226^+$ T cells was also significantly higher than that among the CD226- subset ($P < 0.001$, Figures 1C, 1D).

The CD226 antibody can inhibit CD226 function in some diseases (*8,9*), so the current study sought to determine whether CD226 or TIGIT antibodies influenced their functioning in endometriosis. After CD226 or TIGIT was, cytokines were compared to those in cells with unblocked CD226 or TIGIT. There were no differences in TNF-α and IFN-γ after TIGIT was blocked, but the level of IL-10 was higher in $TIGIT^+$ $CD4^+$ T cells. After CD226 was blocked, TNF-α and IFN-γ were lower while IL-10 was higher (Figures 1E, 1F).

About 10% of women of childbearing age develop endometriosis; the condition especially affects infertile women, and women with chronic pelvic pain often exhibit endometriosis (*10*). The onset of endometriosis is related to sex hormones, immunity, inflammation, heredity, and other factors; and further research is required to elucidate its underlying pathogenesis. One of the most accepted views on the etiology of endometriosis is that abnormal immunity generates ectopic endometrial cells that invade unspecified locations and that are unable to be cleared. Immune-checkpoint molecules appear to regulate immunity in many diseases (*11,12*). For example, PD-1 regulates the actions of lymphocytes in endometriosis (*13*). There are no reports in the literature that mention TIGIT and CD226 in endometriosis, and the current study is thus the first to reveal abnormal expression of CD226 on peripheral $CD4^+$ T cells. CD226 decreased in the current patients with endometriosis. This decrease may preclude $CD4^+$ T cells from secreting enough cytokines to allow clearance of ectopic endometrial cells. Although TIGIT might produce an over-activated immune response (*14*), the current study found no difference in TIGIT between patients with endometriosis and the control group. The mildly negatively regulated molecule TIGIT is presumably replaced with PD-1 or another strongly negative immune-checkpoint molecule.

Results revealed that activated T cells expressed more TIGIT and CD226, a finding similar to that in several other studies (*9,15*). This may indicate that TIGIT and CD226 remain at a consistent level during health. When endometrial cells invade ectopic sites such as the peritoneum or pelvic cavity, however, immune cells are then mobilized, and the immune-checkpoint molecules are activated.

Since the CD226 antibody inhibits CD226 function in some diseases, the current study sought to reveal whether CD226 or TIGIT influenced the functioning of CD226 and TIGIT in endometriosis. Thus, cytokine levels were examined when TIGIT or CD226 was blocked. There were no differences in TNF-α or IFN-γ levels in cells with blocked TIGIT, but after TIGIT was blocked the level of IL-10 was elevated in $TIGIT^+$ $CD4^+$ T cells. Thus, TIGIT did not inhibit $CD4^+$ T cell function by reducing production of the cytokines TNF-α and IFN-γ. When CD226 was blocked before activating $CD4^+$ T cells, TNF-α and IFN-γ decreased while IL-10 increased. CD226 presumably plays a positive role in the effector function of $CD4^+$ T cells (*8*) since levels of the proinflammatory cytokines IFN-γ and TNF-α were consistent with levels of CD226, while levels of the anti-inflammatory cytokine IL-10 were negatively correlated. CD226 decreased significantly in patients with endometriosis, and this decrease may have reduced the secretion of enough TNF-α and IFN-γ effectors by $CD4^+$ T cells to clear endometrial cells in the ectopic environment.

In summary, the current results indicated that there was a diminution of CD226 in $CD4^+$ T cells in patients with endometriosis. CD226 was correlated with the effector role of $CD4^+$ T cells in patients with endometriosis, and blocking CD226 suppressed this function. These results suggest that CD226 in $CD4^+$ T cells has a potential role in diagnosing endometriosis.

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References

1. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. Fertil Steril. 2012; 98:511-519.
2. Delbandi AA, Mahmoudi M, Shervin A, Moradi Z, Arablou T, Zarnani AH. Higher frequency of circulating, but not tissue regulatory T cells in patients with endometriosis. J Reprod Immunol. 2020; 139:103119.
3. Abbas AR, Baldwin D, Ma Y, Ouyang W, Gurney A, Martin F, Fong S, van Lookeren CM, Godowski P, Williams PM, Chan AC, Clark HF. Immune response in silico (IRIS): Immune-specific genes identified from a compendium of microarray expression data. Genes Immun. 2005; 6:319-331.
4. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol. 2009; 10:48-57.
5. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, Sharpe AH, Kuchroo VK. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol. 2011; 186:1338-1342.
6. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory receptors with specialized functions in immune regulation. Immunity. 2016; 44:989-1004.
7. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. J Immunol. 2012; 188:3869-3875.
8. Li W, Deng C, Yang H, Lu X, Li S, Liu X, Chen F, Chen L, Shu X, Zhang L, Liu Q, Wang G, Peng Q. Expansion of circulating peripheral $TIGIT^{+}CD226^{+}$ CD4 T cells with enhanced effector functions in dermatomyositis. Arthritis Res Ther. 2021; 23:15.
9. Deng C, Li W, Fei Y, Wang L, Chen Y, Zeng X, Zhang F, Li Y. Imbalance of the CD226/TIGIT immune checkpoint is involved in the pathogenesis of primary biliary cholangitis. Front Immunol. 2020; 11:1619.
10. Endometriosis Collaboration Group, Chinese Society of Obstetrics and Gynecology, Chinese Medical Association. Guidelines on the diagnosis and treatment of endometriosis (Third edition). Zhonghua Fu Chan Ke Za Zhi. 2021; 56:812-824. (in Chinese)
11. Tang H, Cao Y, Jian Y, Li X, Li J, Zhang W, Wan T, Liu Z, Tang W, Lu S. Conversion therapy with an immune checkpoint inhibitor and an antiangiogenic drug for advanced hepatocellular carcinoma: A review. Biosci Trends. 2022; 16:130-141.
12. Li C, Ying C, Duan Z. The imbalance of T-cell immunoglobulin and ITIM domain and CD226 on regulatory T cell in recurrent spontaneous abortion patients. Reprod Dvlpmt Med. 2022; 6:175-180.
13. Walankiewicz M, Grywalska E, Polak G, Korona-Glowniak I, Witt E, Surdacka A, Kotarski J, Rolinski J. The increase of circulating PD-1- and PD-L1-expressing lymphocytes in endometriosis: Correlation with clinical and laboratory parameters. Mediators Inflamm. 2018; 2018:7041342.
14. Ge Z, Peppelenbosch MP, Sprengers D, Kwekkeboom J. TIGIT, the next step towards successful combination immune checkpoint therapy in cancer. Front Immunol. 2021; 12:699895.
15. Deng C, Chen Y, Li W, Peng L, Luo X, Peng Y, Zhao L, Wu Q, Zhang W, Zhang X, Fei Y. Alteration of CD226/TIGIT immune checkpoint on T cells in the pathogenesis of primary Sjogren's syndrome. J Autoimmun. 2020; 113:102485.

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