

# Aberrant Expression of Fgl-1 and Lag-3 in Adenomyosis

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## Research Article

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# Abstract

**Background:** The presence of ectopic functional endometrial glands and stroma in the myometrium of the uterine cavity is considered as adenomyosis. Various inflammatory, vascular and mechanical factors are involved in the symptoms and evolution of this pathology. Lymphocyte-activation gene 3 (Lag-3) is an immune inhibitory receptor and fibrinogen-like protein 1 (Fgl-1) is a major functional ligand of Lag-3. The binding of Lag-3 and Fgl-1 leads to inhibition of T-cell immunity, which is an important target of immunotherapy. The objective of this study was to evaluate the expression of Lag-3 and Fgl-1 in normal endometrium and adenomyosis.

**Methods:** The expression of the Lag-3 and Fgl-1 in normal endometrium (proliferative phase: n=15; secretory phase: n=15) and adenomyotic endometrium (proliferative phase: n=15; secretory phase: n=15) were determined using immunohistochemistry and immunofluorescence analysis.

**Results:** In normal and adenomyotic endometrium, no significant difference of Fgl-1 expression was noted between proliferative and secretory phases. Compared with normal endometrium, eutopic and ectopic endometrium of adenomyosis showed increased expression of Fgl-1. Lag-3 was almost negative in endometrial glands of normal and adenomyosis. Compared with normal endometrium, Lag-3 positive T-lymphocytes were more common in the stroma of adenomyosis.

**Conclusions:** Our data suggest that aberrant expression of Lag-3 and Fgl-1 is present in the eutopic and ectopic endometrium of adenomyosis. We conclude that Lag-3/Fgl-1 signaling may be involved in the pathogenesis and development of adenomyosis.

## Background

Adenomyosis is a prevalent gynaecologic benign condition characterized by the presence of heterotopic endometrial glands and stroma in the myometrium [1]. Increased dysmenorrhea and menorrhagia are typical symptoms of adenomyosis, while one third of patients are asymptomatic. Patients usually complain of chronic pelvic pain, which lower the quality of life [2]. However, the etiology and pathogenesis of the disease are still unclear.

In recent years, the immunological mechanism of adenomyosis has received more and more clinical attention. A recent study reported that the periglandular areas of the adenomyosis regions are rich in immune cells including macrophages, mast cells and T-lymphocytes [1]. In addition, human leukocyte antigen (HLA) and several cytokines (IL-37, IL-6, IL-8, IL-10, nerve growth factor etc.) have been suggested to be involved in the development of adenomyosis [2-6].

Lymphocyte-activation gene 3 (Lag-3, also known as CD223) was reported by Triebel et al. in 1990 as a transmembrane protein [7]. Lag-3 is expressed on activated T cells, natural killer cells, regulatory T cells (Tregs), Tr1 cells, exhausted T cells, B cells, and dendritic cells [8]. Lag-3 gene is a CD4 homologue which exists on chromosome 12 (12p13) of human and chromosome 6 of mouse. Lag-3 and CD4 have

adjacent chromosomal localization and similar intron/exon structures, but only about 20% of the amino acid sequences in the two molecules are identical [9, 10]. In the past few years, Lag-3 has received extensive attention as an inhibitory receptor. Lag-3 negatively regulates the proliferation, activation, effector function, and homeostasis of both CD8+ and CD4+ T cells [11]. In addition, Lag-3 can cooperate with other inhibitory molecules in tumor tissues to escape tumor immunity, such as PD-1, CTLA-4, TIM-3, etc. [12-16]. Studies have already shown that the aberrant expressions of these inhibitory molecules are essential in the development of various cancers [16, 17]. Therefore, the blocking of Lag-3 and other inhibitory molecules has been a research hotspot of current cancer immunotherapeutic developments, especially Lag-3 and PD-1 [18].

In recent studies, fibrinogen-like protein 1 (Fgl-1) has been identified as the major ligand for Lag-3 which is an immunosuppressive receptor [11]. Fgl-1 belongs to the fibrinogen family and can be secreted by liver cells at low levels under normal physiological conditions [19]. Fgl-1 can interact with the D1/D2 domain of Lag-3, thereby inducing the suppressive function and immune tolerance of T cells. Blocking the Fgl-1/Lag-3 interaction can significantly stimulate the T cell expansion and activation, enhance the anti-tumor T cell response [11].

However, the role of Lag-3 and Fgl-1 in adenomyosis remains unclear. In the present study, we aimed to determine the expression of Lag-3 and Fgl-1 in normal endometrium and adenomyosis.

## Methods

### Materials and tissue collection

In the present study, tissue samples of adenomyosis and their corresponding endometrium (proliferative phase: n=15; secretory phase: n= 15) from 30 patients with histological evidence of adenomyosis were employed. The phase of the cycle was determined according to the last menstruation, the ovarian findings during operation and the histologic phase pattern of the endometrium. Normal endometrium (proliferative phase: n=15; secretory phase: n= 15) were obtained from 30 women of reproductive age. Prior to surgery, all patients were experiencing regular menstrual cycles and none of them received any hormone therapy for at least 3 months. The present study was approved by the Institutional Review Board of Shandong Provincial Hospital Affiliated to Shandong First Medical University and written informed consent was obtained from each participant.

### Immunohistochemistry

Normal endometrium, eutopic and ectopic endometrium of adenomyosis were used in the immunohistochemistry analysis. The fresh tissues were washed with phosphate-buffered saline (PBS) to remove blood. The 4% paraformaldehyde-fixed specimens were embedded in paraffin, cut into 4  $\mu$ m sections and mounted onto glass slides. After deparaffinized and rehydrated, the sections were introduced into ethylenediaminetetraacetic acid solution (EDTA) buffer at pH 9.0 for antigen retrieval. The sections were incubated with 3 % H<sub>2</sub>O<sub>2</sub> for 30 min to block endogenous peroxidase activity. After rinsed in

PBS, the sections were blocked with 10% normal goat serum for 30 min, and then incubated with the primary antibodies, rabbit anti-human Fgl-1 antibody (Abcam, Cambridge, MA), mouse anti-human Lag-3 antibody (Abcam, Cambridge, MA), overnight in a wet chamber at 4°C. An HRP-conjugated goat anti-rabbit or mouse IgG was used as second antibody. HRP activity was detected by measuring the level of the substrate diaminobenzidine tetrahydrochloride (DAB). Finally, sections were counterstained with hematoxylin, then dehydrated and cover-slipped. The sections incubated with non-immune serum instead of primary antibody were used as a negative control. All sections were observed using a Leica DM4000B microscope (Leica), and pictures were acquired using the IM50 image analysis system (Leica).

The immunostaining was expressed as immunoscore, which was semiquantitative as a product of a quantity score and a staining intensity. The quantity score was estimated as follows: no staining-0; 1–10% positively staining cells-1; 11–50% positively staining cells-2; 51–80% positively staining cells-3; and 81–100% positively staining cells-4. The staining intensity of the glandular epithelial cells was estimated as follows: 0–negative; 1–weak staining; 2–moderate staining; and 3–strong staining. Two sections per sample were assessed by two observers. All slides were evaluated blind for immunostaining without any knowledge of the clinical or pathological data.

## **Immunofluorescence**

As previously described, the tissues were fixed and embedded. Sections were cut and mounted, then deparaffinized and rehydrated. The sections were subjected to antigen retrieval by a pressure-cooker for 130 seconds in EDTA buffer at pH 9.0. Following being soaked by PBS buffer for three times (5 min each time), the sections were permeated with 0.5% Triton X-100 at room temperature for 20 min. The sections were rinsed in PBS, blocked with 10% normal goat serum at 37°C for 60 min. Following removing the blocking solution, the diluted primary antibodies were added and incubated overnight at 4°C. The primary antibodies included rabbit anti-human Lag-3 (diluted 1:100 in PBS), mouse anti-human Fgl-1 (diluted 1:100 in PBS), CD4 (diluted 1:100 in PBS), CD8 (diluted 1:100 in PBS) and Foxp3 (diluted 1:100 in PBS) antibodies. After the sections were rinsed with PBS, the sections were incubated with the Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (diluted 1:500 in PBS) and Alexa Fluor 647-conjugated goat anti-mouse secondary antibody (diluted 1:500 in PBS) at 37°C for 60 min. After rinsing with PBS, DAPI was added to stain the nucleus for 5 min in the dark. And then rinsed with PBS again and mounted with containing anti-fluorescence quencher. Finally, the image was captured with a confocal laser scanning microscope.

## **Statistical analysis**

Statistical analyses were performed using two-tailed Student's t-test using SPSS 20.0 (SPSS Inc., Chicago, IL). Values are expressed as means  $\pm$  SD. Differences between two groups were determined by the two-tailed student's t-test. The statistically significant difference was set at  $p < 0.05$ .

## **Results**

## **Fgl-1 expression was upregulated in adenomyosis**

The expression of Fgl-1 in normal endometrial tissues, eutopic and ectopic endometrium of adenomyosis was determined using immunohistochemical analysis. As shown in Figure.1, the immunostaining of Fgl-1 in normal endometrium was weakly positive and usually restricted to the cytoplasm of glandular and stromal cells (Figure.1A, D). In addition, no significant difference in Fgl-1 expression was observed between the normal endometrium in the proliferative and secretory phases ( $p \geq 0.05$ ).

In eutopic endometrium of adenomyosis, the immunostaining of Fgl-1 was positive and was distributed in cytoplasm of glandular and stromal cells (Figure.1B, E). In addition, no significant difference in Fgl-1 expression was identified between endometrium of adenomyosis in the proliferative and secretory phases ( $p \geq 0.05$ ). However, eutopic endometrium of adenomyosis showed significantly increased Fgl-1 expression in both the proliferative and secretory phases compared with normal endometrium (Figure.1G,  $p \leq 0.01$ ,  $p \leq 0.05$ ).

In ectopic endometrium of adenomyosis, the immunostaining of Fgl-1 was positive and was restricted to the cell cytoplasm of epithelial cells and stromal cells (Figure.1C, F). In addition, no significant difference in Fgl-1 expression was observed between ectopic endometrium in the proliferative and secretory phases ( $p > 0.05$ ). Ectopic endometrium in both the proliferative and secretory phases showed significantly increased Fgl-1 expression compared with the normal endometrium (Figure.1G,  $p \leq 0.01$ ,  $p \leq 0.01$ ). However, no significant difference was noted in Fgl-1 expression between eutopic and ectopic endometrium of adenomyosis ( $p \geq 0.05$ ).

These data suggest that Fgl-1 expression was elevated in adenomyosis. In addition, Fgl-1 expression was not altered during the menstrual cycle in either normal endometrium or endometrium of adenomyosis.

## **Increased Lag-3 positive T-lymphocyte infiltration in adenomyosis**

The expression of Lag-3 in normal endometrial tissues and adenomyosis was determined by immunohistochemical analysis. In immunohistochemical analysis, the immunostaining of Lag-3 in endometrial glandular epithelial cells was weak positive to null and no significant difference was noted between normal endometrium and endometrium of adenomyosis (Figure.2A, B, C, E, F, G,  $p \geq 0.05$ ). In adenomyosis, Lag-3-positive (Lag-3+) cells could be noted in the interstitial components (46.7% in proliferative phase, and 40% in secretory phase, Figure.2D, G, Figure.4A). In normal endometrium, Lag-3+ cells could be less noted in the interstitial components (6.7% in proliferative phase, and 3.3% in secretory phase, Figure.2A, E, Figure.4A).

The expression and localization of Fgl-1 and Lag-3 was determined by immunofluorescence analysis. In normal endometrium, CD4+/Lag-3+, CD8+/Lag-3+ and Foxp3+/Lag-3+ cells were very few (6.7% in proliferative phase, and 3.3% in secretory phase, Figure.3A1-6, D4-6). In adenomyosis, Lag-3 colocalized with CD4, CD8 and Foxp3 expression in the interstitial components, and CD4+/Lag-3+, CD8+/Lag-3+ and Foxp3+/Lag-3+ cells were noted in 46.7% of proliferative phase and 40% of secretory phase (Figure.3B, C,

E4-6, F4-6). In immunofluorescence analysis, higher expression of Fgl-1 is noted in adenomyosis compared with normal endometrium (Figure.3D1-3, E1-3, F1-3). The data was consistent with the results of immunohistochemistry analysis.

These data suggest that Lag-3+ T lymphocyte infiltration was involved in the occurrence and development of adenomyosis.

## Discussion

Although adenomyosis is a common benign disease among women of reproductive age, it exhibits similar biological behaviors with malignant tumors and its pathogenesis has not been clearly explained so far, which bring great difficulties to clinical treatment [20]. More and more studies have shown that alterations in the immune mechanism, including the infiltration of a large number of immune cells and abnormal expression of immune factors, might play a significant role in the occurrence and development of adenomyosis. In the present study, we found that aberrant expression of Lag-3 and Fgl-1 is present in the eutopic and ectopic endometrium of adenomyosis, indicating the possible role of Lag-3/Fgl-1 signaling in the pathogenesis and development of adenomyosis. The study provides a theoretical basis for the immunotherapy of adenomyosis.

Fgl-1 is a member of the fibrinogen family and shows marked homology with fibrinogen beta and gamma. Fgl-1 is expressed in the liver and induces hepatocyte proliferation. Fgl-1 also plays a prominent role in the negative regulation of inflammatory immune responses [11, 19]. Fgl-1 expression is decreased in hepatocellular carcinoma (HCC) and Fgl-1 acts as a tumor suppressor in hepatocellular cancer through an Akt dependent mechanism [21]. However, Fgl-1 is upregulated in other human cancers, including gastric cancer, lung cancer, prostate cancer, melanoma and colorectal cancer, and is associated with poor prognosis [11, 22]. In this study, we found that abnormally elevated Fgl-1 expression is present in eutopic and ectopic endometrium of adenomyosis, suggesting its significant role in this disease. In addition, during the immunohistochemical analysis of Fgl-1 expression in adenomyosis, no significant difference of Fgl-1 expression was noted between the proliferation period and secretion period in normal endometrium or in adenomyosis. These data suggested that the expression of Fgl-1 might not be regulated by estrogen and progesterone, which will be further investigated in future studies.

Lag-3 plays a key role in regulating immune response. Lag-3 is mainly expressed in activated T cells and NK cells, and is identified to be a marker of T cell activation [23]. Lag-3 has received widespread attention as a new emerging target in cancer immunotherapy. In various tumors, like melanoma, persistent antigen stimulation increases Lag-3 expression, leading to T cell exhaustion and disability, which inhibits T cell proliferation and cytokine secretion [24, 25]. In head and neck squamous cell carcinoma, oesophageal adenocarcinoma, colorectal cancer, non-small cell lung cancer, hepatoma and other cancers, Lag-3 is abnormally expressed on tumor infiltrating lymphocytes (TILs), which inhibits anti-tumor immunity and promotes tumor escape [8, 17, 26-28]. Lag3 is also abnormally expressed in autoimmune diseases and

chronic viral infections [29, 30]. At present, numerous Lag-3 antibodies, such as REGN3767, IMP321 and LBL-007, have also achieved certain results in clinical trials [31-33].

It is worth noting that Lag-3 is usually co-expressed with other immunosuppressive receptors in tumors, especially PD-1, which synergistically induces T-cell exhaustion to escape tumor immunity [12-16, 34, 35]. In a murine ovarian cancer model, Lag-3 and PD-1 collaborate to mediate T cell signaling and antitumor immunity [13]. And the high expression of these inhibitory molecules is significantly related to the prognosis of patients [16, 17]. Study found that the T cells that co-express Lag-3 and PD-1 are significantly exhausted compared with T cells that express Lag-3 or PD-1 alone [36]. A recent study showed that, in the cancer setting, cells that co-express inhibitory receptors are not only exhausted cells, but also highly activated cells sensitive to treatment [37]. Double blocking PD-1 and Lag-3 showed synergistic anti-tumor immunity [38]. Dual targeting of immune checkpoints PD1 and Lag-3 successfully controlled chronic lymphocytic leukemia development in pre-clinical mouse models [18]. In the present study, through immunofluorescence analysis, we observed typical double immunofluorescence staining of Lag-3 and CD4, CD8, Foxp3 in stroma of adenomyosis. The results showed that the percentage of Lag-3 positive lymphocytes in stroma of adenomyosis was increased significantly compared with normal endometrium. It can be consequently speculated that Lag3 may be involved in the development of adenomyosis through T cell-mediated immunosuppression and immune escape.

A recent study reported that Fgl-1 is an MHC class II-independent high affinity ligand of Lag-3, revealing an immune evasion mechanism. In the tumor microenvironment, Fgl-1 may inhibit T cell response to mediate immune escape by interacting with Lag-3 specifically expressed by tumor infiltrating T cells. Silencing the Fgl-1-Lag-3 interaction by either genetic knockout or antibody blockade can stimulate cell expansion and activation to promote tumor immunity (Fig.4) [11, 22]. Our results suggested that the Lag-3/Fgl-1 signaling was abnormally activated in adenomyosis. It is suggested that Lag-3/Fgl-1 signaling plays an important role in the pathogenesis and development of adenomyosis. The study demonstrates the possibility of immunotherapy for adenomyosis and provides a basis for immunotherapy of adenomyosis.

## Conclusions

The occurrence of dysmenorrhea, menorrhagia and infertility associated with adenomyosis is increasingly common. Therefore, adenomyosis has a great impact on women's life, physical and mental health. In conclusion, the present data suggested that Lag-3/Fgl-1 signaling might be involved in the pathogenesis and development of adenomyosis. This study provides a new understanding of the pathogenesis of adenomyosis and a potential therapeutic target for adenomyosis. In future studies, we will use a variety of experimental techniques to study the role of Lag-3/Fgl-1 signaling in adenomyosis.

## Abbreviations

DAB: Diaminobenzidine tetrahydrochloride; EDTA: Ethylenediaminetetraacetic acid solution; Fgl-1: Fibrinogen-like protein 1; HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen; Lag3: Lymphocyte activation gene 3; PBS: Phosphate-buffered saline; TILs: Tumor infiltrating lymphocytes; Tregs: Regulatory T cells.

## **Declarations**

### **Ethics approval and consent to participate**

The use of human tissues was approved by the Institutional Research Ethics Committees of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Written informed consent was obtained from all participants before being included in the study.

### **Consent for publication**

Written informed consent for publication was obtained from all participants.

### **Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

HZ participated in its design and coordination, and edited the manuscript for submission. XQ and GY carried out the experiments, participated in its design and coordination. WS carried out the experiments, participated in the interpretation of data and drafted the manuscript. NL and JL helped to revise the manuscript and performed the statistical analysis. CL, CL, ML and XZ participated in the design of the study, supervised the study and critically helped to draft the manuscript. All authors read and approved the final manuscript.

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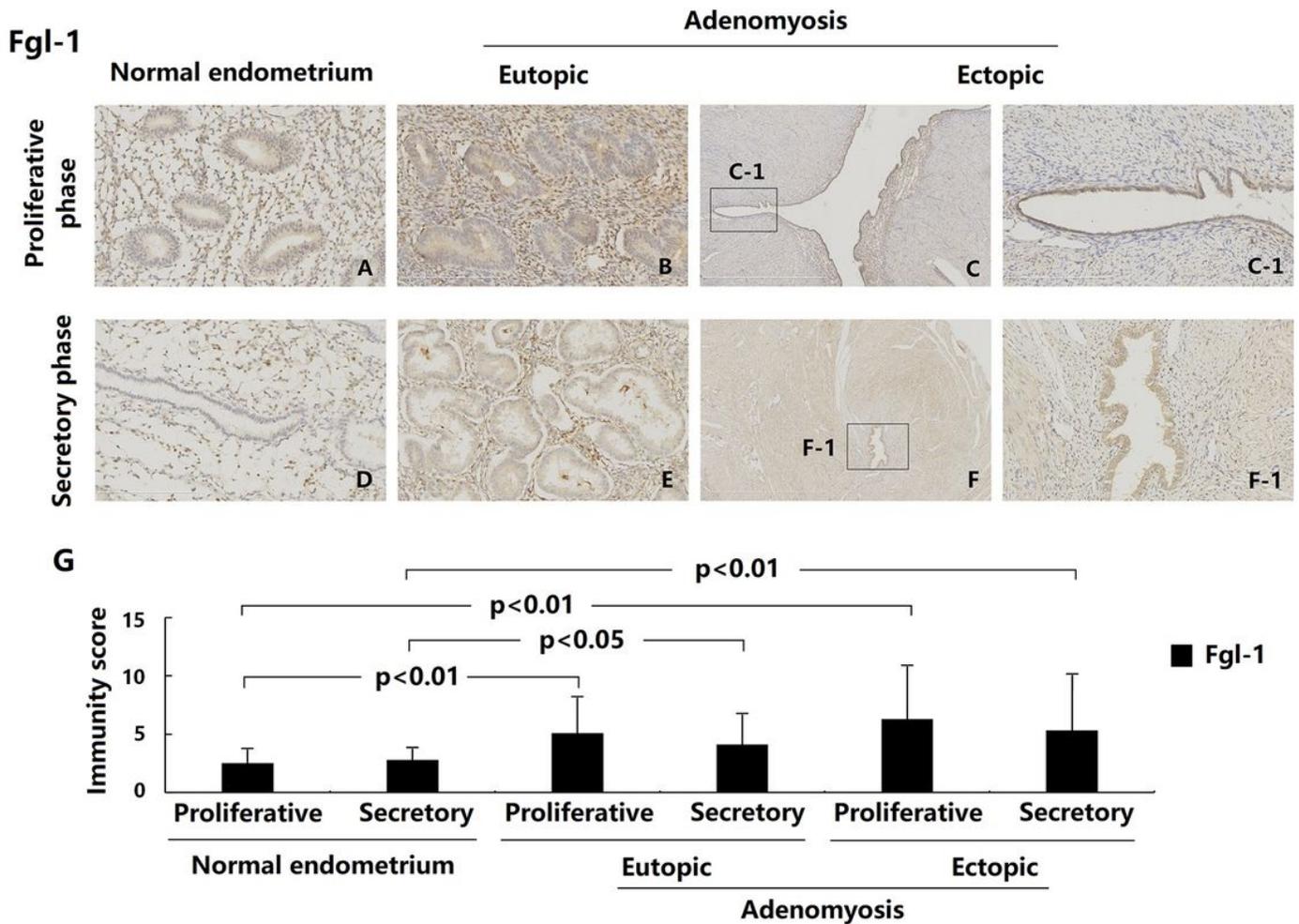
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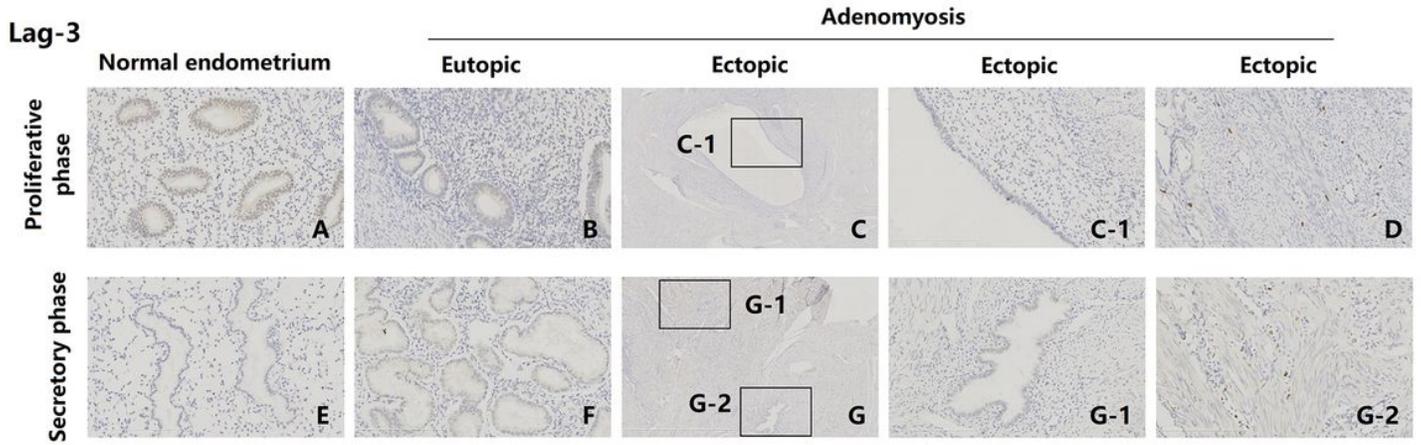
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## Figures



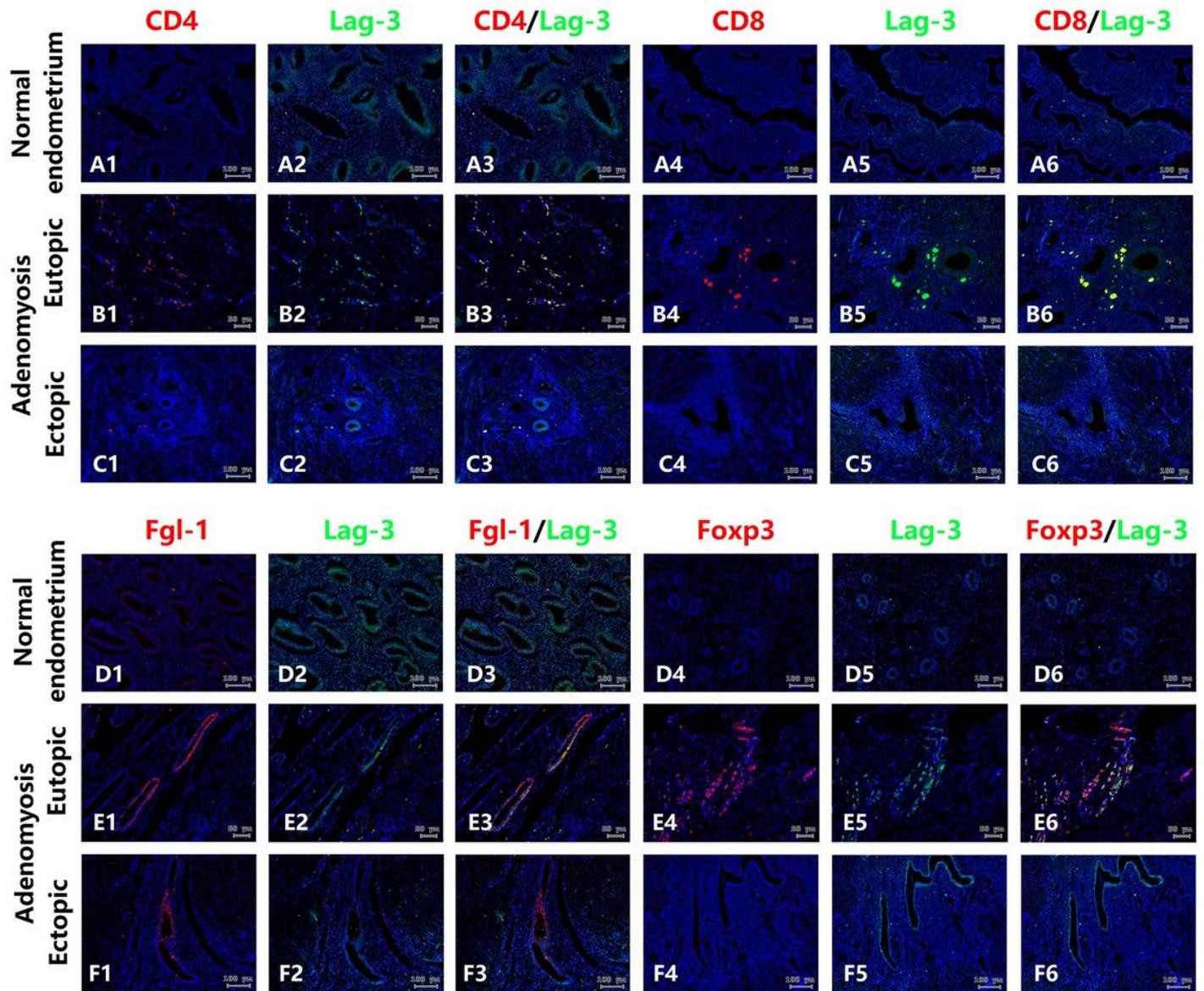
**Figure 1**

Immunoexpression of Fgl-1 in normal endometrium, eutopic and ectopic endometrium of adenomyosis. A: Normal endometrium of proliferative phase (n=15); B: Eutopic endometrium in adenomyosis of proliferative phase (n=15); C: Ectopic endometrium in adenomyosis of proliferative phase (n=15); D: Normal endometrium of secretory phase (n=15); E: Eutopic endometrium in adenomyosis of secretory phase (n=15); F: Ectopic endometrium in adenomyosis of secretory phase (n=15). Magnification:×200; G: Immunoscore of Fgl-1.



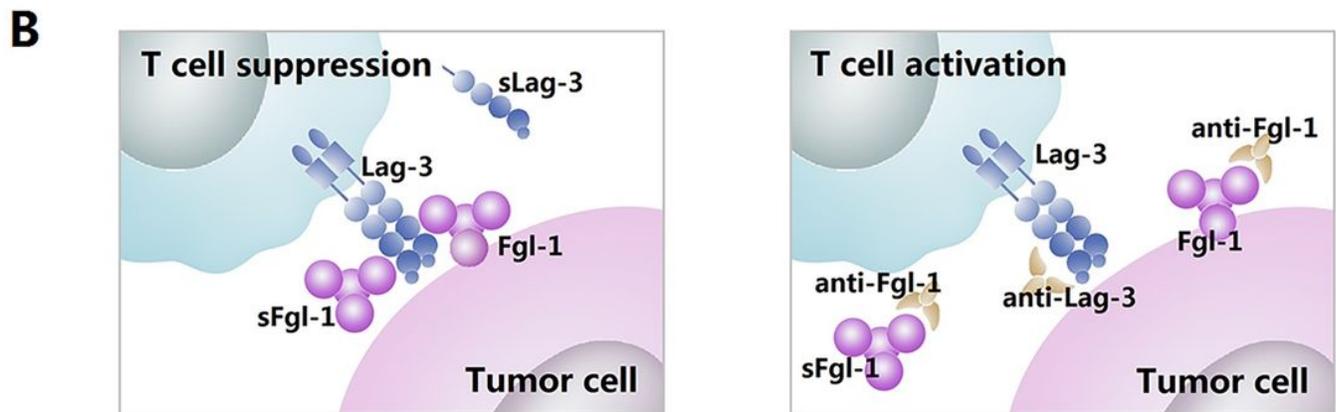
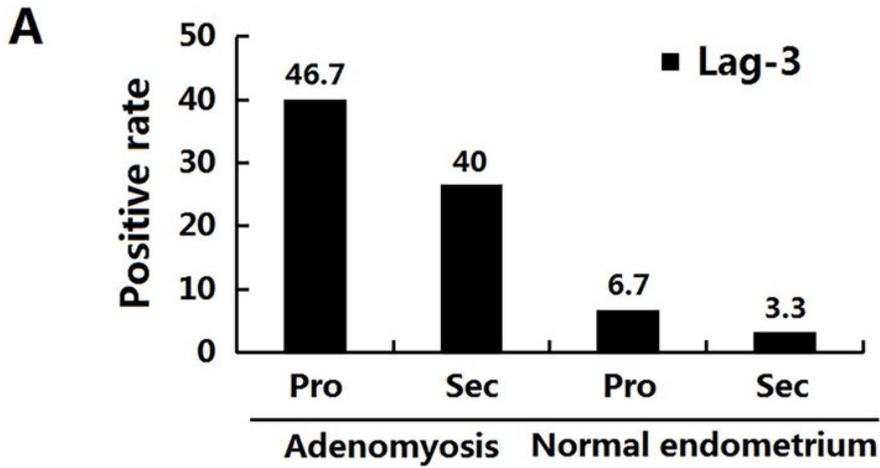
**Figure 2**

Immunoexpression of Lag-3 in normal endometrium and endometrium of adenomyosis. A: Normal endometrium of proliferative phase (n=15); B: Eutopic endometrium in adenomyosis of proliferative phase (n=15); C, D: Ectopic endometrium in adenomyosis of proliferative phase (n=15); E: Normal endometrium of secretory phase (n=15); F: Eutopic endometrium in adenomyosis of secretory phase (n=15); G: Ectopic endometrium in adenomyosis of secretory phase (n=15); Magnification:×200.



**Figure 3**

The expression and localization of Fgl-1 and Lag-3 in normal endometrium and endometrium of adenomyosis determined by immunofluorescence analysis. A, D4-6: CD4+/Lag-3+, CD8+/Lag-3+, and Foxp3+/Lag-3+ cell staining results in normal endometrium; B, C, E4-6, F4-6: Representative images of CD4+/Lag-3+, CD8+/Lag-3+ and Foxp3+/Lag-3+ cell in the interstitial components of adenomyosis; D1-3, E1-3, F1-3: Immunofluorescence expression of Fgl-1 and Lag-3 in normal endometrium, eutopic and ectopic endometrium of adenomyosis; Lag-3 antibody (green), CD4/CD8/Foxp3/Fgl-1 antibody (red); Magnification:×200.



**Figure 4**

A: Lag-3 positive cells rate in the interstitial components of normal endometrium and adenomyosis; B: Schematic representation of the function Lag-3 and Fgl-1 in the tumor immune microenvironment.