LILRA3 is increased in IBD patients and functions as an anti-inflammatory modulator

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April 28, 2020

Abstract

Growing evidence shows that a homozygous 6.7-kb deletion of the novel anti-inflammatory molecule leukocyte immunoglobulin like receptor A3 (LILRA3) is associated with many autoimmune disorders. However, its effects on pathogenesis of inflammatory bowel disease (IBD) have not been clarified yet. LILRA3 is mainly expressed in monocytes, whereas its effects on biological behaviors of monocytes have not been systematically reported. To investigate the association between LILRA3 polymorphism and IBD susceptibility, LILRA3 polymorphism was assessed in 378 IBD patients and 509 healthy controls in our study, quantitative real-time PCR (qRT-PCR), western blot and immunohistochemistry (IHC) were employed to detect the LILRA3 expression in IBD patient blood and intestinal samples. Despite no association of the polymorphism with IBD development was found, LILRA3 expression was markedly increased in IBD patients compared with healthy controls. Human U937 monocyte cell line was employed to establish LILRA3-overexpressing cells and the effects of LILRA3 on the biological behaviors of U937 cells were systematically explored. We found that overexpression of LILRA3 in monocytes led to significant decreases in secretion of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6). Additionally, LILRA3 abated monocyte migration by reducing the expression of several chemokines and enhanced monocyte phagocytosis by increasing CD36 expression. Furthermore, LILRA3 promoted monocyte proliferation through a combination of Akt and MEK/Erk signaling pathways. We report for the first time that LILRA3 is related to IBD and functions as an anti-inflammatory modulator in U937 cells.

Introduction

Inflammatory bowel disease (IBD), which encompasses ulcerative colitis (UC) and Crohn's disease (CD), comprises a group of intestinal chronic disorders characterized by inflammation and periods of remission and relapse[1]. The populations of western countries are more likely to suffer from IBD than those in Asia. However, recent studies have shown that the incidence and prevalence rates in some Asian countries are rapidly increasing[2-4].

Numerous studies have indicated that genetic factors play important roles in the pathogenesis of IBD, with involvement of some of these genes in multiple autoimmune disorders. Leukocyte immunoglobulin like receptors (*LILRs* ; synonyms: ILT, LIR, CD85) are the most conserved genes among those located within the leukocyte receptor cluster on human chromosome 19. The family includes 13 members with activating or inhibiting capacity[5]: LILRs with long cytoplasmic tails bearing tyrosine-based inhibitory motifs are inhibitory receptors (*LILRBs*), whereas LILRs with short cytoplasmic tails have activating functions (*LILRAs*)[6,7]. *LILRA3* (ILT-6, CD85e), located in the centromeric ILT cluster, is a special member of the LILR family. Genomic sequencing of LILRA3 has revealed that LILRA3 is highly homologous to other LILRs such

as LILRB1 and LILRB2[8], suggesting that LILRA3 might act by impairing the function of these LILRBs. In addition, LILRA3 shows presence-absence variation, as opposed to other LILRs, which are conserved genetically[9]. For example, some individuals may carry an aberrant deletion of a 6.7-kb fragment encompassing the first seven exons[8,10], and this variation has been proven to be associated with many autoimmune diseases, such as Sjögren's syndrome (SS), multiple sclerosis (MS) and rheumatoid arthritis (RA)[11-14].

rs103294 and rs410852 are two single-nucleotide polymorphisms (SNPs) of the LILRA3 gene. In a case-control study among the Chinese population, rs103294 was reported to be associated with benign prostatic hyperplasia[15]. A genome-wide association study (GWAS) also identified rs103294 as a new risk locus for prostate cancer[16]. This GWAS also revealed that polymorphism of this locus affects LILRA3 expression. Many recent articles have demonstrated that the 6.7-kb deletion affects LILRA3 mRNA and protein expression, with individuals carrying the wild type (+/+) having much higher levels than those with the homozygous deletion (-/-)[17-19]. Nonetheless, increased LILRA3 is detected in many diseases, such as MS and systemic lupus erythematosus (SLE)[18,19], both of which are autoimmune disorders characterized by excessive inflammation. These findings indicate that LILRA3 is a novel susceptibility gene for autoimmune diseases and might play a crucial role in the pathogenesis of chronic inflammatory diseases. Additionally, it has been reported that interleukin 10 (*IL-10*) or interferon-γ (*IΦN-γ*) sharply upregulates LILRA3 expression in human monocytes, whereas tumor necrosis factor-α (*TNΦ-α*) exhibits the opposite effect[20]. Furthermore, LILRA3 induces proliferation of $CD8^+$ T-cells and NK cells in the presence of pro-inflammatory cytokines[21], suggesting an anti-inflammatory effect of LILRA3. Apart from inflammation, LILRA3 is also reported to function as an antagonist of LILRB2 and to promote synapse formation through the Erk/MEK pathway[22].

Because IBD is an autoimmune disorder characterized by recurrent intestinal inflammation, we hypothesized that LILRA3 might play a role in IBD pathogenesis. Accordingly, in this study, we investigated the interaction between LILRA3 polymorphisms and IBD development. Although no significant association was found, we surprisingly observed increased LILRA3 in IBD patients. LILRA3 is mainly expressed in mono-myeloid cells, such as monocytes, macrophages (Mø) and dendritic cells (DCs)[21,23-25]. Monocytes are critical regulators in immune responses and have important roles in immune surveillance by directly phagocytizing pathogens in circulation[26]. Monocytes secrete many cytokines such as IFN-γ, TNF-α, interleukin 6 (*IL-6*), and IL-10. As these cells also exert many of their functions outside the vascular system, crossing the blood vessel wall and migrating to the site of injury are required[27]. The effects of LILRA3 on monocytes have not been systematically reported. We employed the U937 human monocyte cell line to establish LILRA3-overexpressing cells and then explored the effects of LILRA3 on the above functions of monocytes as well as other biological behaviors such as apoptosis and proliferation.

Materials and Methods

Ethics Statement

Our study has been conducted in accordance with the principles expressed in the Declaration of Helsinki, and was approved by the ethics committee of Zhongnan Hospital of Wuhan University (2014037). Informed consent has been obtained.

Patients and samples collection

Lithium sulfate anti-coagulated peripheral blood samples were obtained from 378 IBD patients (185 CD, 193 UC) and 509 healthy controls. All patients were recruited from the Department of Gastroenterology, Zhongnan Hospital of Wuhan University (Wuhan, China) from September 2014 to January 2016. The patients were sub-genotyped according to Montreal classification criteria. Healthy controls were selected among volunteers from January to November 2015. The demographic and sub-phenotype data for the patients and controls are shown in Supplementary Table 1. Total genomic DNA was extracted from blood using the QIAamp DNA blood midi kit (Qiagen, Shanghai, Germany). Inflamed ileal and/or colonic tissues were collected from 36 CD patients and 52 UC patients who underwent endoscopy at Zhongnan Hospital. All biopsies were taken from inflammatory sites adjacent to ulcerations. Non-inflamed colonic mucosa samples were collected from 14 healthy volunteers; another 20 normal biopsies (Zhongnan Hospital from September

2014 to June 2015) obtained from uninfected sites 20 centimeters from polyps or neoplasms served as controls. All patients and controls were of the Han population from Central China. Diagnosis of CD and UC was based on a combination of clinical symptoms, laboratory and radiological examinations and endoscopy with histology.

6.7-kb deletion genotyping

LILRA3 presence-absence variation was detected by polymerase chain reaction (PCR) using the two sets of primers listed in Supplementary Table 2. With LILRA3 deletion, primer 1 (flanking the deletion) amplified a fragment of 166 bp. When LILRA3 was present, primer 2 (designed within the 6.7-kb region) amplified a fragment of 250 bp. Each DNA sample was tested using the two sets of primers in separate reactions. The amplified products were analyzed on a 1.5% agarose gel. The results of gel electrophoresis are shown in Supplementary Figure 1.

rs103294 and rs410852 genotyping

The genotypes of rs103294 and rs410852 were detected by the PCR-ligation detection reaction (PCR-LDR) method with technical support from Shanghai Biowing Applied Biotechnology Company (Shanghai, China)[28,29]. The sequences of the primers and probes are listed in Supplementary Table 2. The PCR product was used as the template in the LDR reaction, and the LDR products were assessed using the ABI 3730 sequencer (ABI, Carlsbad, USA).

Cell culture

The human U937 monocyte cell line (Cat. FDCC-HLC063) and 293T cells (Cat. FDCC-HSN190) were obtained from Fudan Cell Center (F.D.C.C) of Fudan University (Shanghai, China). Identification of the two cell lines were at the China Centre for Type Culture Collection in Wuhan, China. The U937 cells were cultured with RPMI-1640 medium (HyClone, Logan, USA); 293T cells were cultured in DMEM medium (HyClone, Logan, USA) containing 10%fetal bovine serum (FBS) (HyClone, Logan, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) in humidified 5% CO_2 at 37°C.

Establishment of the LILRA3-overexpressing U937 cell line

The open reading frame (ORF) of LILRA3 was amplified from U937 cells using qRT-PCR and cloned into the expression vector pWSLV-02 (carrying enhanced green fluorescent protein (EGFP) as a reporter gene) (Viewsolid, Beijing, China) to generate the plasmid pWSLV-02-LILRA3. For lentivirus production, 293T cells were co-transfected with the pWSLV-02-LILRA3 plasmid, which encodes the intact sequence of LILRA3 cDNA, or the empty vector pWSLV-02 as a control using Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia, Rockville, USA). Virus was collected at 24 hours and 48 hours after transfection. To prepare the LILRA3-overexpressing cell line, U937 cells were infected with lentivirus for 72 hours, and FCM was then used to select cells with stable LILRA3 lentivirus integration.

RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from peripheral blood, fresh-frozen biopsies and pWSLV-02-LILRA3 plasmid- or pWSLV-02-transfected U937 cells using the Trizol reagent (Invitrogen, Carlsbad, USA). The quantity and quality of the RNA samples were assessed using a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, USA). Total RNA (1 μg) was used to synthesize cDNA using a first-strand cDNA synthesis kit (Thermo Scientific, Waltham, USA). qRT-PCR was subsequently performed using the QuantStudioTM6 Flex Real-Time PCR instrument (ABI, Carlsbad, USA) with the SYBR® Premix Ex TaqTM II mix (Takara, Kusatsu, Japan). The 2^{-CT} method was applied to determine the relative mRNA levels normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting

Total protein was extracted from fresh-frozen intestinal biopsies and pWSLV-02-LILRA3 plasmid- or pWSLV-02-transfected U937 cells using a mixture of RIPA lysis solution containing protease inhibitor and phos-

phatase inhibitor. The protein concentration was determined using a BCA assay kit. RIPA and the BCA kit were purchased from Beyotime Biotechnology (Shanghai, China). A total of 40 μg protein from each sample was separated by 12.5% or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, USA). The membranes were blocked with 5% nonfat milk in TBST (Tris-buffered saline (TBS) with 0.1% Tween 20) at room temperature for 1 hour and then incubated with specific primary antibodies in TBST overnight at 4°C followed by three washes with TBST. The membranes were then incubated with the following antibodies: anti-LILRA3 polyclonal antibody (pAb) (Cat. GTX108819, GeneTex, Alton Pkwy Irvine, USA); anti-CD36 pAb (Cat. 18836-1-AP) and anti-CD206 pAb (Cat. 18704-1-AP) were obtained from Proteintech (Wuhan, China); anti-p-Foxo3a mAb (Cat. ab47285) and anti-Foxo3a mAb (Cat. ab17026) were procured from Abcam (Cambridge, USA); anti-p-Akt monoclonal antibody (mAb) (Cat. #4060), anti-Akt mAb (Cat. #4691), anti-p-MEK1/2 mAb (Cat. #9154), anti-MEK1/2 mAb (Cat. #8727), anti-p-Erk1/2 mAb (Cat. #4370), anti-Erk1/2 mAb (Cat. #4695), anti-p-P38 MAPK mAb (Cat. #4511), anti-P38 MAPK mAb (Cat. #8690), anti-p-PDK1 mAb (Cat. #3438), anti-PDK1 pAb (Cat. #3062), anti-p-c-Raf pAb (Cat. #9421), anti-p-PI3K pAb (Cat. #4228), anti-PI3K mAb (Cat. #4257), anti-p-P65 mAb (Cat. #3033), anti-P65 mAb (Cat. #8242), anti-p-SAPK/JNK mAb (Cat. #4668), anti-JNK2 mAb (Cat. #9258), anti-p-mTOR mAb (Cat. #5536), anti-mTOR mAb (Cat. #2983) were purchased from Cell Signaling Technology (CST, Danvers, USA). GAPDH (Cat. KM9002, Sungene Biotechnology, Tianjin, China) was used as the internal reference. The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse antibody (Cat. LK2001/LK2003, Sungene Biotechnology, Tianjin, China) in TBST for 2 hours at room temperature. After 3 washes with TBST, the blots were visualized using enhanced chemiluminescence kit (Bio-rad, Hercules, USA).

Elisa

Culture media of U937 cells were collected and centrifuged at 20,000 × g for 1 min. The supernatants were aliquoted and stored at -80°C until analysis. The concentration of IFN-gamma, IL-6, TNF-α, CCL2, CCL3, CXCL8/IL-8 and CXCL10 was measured using ELISA kits (NeoBiosciences, Shenzhen, China), according to the manufacturers' protocol.

Histopathology, immunohistochemistry (IHC) and immunofluorescence

Colon biopsies for histopathology were fixed overnight in 10% formalin. After paraffin embedding, 3-μm sections were cut and stained with hematoxylin and eosin (H&E) in accordance with standard protocols for microscopic examination. IHC was performed on 3 μm sections of paraffin-embedded fresh-frozen endoscopic colon tissues from patients with IBD and control individuals. The sections were deparaffinized, and antigen was retrieved by microwave oven irradiation using sodium citrate buffer, followed by blocking with 5% goat serum for 30 minutes at room temperature. The sections were incubated with the primary antibody overnight at 4°C. After careful washing, the sections were then incubated for 1 hour at room temperature with a secondary antibody. Sections treated with phosphate-buffered saline (PBS) instead of the primary antibody were used as a negative control. Images were acquired by a microscope (Olympus, Cat. #IX73). Image-Pro Plus version 6.0 (Media Cybernetics, BetheSEMa, MD) was used to calculate the integrated option density (IOD).

Apoptosis assay

Flow cytometry (FCM) was applied to detect the involvement of LILRA3 in apoptosis of U937 cells. U937 cells (4×10^5cells/well) harboring the null vector (pWSLV-02) and overexpression plasmid (pWSLV-02-LILRA3) were plated in 6-well plates. After 48 hours of culture, the cells were harvested and washed twice with PBS. The cells were then stained using an annexin V-PE/7-amino-actinomycin D (7-AAD) kit (BD, San Jose, USA) following the manufacturer's protocols. Annexin V-PE was used to detect phosphatidylserine externalization, a hallmark of early apoptosis, and 7-AAD was used to label DNA fragments, a sign of cell death. The Q4 area (annexin V-PE positive and 7-AAD negative) represents early apoptotic cells, and Q2 (both annexin V-PE and 7-AAD positive) represents late apoptosis.

Cell migration

A migration assay was performed using transwell 24-well Boyden chambers (Corning, Corning, USA) with an 8.0 μm pore size membrane without matrigel. Cells were seeded in the upper chamber at a density of 5×10^4cells/well in 200 μl serum-free RPMI-1640 medium. As a chemoattractant, 600 μl 10% FBS containing complete medium was added to the lower chamber. After incubation for 6 hours at 37°C in humidified 5% CO_2, the upper chamber was detached from the well, and the cells at the lower chamber, representing migrated cells, were counted using a cell counting chamber.

Phagocytosis assay

Latex beads (L3030, Sigma, St. Louis, USA) were used to detect the potential influence of LILRA3 on the phagocytosis capacity of U937 cells. U937 cell lines harboring pWSLV-02-LILRA3 or pWSLV-02 were seeded in 6-well plates (1.0×10^5cells/well) in RPMI-1640 medium containing 1% FBS, followed by incubation at 37°C in humidified 5% CO_2 for 20 hours. A mixture of FBS and latex beads (FBS: latex beads = 20:1) was added to each well. The percentage of GFP^+ Texas Red^+ cells was then analyzed by FCM at 0.5, 6, 12 and 24 hours.

Cell proliferation assay

Cell proliferation was detected by the cell counting kit-8 (CCK-8) assay according to the manufacturer's instructions. U937 cells harboring the LILRA3 overexpression plasmid or the null vector were plated in 96-well plates (3.0×10^3cells/well) for 0, 12, 24, 48 and 72 hours. To each sample, 10 μl CCK-8 solutions (Dojindo, kamimashiki gun Kumamoto, Japan) were added at the indicated time. After incubation at 37°C in humidified 5% CO_2 for 1 hour, the optical density (OD) of each sample was measured at 450 nm using an auto-microplate reader (Infinite M200, Tecan, Manedorf, Switzerland). Three parallel replicates were employed for each sample.

Statistical analysis

All analyses were performed using SPSS Version 17.0 and GraphPad Prism software version 5.0. Among the healthy controls, the distributions of the 6.7-kb deletion genotype, rs103294 and rs410852 were in Hardy-Weinberg Equilibrium (HWE) (p=0.55, p=0.31, p=0.55 respectively). The $\chi 2$ test was used to analyze the frequencies of alleles and genotypes between the cases and controls and the differences between the sub-phenotypes of the patient groups with the controls. Odds ratios (ORs) are provided with 95% confidence interval (CI) to estimate the relative risk of developing CD and UC. Differences in LILRA3 mRNA and protein expression between the patient groups and controls were analyzed using one-way analysis of variance (ANOVA). Differences between the U937 cells harboring the null vector and overexpression plasmid were analyzed using an unpaired t-test. A p value less than 0.05 was considered to be statistically significant. Data are presented as mean ± SD from three independent experiments and representative data are shown.

Results

The association between LILRA3 gene polymorphism and the development of IBD

The deletion rate among 877 subjects enrolled in our study (both patients and healthy controls) was 89.4%, which was higher in comparison with that reported for the Han Chinese population in Beijing (CHB) (89.4% *vs.* 70%)[30]. The deletion ratio in three groups was 88.4% for healthy controls, 91.4% for CD and 90.7% for UC, and no association was found between the 6.7-kb deletion and IBD development (p=0.27, p_{FDR}=0.41, OR=1.39, 95%CI=0.78-2.47 for CD; p=0.39, p_{FDR}=0.96, OR=1.28, 95%CI=0.73-2.22 for UC). In addition, no difference at the allele level ("-" *vs.* "+") was found (p=0.70, p_{FDR}=0.90, OR=1.05, 95%CI=0.82-1.35 for CD; p=0.87, p_{FDR}=0.87, OR=1.02, 95%CI=0.80-1.30 for UC) (Table 1), and the deletion did not affect the phenotype frequencies of CD or UC (Table 2). These data indicate that the 6.7-kb deletion has no association with CD and UC development among the Han population of Central China.

Limited association was detected between the rs103294 and rs410852 genotypes and disease susceptibility. (p=0.94 for rs103294; p=0.17 for rs410852). Nonetheless, at the allele level, the allele T of rs103294 was

found to be a risk locus for CD (p=0.04, p_{FDR}=0.12, OR=1.32, 95%CI=1.01-1.73) (Table 1). Relationships between the phenotypes of patient groups and the controls were then analyzed, revealing that the CD patients carrying the rs410852 genotype were less likely to develop intestinal stricturing or penetrating complications (Table 2). However, no association for the genotypes and allele frequencies between the UC patients and controls was observed for the groups as a whole or after stratified.

IBD patients exhibit increased LILRA3 expression compared with healthy controls

The effect of LILRA3 variation on its expression in peripheral blood was assessed for 36 patients with CD, 48 patients with UC and 53 healthy controls. The subjects enrolled were derived from samples with available genotyping data. As shown in Figure 1A, LILRA3 was almost undetectable in subjects homozygous for the 6.7-kb deletion, whereas its expression was significantly increased in those heterozygous for the deletion and with the wild-type gene. In addition, when the homozygous deletion subjects were excluded, we found remarkable increases in the levels of LILRA3 mRNA in both the CD (2.11±1.47) and UC (1.72±1.10) patients compared with the controls (0.98±0.59) (p=0.005 for CD; p=0.014 for UC) (Figure 1B).

Considering that IBD is a chronic inflammatory disease that primarily involves the gut, we then carried out qRT-PCR and western blotting to investigate intestinal LILRA3 expression. Based on our finding that samples with undetectable LILRA3 expression are homozygous for the deletion genotype, intestinal samples with undetectable LILRA3 were directly excluded from this analysis. Overall, a total of 14 healthy controls, 36 CD and 52 UC samples were included. Increased LILRA3 mRNA levels were observed in the patient groups compared with healthy controls. ($p < 0.001$ for both CD and UC group) (Figure 1C). Total protein from another 8 non-IBD, 7 CD and 10 UC samples was extracted, and in accordance with the qRT-PCR data, western blotting showed a noteworthy increase in LILRA3 expression in both CD (2.50±1.68) and UC (2.26±1.72) group in comparison with healthy controls (0.39±0.27) ($p < 0.05$ for both CD and UC group) (Figure 1D).

To further detect pathological changes and in situ expression of LILRA3 in intestinal biopsies, H&E staining and IHC were applied. The morphology and structure of mucosa from the healthy controls were normal. In contrast, the sub-mucosa and lamina propria (LP) of the CD and UC biopsies were saturated with lymphocytes, plasma cells and neutrophils (Figure 1E). LILRA3 is reported to exist in a soluble form, and we consistently found LILRA3 in the cytoplasm of cells located in the LP (Figure 1E). Compared with the non-IBD controls (0.16±0.009), many more LILRA3-positive cells were detected in the LP of samples from the CD (0.21±0.03) and UC patients (0.19±0.03) ($p < 0.01$ for CD; $p < 0.05$ for UC) (Figure 1E, 1G). According to previous study, LILRA3 is mainly expressed in myeloid cells. We then use CD68 and LILRA3 antibody to stain the macrophages in the LP. Immunofluorescence assay revealed that the LILRA3 protein were expressed in CD68 positive macrophages and CD patients possessed more $CD68^{+}LILRA3^{+}$ cells than healthy controls (16.09±5.03 for CD, 6.57±1.96 for HC, $p < 0.01$) (Figure 2A-2C). In summary, these findings demonstrate that the overwhelming majority of LILRA3 protein is expressed in macrophages and is remarkably increased in IBD patients, identifying a definite role of LILRA3 in IBD development.

Overexpression of LILRA3 downregulates secretion of many cytokines by U937 cells

Establishment of LILRA3-overexpressing U937 cells was verified at the mRNA and protein levels. As shown in Figure 3A, significant increases in LILRA3 were detected in the overexpressing U937 cells. To explore the potential effect of LILRA3 on cytokine secretion and further elucidate its role in inflammation, the supernatant of the two established cell lines were collected. Elisa assay demonstrated that the presence of LILRA3 could markedly downregulate secretion of IFN-γ ($p < 0.001$), TNF-α ($p < 0.001$), and IL-6 ($p < 0.001$) (Figure 3B). In view of that the LILRA3 is a soluble protein, we use LILRA3-overexpressing U937 cell lines supernate to culture the U937 cells for 12 and 24 hours, and in consistent with the previous results, we detected significantly decreased in the secretion of IFN-γ, TNF-α as well as IL-6. Cells cultured for 12 hours showed a more significant effect of inhibition (Figure 3C). Previous studies have demonstrated an anti-inflammatory role for LILRA3 based on findings that IL-10 upregulates but TNF-α downregulates LILRA3 production in vitro. Our results support the growing body of evidence that LILRA3 participates

in inflammation as an anti-inflammatory molecule.

The presence of LILRA3 has limited effects on U937 cell apoptosis

The impact of LILRA3 on apoptosis has not been reported. After culturing for 48 hours, cells were harvested and stained with 7-AAD kit, and the apoptosis ratio was calculated for GFP^+ cells. According to the data shown in Figure 3D and 3E, we conclude that although LILRA3 obviously decreased the late apoptosis rate compared with the null vector cells (3.64%±0.11% *vs.* 4.41%±0.20%, $p < 0.01$), it had little effect on early apoptosis and total apoptosis frequencies ($p > 0.05$). This result indicates that LILRA3 is not involved in regulating apoptosis in U937 cells.

LILRA3 attenuates U937 cell migration by decreasing expression of certain chemokines

A transwell test was applied to investigate cell migration. We found that after 6 hours of incubation in 10% FBS containing RPMI-1640, few of the U937 cells overexpressing LILRA3 had migrated into the lower chamber compared with cells harboring the null vector ($p < 0.001$) (Figure 3F). Chemokines and their seven-transmembrane, G-protein coupled receptors are recognized as key mediators physiologically directing cell migration. We then performed Elisa assay to detect expression of two CC-type chemokines (CCL2, CCL3) and other two CXC-type chemokines (CXCL8/IL-8, CXCL10), which were predominantly expressed in monocytes. Interestingly, we found that CCL2, CCL3, IL-8, CXCL10 were significantly suppressed by LILRA3 ($p < 0.001$) (Figure 3G). To verify our findings, we then added a certain amount of recombinant CCL2 or CXCL8 (Peprotech, Rocky Hill, USA) to the upper chamber, and the migration assay results showed that the migration capacity, inhibited by LILRA3, was reversed by the exogenous IL-8 ($p<0.01$) (Figure 3H). The data suggest that LILRA3 attenuates the migration capacity of U937 cell lines mainly by suppressing IL-8 secretion.

LILRA3 promotes phagocytosis of U937 cells by upregulating CD36 expression

The phagocytosis efficiency was detected by FCM at 0.5, 6, 12 and 24 hours by analyzing the percentage of GFP^+ Texas Red^+ cells. As shown in Figure 4A and 4B, LILRA3-overexpressing U937 cells showed a strong ability to engulf beads beginning at 6 hours in comparison with cells expressing the null vector (5.23%±0.21% *vs.* 3.80%±0.35%, $p < 0.01$ at 6 hours; 26.00%±0.17% *vs.* 20.47%±0.42%, $p < 0.001$ at 12 hours; 30.03%±0.83% *vs.* 26.43%±0.64%, $p < 0.01$ at 24 hours). Pattern recognition receptors (PRRs), mainly including mannose receptor (MR) and scavenger receptor (SR), play important roles in phagocytosis. CD206/*MRC1* (an important MR) and CD36/*SCARB3* (an important class B SR) are two highly effective endocytic receptors expressed on monocytes[31,32]. qRT-PCR and western blotting were employed to further explore whether LILRA3 could alter expression of these two receptors on the plasma membrane (for primers used in the reaction, see Supplementary Table 2). We found that LILRA3 could significantly promote expression of CD36 yet had no influence on CD206 (Figure 4C, 4D, 4E). CD36 specific antibody (BD, San Jose, USA) was added to the culture of LILRA3-overexpressing cell lines, and the phagocytosis efficiency was detected again. The specific antibody could significantly decrease the phagocytosis ability of LILRA3-overexpression cell lines at a concentration dependent manner ($p<0.01$ for both groups at 12h culture, $p<0.01$ for 1mg/ml and $p<0.001$ for 10mg/ml at 24h culture) (Figure 4F). Taken together, our results reveal that LILRA3 enhances the phagocytosis ability of U937 cells by increasing CD36 expression.

LILRA3 enhances the proliferation of U937 cells through a combination of Akt and MEK/Erk signaling pathways

The proliferation capacity of the two established U937 cell lines was evaluated using CCK-8 assay by detecting OD values at 450 nm at 12, 24, 48 and 72 hours. After 24 hours of culturing, enhanced proliferation was observed for the LILRA3-overexpressing U937 cells compared with the cells expressing the null vector, and the difference was quite significant at 48 and 72 hours ($p=0.016$ for 48 hours; $p=0.003$ for 72 hours) (Figure 5A). Western blotting was applied to investigate the potential signaling pathway, and we found that overexpression of LILRA3 significantly increased the levels of phosphorylated *Akt* , *MEK* and *P38 MAPK*(Figure 5B-5D). Given that the MEK and Akt pathways are closely related to proliferation, our

ensuing research mainly focused on exploring the upstream and downstream factors of these two kinases. The levels of phosphorylated *Erk* (the accepted downstream protein of MEK),*PDK1* and *c-Raf* (the classical kinases for Akt and MEK, respectively), *PI3K* (the conventional upstream protein of both Akt and MEK), and *Foxo3a* (the shared downstream protein of MEK and Akt) were further examined. Intriguingly, we found that phosphorylation of Erk, PDK1, c-Raf and PI3K was significantly increased but that of Foxo3a decreased in the presence of LILRA3 (Figure 5C, 5E, 5F and 5G). Phosphorylation of *P65* , *SAPK/JNK* and*mTOR* was also investigated, but no difference was found (see Supplementary Figure 2). Because both Akt and MEK can be activated by PI3K, we speculated that the two pathways might function synergistically to regulate U937 cell proliferation. To confirm our hypothesis, LILRA3-overexpressing cells were seeded into 6-well plates and pretreated with specific inhibitors of MEK (GSK1120212, 1 μmol/L), Akt (MK-2206 2HCL, 5 μmol/L) or PI3K (LY294002, 20 mmol/L); the three inhibitors were purchased from Selleckchem (Houston, USA). Western blotting was used to evaluate the inhibitory efficiency, and the CCK-8 assay was carried out at 24, 48 and 72 hours to detect cell viability. We found that the three inhibitors could dramatically decrease phosphorylation of their targeted proteins (Figure 6A-6C) and could sharply reduce cell viability after 24 hours of culture compared with cells treated with dimethyl sulfoxide (DMSO) (Figure 6D). Most importantly, as shown in Figure 6C and 6D, LY294002 (a specific inhibitor of PI3K) also markedly decreased phosphorylation of Akt and MEK, with the highest inhibitory efficiency on cell proliferation compared with the other inhibitors. These results were consistent with our speculation. Foxo3a is a well-known critical transcription factor involved in cell growth. Although we detected a noteworthy decline in phosphorylation of this protein in cells pretreated with the three inhibitors, this result was not in accordance with our finding when the level of phosphorylated Foxo3a was detected between LILRA3-overexpressing and null vector cells. Indeed, Foxo3a phosphorylation should be enhanced by activated Akt and MEK; in contrast, we detected a decreased level when LILRA3 was overexpressed. A possible hypothesis to explain this phenomenon is that LILRA3 might have a direct/indirect inhibitory effect on Foxo3 phosphorylation; further investigations are needed to verify this speculation. In conclusion, our data indicate that LILRA3 regulates cell proliferation, most likely through PI3K/Akt and PI3K/MEK/Erk signaling pathways.

A schematic of the possible signaling pathway for LILRA3

The exact receptor for LILRA3 remains unknown. The classical human leukocyte antigen-I (HLA-I): *HLA-C* and the non-classical HLA-I molecule *HLA-G* as well as *Nogo 66* (a highly conserved 66-amino acid loop of *Nogo A*) are reported as possible receptors for LILRA3[22,33,34]. Based on these findings, we hypothesize that LILRA3 might activate the above signaling pathway by interacting with one of the above or unknown receptors (Figure 7).

Discussion

Our initial goal was to clarify the association between LILRA3 polymorphism and IBD susceptibility. Although no significant association with IBD development was observed between rs103294, rs410852 and the 6.7-kb deletion genotypes, we demonstrated that homozygous 6.7-kb deletion results in undetectable LILRA3 levels, which is consistent with previous studies[18,19]. In addition, we detected a much higher deletion ratio among Han population of Central China compared with CHB. Unexpectedly, we found LILRA3 expression in IBD patients was significantly higher than in healthy controls. LILRB1 and LILRB2 are two members of LILRB family. LILRB1 was reported to block the combination of CD8 molecular with HLA-I hence to regulate $CD8^+$ T cells. LILRB2 promoted hematopoietic stem cell (HSC) proliferation. Considering that LILRA3 is homologous to LILRB1 and LILRB2, additionally, LILRA3 alleles were in strong linkage disequilibrium with LILRB2 alleles. Previous study reported that LILRA3 might affect functions of LILRB2 and LILRB1. Based on these findings, we speculate that LILRA3 might play certain roles in immune system.

LILRA3, a member of the highly homologous family of receptors primarily co-expressed on mono-myeloid leukocytes, is increasingly recognized as regulating innate immune responses[24,35]. In vitro, recombinant LILRA3 shows strong and specific binding to the surface of primary peripheral blood mononuclear cells (PBMCs), U937 monocyte cells and B cells[23]. Additionally, recombinant LILRA3 could dramatically abrogate lipopolysaccharide (LPS)-induced TNF-α secretion by monocytes, and increases in LILRA3 have been de-

tected in MS and SLE patients. All these data indicate a critical role for LILRA3 in inflammatory diseases. The intestinal LP contains a diverse array of mononuclear phagocyte (MNP) subsets, including conventional dendritic cells, monocytes and tissue-resident macrophages that collectively play an essential role in mucosal homeostasis, infection and inflammation. In our study, we also found that LILRA3 is mainly expressed on macrophages located in the LP of intestinal and CD patients possessed more $CD68^{+}LILRA3^{+}$cells. Intestinal macrophages are mainly derived from monocytes in circulation, and LILRA3 was reported to mainly expressed on monocytes. Since IBD is characterized by immunologic disorder and monocytes are critical regulators in immune responses and played important roles in IBD pathogenesis. We speculate that LILRA3 might exert certain function on monocytes. To deduce the effect of LILRA3 on immune responses, in our study, human U937 monocytes were employed to establish LILRA3-overexpressing cells, and we observed that LILRA3 could markedly decrease IFN-γ, TNF-α and IL-6 secretion and increase IL-10 secretion. This finding further confirms an anti-inflammatory effect of LILRA3 in immune responses.

Monocytes account for 2–10% of all leukocytes in human body. These cells circulate through the blood and lymphatic system and are recruited to damaged tissue or infected sites to differentiate into Mø and DCs, thus triggering adaptive immune responses[36]. Therefore, stimulation of monocyte migration can help to boost inflammatory responses. In vivo, migration is largely mediated by interaction between chemokines and chemokine receptors. We found that the migration ability of U937 cells was sharply attenuated by LILRA3. CCL2, CCL3, CXCL8 and CXCL10 are the main chemokines secreted by monocytes, and LILRA3 was able to conspicuously reduce expression of these molecules on U937 cells. This finding is in agreement with our transwell assay, which demonstrated impaired migration capacity of U937 cells when LILRA3 was overexpressed. Based on these results, we speculate that LILRA3 might prevent monocyte migration from the circulation into tissues, thereby suppressing inflammation.

In this study, LILRA3-overexpressing cells engulfed more latex beads than cells harboring the null vector. This result indicates that LILRA3 can promote the phagocytosis capacity of U937 cells. Phagocytosis is predominantly mediated by endocytic receptors present on the phagocyte membrane[37]. SR and MR are important receptors associated with endocytosis. SR consists of a family of receptors with many ligands and can be divided into two subgroups: class A and class B. Class A and class B can be further divided into many types including SR-AI, SR-AII, SR-AIII, SR-BI, SR-BII, and SRBIII[29,37]. SRBIII, also termed CD36, is mainly expressed on cells of lymphoid and hematopoietic lineages, such as monocytes, Mø, platelets, endothelial cells and a variety of cultured cell lines[38,39]. MR is a carbohydrate-binding receptor mainly expressed by Mø and DCs[32]. In our study, both CD36 and CD206 were detected on U937 cells, and LILRA3 increased CD36 expression but had a limited effect on CD206 expression. These results are consistent with our phagocytosis assay.

Similar to previous studies reporting that recombinant LILRA3 could induce the proliferation of $CD8^{+}$ T-cells and NK cells in mixed lymphocyte reactions (MLR)[21], we observed that increased LILRA3 expression could enhance U937 cells proliferation in vitro. PI3K/Akt and PI3K/MEK/Erk signaling are two essential pathways involved in cell survival and growth[40,41], and we found that LILRA3 activated these two pathways to regulate cell proliferation. Foxo3a, a tumor-suppressive transcriptional factor, is known to regulate various cellular events such as metabolism, proliferation, tumorigenesis, cell cycle arrest, apoptosis and longevity[42-44]. Previous studies have shown that the two key kinases Akt and Erk have the ability to regulate the transcriptional activity of Foxo3a via specific phosphorylation[45-47]. Akt- or MEK-mediated phosphorylation causes Foxo3a to bind to the 14-3-3 protein, followed by translocation from the nucleus to the cytoplasm, with inhibition of transcriptional activity[43,44,48]. The Ras/Erk pathway also regulates cell growth by inhibiting Foxo3a via MDM2-mediated degradation[44]. In our study, we observed that Foxo3a phosphorylation was decreased when LILRA3 was overexpressed. In contrast, phosphorylation of Foxo3a was conspicuously reduced when LILRA3-overexpressing cells were pre-incubated with specific inhibitors of MEK, Akt and PI3K. One possible explanation for this inconsistency is that LILRA3 might directly/indirectly affect Foxo3a phosphorylation through an unknown mechanism. Further experiments are needed to elucidate this mechanism.

Taken together, we found that LILRA3 might function as an anti-inflammatory molecule in U937 cells based on the following: 1) LILRA3 directly inhibited inflammation by decreasing proinflammatory cytokine secretion; 2) LILRA3 restrained monocytes in circulation system by attenuating their migration ability and accordingly reduced Mø and DCs in tissues, thereby restricting activation of adaptive immunity; 3) increased LILRA3 enabled monocytic cells to engulf more pathogens in circulation; 4) LILRA3 increased the proliferation of immunocytes, such as monocytes, $CD8^+$ T-cells and NK cells, thus allowing these cells to kill more pathogens and microorganisms.

Collectively, for the first time, we report that LILRA3 expression is markedly increased in IBD patients. LILRA3 might function as an anti-inflammatory modulator in innate immune responses and regulate cell proliferation through a combination of Akt and MEK/Erk signaling pathways. Further efforts are needed to explore the exact role of LILRA3 in adaptive immunity.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (grant number 81470823, 81800488).

The authors thank all individuals who participate in this study.

Conflicts of interest statement

There is no financial conflict of interest to declare for any of the authors.

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Table 1: Association between LILRA3 polymorphism and IBD development

Name	[a]HC (N=509) N (%)	CD (N=185) N (%)	CD (N=185) *P*	CD (N=185) [b]P_{FDR}	CD (N=185) OR (95%CI)	UC (N=193) N (%)	UC (N=193) N (%)	UC (N=193) *P*	UC (N=193) P_{FDR}	UC (N=193) P_{FDR}	UC (N= OR (95%
6.7 kb deletion	450(88.4)	169(91.4)					175(90.7)				
-/-,-/+											
+/+[c]*	59(11.6)	16(8.6)	0.27	0.41	1.39(0.78-2.47)	18(9.3)	18(9.3)	0.39	0.96	0.96	1.28(2.22)
Allele											
-	660(64.8)	244(65.9)				252(65.3)	252(65.3)				
+*	358(35.2)	126(34.1)	0.70	0.90	1.05(0.82-1.35)	134(34.7)	134(34.7)	0.87	0.87	0.87	1.02(1.30)
rs103294	453(89.0)	165(89.2)	0.94	0.94		172(89.1)	172(89.1)	0.96	0.96	0.96	
TT, CT	56(11.0)	20(10.8)	**0.04**	0.12	1.02(0.59-1.75)	21(10.9)	21(10.9)	0.52	0.78	0.78	1.01(1.72)
CC*	696(68.4)	274(74.1)	0.17	0.40		257(66.6)	257(66.6)	0.80	0.96	0.96	
Allele	322(31.6)	96(25.9)	0.90	0.90	**1.32(1.01-1.73)**	129(33.4)	129(33.4)	0.30	0.78	0.78	0.92(1.18)
T	495(97.2)	176(95.1)				187(96.9)	187(96.9)				
C*	14(2.8)	9(4.9)			0.55(0.24-1.30)	6(3.1)	6(3.1)				0.88(2.33)
rs410852	864(84.9)	315(85.1)				336(87.0)	336(87.0)				
GG, AG	154(15.1)	55(14.9)			1.02(0.73-1.43)	50(13.0)	50(13.0)				1.20(1.69)
AA*											
Allele											
G											
A*											

[a]HC: healthy controls

[b]P_{FDR}: represents the Pvalue adjusted by false discovery rate correction.

[c]*: represents the wild genotype or wild allele.

Table 2: Association between LILRA3 polymorphism and IBD in subphenotype-control cohort

Phenotype	6.7 kb deletion	6.7 kb deletion	6.7 kb deletion	6.7 kb deletion	rs103294	rs103294	rs103294	rs103294	rs103294	rs103294	rs103294	rs410852	rs410852	rs410852	rs410…
	-/-,-/+ *VS* +/+	-/-,-/+ *VS* +/+	*P*	OR(95%CI)	TT,CT *VS* CC	TT,CT *VS* CC	*P*	*P*	OR(95%CI)	OR(95%CI)			GG,AG *VS* AA	GG,AG *VS* AA	GG, *VS* AA
[d]**HC (N)**	450	59	Ref	Ref	453	56	56	Ref	Ref	Ref	Ref			495	14
CD (N)	169	16			165	20	20							176	9
Diagnosis age [e](yr) <17	13	2	1.00	0.79(0.19-3.87)	12	2	2	1.00	1.00	0.74(0.16-3.40)	0.74(0.16-3.40)			14	0
17-40	122	10		1.60(0.80-3.22)	113	13	13	0.83	0.83	1.08(0.57-2.03)	1.08(0.57-2.03)			120	6
>40	34	4	1.00	1.11(0.38-3.25)	40	5	5	1.00	1.00	0.99(0.38-2.61)	0.99(0.38-2.61)			42	3
Site of disease															
L1 Ileal	45	3	0.26	1.97(0.59-6.53)	43	5	5	0.90	0.90	1.06(0.40-2.80)	1.06(0.40-2.80)			45	3
L2 Colonic	51	8	0.66	0.84(0.38-1.85)	51	8	8	0.56	0.56	0.79(0.36-1.75)	0.79(0.36-1.75)			56	3
L3 Ileocolitis	69	5	0.21	1.81(0.70-4.67)	67	7	7	0.69	0.69	1.18(0.52-2.70)	1.18(0.52-2.70)			71	3
L4 Upper [f]GI	4	0	1.00	1.13(1.10-1.17)	4	0	0	1.00	1.00	1.12(1.09-1.16)	1.12(1.09-1.16)			4	0
Disease behavior															

B1 Non-stricturing Non-penetrating	114 33 22 26 175	11 4 1 1 18	0.37 1.00 0.46 0.34	1.36(0.69-2.67) 1.08(0.37-3.16) 2.88(0.38-21.79)	113 31 21 25 172	12 6 2 2 21	12 6 2 2 21	0.65 0.49 0.99 0.79	0.65 0.49 0.99 0.79	1.16(0.60-2.25) 0.64(0.26-1.60) 1.30(0.30-5.68)	1.16(0.60-2.25) 0.64(0.26-1.60) 1.30(0.30-5.68)		122 33 21 26 187	3 4 3 1 6
B2 Stricturing				3.41(0.45-25.59)						1.55(0.36-6.70)	1.55(0.36-6.70)			
B3 Penetrating														
Perianal disease														
UC (N)														
Diagnosis age (yr)	3	0	1.00	1.13(1.10-1.17)	22	0	0	0.20	0.20	1.12 (1.09-1.16)	1.12 (1.09-1.16)		2	0
<17	96	8	0.25	1.57(0.73-3.40)	73	10	10	0.78	0.78	0.90 (0.44-1.85)	0.90 (0.44-1.85)		101	2
17-40	76	10	0.99	1.00(0.49-2.03)	77	11	11	0.68	0.68	0.87 (0.43-1.73)	0.87 (0.43-1.73)		84	4
>40	19	4	0.61	0.62(0.21-1.89)	19	4	4	0.54	0.54	0.59 (0.19-1.79)	0.59 (0.19-1.79)		22	1
Disease location	70	8	0.73	1.15(0.53-2.50)	68	10	10	0.64	0.64	0.84 (0.41-1.73)	0.84 (0.41-1.73)		76	2
E1 Proctitis	86	6	0.15	1.88(0.79-4.49)	85	7	7	0.33	0.33	1.50 (0.62-3.41)	1.50 (0.62-3.41)		89	3
E2 Left-sided														
E3 Extensive														

[d]HC: healthy controls

[e]yr: year

[f]GI: gastrointestinal

Figure Legends

Figure 1: LILRA3 is increased in IBD patients compared with healthy controls. (A) Impact of the 6.7-kb deletion on LILRA3 mRNA expression in peripheral blood. mRNA expression was evaluated by

qRT-PCR assay. Data are expressed as the mean ± SD. Sample groups and genotypes were indicated. ** $p < 0.01$,*** $p < 0.001$ versus same genotypes in healthy controls (HC). (B) mRNA expression of LILRA3 in blood when samples homozygous for the deletion (-/-) were excluded. (C) mRNA expression of LILRA3 in the intestine. Subjects possessing undetectable LILRA3 levels were excluded. (D) Protein expression of LILRA3 in the intestine. Protein was extracted from 8 non-inflammatory bowel disease (NIBD), 7 CD and 10 UC samples. GAPDH was used as a loading control. (E) H&E staining (the left two columns) and immunohistochemistry for LILRA3 (the right column) were performed on 3 μm colon sections, and representative pictures are shown. Scale bar is 50 μm for the left column, 30 μm for the right two columns. (F) Negative control staining for immunohistochemistry. Sections were treated with PBS instead of the primary antibody. Scale bar is 30 μm. (G) Quantification of LILRA3$^+$ cells in intestinal biopsies. A total of 20 NIBD, 14 CD and 14 UC samples were enrolled. Each experiment was repeated for at least three times. $^{*}p < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.001$ versus HC or NIBD groups.

Figure 2: LILRA3 is mainly expressed on CD68$^+$macrophages in human intestinal. (A) Localization of LILRA3 expression by Immunofluorescence. CD68 monoclonal antibody was used to mark macrophages. LILRA3 protein were expressed in CD68$^+$macrophages and CD patients possessed more CD68$^+$LILRA3$^+$ cells than Non-IBD group. Scale bar is 20 μm for each column. (B) Negative control staining for immunofluorescence. Sections were treated with PBS instead of the primary antibody. (C) Quantification of LILRA3$^+$CD68$^+$ cells in intestinal biopsies. Each group includes 5 samples. Each experiment was repeated for at least three times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 3: Effect of LILRA3 on U937 cells and related mechanisms. (A) Verification of LILRA3 expression in the two established cells by qRT-PCR, Elisa and western blotting. (B) Effect of LILRA3 on cytokine secretion by U937 cells. Expression of IFN-γ, TNF-α and IL-6 was assessed by Elisa. Data are expressed as mean ± SD. (C) Supernate collected from the LILRA3-overexpressing U937 cell lines was used to culture the U937 cells, and secretion of IFN-γ, TNF-α as well as IL-6 were significantly decreased. Cells cultured for 12 hours showed a more significant effect of inhibition. (D) Representative flow cytometry analysis of apoptosis stained with annexin V-PE and 7-AAD among LILRA3-overexpressing U937 cells and U937 cells expressing the null vector. (E) Statistical analysis of apoptotic rate (%) in the two established cell lines. (F) Effect of LILRA3 on U937 cell migration. Cell counts migrated into the lower chamber were analyzed between the two cell lines. (G) Impact of LILRA3 on chemokine secretion in U937 cells by Elisa assay. CCL2, CCL3, IL-8 and CXCL10 were dramatically decreased by LILRA3. (H) Recombinant CCL2 and CXCL8 were added to the upper chamber in the migration assay, and LILRA3 induced impaired cell migration was reversed by the exogenous IL-8. Data are expressed as mean ± SD. Each experiment was repeated for at least three times.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 4: LILRA3 enhances the phagocytosis ability of U937 cells by upregulating CD36 plasma membrane expression . (A) Representative FCM analysis of phagocytosis by U937 cells at 0.5, 6, 12 and 24 hours. The percentage of GFP$^+$ Texas Red$^+$ cells was analyzed. (B) Phagocytosis rates were statistically analyzed between LILRA3-overexpressing U937 cells and U937 cells expressing the null vector. (C, D and E) Effects of LILRA3 on CD36 and CD206 expression according to qRT-PCR and western blotting. (F) The specific antibody of CD36 could significantly decrease the phagocytosis ability of LILRA3-overexpression cell lines at a concentration dependent manner. Each experiment was repeated for at least three times.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 5: LILRA3 increases U937 cell proliferation and the possible signaling pathway . (A) Proliferation of the LILRA3-overexpressing U937 cells and null vector U937 cells were analyzed by CCK-8 assay. OD at 450 nm was detected at 0, 12, 24, 48 and 72 hours. (B, C and D) Western blot analysis of p-Akt, Akt, p-MEK, MEK, p-Erk, Erk, p-P38 MAPK and P38 MAPK in LILRA3 overexpressing cells (OE) and null vector cells (NV). (E, F and G) Western blot analysis of p-PDK1, PDK1, p-c-Raf, p-PI3K, PI3K, p-Foxo3a and Foxo3a, p-Foxo4, p-Foxo1 in LILRA3 overexpressing cells (OE) and null vector cells (NV). Each experiment was repeated for at least three times.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus null vector cells. GAPDH abundance was used as a control.

Figure 6: LILRA3 might regulate U937 cells proliferation through PI3K/Akt and PI3K/MEK/Erk signaling pathways . (A-C) Western blot analysis for the inhibitory efficiency of GSK1120212, MK-2206-2HCL and LY294002 and the expression of the related downstream proteins (p-Foxo3a and Foxo3a for (A) and (B), p-Akt, Akt, p-MEK, MEK, p-Foxo3a and Foxo3a for (C)). GAPDH abundance was used as a control. (D) CCK-8 assay to detect the proliferation of LILRA3-overexpressing U937 cells (OE) after pretreated with three specific inhibitors for indicated time. NV: Null vector U937 cells. Each experiment was repeated for at least three times. $^{*}p < 0.05$, $^{\#}p < 0.01$ versus LILRA3-overexpressing U937 cells pretreated with DMSO.

Figure 7: A schematic of the possible signaling pathway of LILRA3 . LILRA3 might interact with one of the listed or unknown receptors and trigger PI3K/Akt and PI3K/MEK/Erk signaling to regulate U937 cell proliferation. LILRA3 might directly/indirectly inhibit Foxo3a phosphorylation. Whether Foxo3a plays a role in LILRA3-induced cell proliferation needs to be further investigated.

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