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MicroRNA-155 targets p65 to regulate PD-L1 expression in the early pregnancy endometrium

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Reproductive disorders in dairy cows represent a significant challenge to the advancement of the dairy industry. Pregnancy success is closely related to the mechanism of immune tolerance, with the PD-1/PD-L1 signaling pathway playing a role in immune regulation, which is associated with immune tolerance and pregnancy maintenance. MicroRNAs can regulate pivotal molecules within the signaling pathway, physiological activities, and disease processes. p65 and PD-L1 expression was significantly increased in the early pregnant uterine epithelium. In contrast, the expression of p65 and PD-L1 was homogeneous in the endometrial epithelium of E_2 and P_4 co-stimulated bEECs and changed with the stimulation time and concentration. MiR-155 expression was significantly reduced in the early pregnant uterine epithelium. p65 was identified as a molecular target of miR-155 using a dual luciferase assay and mimics/inhibitor transfection, and miR-155 inhibited p65 expression by binding to the 3′-UTR of p65 mRNA. The regulation of PD-L1 expression by p65 was confirmed through the knockdown of p65 by si-p65 and the overexpression of p65 by pcDNA3.1-p65. In the context of pregnancy, miR-155 was observed to target p65, thereby regulating PD-L1 expression at the endometrial epithelium.

Keywords Cows, Early pregnant, Uterine epithelium, PD-L1, miR-155, p65

Reproductive disorders in dairy cows represent a significant challenge to the advancement of the dairy industry, embryo loss and early fetal abortion are pivotal factors contributing to pregnancy failure in dairy cows[1,2]. Most embryo loss in dairy cows occurs during the initial 60 days of pregnancy[3–5]. Severe embryo loss during this early stage results in a calving rate of less than 30% for artificial insemination on numerous intensive dairy farms, which significantly impacts the reproductive performance of dairy cows and the economic benefits derived from them[3–5].

The success of pregnancy is inextricably linked to the mechanism of maternal uterine immune tolerance to "homozygous hemizygous" embryos[6]. A healthy pregnancy is contingent upon the maternal immune system's recognition of the paternally expressed antigens of the fetus, thereby developing maternal immune tolerance to the fetus[6–8]. The differential expression of chemokines at the maternal-fetal interface in early pregnancy has been demonstrated to selectively recruit maternal immune cells, participate in embryonic trophoblast invasion and placental angiogenesis, and contribute to the formation of a favorable uterine immune microenvironment in the embryo[9,10]. A failure of recognition mechanisms and an imbalance of cytokine homeostasis may result in a reduction of maternal immune tolerance, which could potentially lead to the development of abnormal pregnancies or even pregnancy failure[11]. It is therefore evident that the establishment of immune tolerance at the maternal-fetal interface in the early stages of pregnancy, and the maintenance of this tolerance throughout the remainder of the gestation period, are critical to the overall outcome of the pregnancy. Estrogens play a crucial role in endometrial receptivity during the periconceptional period[12]. In pregnancies conceived naturally, lower estrogen levels are hypothesized to trigger abnormal placentation. Conversely, an estrogen excess appears to worsen pregnancy development and the ensuing outcomes[13]. Progesterone plays a significant role in the maintenance of pregnancy[14]. Progesterone inhibits maternal immune reactions at the uteroplacental interface and promotes maternal immune tolerance to fetal alloantigens[15,16].

Programmed death ligand 1 (PD-L1) is a type I transmembrane surface glycoprotein belonging to the B7 family of co-stimulatory molecules, PD-L1 acts as a ligand for programmed cell death protein 1 (PD-1)[17]. Involvement of PD-1/PD-L1 signaling pathway in the induction of maternal-fetal immune tolerance and pregnancy maintenance[18]. The PD-1/PD-L1 signaling pathway plays a pivotal role in maintaining equilibrium between

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Th1/Th2 cytokine levels in various cells at the maternal-fetal interface, thereby fostering an immune-tolerant environment throughout the gestational period[19,20]. The PD-1/PD-L1 signaling pathway exerts a regulatory influence on the innate immune response and has been demonstrated to regulate macrophage differentiation and function effectively[21,22]. An imbalance in the PD-1/PD-L1 signaling pathway results in the dysregulation of maternal tolerance, which can subsequently lead to the development of pregnancy abnormalities, including embryo loss, pre-eclampsia, and miscarriage[23]. A recent study has revealed that the fetus and placenta exhibit a high expression of PD-L1 during pregnancy[24]. Consequently, the uterine epithelium, as a constituent of the gestational milieu, is also required to maintain elevated PD-L1 expression throughout pregnancy to sustain immune tolerance. Furthermore, it should enhance PD-L1 expression during the initial stages of pregnancy to facilitate the formation of gestational immune tolerance. Macrophage colony-stimulating factor (M-CSF) and interleukin-34 (IL-34) are unique cytokines that regulate the maturation and differentiation of monocytes and macrophages, they are two ligands for CSF-1 receptor (CSF1R)[25]. It is hypothesized that M-CSF and IL-34 play a role in the development of cancers associated with macrophages, such as malignant pleural mesothelioma[26]. The uterine epithelium should express high levels of M-CSF and IL-34 to regulate the immune process of macrophages in the uterus during pregnancy.

The NF-κB signaling pathway represents a classical and pivotal cellular signaling pathway, exerting a profound influence on a multitude of physiological and pathological processes, p65 and p50 heterodimers are responsible for regulating the transcription of target genes when the classical NF-κB pathway is activated[27,28]. The coordinated expression of NF-κB transcription factors throughout pregnancy is of critical importance for the completion of the gestational period. Embryo implantation, vascular remodeling, placental formation, fetal development, and delivery all require NF-κB transcription factors at appropriate levels to ensure that the pregnancy proceeds without complications[29,30]. The dysregulation of NF-κB transcription factors at various stages has been linked to a range of pregnancy-related complications, including pre-eclampsia[31,32], miscarriage[33], and preterm labour[29]. Additionally, NF-κB transcription factors have been associated with several pregnancy-related disorders, including gestational diabetes mellitus[34], essential fetal hypertension[35], and endometrial fibrosis[36].

MicroRNAs (miRNAs) are endogenous, cellular, and evolutionarily conserved non-coding RNAs of approximately 20–25 nucleotides in length, and miRNA inhibits target mRNA translation, induce shearing, and rapid nuclear deadenylation by binding to the 3' non-coding region of the target gene, thereby reducing the stability of the target mRNA or negatively regulating the expression of the gene by inhibiting the translation of the mRNA[37,38]. MiRNAs are involved in several key processes within the immune system, including the proliferation and differentiation of immune cells, intracellular signal transduction, cytokine release, and both natural and acquired immune responses, and play an important regulatory role in immune responses and disease development[39]. The involvement of miRNAs in the process of pregnancy, the regulation of the expression of genes associated with pregnancy, and the regulation of immune processes during pregnancy[40–42]. MiR-155 is a multifunctional miRNA that regulates key molecules in multiple signaling pathways and plays a critical role in a variety of diseases and physiological processes. It has been demonstrated that miR-155 regulates insulin sensitivity in tissues and organs such as the liver and skeletal muscle, and plays a pivotal role in the pathogenesis of diabetes and its associated complications[43]. Additionally, miR-155 has been identified as a critical mediator in the preeclampsia[44,45].

The objective of this experiment was to identify differentially expressed genes in the endometrium of dairy cows in early pregnancy and to investigate the role of miR-155 in the regulation of PD-L1 expression through p65. The findings provide insights into the study of immune tolerance in pregnancy and a theoretical basis for the reduction of reproductive disorders in dairy cows.

Result

PD-L1 expression was increased in the early pregnant endometrial tissue

Endometrial epithelial cells were monolayer/pseudo-complex columnar epithelial cells with rod-shaped nuclei, and early pregnancy endometrial epithelium was thickened with round nuclei (Fig. 1A). The results of immunohistochemistry demonstrated that the expression levels of p65 and PD-L1 at the endometrial epithelium were significantly higher in the early pregnancy group than in the non-pregnancy group (Fig. 1B). The qPCR result demonstrated that the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA were significantly higher in the early pregnancy group than in the non-pregnancy group (Fig. 1C). The expression levels of p65, phosphorylated p65, and PD-L1 protein were also significantly higher in the early pregnancy group than in the non-pregnancy group (Fig. 1D).

The expression levels of PD-L1 and p65 in cells stimulated with E_2 or P_4

We used E_2 and P_4 to simulate pregnancy and investigate the expression of relevant genes in the context of pregnancy. bEECs were stimulated with different concentrations of E_2 (12.5 pg/mL, 25 pg/mL, 50 pg/mL) at different times (6 h, 12 h, 24 h). qPCR results demonstrated that the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA were influenced by both time and concentration (Fig. 2A–C). The changes in the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA were more similar after 24 h of E_2 stimulation, 50 pg/mL E_2 stimulation significantly promoted the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA (Fig. 2C). bEECs were stimulated with different concentrations of P_4 (1.3 ng/mL, 2.5 ng/mL, 5 ng/mL) at different times (6 h, 12 h, 24 h). qPCR results showed that the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA changed with different time and concentration (Fig. 2D–F). The changes in the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA were more similar after 12 h of P_4 stimulation, the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA gradually increased with increasing P_4 concentration (Fig. 2E). At the protein level, after 6–24 h of stimulation, the expression levels of p65, phosphorylated p65, and PD-L1 protein demonstrated a decline with elevated E_2 concentrations and an increase with rising P_4 concentrations. (Fig. 2G–I). After 12 h of stimulation,

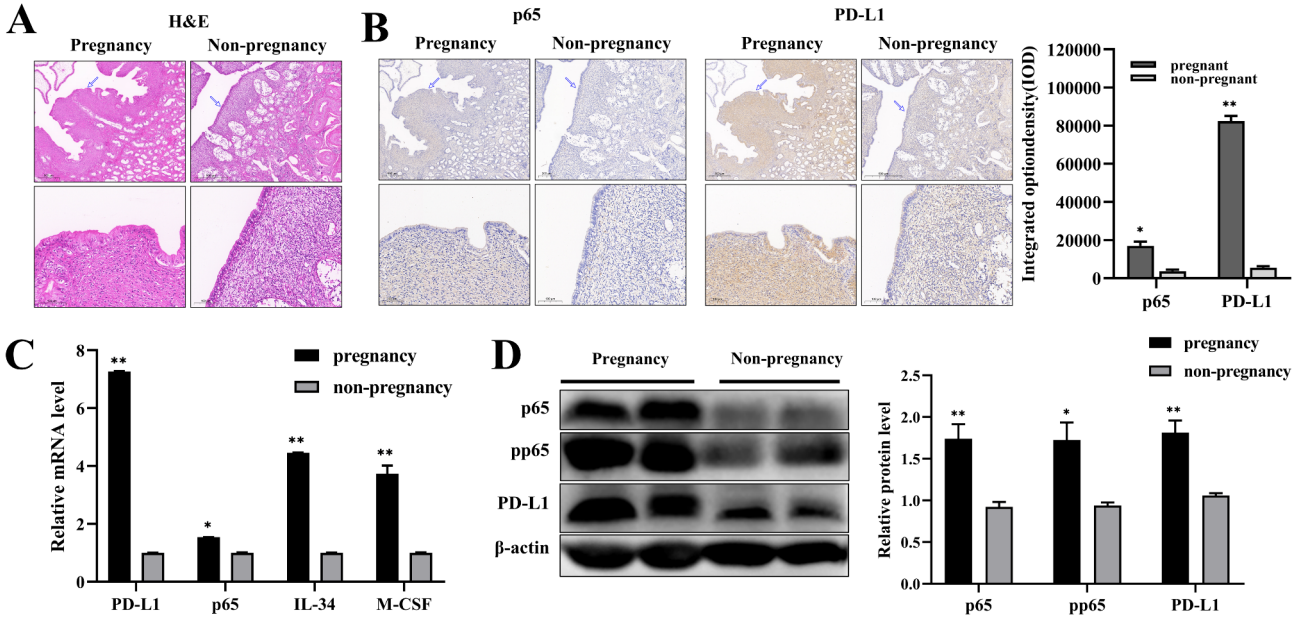

Fig. 1. PD-L1 expression was increased in the early pregnant endometrial tissue. (**A**) H&E staining of uterine tissues ($n = 3$). (**B**) The expression levels of p65 and PD-L1 were measured by immunohistochemical staining in the uterine horn ($n = 3$). (**C**) The mRNA expression levels of p65, PD-L1, M-CSF, and IL-34 were detected by qPCR. GAPDH was used as an endogenous control. (**D**) The protein expression levels of p65, pp65, and PD-L1 were measured by Western blot. β-Actin was used as an endogenous control. Data are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

the expression levels of p65, phosphorylated p65, and PD-L1 protein were slightly but not significantly increased (Fig. 2H).

The expression levels of PD-L1 and p65 in cells co-stimulated with E_2 and P_4

To investigate the combined effects of E_2 and P_4 on the genes involved, we used E_2 and P_4 co-stimulation for further experiments. bEECs were co-stimulated with different concentrations of E_2 (12.5 pg/mL, 25 pg/mL, 50 pg/mL) and P_4 (1.3 ng/mL, 2.5 ng/mL, 5 ng/mL) at different times (6 h, 12 h, 24 h). E_2 and P_4 co-stimulation could affect the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA. However, the trend of p65, PD-L1, M-CSF, and IL-34 mRNA expression was found to be different (Fig. 3A-C). After 6 h of 12.5 pg/mL E_2 and 1.3 ng/mL P_4 co-stimulation, the expression levels of p65, phosphorylated p65, and PD-L1 protein were all slightly increased but not significantly, the remaining group protein expressed similarly to the NC group (Fig. 3D). After 12 h of co-stimulation, there was an increase in the expression levels of p65, phosphorylated p65, and PD-L1 protein with elevated E_2 concentrations and P_4 concentrations (Fig. 3E). After 24 h co-stimulation, the expression levels of p65, phosphorylated p65, and PD-L1 protein were all slightly but not significantly decreased (Fig. 3F).

p65 was the molecular target of miR-155

We used TargetScan to predict the binding potential of miR-155 and p65. We found that p65 is one of the molecular targets of miR-155 and the miR-155 binding site is located at the 3'-UTR of p65 mRNA, then, we constructed wild/mutant types of the 3′-UTR with psiCHECK™-2 (Fig. 4A). The expression level of miR-155 in the early pregnancy group was found to be significantly lower than in the non-pregnancy group (Fig. 4B). The dual-luciferase reporter assay showed that miR-155 mimics significantly suppressed luciferase activity in comparison to the control mimic (Fig. 4C). The qPCR result showed the transfection efficiency of the transfected miR-155 mimics (Fig. 4D). MiR-155 mimics significantly reduced the expression level of p65 protein (Fig. 4E). MiR-155 inhibitors significantly increased the expression level of p65 protein (Fig. 4F). These results indicated that miR-155 directly bound to the 3′-UTR of the p65 mRNA, thereby regulating its protein expression.

p65 regulated PD-L1 protein expression

In comparison to siRNA NC (si-NC), knockdown of p65 protein expression with p65 siRNA (si-p65) reduced the expression levels of phosphorylated p65 and PD-L1 protein (Fig. 5A). The qPCR result showed the transfection efficiency of transfected pcDNA3.1-p65 (Fig. 5B). In comparison to pcDNA3.1-NC, overexpression of p65 protein expression with pcDNA3.1-p65 increased the expression levels of phosphorylated p65 and PD-L1 protein (Fig. 5C). These results showed that p65 regulated PD-L1 protein expression.

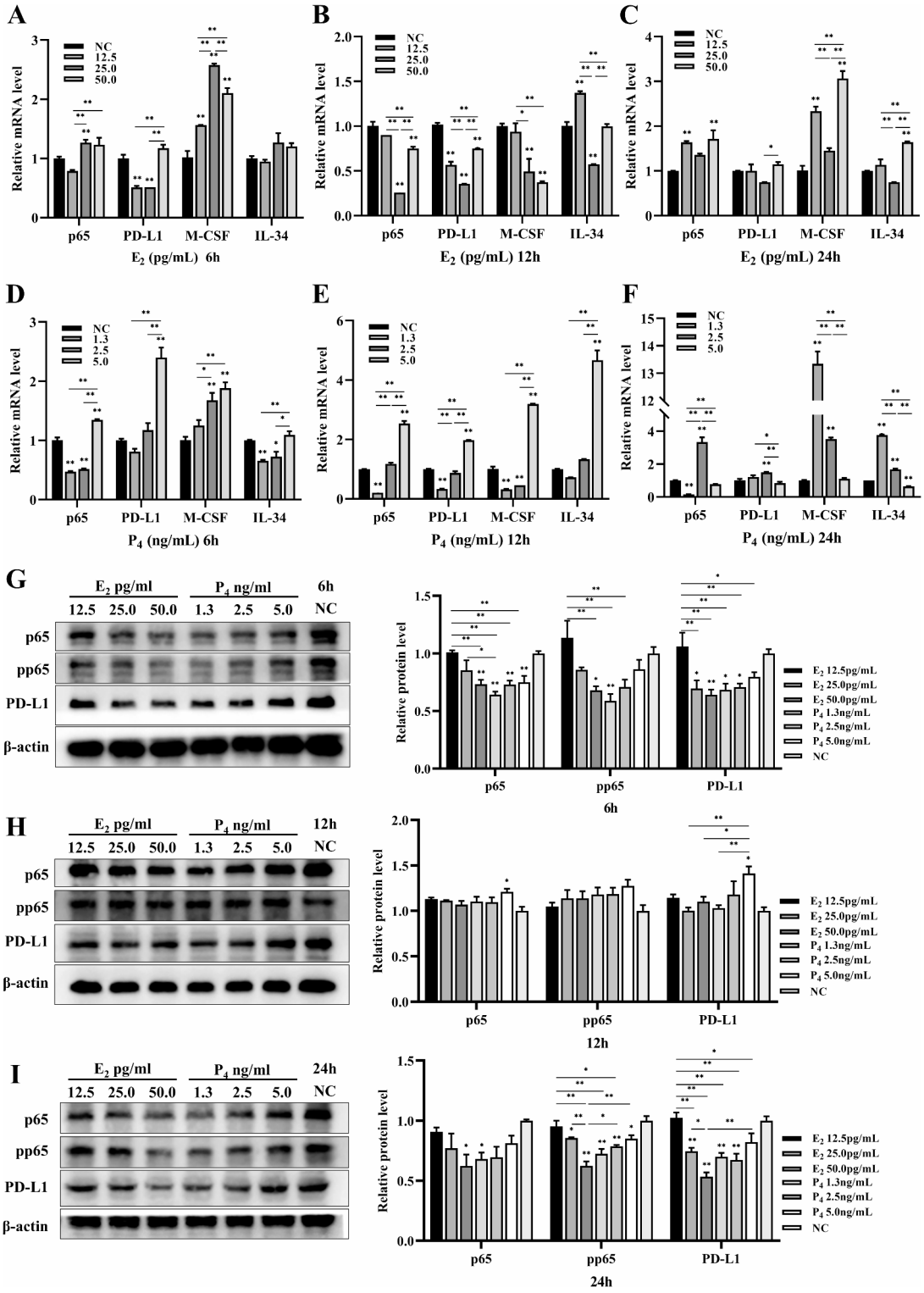

Fig. 2. The expression levels of PD-L1 and p65 in cells stimulated with E_2 or P_4. (**A**–**C**) The mRNA expression levels of p65, PD-L1, M-CSF, and IL-34 in bEECs after stimulation with different concentrations of E_2 (12.5 pg/mL, 25 pg/mL, 50 pg/mL) for 6 h (**A**), 12 h (**B**), 24 h (**C**) were detected by qPCR. GAPDH was used as an endogenous control. (**D**–**F**) The mRNA expression levels of p65, PD-L1, M-CSF, and IL-34 in bEECs after stimulation with different concentrations of P_4 (1.3 ng/mL, 2.5 ng/mL, 5 ng/mL) for 6 h (**D**), 12 h (**E**), 24 h (**F**) were detected by qPCR. GAPDH was used as an endogenous control. (**G**–**I**) The protein expression levels of p65, pp65, and PD-L1 in bEECs after stimulation with different concentrations of E_2 (12.5 pg/mL, 25 pg/mL, 50 pg/mL), P_4 (1.3 ng/mL, 2.5 ng/mL, 5 ng/mL) for 6 h (**G**), 12 h (**H**), 24 h (**I**) were detected by Western blot. β-Actin was used as an endogenous control. Data are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

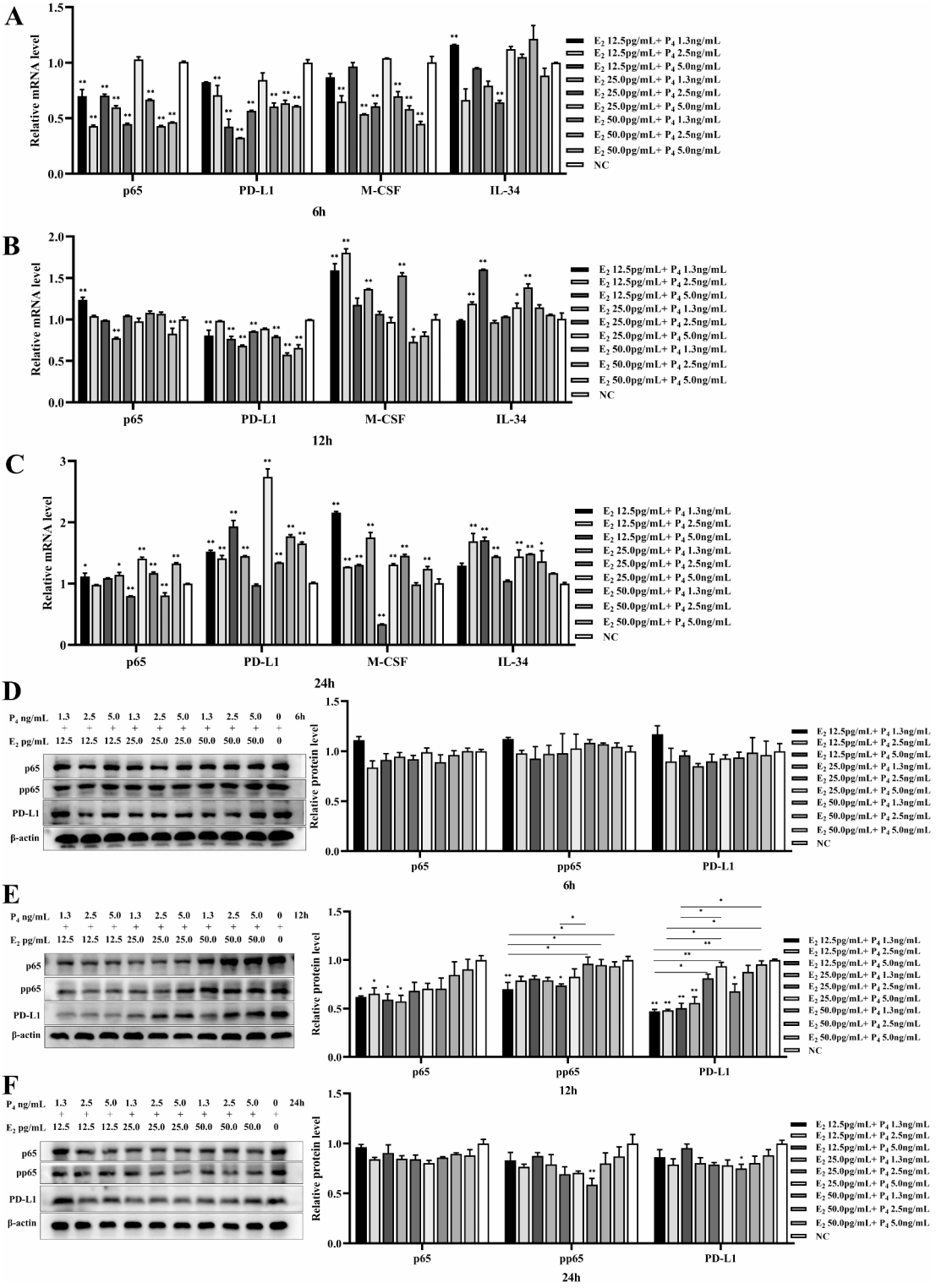

Fig. 3. The expression levels of PD-L1 and p65 in cells co-stimulated with E_2 and P_4. (**A**–**C**) The mRNA expression levels of p65, PD-L1, M-CSF, and IL-34 in bEECs after co-stimulation with different concentrations of E_2 (12.5 pg/mL, 25 pg/mL, 50 pg/mL) and P_4 (1.3 ng/mL, 2.5 ng/mL, 5 ng/mL)for 6 h (**A**), 12 h (**B**), 24 h (**C**) were detected by qPCR. GAPDH was used as an endogenous control. The statistical differences between treatment groups can be found in the Supplementary Table. (**D**–**F**) The protein expression levels of p65, pp65, and PD-L1 in bEECs after co-stimulation with different concentrations of E_2 (12.5 pg/mL, 25 pg/mL, 50 pg/mL) and P_4 (1.3 ng/mL, 2.5 ng/mL, 5 ng/mL) for 6 h (**D**), 12 h (**E**), 24 h (**F**) were detected by Western blot. β-Actin was used as an endogenous control. Data are expressed as the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

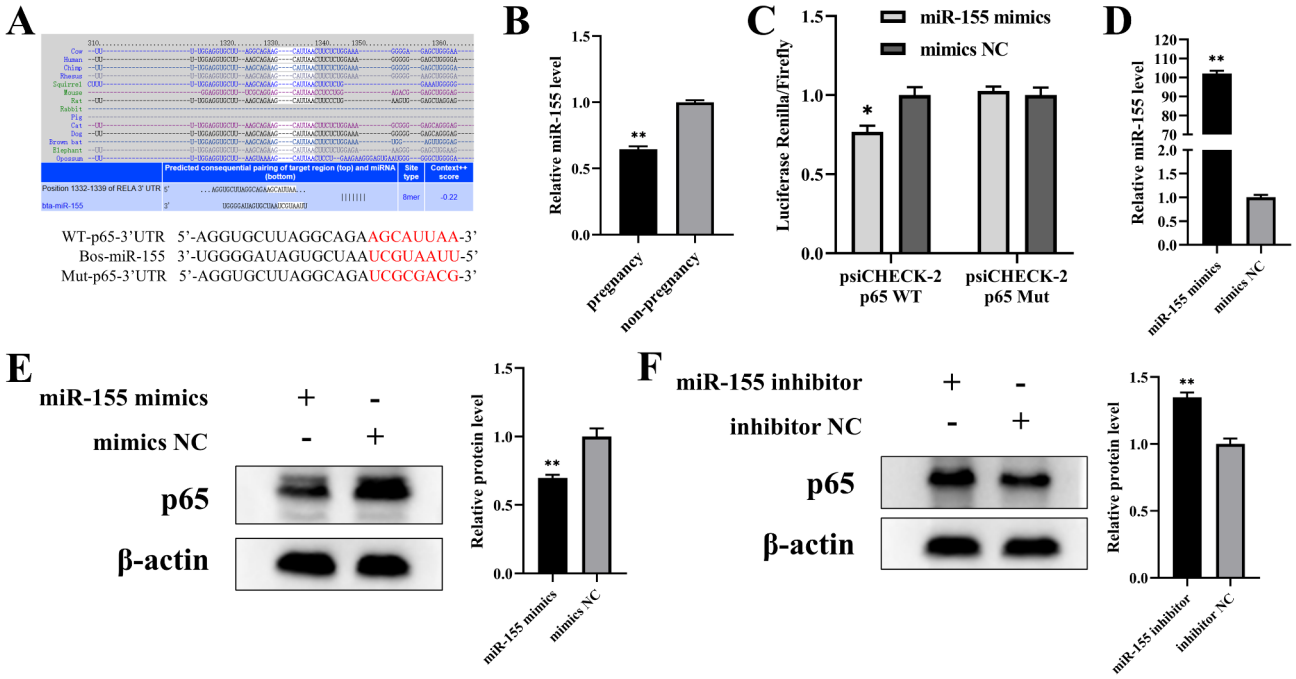

Fig. 4. p65 was the molecular target of miR-155. (**A**) TargetScan was used for the binding prediction of miR-155 and p65 mRNA 3′-UTR. The mutant sequence was designed thereby. (**B**) The expression level of miR-155 was detected by qPCR. U6 was used as an endogenous control. (**C**) Luciferase activity of psi-CHECK2-p65 WT vector and psi-CHECK2-p65 MuT vector in the mimics group and mimics NC group were detected. The dual-luciferase reporter assay was carried out in HEK293T cells. The luciferase activities were normalized by the ratio of frefly and Renilla luciferase signals. (**D**) MiR-155 mimics and mimics NC were transfected into bEECs. After transfection for 24 h, the expression level of miR-155 was measured by qPCR. U6 was used as an endogenous control. (**E**) MiR-155 mimics and mimics NC were transfected into bEECs. After transfection for 48 h, the protein expression level of p65 was measured by Western blot. β-Actin was used as an endogenous control. (**F**) MiR-155 inhibitor and inhibitor NC were transfected into bEECs. After transfection for 48 h, the protein expression level of p65 was measured by Western blot. β-Actin was used as an endogenous control. Data are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

Discussion

The inability to establish gestational immunological tolerance, or the presence of reduced or imbalanced immunological tolerance, can result in embryo loss or abortion, which can have a significant impact on the reproductive performance of dairy cows, affecting the calving rate of AI in intensive dairy farms and reducing economic benefits[3]. The PD-1/PD-L1 signaling pathway plays a pivotal role in the induction of maternal-fetal immune tolerance and the maintenance of pregnancy, regulating the immune response process[18]. The results of this experiment demonstrated a notable elevation in PD-L1 expression within the context of early pregnant endometrial tissue. It can be posited that the endometrial epithelium, as an integral component of the pregnancy environment, sustains elevated PD-L1 expression throughout gestation to facilitate the establishment and sustenance of pregnancy-specific immune tolerance. The PD-1/PD-L1 signaling pathway also exerts regulatory control over the differentiation and function of macrophages, furthermore, macrophages are instrumental in the establishment and maintenance of immune tolerance during pregnancy[21], M-CSF and IL-34 are essential proteins for the proliferation and differentiation of monocytes/ macrophages[25]. The increased expression levels of M-CSF and IL-34 mRNA observed in the early pregnant endometrial tissue were intended to meet the demands of monocyte/macrophage proliferation and differentiation in the pregnancy environment.

p65 is a pivotal protein in the NF-κB signaling pathway, regulating the transcription of a multitude of genes[27]. p65 undergoes phosphorylation in the nucleus, where it binds to DNA and regulates gene transcription[46]. Furthermore, a notable elevation in the expression levels of p65 and pp65 was discerned in the early pregnant endometrial tissue. It was postulated that p65 might regulate PD-L1 expression, thereby establishing or maintaining immune tolerance during pregnancy. The immunohistochemical examination of tissue sections revealed a significant increase in p65 and PD-L1 expression in the early pregnant endometrial tissue.

The use of E_2 and P_4 to stimulate bEECs to create a pregnancy-like environment revealed that the expression levels of p65, PD-L1, M-CSF, and IL-34 mRNA were influenced by both the stimulation concentration and the stimulation time. In contrast, the expression levels of p65, pp65, and PD-L1 protein demonstrated greater uniformity in response to varying stimulation concentrations and stimulation times. The convergence of p65, pp65, and PD-L1 protein expression suggested a potential link between them. It was proposed that p65 may be involved in the regulation of PD-L1 expression.

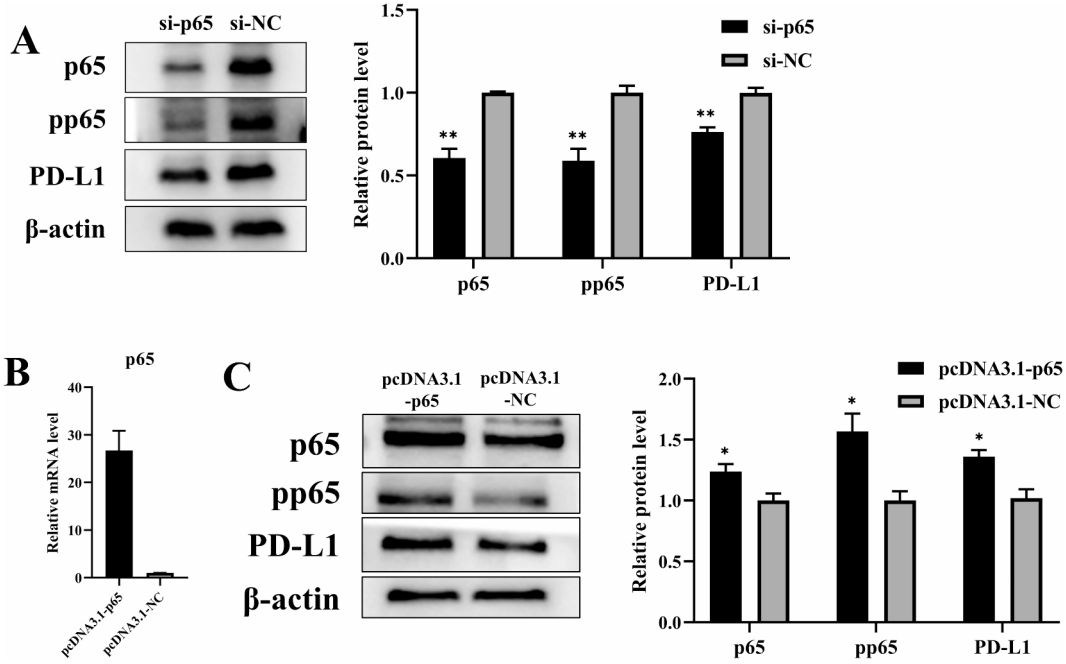

Fig. 5. p65 regulated PD-L1 protein expression. (A) Si-p65 and si-NC were transfected into bEECs. After transfection for 48 h, the protein expression levels of p65, pp65, and PD-L1 were measured by Western blot. β-Actin was used as an endogenous control. (B) pcDNA3.1-p65 and pcDNA3.1-NC were transfected into bEECs. After transfection for 24 h, the mRNA expression levels of p65 were measured by qPCR. GAPDH was used as an endogenous control. (C) pcDNA3.1-p65 and pcDNA3.1-NC were transfected into bEECs. After transfection for 48 h, the protein expression levels of p65, pp65, and PD-L1 were measured by Western blot. β-Actin was used as an endogenous control. Data are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

MiRNAs are small non-coding RNAs that play a pivotal role in post-transcriptional protein regulation and are involved in the control of numerous cellular pathways[47]. MiRNAs play a pivotal role in the regulation of physiological processes and the development of disease, they are involved in the process of pregnancy, regulating the expression of genes associated with pregnancy and influencing the immune response during this period[40,42]. Additionally, miRNAs can serve as diagnostic markers for disease and may also be employed as therapeutic agents in disease treatment[47]. It seems reasonable to posit that miRNA is also involved in the regulation of p65 during pregnancy. A potential targeting relationship between miR-155 and p65 was identified through TargetScan prediction. miR-155 is a multifunctional miRNA that regulates key molecules in multiple signaling pathways and plays a critical role in a variety of diseases and physiological processes. To validate the prediction, miR-155 was examined in the early pregnant uterine epithelial tissues and was found to be significantly reduced in the early pregnant uterine epithelial tissues, in contrast to the significant increase in p65. The results of the dual luciferase reporter gene assay indicated that p65 is a molecular target of miR-155. The results of the miR-155 mimics and inhibitor assay further demonstrated the effect of miR-155 on p65, which was a molecular target of miR-155, and the ability of miR-155 to repress the expression of p65.

To ascertain the regulatory effect of p65 on PD-L1, siRNA knockdown of p65 demonstrated that the knockdown of p65 protein markedly reduced the expression level of PD-L1. pcDNA3.1-p65 overexpression of p65 revealed that the overexpression of p65 protein significantly enhanced the expression level of PD-L1. In conclusion, it can be stated that p65 played a regulatory role in the expression of PD-L1.

During the early stages of pregnancy, the intrauterine immune environment undergoes a gradual transformation, shifting from a pro-inflammatory to an anti-inflammatory state[48]. Macrophages undergo a transformation from pro-inflammatory M1 to anti-inflammatory M2 during pregnancy[49]. This transition occurs during pregnancy and is maintained after the establishment of the maternal-fetal bond, ensuring a normal pregnancy. The NF-κB signaling pathway is widely regarded as highly correlated with inflammation[28]. In contrast, PD-L1 is associated with the immunosuppressive milieu and correlates with the macrophage M2 phenotype[50]. In contrast, our findings revealed that p65 and PD-L1 protein expression were elevated at the endometrial epithelium in the early stages of pregnancy. Additionally, our experimental observations indicated that p65 played a regulatory role in PD-L1 expression, which was in line with the coexistence of pro-inflammatory and anti-inflammatory conditions in the uterus during early pregnancy. The mechanism underlying the establishment of this distinctive immune environment might be associated with the regulation of PD-L1 by p65. This study was designed to investigate the changes in PD-L1 expression in the endometrial epithelium during pregnancy, as well as the underlying mechanisms of gene regulation. However, this study did not address the role of PD-L1 on immune cells in the immune milieu of pregnancy. The following trial will investigate the role of PD-L1 on uterine immune cells.

When all the results were considered collectively, it could be stated that miR-155 targets p65 to regulate the expression of PD-L1 within the context of pregnancy. The aforementioned regulatory pathways might provide a theoretical basis for the study of immune tolerance in pregnancy and potential strategies for the reduction or treatment of clinical reproductive disorders in dairy cows.

Materials and methods

Bovine uterine tissues collection

Bovine uterine tissues from cows were obtained from a local slaughterhouse. Uterines were obtained within 30 min of slaughter and then transported to the laboratory within 1 h on ice for immediate processing. The classification ($n=3$) of tissues was dependent upon the morphology of the corpus luteum and uterus[51]. All experiments were performed according to protocols and guidelines approved by the Huazhong Agriculture University Animal Care and Use Committee (Wuhan, China).

Histological analysis and immunohistochemistry

Uterine tissues were excised and fixed with 4% paraformaldehyde for 24 h. Thereafter, the fixed tissues were embedded in paraffin, sliced to a thickness of 4–6 μm, and stained with Hematoxylin and eosin (H&E) and immunohistochemistry. The integrated option density (IOD) of immunohistochemistry was quantified using Image J (National Institutes of Health, Bethesda, Maryland, USA) and the Image J's plug-in IHC Toolbox (The University of Nottingham, UK).

Cell culture

The bovine endometrial epithelial cells line (bEECs) and HEK293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEK293T was maintained in DMEM (Gibco, USA), and bEECs were maintained in DMEM/F12 (Gibco, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum. All cells were cultured at 37 ℃ in a humid incubator with 5% CO_2. Estradiol (E_2) and progesterone (P_4) were from TCI (Shanghai, China).

Cell transfection

MiR-155 mimics, miR-155 inhibitors, p65 siRNAs (si-p65), and corresponding negative controls (NC) were designed and synthesized by GenePharma (Shanghai, China). These sequences are listed in Table 1. pcDNA3.1-p65 and pcDNA3.1-NC were synthesized by GeneCreate (Wuhan, China). The miRNA, siRNA, and pcDNA3.1 were transfected in cells at 5 μg using ExFect Transfection Reagent (Vazyme, Nanjing, China) according to the manufacturer's instructions in 6 well cell culture plates.

RNA isolation and qPCR analysis

Total RNA from the bEECs or uterine tissues was extracted by TRIzol (Solarbio, Beijing, China). cDNA was generated by using a reverse transcription kit (Vazyme, Nanjing, China) for reverse transcription. MiRNA was reversed by using a miRNA 1st Strand cDNA Synthesis (Vazyme, Nanjing, China) with a special stem-loop reverse transcription primer for miR-155. Quantitative real-time PCR analysis was performed by the LightCycler® 96 system (Roche Applied Science, Mannheim, Germany) with the qPCR SYBR Green Master kit (Yeasen, Shanghai, China) in a 20 μL reaction system. Results were shown with the $2^{-\Delta\Delta Ct}$ comparative method. U6 and GAPDH were used for the normalization. The primers are listed in Table 2.

Western blot experiments

Proteins were extracted using a radioimmunoprecipitation lysis buffer (RIPA) (BioSharp, China) containing a phosphatase inhibitor (Applygen, Beijing, China) and a protease inhibitor PMSF (BioSharp, China). The concentrations of the proteins were measured using a Pierce BCA assay protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples were separated by SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in the blocking buffer for two hours, and then the membranes were incubated with specific primary antibodies at 4℃ overnight. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the enhanced

Gene	Sequence
Mimics NC	Sense: UUCUCCGAACGUGUCACGUTT
	Anti-sense: ACGUGACACGUUCGGAGAATT
miR-155 mimics	Sense: UUAAUGCUAAUCGUGAUAGGGGU
	Anti-sense: CCCUAUCACGAUUAGCAUUAAUU
Iinhibitor NC	Sense: CAGUACUUUUGUGUAGUACAA
miR-155 inhibitor	Sense: ACCCCUAUCACGAUUAGCAUUAA
si-p65	Sense: GGACGUACGAGACCUUCAATT
	Anti-sense: UUGAAGGUCUCGUACGUCCTT
si-NC	Sense: UUCUCCGAACGUGUCACGUTT
	Anti-sense: ACGUGACACGUUCGGAGAATT

Table 1. Sequences for miR-155 mimics, miR-155 inhibitor, and si-p65.

Gene	Sequence
miR-155	RT:5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCCCTAT-3′
	Forward: 5′-GCCGAGTTAATGCTAATCG-3′
	Reverse: 5′-GTGTCGTGGAGTCGGCAAT-3′
U6	Forward: 5′-CGAGCACAGAATCGCTTCA-3′
	Reverse: 5′-CTCGCTTCGGCAGCACATAT-3′
p65	Forward: 5′-AGCCCTTTCAATGGACCCAC-3′
	Reverse: 5′-TGATGGTGCTGAGAGATGGC-3′
M-CSF	Forward: 5′-CCTGGTGAGCAATGGTGCTA-3′
	Reverse: 5′-CACGGGATCATCCAACTGCT-3′
PD-L1	Forward: 5′-TCCAAGGACCTGTATGTGGTAG-3′
	Reverse: 5′-GTGCTGAACGTTTGGGTCTT-3′
IL-34	Forward: 5′-ATCTGTGGGTCCTGGTGAGT-3′
	Reverse: 5′-GACGTCCAGCAGTAGTGTGT-3′
GAPDH	Forward: 5′-AAGGTCGGAGTGAACGGATT-3′
	Reverse: 5′-ATGACGAGCTTCCCGTTCTC-3′

Table 2. Sequences for qPCR.

chemiluminescence reagents (MeilunBio, Dalian, China). Primary antibodies against p65 (1:2000), pp65 (1:2000), and β-Actin (1:50000) were from Abclonal (Wuhan, China). Primary antibodies against PD-L1 (1:800) were from Servicebio (Wuhan, China). Secondary antibodies (1:8000) were from Abclonal (Wuhan, China). The grey value of each band was quantified using Image J (National Institutes of Health, Bethesda, Maryland, USA), and the relative expression levels of the indicated proteins were normalized to an endogenous control, β-actin.

Luciferase reporter assay

The TargetScan (http://www.targetscan.org/vert_72/) tool was employed to predict the potential binding sites between the target gene p65 and miR-155. The psi-CHECK2-p65 WT vector and the psi-CHECK2-p65 MuT vector were synthesized by GeneCreate (Wuhan, China). HEK293T cells were co-transfected with miR-155 mimics or mimic NC (3 μg)and luciferase reporter plasmids (5 μg) using the ExFect Transfection Reagent (Vazyme, Nanjing, China) in 6 well cell culture plates. The luciferase activity was measured by a Lumat LB 9507 Ultra Sensitive Tube Luminometer (Titertek Berthold, Nanjing, China) after using the Dual-Luciferase Reporter Gene Assay Kit (Shanghai, China). The firefly luciferase activity was for normalization.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.3.0 (GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA with Dunnett's multiple comparison test was used to compare the differences among multiple groups. Student's t-test was used to determine the statistical significance of differences between groups. Data are expressed as mean ± SEM. $*p < 0.05$ means significantly different; $**p < 0.01$ means extremely significantly different.

Data availability

This published article and its supplementary information file include all data generated during this study. Any additional inquiries are available upon request to the corresponding author.

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Author contributions
JZ and GD conceived and designed the experiments; JZ, YG, and HZ carried out the experiments; JZ, NC, and WF analyzed the data; JZ, XF, and WJ wrote the manuscript. All authors agree with the experimental content.

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Declarations

Competing interests
The authors declare no competing interests.

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