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Identification of programmed cell death 1 and its ligand in the testicular tissue of mice

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Problem: This study aims to determine the expression and localization of programmed cell death 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) in the testes of mice at different developmental stages.

Method of study: By means of RT-qPCR, Western blot and immunofluorescence, the expression and localization of PD-1 and PD-L1 were detected in the testicular tissues of mice at different postnatal times: P7, P14, P21, P28, P35, and adulthood. Meanwhile, the level of soluble PD-L1 (sPD-L1) was evaluated by ELISA in the testicular interstitial fluid (IF) of the adult mice, culture supernatants of TM4 cell lines (Sertoli cells lines), and primary Sertoli cells at P14.

Results: *Pd-1* mRNA levels were unexpectedly low. From P7 to P21, there was limited PD-1 protein detected while PD-1 was evident at P28 and afterward at significantly higher levels than at P14 and P21 ($P < 0.05$). Despite being found in the interstitial area at P7, P14, and P21, PD-1 was also detected in the germ cells of the seminiferous tubules after P28. *Pd-l1* mRNA exhibited age-related changes, peaking at P21, while PD-L1 protein was constitutively expressed at any stage, specifically localized in the nucleus of Sertoli cells. Moreover, the level of sPD-L1 in IF was significantly higher than that in the culture supernatants of both TM4 and primary Sertoli cells at P14.

Conclusions: PD-1 and PD-L1 were present in the testicular tissue of adult mice. The expression and localization of PD-1 fluctuated with age, and PD-1 was mainly localized to advanced germ cells, suggesting that it may play a role in spermiogenesis. PD-L1 was constitutively expressed in the nucleus of Sertoli cells, which could secrete sPD-L1 into the testicular interstitial space and thus may be involved in testicular immune privilege.

KEYWORDS
immune privilege, mouse, PD-1, PD-L1, spermatogenesis, testis

1 | INTRODUCTION

As male reproductive organs, the testes have the specific function of producing androgens and sperm. This process occurs in the interstitial area and within the seminiferous epithelium, respectively. Spermatogenesis is a highly complex process, involving diploid germ cells developing into haploid spermatozoa, which can be divided into three main stages: mitotic proliferation and differentiation of spermatogonia, meiotic differentiation of spermatocytes, and spermiogenesis.[1,2] Successful spermatogenesis depends on a stable intra-testicular immune microenvironment. Clinical data and

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1 of 9

research results indicate that an abnormal systemic and local immune status associated with infection and inflammation can inhibit spermatogenesis within the testes and lead to male infertility.[3-5] The incidence of male infertility due to infection ranges from 6% to 15% in different reports[6]; however, the underlying mechanism has not been completely elucidated.

Immune-privileged sites are unique places where foreign antigens or grafts are tolerated without triggering intense immune responses. Under physiological conditions, the testis is an immune-privileged organ where the immune system does not attack neo-antigen-expressing advanced spermatocytes that arise in puberty after the establishment of self-tolerance. Other immune-privileged sites include the anterior chamber of the eye, the central nervous system, and the placenta.[7] Although the exact mechanism is not yet clear, an increasing number of recent studies agree that immune privilege is an active process involving multiple mechanisms and not just immunological suppression.[8-10] In terms of the testes, multiple mechanisms are involved in maintaining the immune-privileged status. First, the blood-testis barrier effectively protects germ cells from various leukocytes in the interstitial space.[9] Second, testicular somatic cells, including Sertoli cells (SCs), peritubular cells, and Leydig cells, contribute to create an immune-privileged environment. As an example, SCs can produce several types of immuno-regulatory molecules, including tumor growth factor β, indoleamine 2,3-dioxygenase, galectin-1, activin A, and complement inhibitors.[11,12] In addition, Leydig cells express three members of the receptor tyrosine kinase family (TYRO3, AXL, and MER) and their ligands, which negatively regulate the inflammatory signal mediated by Toll-like receptors[12] and synthesize testosterone with anti-inflammatory properties.[13] Dendritic cells,[14,15] regulatory T cells (Tregs),[16] and alternatively activated (M2) macrophages[17] in the interstitial space help convert the environment to an immune-skewing milieu that is advantageous for developing germ cells. Collaboration between these processes is beneficial to normal testicular function. However, the breakdown of this elaborate physiological status can lead to orchitis (an etiological factor of male infertility), followed by impaired androgen synthesis and diminished spermatogenesis.[12]

Programmed cell death 1 (PD-1, or CD279) was initially identified in 1992 and is related to programmed cell death in T cells.[18] Its immuno-regulatory role, not recognized until later, is of intense current interest. PD-1 was reported to be mainly expressed on hematopoietic cells, including activated T cells, B cells, myeloid dendritic cells, activate monocytes/macrophage, and natural killer T cells.[19] PD-L1, a ligand of PD-1, together with PD-1 belongs to the B7/CD28 family and is predominantly found on antigen presenting cells (APCs) and many nonhematopoietic cell types.[20] Engagement of PD-1/PD-L1 delivers co-inhibitory signals into PD-1^{+} T cells, mainly promoting the development and function of Tregs and suppressing effector T-cell-mediated immune responses.[21] The PD-1/PD-L1 inhibitory pathway plays a vital role in the maintenance of peripheral tolerance and is involved in the pathogenesis of multiple diseases, including cancers, chronic infections, and autoimmunity.[21] Aside from their membrane-bound forms, PD-1 and PD-L1 also have soluble forms: soluble PD-1 (sPD-1) and soluble PD-L1 (sPD-L1).[22] The sPD-L1 can be detected in physiological and pathological conditions and is mainly produced by matrix metalloproteinase (MMP) cleavage from the cell surface.[23] Both molecules can regulate the PD-1/PD-L1 pathway in that sPD-1 can inhibit negative signaling mediated by the PD-1/PD-L pathway in activated CD8^{+} T cells[24] while sPD-L1 has the opposite effect.[25]

Emerging evidence points to the supporting role of the PD-1/PD-L1 pathway at immune-privileged sites. Human corneal endothelial (HCE) constitutively expresses PD-L1 and PD-L2 (another ligand of PD-1). The proliferation of activated T helper 1 (Th1) cells that overexpress PD-1 can be efficiently inhibited in a co-culture system with HCE in vitro, and administration of α-PD-L1 mAb, but not PD-L2, blocks the suppressive effect of HCE on Th1 cells.[26] In a rodent model of ischemic stroke, adoptively transferred Tregs suppress peripheral neutrophil-derived matrix metallopeptidase-9 production by PD-1/PD-L1 interaction, preventing damage to the blood-brain barrier and reducing acute ischemic brain injury.[27,28] At the maternal-fetal interface, PD-L1 has been found to be expressed on various cell types, including trophoblast cells, decidual stromal cells, and immune cells.[29] Decidual stromal cells can suppress CD4^{+} T-cell pro-inflammatory cytokine production via the PD-1/PD-L pathway, which helps to regulate a balanced cytokine milieu at the maternal-fetal interface.[30] Moreover, an impaired PD-1/PD-L1 pathway has been found to be involved in pregnancy-related complications, including preeclampsia and recurrent spontaneous abortion.[29] Several researchers have also focused on the PD-1/PD-L1 inhibitory pathway in the testes. Dal Secco et al[31] reported that murine SCs inducibly express PD-L1 and MHC-II in response to IFN-γ in vitro, directly inhibiting CD8^{+} T-cell proliferation through PD-L1. In addition, the interaction of PD-1 and PD-L1 reportedly prolongs the survival of intra-testicular islet allografts, with less proliferation and more apoptosis of infiltrating T cells.[32] These data suggest that the interaction between PD-1 and PD-L1 may contribute to testicular immune privilege.

However, the expression and localization of PD-1 and PD-L1 remain controversial. In the study by Dal Secco et al,[31] PD-L1 was inducibly expressed on SCs but constitutively expressed on peritubular cells in the testes of mice. However, spermatocytes and spermatids were the main cell types that expressed PD-L1 in the seminiferous tubules in an allograft study.[32] In addition, recent data showed that there was little or no PD-L1 expression,[33,34] and PD-1 expression was also not detected in normal human testes.[34] This ambiguity surrounding expression made it difficult to identify the exact function of the PD-1/PD-L1 pathway in testicular functions.

The goal of this study was to determine the precise expression and localization of PD-1 and PD-L1 in the testes of mice at different developmental stages. The results would provide a theoretical basis for further investigation of the effect of the PD-1/PD-L1 pathway on testicular physiological functions.

2 | MATERIALS AND METHODS

2.1 | Animals and sample collection

Adult ICR mice were purchased from the Provincial Center for Disease Control and Prevention, Wuhan, China, and young mice were secured via in-house breeding at the Animal Center of Huazhong University of Science and Technology. The Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology, approved the animal use protocol.

The mice were maintained in a specific pathogen-free facility with a 12/12 hours light/dark cycle. For the collection of testes from young postnatal mice (days P7, P14, P21, P28, and P35), timed, natural mating was performed by placing two wild-type adult female mice (6-8 weeks old) into a cage with one wild-type adult male mouse (6-8 weeks old). Female mice were examined for plugs at 8:00 AM every morning. Once a plug was observed, the female was housed individually. At six time points (P7, P14, P21, P28, P35, and adult), male mice were euthanized to collect testes and spleen. One testicle and spleen from each animal were stored at −80°C prior to RNA isolation and Western blot analysis. The contralateral testicle was fixed in 4% paraformaldehyde for 2-3 hours, dehydrated using graded sucrose solutions, and then embedded in optimal cutting temperature compound (OCT, USA) for immunofluorescence examination.

2.2 | Real-time quantitative RT-PCR (RT-qPCR)

Total RNA was extracted with TRIzol reagent (Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA quantity and quality were assessed using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). An equal amount of total RNA (1 μg) was treated with gDNA Eraser reagent to eliminate potential genomic DNA and then used for cDNA synthesis in a 20 μL reaction system (Takara Bio, Shiga, Japan). RT-qPCR amplification analysis was performed with 2 μL of cDNA using a SYBR® Premix Ex Taq™ II kit (Takara Bio, Shiga, Japan) on a LightCycler® 96 (Roche, Basel, Switzerland). Primer sequences (5′-3′) were as follows: *Pd-1-For*: GTCCCTCACCTTCTACCC; *Pd-1-Rev*: GGTTCCAGTTCAGCATAAGA; *Pd-l1-For*: TATCACGGCTCCAA AGGACT; *Pd-l1-Rev*: ACCACTAACGCAAGCAGGTC; *β-actin -For*: AACAGTCCGCCTAGAAGCAC; and *β-actin-Rev*: CGTTGACATCC GTAAAGACC. Relative mRNA levels to calibrator were computed using the $2^{-\triangle\triangle CT}$ method.

2.3 | Western blot analysis

The testes were lysed with lysis buffer (Beyotime Biotechnology, Wuhan, China), and protein concentrations were detected using a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology). Proteins (30 μg/lane) were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands were then transferred to polyvinylidene difluoride membranes (Millipore,

Bedford, MA, USA). After blocking with PBST containing 5% nonfat milk at room temperature for 2 hours, the membranes were incubated overnight at 4°C with primary antibodies against PD-1 (1:1000, Novus Biologicals, Littleton, CO, USA), PD-L1 (1:500; Novus Biologicals), and β-actin (1:1000, Santa cruz Technology, Dallas, TX, USA), followed by horseradish peroxidase-conjugated secondary antibody (Santa cruz Technology, USA) at room temperature for 2 hours. The target bands were detected using an enhanced chemiluminescence detection kit (Beyotime Biotechnology, Wuhan, China). Relative protein levels were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-actin was used as the loading control.

2.4 | Immunofluorescence staining

Sections (5 μm thick) were cut using a freezing microtome (Leica1850; Leica, Nussloch, Germany), and the slides were then fixed with −20°C precooled methanol for 5 minutes. After soaking in citrate buffer and microwaving at 100°C for 10 minutes to retrieve antigens, the slides were blocked with 5% bovine serum albumin in PBS for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies against PD-1 (1:50, Novus Biologicals), PD-L1 (1:50, Novus Biologicals), and WT1 (1:20, Novus Biologicals). Tissue sections were incubated with fluorescent secondary antibodies (Abbkine, USA) in the dark for 2 hours at room temperature. Finally, the slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature, mounted with glycerinum (Sigma-Aldrich, St. Louis, MO, USA) and observed under a fluorescence microscope (TH4-200 Olympus, Tokyo Japan). Indirect immunofluorescence of cells was performed as previously described.[35]

2.5 | Collection of testicular interstitial fluid (IF)

Testicular IF was collected from adult testes of ICR mice (6-8 weeks old). The testes were removed from the mice and washed with cold PBS to clear residual blood. Excess water was removed from the tissue by blotting dry with filter paper. A small incision was made in the distal end of the testicular capsule avoiding damage to the seminiferous epithelium. Approximately 3-4 testes were hung in a 15-mL tube containing 1 μL PMSF/50 μL PBS for 16 hours at 4°C. Testicular IF samples were then removed and centrifuged at 10 000 g for 15 minutes. The supernatant was transferred to a 1.5-mL tube and stored at −80°C.

2.6 | Cell isolation and culture of primary SCs and TM4 cell lines

SCs were isolated as described previously.[35,36] Briefly, the testes from 2-week-old ICR male mice were collected and washed with PBS. The tunica-free testes were then incubated with 2.5 mg/mL trypsin (Gibco, Grand Island, NY, USA) and 10 μg/mL DNase I (Sigma, St. Louis, MO, USA) at 32°C for 5 minutes with gentle oscillation. Trypsin digestion was stopped by adding DMEM/F12 (HyClone, Logan, UT, USA) with 10% FBS (HyClone). After 5 minutes, the tubules were settled by unit gravity; the supernatants were carefully removed, and the tubules were washed

for 9 times with PBS to remove contaminating interstitial cells. Then, seminiferous tubules were resuspended in 1 mg/mL collagenase (Sigma, USA), 1 mg/mL hyaluronidase (Sigma, USA), and 10 μg/mL DNase I (Sigma, USA) at 32°C for 10 minutes with gentle oscillation to separate the peritubular myoid cells, germ cells, and SCs. The cell suspensions were cultured with DMEM/F12 supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) at 35°C with 5% CO_2 for day 7. When the purity of the SCs was more than 85% based on the staining for WT1 on day 7, the SCs can be used in this study. Then, the cells with high purity were cultured for another 24, 48, and 72 hours, respectively.

TM4 cell lines were obtained from ATCC and cultured in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell lines were grown at 37°C with 5% CO_2 and were cultured for 24, 48, and 72 hours, respectively.

After primary SCs with high purity and TM4 cell lines were cultured for 24, 48, and 72 hours, the supernatants were harvested, respectively. Supernatants samples were centrifuged at 10 000 g for 15 minutes to removed cellular contaminants and then stored at −80°C for further analysis.

2.7 | ELISA for detection of sPD-L1

The levels of sPD-L1 in the testicular IF and the culture supernatants of the primary SCs and TM4 cell lines were quantified using a PDCD1LG1 ELISA Kit (USCN Life Science, Wuhan, China) following the manufacturer's protocol.

2.8 | Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 22.0 software; SPSS Inc, Chicago, IL, USA), and graphs were drawn using GraphPad Prism software, version 5. Data are presented as the mean ± SEM. One-way ANOVA was used for multiple comparisons. A P-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Expression and localization of PD-1 and PD-L1 in testicular tissue of normal adult mice

To examine whether PD-1 and PD-L1 proteins were expressed, Western blot and immunofluorescence were performed on testicular tissue from adult mice. As shown in Figure 1A, Western blot analysis indicated that PD-1 and PD-L1 proteins were indeed present in the testicular tissue of adult mice. In the tissue sections, PD-1 staining occurred in clusters of germ cells in the adluminal compartment of the seminiferous tubules and was also observed occasionally in the interstitial area (Figure 1B). PD-L1 was mainly localized on the periphery of the seminiferous tubules, and there was limited PD-L1[+] staining in the interstitial space. To assess the cell type expressing PD-L1, we used a stable marker of SCs, Wilms tumor nuclear protein 1 (WT1, constitutively expressed in the nucleus throughout all developmental ages).[37] Co-localization of PD-L1 and WT1 in the nucleus indicated that it was the SCs that mainly expressed PD-L1 protein in the adult testicular tissues (Figure 1C).

3.2 | Expression pattern and localization of PD-1 and PD-L1 in the developing mouse testes

To evaluate the expression levels of the molecules, we performed real-time RT-qPCR, Western blot, and immunofluorescence to examine testicular tissues harvested at different postnatal times: P7,
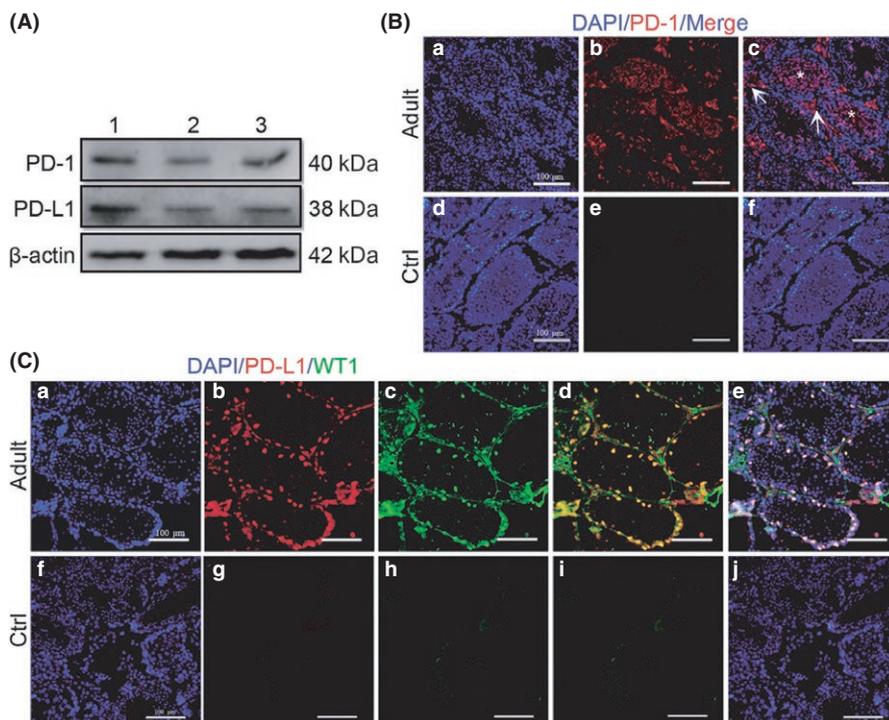

FIGURE 1 Expression and localization of PD-1 and PD-L1 in normal testicular tissue of adult mice (A) Western blot results of PD-1 and PD-L1 protein in 8-week-old ICR male mice testicular tissue. β-actin was used as the loading control (n = 3, three independent experiments). B, Immunofluorescence for DNA (DAPI, blue in a, d), PD-1(red in b, e), and negative control (Ctrl, d-f) in adult testes. PD-1 staining occurred in clusters of germ cells in the adluminal seminiferous tubule compartment (asterisk) and was also observed occasionally in the interstitial area (arrow). C, Immunofluorescence for DNA (blue in a, f), PD-L1 (red in b, g) and WT1 (Sertoli cell marker, green in c, h) in adult male testes (a-e) and negative control (f-j). Image represents at least three independent experiments. Scale bar, 100 μm
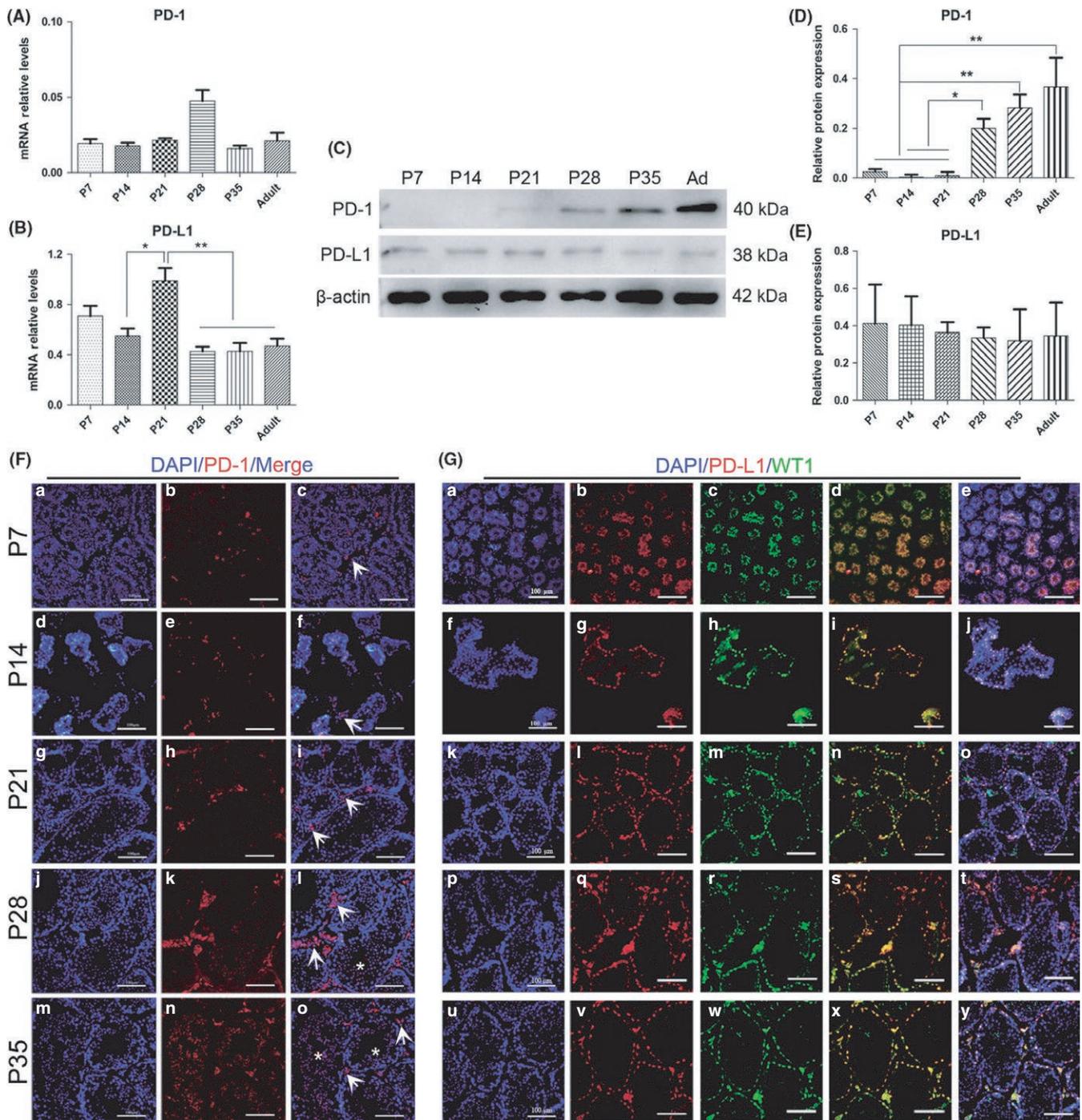
FIGURE 2 The expression pattern and localization of PD-1 and PD-L1 in mice testes at different developmental stages (A, B) *Pd-1* and *Pd-l1* mRNA expression levels were verified by RT-qPCR at different developmental stages of mice testes (n = 4). The *Pd-1* mRNA and *Pd-l1* mRNA expression levels in the spleen tissue of adult mice were set as controls. C, Representative image of Western blot results. (D, E) Relative PD-1 protein and PD-L1 protein expression levels were measured in each group (n ≥ 4). F, The immunofluorescence staining for PD-1 (red) in the testicular tissue in each group. The nucleus was counterstained by incubating the sections with DAPI. G, The immunofluorescence staining for PD-L1 (red), WT1 (green), and DNA (blue, DAPI) of the testes tissue for each group. Asterisk and arrow indicated the adluminal compartment and interstitial area of seminiferous tubule, respectively. Scale bar: 100 μm. P14: postnatal day 14; P21: postnatal day 21; P28: postnatal day 28; P35: postnatal day 35; Ad: Adulthood. Data are presented as the mean ± SEM, *$P < 0.05$, **$P < 0.01$.

P14, P21, P28, P35, and adulthood. The real-time RT-qPCR results showed that the *Pd-1* mRNA levels were unexpectedly low in testicular tissue throughout life, slightly increased at P28, and not significantly different ($P > 0.05$; Figure 2A). *Pd-l1* mRNA exhibited age-related changes, peaking at P21 (the level at P21 was significantly higher than that at P14 [$P < 0.05$], P28, P35, and adulthood [$P < 0.01$]; Figure 2B). The *Pd-1* mRNA and *Pd-l1* mRNA expression levels in the spleens of the adult males were used as controls.

Western blot analysis was performed to examine the protein expression of PD-1 and PD-L1 levels in testicular tissues (Figure 2C). While there was limited PD-1 protein detected from P7 to P21, PD-1 was evident at P28 at significantly higher levels than at P14 and P21 ($P < 0.05$), showing gradual upregulation. PD-1 protein levels at P35 and in adult testes were also obviously higher than those at P7, P14, and P21 ($P < 0.01$; Figure 2D), suggesting that PD-1 was predominantly expressed in advanced spermatocytes. In contrast, PD-L1 was constitutively expressed in the testicular tissues at different developmental stages, but there were no statistically significant differences between groups ($P > 0.05$; Figure 2E). The immunofluorescence results confirmed that the localization of PD-1 changed with age in the testicular tissues. PD-1 staining was only found in the interstitial area at P7, P14, and P21 but was also detected in the germ cells of the seminiferous tubules after P28 (Figures 2F, 1B). PD-L1 was expressed in the nucleus of SCs, as indicated by the co-localization of WT1 and PD-L1 surrounding the seminiferous tubules at any phase (Figures 2G, 1C), which was also consistent with the Western blot results.

3.3 | Levels of sPD-L1 in the testicular IF of adult mice and culture supernatants of TM4 cell lines and primary SCs

Based on the localization of PD-L1 in the testicular tissues and the immunosuppressive function of sPD-L1,[25] we speculated that PD-L1, mainly expressed in the SCs, probably played an immuno-inhibitory role by interacting with PD-1+ immune cells in the interstitial area via its soluble form, sPD-L1. To verify this hypothesis, the testicular IF of the adult mice and culture supernatants of TM4 and primary SCs were collected and analyzed for levels of sPD-L1 by ELISA. In addition, immunofluorescence staining was conducted to detect PD-L1 expression in the TM4 cell lines and primary SCs. The staining results showed that PD-L1 was mainly localized in the nucleus of the TM4 cell lines and primary SCs (Figure 3A). The concentration of sPD-L1 in the testicular IF of adult mice was 6.608 ± 1.814 ng/mL, which was significantly higher than that in TM4 culture supernatants (D1: 0.102 ± 0.067 ng/mL after culturing for 24 hours; D2: 0.089 ± 0.031 ng/mL after culturing for 48 hours; and D3: 0.093 ± 0.028 ng/mL after culturing for 72 hours) and in culture supernatants of primary SCs (0.048 ± 0.005 ng/mL, 0.121 ± 0.047 ng/mL, and 0.061 ± 0.009 ng/mL for 24, 48, and 72 hours, respectively) ($P < 0.001$). A slight increase in sPD-L1 levels was observed at 48 hours, compared with 24 hours in culture supernatants of primary SCs. However, there were no significant differences in the culture supernatants between other groups ($P > 0.05$) (Figure 3B).

4 | DISCUSSION

In the present study, we found that the PD-1 and PD-L1 proteins were present in the testes of mice. Furthermore, we demonstrated for the first time that PD-1 was frequently localized to the germ cells of the seminiferous tubules and that PD-1 staining was also seen occasionally in the interstitial area. Additionally, we found that PD-L1 was constitutively expressed by SCs, which was
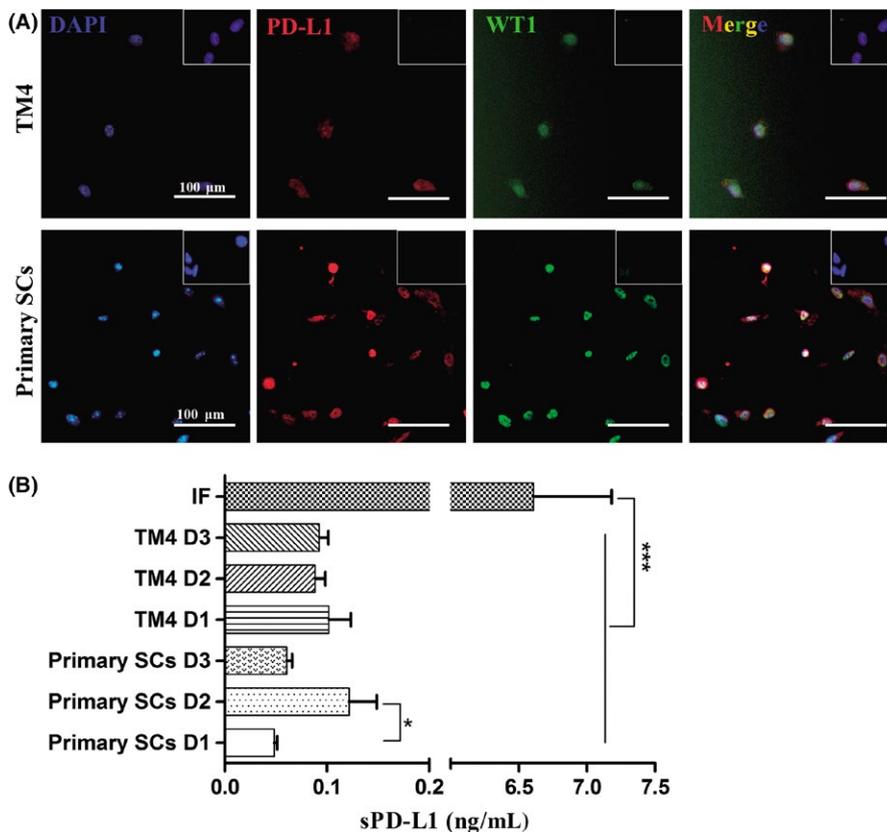

FIGURE 3 sPD-L1 levels in the testicular IF of adult mice and culture supernatants of TM4 cell lines and primary SCs at P14 (A) The immunofluorescence staining for PD-L1 (red), WT1 (green), and DNA (blue, DAPI) in the TM4 cell lines and primary SCs (the upper right represents the negative control). Scale bar: 100 μm. B, The levels of sPD-L1 in the testicular IF of adult mice (n = 10) and culture supernatants of TM4 (n = 10) and primary SCs (n = 3). IF: interstitial fluid. D1: culture supernatants for 24 h, D2: culture supernatants for 48 h, D3: culture supernatants for 72 h. Data are presented as the mean ± SEM, *$P < 0.05$, ***$P < 0.001$.

supported by the co-localization of PD-L1 and WT1 in the seminiferous tubules.

Until recently, only two studies have reported no detectable PD-1 expression in normal human testicular tissue by immunohistochemical staining.[34,38] However, there has not been any research focusing on PD-1 expression in the testes of mice. In our study, we first detected PD-1 expression in the germ cells of adult mice by immunofluorescence staining. Spermatogenesis is a complex process involving the apoptosis of germ cells; however, the exact mechanisms underlying this process are not clear. PD-1 was initially found on T cells and associated with programmed cell death.[18] Therefore, we proposed the hypothesis that PD-1 expression in germ cells may be involved in programmed cell death. However, this supposition needs further verification in PD-1 knockout mice.

We also found that SCs constitutively express PD-L1, which is obviously inconsistent with previous research. As mentioned previously, mouse SCs inducibly express the negative co-stimulatory ligand PD-L1 in vitro. When CD8$^+$ T cells were co-cultured with PD-L1$^+$ SCs, the proliferation of CD8$^+$ T cells was evidently inhibited. Blockade of PD-L1 on the surfaces of SCs reversed the inhibitory effect. In addition, it has been shown that PD-L1 is constitutively expressed on peritubular cells surrounding seminiferous tubules via immunofluorescence.[31] In an allogeneic murine transplantation experiment, the PD-1/PD-L1 pathway was essential for prolonged survival of intra-testicular allografts, and spermatocytes and spermatids were mainly PD-L1$^+$ cells in the seminiferous tubules of the testes based on immunohistochemical staining.[32] However, controversial results were obtained in human testes. Based on immunohistochemical staining, researchers claimed that there was no[33] or little[34] PD-L1 expression in normal human testes. This difference in study results may be due to the use of diverse antibodies, mouse strains with different genetic backgrounds, or different detection methods.

For further assessment of the expression patterns and localizations during different developmental stages, we chose six different time points: P7, P14, P21, P28, P35, and adulthood. Major cellular changes occur at the six time points during the development of germ cells in male mice. At P7, spermatogonia are the only germ cell type within the testes. In P14 testes, germ cells enter meiosis, and mid-pachytene spermatocytes appear in the seminiferous tubules. Male germ cells develop into round spermatids at P21 and into elongated spermatids at P28; by P35, sperm are present in the testis.[39,40] Complete spermatogenesis is established in the testes of adult mice.

In our study, we detected minimal Pd-1 mRNA at the six different time points. However, Western blot results suggested that PD-1 protein evidently appeared at P28 and then gradually increased. In line with Western blot results, immunofluorescence staining showed that there were PD-1$^+$ cells in the seminiferous tubules of the testes until P28, which was inconsistent with the Pd-1 mRNA results. The relationship between protein and mRNA expression levels in a given cell or tissue is influenced by the transcription, mRNA stability, translation efficiency, and protein degradation.[41] Besides, spermatogenesis consists of a series of highly regulated events through which germ cells eventually develop into sperm with minimal cytoplasm and highly condensed and compact nucleus. Novel mRNA and proteins are produced during the process.[42] During spermiogenesis, most of the spermatid cytoplasm containing RNA is extruded as a residual body, and cytoplasmic droplets are phagocytosed by SCs.[43] The reason for the inconsistencies between PD-1 protein and mRNA may be due to the following aspects: (a) it may be related to the instability of RNA compared to proteins and/or transcription/translation regulation; (b) Pd-1 mRNA is depleted as a residual body or cytoplasmic droplet; (c) we failed to make assessments at the optimal time considering temporal and spatial differences for the appearance of mRNA and proteins within cells; and (d) we used Pd-1 mRNA and Pd-l1 mRNA expression levels in the spleens of adult males as controls.

Also, our data suggested that PD-1 was mainly localized in the elongating spermatids and sperm, and higher level of PD-1 proteins was mainly examined in the adult mice, which indicated PD-1 might have a previously unrecognized role in spermiogenesis. In male adult mice, testosterone and follicle-stimulating hormone are the principal hormonal regulators of spermatogenesis. And androgens (including dihydrotestosterone and testosterone) have the anti-inflammatory property and can influence the functions of immune cells such as macrophage and T cells.[13,44] Moreover, some data showed that 17β-estradiol (E2) and endogenous glucocorticoid could upregulate PD-1 expression on immune cells, such as Tregs, APCs, and NK cells, protecting mice from pathological damage.[45-47] However, there is no relevant research on whether androgens modulate the PD-1 expression or not. Therefore, it still keeps a mystery and needs further investigations. In addition, PD-1 staining was also seen in the interstitial area. There are various immune cells, including macrophages, T cells, dendritic cells, and mast cells, in the testicular interstitial area of mice.[9,12] Therefore, PD-1$^+$ cells might be the immune cells involved in the maintenance of testicular immune privilege; however, an understanding of the exact underlying mechanism requires further study.

It has been shown that mRNA levels of PD-L1 represent age-related changes and peak in P21 testes. Western blot analysis showed that PD-L1 proteins had similar expression levels without statistically significant differences at six different time points. Co-localization of PD-L1 and WT1 indicated that PD-L1 was mainly localized in the nucleus of SCs. Contrary to previous studies,[31,32] it was the SCs that constitutively expressed PD-L1 regardless of testis developmental phase. SCs play a central role in the development of functional testes, including promoting gonadal differentiation, supporting spermatogenesis, and creating an immune-privileged environment.[37,48] The role played by PD-L1 expressed in the nucleus of SCs requires further investigation.

Recent studies agree that PD-L1 is a membrane-bound cell-surface molecule or exists in the form of sPD-L1 in the plasma. Chen et al[23] showed that sPD-L1 exists in human serum and that the concentration increases in an age-dependent manner. Moreover, it is mainly produced via cleavage from cell surface and is then released by MMP. Emerging data indicate that higher levels of sPD-L1 in serum (or plasma) are related to the poor outcome or disease severity of non-small-cell lung cancer,[49] hepatocellular carcinoma,[50]

and systemic sclerosis.[51] Additionally, sPD-L1 can specifically bind to PD-1 and deliver inhibitory signaling into PD-1^{+} T cells.[23,25] Based on the traditional role of PD-L1 or sPD-L1, we speculated that SCs could secrete sPD-L1 into the interstitial space in physiological conditions, interacting with PD-1^{+} immune cells, thereby playing an immunosuppressive role. To verify this hypothesis, we performed immunofluorescence staining of TM4 cell lines and primary SCs, and conducted ELISA for the detection of sPD-L1 in testicular IF and culture supernatants.

The results of immunofluorescence staining showed that PD-L1 was mainly localized in the nucleus of the TM4 cell lines and primary SCs. The concentration of sPD-L1 in the testicular IF of adult mice was 6.608 ± 1.814 ng/mL, which was significantly higher than that in culture supernatants of both TM4 and primary SCs. The results showed that other PD-L1^{+} interstitial cells might also secrete sPD-L1 into the testicular interstitial area apart from SCs. Testicular macrophages (TM), as main immune cells, comprised about 80% of leukocyte in the interstitium and mainly showed an alternatively activated phenotype (including increased expression of CD163, high secretion of IL-10, and low secretion of TNF-α) under the influence of the testicular environment, such as corticosterone in the IF.[17,52] Therefore, we speculate that sPD-L1 in IF mainly comes from membrane PD-L1 expressed on TM. In addition, there might be other ways to produce sPD-L1 besides proteolysis of membrane-bound PD-L1 by MMP. Nevertheless, the exact role of PD-L1 expression in the nucleus of SCs and the immunological function of sPD-L1 in the physiological and inflammatory conditions of the testes need further investigation.

In summary, in the present study, we found that PD-1 and PD-L1 were present in the testicular tissues of adult mice. The expression and localization of PD-1 obviously fluctuated with age, suggesting that PD-1 might play a role in spermiogenesis. PD-L1 was mainly localized to the SCs, which could secrete sPD-L1 into the testicular interstitial space. Thus, it might be involved in testicular immune privilege; however, determining the precise function requires further investigation in mouse and human testes.

5 | CONCLUSION

For the first time, our findings indicate that PD-1 and PD-L1 proteins exist in the testes of adult mice. PD-1 was mainly localized to the germ cells and was dependent on the developmental stage of the mouse. Consequently, it may play a role in spermiogenesis, which was not previously recognized. PD-L1 was constitutively expressed in the SCs and is likely related to testicular immune privilege. However, the explanation of the exact role of the PD-1/PD-L1 pathway in the testes is far from complete.

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CONFLICT OF INTEREST

The author declared no conflict of interest.

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