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The Programmed Cell Death Ligand-1/Programmed Cell Death-1 Pathway Mediates Pregnancy-Induced Analgesia via Regulating Spinal Inflammatory Cytokines

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BACKGROUND: The maternal pain threshold gradually increases during pregnancy, especially in late pregnancy. A series of mechanisms underlying pregnancy-induced analgesia have been reported. However, these mechanisms are still not completely clear, and the underlying molecular mechanisms need further investigation. We examined the relationship between the antinociceptive effect and the expression level of programmed cell death ligand-1 (PD-L1) during pregnancy and further observed the changes in pain thresholds and expression levels of cytokines in late-pregnant mice before and after blockade of PD-L1 or programmed cell death-1 (PD-1).

METHODS: Part 1: Female mice were assigned to 3 groups (nonpregnant, late-pregnant, and postpartum). Part 2: Late-pregnant mice were assigned to 3 treatment groups (control [phosphate buffer solution], RMP1-14 [mouse anti–PD-1 antibody], and soluble PD-1 [sPD-1]). Behavioral testing (mechanical and thermal) and tissue (serum and spinal cord) analysis were performed on all groups. PD-L1, interleukin (IL)-10, tumor necrosis factor-α (TNF-α), and IL-6 expression levels in tissue were examined via reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and Western blot analysis.

RESULTS: The mechanical and thermal pain thresholds were significantly increased in late pregnancy and decreased after delivery. PD-L1 expression was also elevated in late pregnancy and decreased after delivery. In addition, in the late stage of gestation, the maternal inflammatory microenvironment was dominated by anti-inflammatory factors. After administration of RMP1-14 or sPD-1, the pain thresholds of late-pregnant mice were significantly reduced. In late-pregnant mice, the high level of IL-10 was obviously reduced, and the low levels of TNF-α and IL-6 were elevated.

CONCLUSIONS: The PD-L1/PD-1 pathway mediates pregnancy-induced analgesia, partially via the regulation of cytokines. (Anesth Analg 2021;133:1321–30)

KEY POINTS

- **Question:** The mechanisms of pregnancy-induced analgesia are not completely clear; are there any other molecular mechanisms that mediate pregnancy-induced analgesia?
- **Findings:** The programmed cell death ligand-1/programmed cell death-1 (PD-L1/PD-1) pathway mediates pregnancy-induced analgesia by regulating the inflammatory cytokine microenvironment in the spinal cord.
- **Meaning:** The application of PD-L1 for the treatment of acute or chronic pain in pregnant women and for labor analgesia appears promising.

GLOSSARY

ANOVA = analysis of variance; **APC** = antigen-presenting cell; **BL** = basal line; **CCI** = chronic constriction injury; **CNS** = central nervous system; **DRG** = dorsal root ganglion; **ECL** = enhanced chemiluminescent; **ELISA** = enzyme-linked immunosorbent assay; **GAPDH** = glyceraldehyde-3-phosphate dehydrogenase; **IASP** = International Association for the Study of Pain; **IL** = interleukin; **PD-1** = programmed cell death-1; **PD-L1** = programmed cell death ligand-1; **PIA** = pregnancy-induced analgesia; **PNS** = peripheral nervous system; **RT-PCR** = reverse transcription-polymerase chain reaction; **SDS-PAGE** = sodium dodecyl sulfate poly acrylamide gel electrophoresis; **SEM** = standard error of mean; **sPD-1** = soluble PD-1; **TFL** = tail-flick latency; **TBST** = Tris-buffered saline with Tween 20; **TNF-α** = tumor necrosis factor-α

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The safe management of analgesia in pregnant women with acute and chronic pain is a unique clinical challenge. Traditional methods of pain management, such as over-the-counter analgesics, opioids, and antidepressants, are limited due to their side effects. Therefore, there is an urgent need to develop new drugs and methods suitable for the treatment of pain in pregnant women.

Although pregnancy is an important risk factor that exacerbates pain progression, previous animal studies demonstrated that the maternal antinociceptive ability (mechanical, thermal, and visceral pain thresholds) progressively improves during pregnancy, especially in late pregnancy[1–5]; this improvement has also been found in pregnant women.[6–9] Moreover, the mechanisms underlying pregnancy-induced analgesia (PIA), first reported in 1980, are intricate and include an opioid receptor–mediated mechanism,[1,3,5,10] a hormone-mediated mechanism,[11,12] a hypogastric nerve mechanism[2] and a T-cell–mediated mechanism.[13] Evidence has shown that pregnancy reduces the level of tumor necrosis factor-α (TNF-α) to reduce neuropathic pain in rats with chronic constriction injury (CCI).[14] However, the detailed immunosuppressive molecular mechanisms underlying PIA have not been investigated.

Programmed cell death ligand-1 (PD-L1, CD274), a checkpoint inhibitor protein, and a novel immunosuppressive analgesic molecule can regulate T-cell homeostasis and induce peripheral immune tolerance via its receptor, programmed cell death-1 (PD-1).[15–17] The PD-L1/PD-1 pathway plays a highly important role in the induction of maternal and fetal immune tolerance and maintains normal pregnancy.[15,18,19] In addition, evidence suggests that PD-L1 is expressed in not only malignant tissues (melanoma) but also normal tissues (the dorsal root ganglion [DRG], spinal cord, and placenta). PD-L1 can regulate DRG neuron excitability in the peripheral and spinal nervous systems in mice and humans via PD-1, suppressing inflammatory, neuropathic, and bone cancer pain.[20] PD-L1 deficiency was found to increase inflammation and neuropathic pain in mice.[21] Interestingly, the maternal peripheral level of PD-L1 during pregnancy and the increased maternal mechanical pain, heat, and visceral pain thresholds follow the same trend.[18] Therefore, the relationship between PD-L1 and PIA is a vital issue that requires further investigation.

In this study, we assessed the expression of PD-L1 in the spinal cord of nonpregnant mice, late-pregnant mice, and postpartum mice and further examined the expression of TNF-α, interleukin (IL)-6, and IL-10 in the spinal cord. We discovered that the maternal pain threshold exhibits changes similar to those in the maternal PD-L1 level during pregnancy and postpartum and that late-pregnant mice exhibited increased levels of anti-inflammatory cytokines and decreased levels of proinflammatory cytokines. Moreover, we revealed that PD-L1 regulates PIA via PD-1 in a manner at least partially due to variations in the maternal inflammatory factor microenvironment. Based on previous experimental evidence and our experimental results indicating that PD-L1 is an effective analgesic and plays an important role in the maintenance of pregnancy, to the clinical application of PD-L1 for the treatment of acute or chronic pain in pregnant women and for labor analgesia appears promising.

METHODS

Animals

Naive, adult (8-week-old) male and female mice were housed 3 to 5 mice per cage for 2 weeks before initiation of the study. All mice used in the experiments were provided by Hunan SJA Laboratory Animal Co, Ltd. Mice were maintained in a temperature-controlled (24–25 °C) and humidity-controlled (50%–60%) environment (12/12 hours light/dark cycle) with free access to water and food. After mating, the female mice (3 per cage) were housed according to their gestational day. Beginning on gestational day 10, the pregnant females were housed individually. We obtained approval from the animal experimentation committee of Xiangya Hospital to perform this study (examination and approval no: 2018121127).

Pregnancy Model

Before mating, the baseline mechanical and thermal pain thresholds of the female mice were determined using von Frey filaments and the tail-flick latency (TFL) test, respectively, between 10:00 AM and 12:00 AM. Due to the ratio at which the mice were placed cages for mating (female: male 2:1), we defined the time at which a vaginal plug was discovered as one half-day (0.5 days) of pregnancy. Early-pregnant females were defined as those 5 to 7 days into the 20 ± 1 day gestation period (≈P6), midpregnant females were defined as those 8 to 15 days into the gestation period (≈P12), late-pregnant females were defined as those 16 to 21 days into the gestation period (≈P19),[13] and postpartum females were defined as those 2 to 7 days (≈D7) after delivery.

Von Frey Test for Evaluating the Mechanical Pain Threshold

Animals were habituated to boxes on an elevated metal mesh floor daily beginning 2 days before baseline testing. We confined individual mice in boxes on the elevated metal mesh floor for a 1-hour habituation period. Then, we stimulated the left hind paws of the mice with a series of von Frey hairs of increasing mass (0.16, 0.4, 0.6, 1.0, 1.4, 2.0, and 4.0 g) applied vertically

to the central plantar surface. Testing started with a 0.4-g monofilament, and a positive response was defined as 3 or more paw withdrawals among 6 applications. If the response was negative, we applied the next monofilament. The monofilament that evoked the first positive response was designated the threshold. Mechanical withdrawal thresholds were measured at baseline, early-pregnant, midpregnant, and late-pregnant and again at 90 minutes after separate administration of the clone RMP1-14 mouse anti–PD-1 antibody (RMP1-14) and soluble PD-1 (sPD-1). Animal experiments were conducted in accordance with the International Association for the Study of Pain (IASP) ethics guidelines.

TFL Test for Thermal Pain

Mice were placed in a mouse fixator for 10 minutes. After the mice were quiet, the lower 1/3 of their tail of each mouse was immersed vertically in water bath, which was heated to and maintained at 48.0 ± 0.5 °C. The TFL was defined as the time from entry of the tail into the water past its surface to retraction of the tail from the surface of the water, with a cutoff time of 15 seconds was established to prevent tissue damage. We assessed 3 times per animal at intervals of 5 minutes per animal, and the average value was defined the TFL of per mice. TFL values were measured at baseline, early-pregnant, mid pregnant, and late pregnant, and again at 90 minutes after separate administration of RMP1-14 and sPD-1.

Drugs and Injections

The mouse anti–TNF-α antibody (catalog: ab215188) was purchased from Abcam. The mouse anti–IL-10 (catalog: DF6894) and anti–IL-6 (catalog: DF6087) antibodies were purchased from Affinity. A mouse PD-L1 (CD274) enzyme-linked immunosorbent assay (ELISA) kit (catalog: CSU-EL004911MO) was purchased from CUSABIO. Mouse PD-1 (5 μg, catalog: 1021-PD-100) was obtained from R&D Systems. RMP1-14 (20 μg, catalog: BE0146) was purchased from Bio X Cell. For intrathecal injection to deliver reagents (20 μL) into the cerebrospinal fluid,[20] a lumbar puncture was performed with a 30-gauge needle between the L5 and L6 vertebrae.[20]

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

The dorsal lumbar (L4–L6) spinal cords of euthanized mice were collected, after which total RNA was extracted using an RNA extraction kit (catalog: MK020001-050, MultiSciences), and 1000 ng of RNA was reverse transcribed. Specific primers for amplification of GAPDH, and PD-L1 were designed, and TNF-α, IL-6, and IL-10 levels were determined from quantitative polymerase chain reaction (PCR) amplification samples containing an equal amount of the reverse transcription (RT) product, 5 μL of 2× iQSYBR-Green mix, 0.2 μL of ROX, and 100 μM forward and reverse primers in a final volume of 10 μL. The primer sequences are shown in the Table. Relative quantification was performed with the $2^{-\triangle\triangle CT}$ method.

Western Blot Analysis

The dorsal lumbar (L4–L6) spinal cords of euthanized mice were collected and stored at −80 °C. The tissue was homogenized in protein lysis buffer containing a phosphatase inhibitor and a protease inhibitor (100:1:1) with an ultrasonic homogenizer, and the supernatant (protein) was obtained by refrigerated centrifugation (4 °C) at 12,000×g for 30 minutes. Then, the protein concentration in the supernatant was determined using a BCA protein quantitation kit (catalog: 70-PQ0012, MultiSciences). Protein samples (30–50 μg) were dissolved in loading buffer and denatured at 100 °C for 8 minutes. The protein samples were individually added to each lane of 12% sodium dodecyl sulfate poly acrylamide gel electrophoresis (SD-PAGE) gels and separated. After the proteins were transferred to membranes, the membranes were blocked with Quick Block solution for 10 minutes at room temperature and were then incubated with primary antibodies (rabbit anti–TNF-α, 1:1000; rabbit anti–IL-10, 1:1000; and rabbit anti–IL-6, 1:1000) overnight at 4 °C. After 3 washes with Tris-buffered saline with Tween 20 (TBST), the membranes were further incubated with secondary antibodies (1:10,000) for 90 minutes at room temperature. Finally, the membranes were washed 3 times, and immunoreactions were detected by incubation in enhanced chemiluminescent (ECL) solution. The intensity of specific bands was analyzed using Image Lab software.

Enzyme-Linked Immunosorbent Assay

A mouse PD-L1 (CD274) ELISA kit (catalog: CSU-EL004911MO) was purchased from CUSABIO. Tissue samples were extracted as described for Western blot analysis. Serum was extracted from whole blood collected by enucleation. After 2 hours of incubation at room temperature, the supernatant (serum) was

Table. Primer Sequence

Target gene	Primer (5′–3′)
PD-L1	Forward 5′-CAGAGGGGATGCTTCTCAAT-3′
	Reverse 5′-GGTTCAACACTGCTTACGTC-3′
IL-10	Forward 5′-TATGATGGGAGGGGTTCTTCCT-3′
	Reverse 5′-GGTTTCTCTTCCCAAGACCCAT-3′
GAPDH	Forward 5′-AGGTCGGTGTGAACGGATTTG-3′
	Reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′
IL-6	MQP036632
TNF-α	MQP077890

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; PD-L1, programmed cell death ligand-1; TNF-α, tumor necrosis factor-α.

obtained by refrigerated centrifugation at 1000×g for 20 minutes. For ELISAs of serum, spinal cord tissue, and placental tissue, 100 μL of serum and samples containing 50 μg of protein were used. ELISA was conducted according to the instructions of the ELISA kit.

Statistical Analyses

Statistical analyses of the data from this study were conducted with GraphPad Prism 5 software. All values and the behavioral data from the von Frey and TFL tests, which indicate the mechanical pain threshold and thermal pain threshold, respectively, are expressed as the means ± standard errors of mean (SEMs). The behavioral and biochemical data were analyzed using the normality test followed by the Kolmogorov-Smirnov test.

For behavioral data, a 2-way analysis of variance (ANOVA) with repeated measures and post hoc multiple comparison Bonferroni tests was used to compare differences between the pregnant group and the nonpregnant group.

The pain thresholds of basal line (BL) was compared with at 4 time points of P6, P12, P19, and D7 separately in pregnant group, and comparing the changes of pain threshold before and after administration of medicine in maternal mice in late pregnancy among the control, RMP1-14, and sPD-1 groups by using repeated measures, paired *T* test and post hoc multiple comparisons Bonferroni tests were used, the significant level of repeated test was $\alpha' = 0.05/k$, k is defined as numbers of comparison.

For biochemical data, a 1-way ANOVA with repeated measures and post hoc multiple comparison Bonferroni tests was used to compare differences among the nonpregnant, late-pregnant, and postpartum groups. A 2-tailed *t* test with or without Welch's correction was used to compare differences between the control group and RMP1-14 group, and between the control group and sPD-1 group. $P < .05$ was considered to be statistically significant.

The sample size in the early stage of the experiment was determined mainly by referring to the sample size reported in previously reported studies. Besides, the sample size was also calculated through experimental design and conventional numerical settings (the effect size of 0.3, a type 1 error [$P = .05$], and a 90% power) using G Power. For example, in part 1, the behavioral experiment to measure mechanical sensitivity between nonpregnant and pregnant groups, the total sample size (n = 10 per group) was calculated.

RESULTS

The Maternal Pain Threshold and Expression of PD-L1 Increase During Pregnancy

As a first step to verify PIA, we observed changes in pain thresholds throughout pregnancy and postpartum and compared the pain thresholds of nonpregnant, early-pregnant, midpregnant, late-pregnant, and postpartum mice. The maternal pain thresholds significantly increased during late pregnancy but returned to baseline after parturition.

In addition, we selected mice at 3 different time points according to the maternal pain threshold (nonpregnant, late-pregnant, and postpartum mice). We first performed quantitative RT-PCR of spinal cord samples obtained at 3 different time points and further evaluated the blood and spinal levels of PD-L1 by ELISA, after which we compared the expression of PD-L1 among nonpregnant, late-pregnant, and postpartum mice (serum level: nonpregnant [259.8 ± 88.66 pg/mL] versus late-pregnant [982.2 ± 243.4 pg/mL] versus postpartum [227.8 ± 17.14 pg/mL]; spinal level: nonpregnant [79.80 ± 14.48 pg/50 μg] versus late-pregnant [113.2 ± 5.376 pg/50 μg] versus postpartum [72.24 ± 13.98 pg/50 μg]; placental level: late-pregnant [102.9 ± 15.81 pg/50 μg]). The spinal level of PD-L1 mRNA in late-pregnant mice was significantly higher than that in nonpregnant mice and postpartum mice. Interestingly, the trends in the maternal pain thresholds and maternal expression levels of PD-L1 during pregnancy and postpartum were remarkably similar (Figure 1).

Late-Pregnant Mice Exhibit Increased Levels of Anti-inflammatory Cytokines and Decreased Levels of Proinflammatory Cytokines

We evaluated and compared the maternal inflammatory factor microenvironment among late-pregnant, postpartum, and nonpregnant mice. In the present study, we examined the levels of the proinflammatory factors TNF-α and IL-6 and the anti-inflammatory factor IL-10 in the spinal cord horn. Proinflammatory and anti-inflammatory factor expression in the spinal cord was compared among the 3 groups using 1-way ANOVA. The levels of anti-inflammatory factors were significantly higher and the levels of proinflammatory factors were significantly lower in late-pregnant mice than in nonpregnant mice and postpartum mice. However, the levels of proinflammatory and anti-inflammatory factors did not differ between nonpregnant mice and postpartum mice (Figure 2). These results indicate that late-pregnant mice exhibit a stronger anti-inflammatory microenvironment, which may explain the reversal of allodynia in late-pregnant mice.

PD-L1 Mediates PIA via PD-1

Interestingly, PD-L1 is not only a checkpoint inhibitor protein that participates in the induction of maternal and fetal immune tolerance and the maintenance of pregnancy but also an endogenous pain inhibitor that regulates DRG neuron excitability in the peripheral

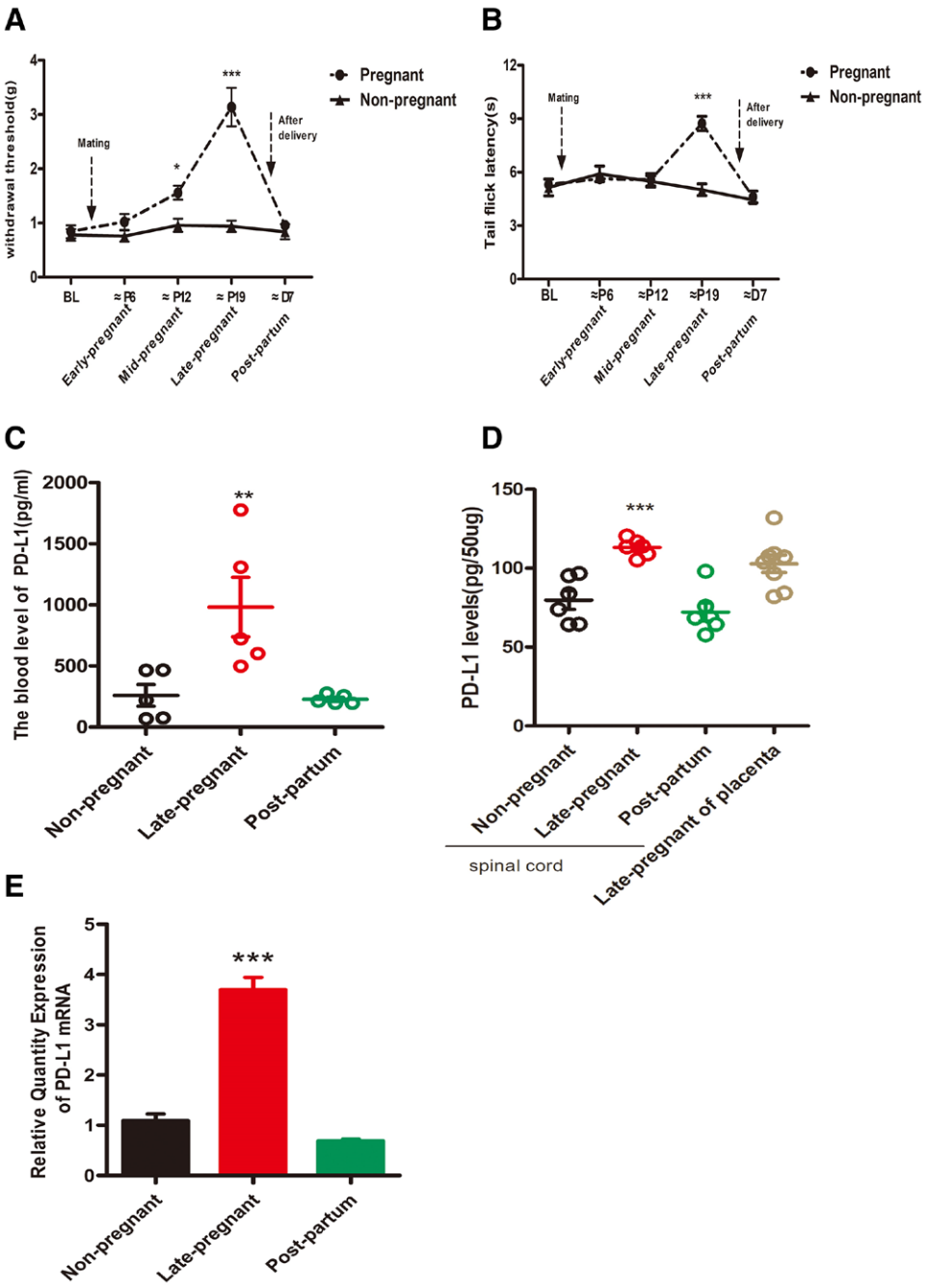

Figure 1. The maternal pain threshold and the maternal expression of endogenous PD-L1 increase in late pregnancy and decrease after delivery. A and B, The withdrawal time and thermal threshold in mice obviously increase during pregnancy, especially in late pregnancy (***P < .001); *P < .05 (at midpregnant time point) for comparison of the nonpregnant group with the pregnant group at the same time point by using 2-way repeated measures ANOVA with the Bonferroni post hoc test, n = 10 per group. The pain thresholds of BL were compared at 4 time points of P6, P12, P19, and D7 separately in pregnancy group by using repeated measures, paired T test, and post hoc multiple comparisons Bonferroni tests, P12 versus BL, P = .005; P19 versus BL, P = .000; (C, D) ELISA results showing the levels of endogenous PD-L1 ([C] serum; [D] spinal cord and placenta) in nonpregnant, late-pregnant, and postpartum mice. Serum: P = .0059, **P < .01, n = 5 per group; nonpregnant group versus late-pregnant group, *P <.05; nonpregnant group versus postpartum group, P > .05; late-pregnant group versus postpartum group, *P < .05. Spinal cord: ***P < .0001, n = 6 per group; 1-way ANOVA with the Bonferroni post hoc test; nonpregnant group versus late-pregnant group, **P < .01; nonpregnant group versus postpartum group, P > .05; late-pregnant group versus postpartum group, ***P < .001. E, Relative expression of PD-L1 mRNA in the lumbar (L4 -L6) spinal cord of nonpregnant, late-pregnant, and postpartum mice. The relative expression level was normalized to the level in nonpregnant mice. ***P < .0001, n = 5 per group, 1-way ANOVA with the Bonferroni post hoc test; nonpregnant group versus late-pregnant group, ***P < .0001; nonpregnant group versus postpartum group, P > .05; late-pregnant group versus postpartum group, ***P < .0001. ANOVA indicates analysis of variance; BL, basal line; ELISA, enzyme-linked immunosorbent assay; PD-L1, programmed cell death ligand-1.

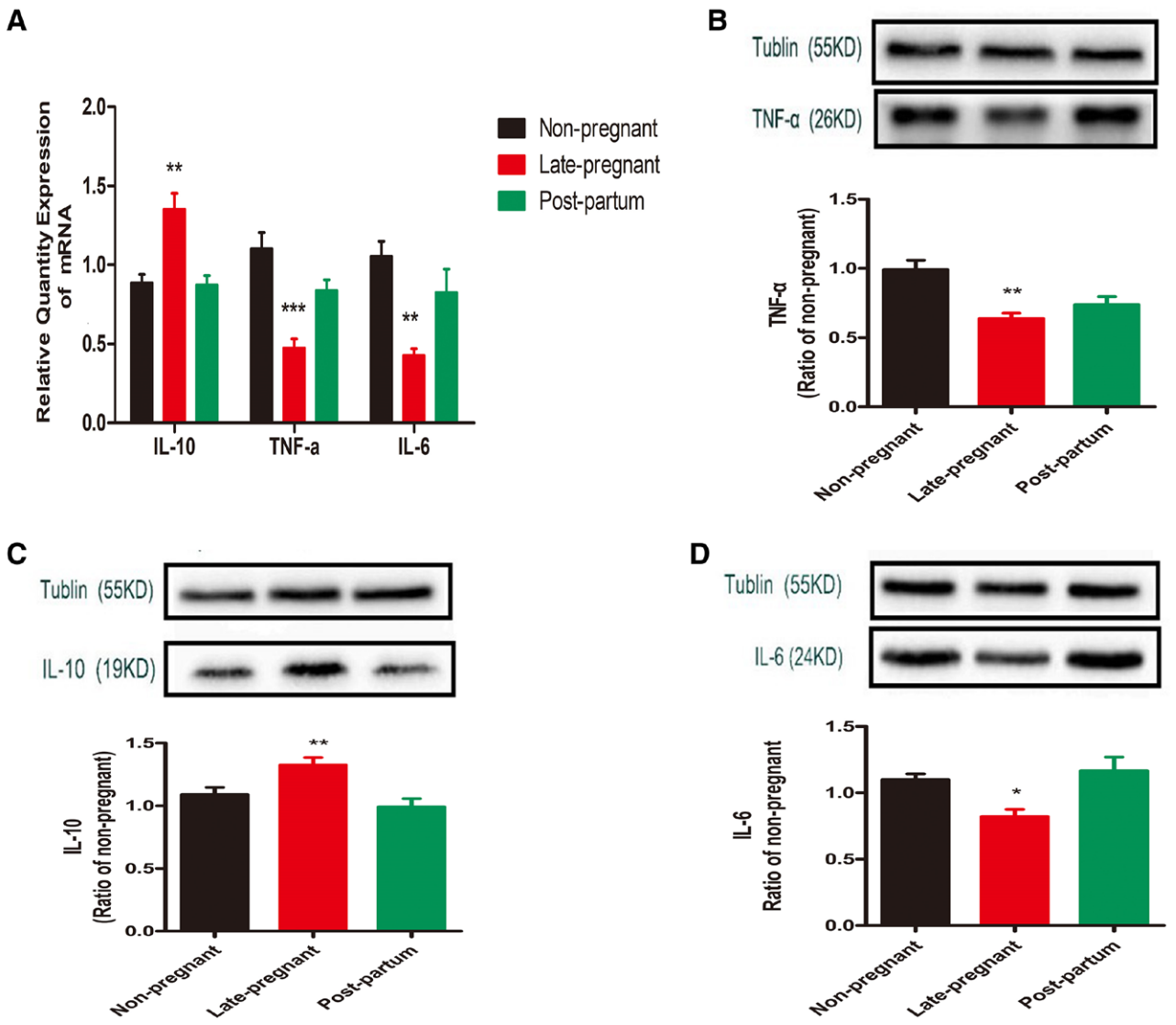

Figure 2. Late-pregnant mice exhibit a stronger anti-inflammatory microenvironment. A, Relative expression of IL-10, TNF-α, and IL-6 mRNA in the dorsal lumbar (L4 -L6) spinal cord of nonpregnant, late-pregnant, and postpartum mice. The relative expression level was normalized to the level in nonpregnant mice. IL-10 expression was higher in late-pregnant mice than in nonpregnant and postpartum mice; TNF-α and IL-6 expression was lower in late-pregnant mice than in nonpregnant and postpartum mice. IL-10, $^{**}P = .001$, <.01; TNF-α, $^{***}P = .0004$, <.001; IL-6, $^{**}P = .0038$, <.01, n = 5 per group, 1-way ANOVA with the Bonferroni post hoc test. B, C, and D, Western blot analysis showing the relative protein expression levels of TNF-α (B), IL-10 (C), and IL-6 (D) in the dorsal lumbar (L4 -L6) spinal cord of nonpregnant, late-pregnant, and postpartum mice. The relative expression levels were normalized to the level in nonpregnant mice. IL-10 protein expression was higher and TNF-α and IL-6 protein expression was lower in late-pregnant mice than in nonpregnant and postpartum mice. IL-10, $^{**}P = .0032$, <.01; TNF-α, $^{**}P = .0013$, <.01; IL-6, $^{*}P = .0124$, <.05; n = 5 per group, 1-way ANOVA with the Bonferroni post hoc test. TNF-^{a} mRNA: $^{*}P < .05^{a}$, $P > .05^{b}$, $^{*}P < .05^{c}$; IL-6 mRNA: $^{*}P < .05^{a}$, $P > .05^{b}$, $P > .05^{c}$; IL-10 mRNA: $^{*}P < .05^{a}$, $P > .05^{b}$, $^{*}P < .05^{c}$. TNF-α protein: $^{*}P < .05^{a}$, $P > .05^{b}$, $P > .05^{c}$; IL-6 protein: $^{*}P < .05^{a}$, $P > .05^{b}$, $^{*}P < .05^{c}$; IL-10 protein: $^{*}P < .05^{a}$, $P > .05^{b}$, $^{*}P < .05^{c}$. a: Nonpregnant group versus late-pregnant group; b: Nonpregnant group versus postpartum group; c: late-pregnant group versus postpartum group. ANOVA indicates analysis of variance; IL, interleukin; TNFα, tumor necrosis factor-α.

and spinal nervous systems. Most importantly, PD-L1 is obviously elevated in the third trimester. Thus, we boldly hypothesized that PD-L1 is likely involved in the regulation of PIA by altering the maternal analgesic microenvironment—the levels of proinflammatory and anti-inflammatory factors.

To determine whether endogenous PD-L1 expressed in the maternal spinal cord regulates PIA, we evaluated the mechanical and thermal pain thresholds in late-pregnant mice after pharmacological blockade of either PD-L1 or PD-1. RMP1-14 is a mouse anti–PD-1 antibody, and sPD-1 can neutralize PD-L1 in the body. The maternal pain thresholds (mechanical and thermal pain thresholds) were obviously decreased by intrathecal injection of sPD-1 (5 μg) and RMP1-14 (20 μg) (Figure 3), suggesting that PD-L1 plays a crucial role in the maternal analgesic effect during pregnancy and mediates PIA via PD-1. Next, we further examined the levels of proinflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10) factors in the spinal cord in

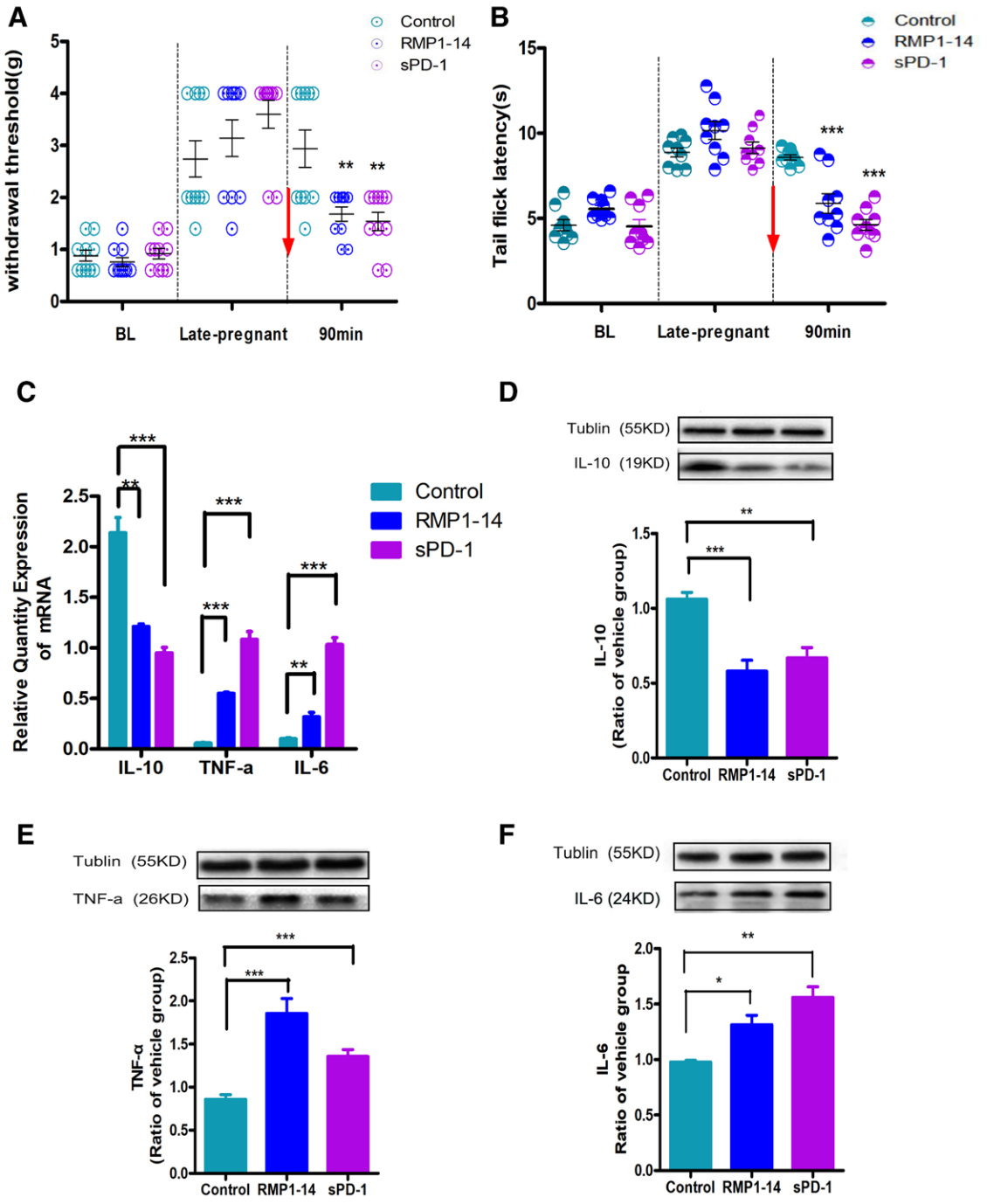

Figure 3. The PD-L1/PD-1 pathway mediates pregnancy-induced analgesia by regulating the inflammatory factor microenvironment in the maternal spinal cord. A and B, Inhibition of endogenous PD-L1 (sPD-1) or PD-1 (RMP1-14, a mouse anti -PD-1 antibody) abolished pregnancy-induced mechanical (A) and thermal (B) analgesia 90 min after intrathecal injection of RMP1-14 and sPD-1. A, RMP1-14 group vs vehicle group, $^{**}P$ = .0087, <.01; sPD-1 group vs vehicle group, $^{**}P$ = .0032, <.01; (B)RMP1-14 group versus control group, $^{***}P$ < .001; sPD-1 group versus control group, $^{***}P$ < .001 by using repeated measures, paired T test, and post hoc multiple comparisons Bonferroni tests; (C) RT-PCR analysis showing that after pharmacological blockade of either PD-L1 or PD-1 in late-pregnant mice, the maternal level of IL-10 was significantly decreased (RMP1-14 group versus control group, $^{**}P$ = .0039, <.01; sPD-1 group versus control group, $^{***}P$ < .001, n = 5 per group) and that the levels of TNF-α and IL-6 were obviously increased (RMP1-14 group versus control group –TNF-α: $^{***}P$ < .0001, IL-6: $^{**}P$ = .0015, <.01; sPD-1 group versus control group –TNF-α: $^{***}P$ < .0001; IL-6: $^{***}P$ < .0001, n = 5 per group) in the spinal cord. D-F, Western blot analysis showing that 90 min after intrathecal injection of RMP1-14 and sPD-1 in late-pregnant mice, IL-10 protein expression in the dorsal lumbar (L4 -L6) spinal cord was reduced (RMP1-14 group compared with control group, $^{***}P$ = .0005, <.001, n = 5 per group); sPD-1 group versus control group, $^{**}P$ = .0016, n = 5 per group and TNF-α and IL-6 protein expression was increased (RMP1-14 group versus control group –TNF-α: $^{***}P$ = .006; *IL-6: P = .0167, <.05, n = 5 per group; sPD-1 group versus control group –TNF-α: $^{***}P$ = .0007, <.001; IL-6: $^{**}P$ = .0037, <.01, n = 5 per group). These data were analyzed using a 2-tailed t test with or without Welch's correction. Arrow indicates drug injection. PD-L1/PD-1 indicates programmed cell death ligand-1/programmed cell death-1; RT-PCR, reverse transcription-polymerase chain reaction; sPD-1, soluble PD-1; TNF-α, tumor necrosis factor-α; IL, interleukin.

late-pregnant mice on intrathecal injection of sPD-1 or RMP1-14 by RT-PCR and Western blot analysis. In late-pregnant mice, intrathecal injection of sPD-1 or RMP1-14 increased the levels of proinflammatory cytokines (TNF-α, IL-6) and decreased the levels of an anti-inflammatory cytokine (IL-10) in the spinal cord.

DISCUSSION

Principal Findings

Our study showed that late-pregnant mice exhibited hypoalgesia and that both the spinal cord and peripheral levels of PD-L1 in mice were significantly increased in late pregnancy and decreased after delivery. Furthermore, in this study, we observed that in normal late-pregnant mice, the spinal levels of proinflammatory cytokines (TNF-α and IL-6) were decreased, while that of an anti-inflammatory cytokine (IL-10) was increased. Lesioning a tissue can lead to a local increase of proinflammatory cytokines; TNF-α and IL-6 have been shown to be algesic in various pain models. TNF-α is the most common and important proinflammatory factor that mediates pain, especially neuropathic pain, which prompts leukocyte transmigration into the nerves.[14,22] IL-6 is an emerging regulator of pathological pain; a variety of pain models has shown elevated expression levels of IL-6 in the spinal cord and dorsal root ganglia. Besides, the administration of IL-6 protein can result in mechanical allodynia and thermal hyperalgesia.[23] In contrast, anti-inflammatory cytokines, such as IL-10, are able to relieve pain, researches have shown that intrathecal administration IL-10 protein can briefly reverse mechanical allodynia and thermal hyperalgesia.[24,25] These results suggest that pregnancy, a physiological process, can alter the inflammatory microenvironment of the maternal spinal cord, affecting the maternal antinociceptive ability. Importantly, intrathecal injection of RMP1-14 and sPD-1 reversed the maternal antinociceptive ability induced in late pregnancy, suggesting that the PD-L1/PD-1 pathway mediates PIA via a mechanism at least partially due to decreased expression of proinflammatory cytokines (TNF-α and IL-6) and increased expression of an anti-inflammatory cytokine (IL-10) in the spinal cord.

Results

A series of experiments have proven that late-pregnant mice exhibit efficient resistance to mechanical, thermal and visceral pain, indicating a condition of PIA.[1,8,13,14,26] The increase in the nociceptive threshold in the late stage of gestation is beneficial for the mother. Previous studies have shown the effect of maternal antinociception on central and peripheral processing.[11,13,14,27] Several mechanisms of PIA have been reported; these mechanisms include an opioid-mediated mechanism,[1,3,5,10] an α(2)-noradrenergic receptor system–mediated mechanism,[26] a hypogastric nerve mechanism,[2] hormone (estrogen, progesterone)-related mechanisms[11,12] and a T-cell–mediated mechanism.[13] T cells are involved in PIA, suggesting the importance of the immune system in mediating maternal hypoalgesia. As the maternal pain thresholds and the maternal expression of PD-L1 during pregnancy and postpartum followed similar trends, we focused on determining whether the PD-L1/PD-1 pathway plays a role in pregnancy-induced hypoalgesia. PD-L1, a checkpoint inhibitor protein and neuromodulator, is expressed not only in malignant tissues (melanoma) but also in normal tissues such as the DRG, spinal cord, and placenta and in a variety of cells, such as T cells, B cells, and professional antigen-presenting cells (APCs). PD-1 is a coinhibitory receptor expressed on spinal cord cells, the primary neurons of the DRG, and activated immune cells (T cells, B cells, and APCs).[21] The PD-L1/PD-1 pathway not only induces maternal and fetal immune tolerance and maintains pregnancy[28] but also regulates DRG neuron excitability in both the peripheral and spinal nervous systems and inhibits pain.[20] Evidence suggests that PD-L1 can inhibit T cell proliferation and cytokine production to maintain immune homeostasis and reduce inflammatory reactions by binding with PD-1.[29] In this study, we observed that late-pregnant mice with high PD-L1 levels harbor a powerful analgesic anti-inflammatory microenvironment and found that PD-L1 performs an analgesic function and regulates the inflammatory microenvironment to influence central processing.

Clinical Implications

PD-L1 is an effective analgesic and plays an important role in the maintenance of pregnancy. Clinical application of PD-L1 for the treatment of acute or chronic pain in pregnant women and for labor analgesia appears promising.

Research Implications

This study provides an overview of the mechanism by which the PD-L1/PD-1 pathway mediates PIA by regulating the levels of inflammatory cytokines in the maternal spinal cord. However, future research on the mechanism of PIA is encouraged. Several interesting lines of mechanistic evidence about PIA have been revealed. For example, the expression of the opioid genes Oprm1 (μ), Oprd1 (δ), and Opkm1 (κ) in the spinal cord and DRG was shown to be dependent on T cells.[13] T cells, especially CD4+ T cells, affect the activation of opioid receptor systems to exert antinociceptive effects.[30] In addition to mediating the regulation of cytokine secretion by CD4+ T cells, the PD-L1/PD-1 pathway can significantly decrease the levels of Th1-type cytokines secreted by CD4+ T cells (TNF-α,

INF-γ, IL-12) and promote the secretion of Th2-type cytokines (IL-4, IL-10).[31] Furthermore, research on PD-L1 in the central nervous system (CNS) and peripheral nervous system (PNS) showed that in the CNS, PD-L1 expression on microglia is associated with inhibition of T cell proliferation and reduced production of proinflammatory cytokines, such as TNF-α.[32,33] In the PNS, phagocytes are the major cellular source of PD-L1, which suppresses the inflammatory response and alleviates neuropathic pain after peripheral nerve injury; thus, microglia and phagocytes play an important role in models of pain.[21,34] Therefore, the means by which the relationship among the spinal cord and peripheral opioid receptor systems, CD4+ T cells, microglia, phagocytes and the PD-L1/PD-1 pathway collectively mediate PIA warrants further investigation.

Strengths and Limitations

In this study, we further confirmed that PD-L1 mediates PIA by finding that the both the maternal pain threshold and the expression of PD-L1 increase during pregnancy. We showed for the first time that PD-L1 plays an important role in PIA. However, we focused on the role of the PD-L1/PD-1 pathway in mediating PIA via the regulation of inflammatory cytokines only in the spinal cord, not in the periphery.

DISCLOSURES

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