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Immunosuppressive mechanism of *Hypoderma lineatum* secreted serine esterase, a potential modulatory method used to inhibit transplant rejection

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ABSTRACT

Background: Although immunosuppressive therapies have made organ transplantation a common medical procedure worldwide, chronic toxicity has a major issue for long-term treatment. One method to improve therapies and methods is the application of immunomodulatory agents from parasites such as *Hypoderma lineatum*. Hypodermin A (HA) is a serine esterase secreted by the larvae of *Hypoderma lineatum*, several studies demonstrated its immunosuppressive mechanism *in vitro*, and recently we discovered that HA inhibits the expression of interferon (IFN)-γ and interleukin (IL)-2 and activates IL-10 expression. Therefore, we hypothesized that it might be a potential agent used to block allograft rejections. However, most studies of the immunosuppressive mechanisms associated with HA were undertaken at the cellular level. In order to augment these studies, we evaluated the immunosuppressive effects of HA *in vivo* using an HA transgenic mouse model.
Result: Our results revealed similar findings to those reported by *in vitro* studies, specifically that HA induced prostaglandin E_2 expression, downregulated IFN-γ and IL-2 expression, and promoted IL-10 secretion *via* E-type prostanoid receptor 4. Additionally, we observed that HA overexpression inhibited lipopolysaccharide-induced TLR4 activation. These findings provide insight into a new potential agent capable of blocking graft rejection.
Conclusion: Our founding suggested that HA-related treatment could be a promising option to improve the viability of grafts in human.

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1. Introduction

Organ transplantation is an effective way to treat many end-stage organ diseases; however, immunological rejection of the graft remains the primary reason for transplant failure. Therefore, to improve graft-viability rates, improved therapies and methods are required to inhibit transplant rejection. One method involves application of immunomodulatory agents from parasites, such as *Hypoderma lineatum*. *H. lineatum* infestation of cattle is difficult to circumscribe in the northern hemisphere due in part to the insect larvae modulating and evading the host immune system following invasion of host connective tissue.

Hypodermin A (HA), which is secreted by *H. lineatum* larvae, contains 771 bp and encodes 257 amino acid residues. HA has serine protease activities and plays an important role in immunosuppression during *H. lineatum* infestation [1]. Our previous study demonstrated that HA downregulates interferon (IFN)-γ and interleukin (IL)-2 expression and promotes IL-10 secretion *in vitro* [2]. The effect of HA on cytokine expression led us to hypothesize that HA might be a potential agent capable of preventing allogeneic rejection.

To investigate the role of HA in host immune response *in vivo*, we established a HA transgenic mouse using pronucleus microinjection and evaluated the effect of HA on cytokine expression related to graft-rejection response. Our results indicated that compared to the control, high levels of prostaglandin E_2 (PEG_2) and IL-10 and low levels of IFN-γ and IL-2 were detected in transgenic mice. Previous studies showed that toll-like receptor 4 (TLR4) activation regulates graft rejection. Here, we found that HA overexpression significantly

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inhibited lipopolysaccharide (LPS)-inducted TLR4 activation both *in vitro* and *in vivo*. Our findings suggested that HA could be a potentially effective immunosuppressant agent for reducing organ-transplant rejection.

2. Materials and methods

2.1. Animals, cell cultures, and lentivirus construction

Friend virus B-type (FVB) mice were purchased from the Animal Center of Xuzhou Medical University, Xuzhou, China. HA-overexpressing FVB transgenic mice were generated by Cyagen Biosciences (Santa Clara, CA, USA), and subsequent HA expression was verified *in vivo* [3]. Normal 6- to 8-week-old female mice were used in the experiments and were maintained under clean conditions. RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA), 0.25 μg/mL fungizone, 100 U/mL penicillin, 10 μg/mL streptomycin sulfate, and 5 μg/mL gentamicin and incubated at 37°C in 5% CO_2.

2.2. Protein analyses via western bloting

Back skin samples taken from mice were homogenized using a PK-02200PMGXL (Proscientific, USA), protein was isolated using lysis buffer (65 mM Tris–HCl, 4% sodium dodecyl sulfate [SDS], 3% DL-dithiothreitol, and 40% glycerol). 30 μg of protein was separated on a SDS-polyacrylamide electrophoresis gel and transferred onto nitrocellulose membranes. Membranes were blocked in 5% skim milk, and probed with a rabbit polyclonal antibody against HA, or a rabbit monoclonal antibody specific to β-actin overnight. Membranes were then washed three times in TBST buffer (20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20), and incubated with horseradish peroxidase-conjugated anti-rabbit (Beyotime Biotechnology, China) (diluted 1:1000) at room temperature, and visualized with Odyssey® CLx Infrared Imaging System (LI-COR, Lincoln, NE, USA).

2.3. RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)

A lentiviral vector containing a green-fluorescent protein tag and the full-length coding sequence for the HA gene (Lv-HA) was generated by GenePharma (Shanghai, China). Our previous study demonstrated its ability to be transduced into RAW264.7 cells and effectively express the HA protein [2]. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total cellular RNA from RAW264.7 cells, followed by conversion to cDNA using reverse transcriptase (Roche Applied Science, Mannheim, Germany). cDNA samples were standardized based on the content of GAPDH cDNA as a housekeeping gene. The sequences of both the forward and reverse primers used in this study were designed with Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA) and are listed in Table 1.

Table 1
Primers used in this study.

Primer	Sequence 5′ → 3′
IL-1β-F	GCAACTGTTCCTGAACTCAACT
IL-1β-R	ATCTTTTGGGGTCCGTCAACT
TNF-α-F	GTGGAACTGGCAGAAGAGGC
TNF-α-R	AGACAGAAGAGCGTGGTGGC
GAPDH-F	GGCAAATTCAACGGCACAGT
GAPDH-R	TAGGGCCTCTCTTGCTCAGT

2.4. Enzyme-linked immunosorbent assay (ELISA)

Blood was obtained from the eyeballs of mice, and the concentrations of IL-2, IFN-γ, IL-10, and PGE_2 in serum were quantified using specific ELISAs (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. RAW264.7 cells were cultured in DMEM and after a series of treatments, supernatants were collected and levels of IL-β1 and tumor necrosis factor (TNF)-α measured by ELISA (R&D systems). Absorbance was determined with an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

2.5. Statistical analysis

All experiments were conducted in triplicate to ensure that the results were reproducible. Data are presented as the mean ± standard deviation. A $P < 0.05$ was considered significant, whereas a $P < 0.01$ was considered highly significant.

3. Results

3.1. HA downregulates the production of IL-2 and IFN-γ and promotes IL-10 production in vivo

Our previous study showed that HA overexpression in RAW264.7 cells downregulates IL-2 and IFN-γ expression and upregulates IL-10 expression. To extend our study *in vivo*, we generated FVB transgenic mice capable of overexpressing HA, and western blot was performed to detect the expression of HA. The result showed that HA was expressed successfully in the transgenic mice (Fig. 1a). Then we measured the concentrations of IL-2, IFN-γ, and IL-10 in serum of mice. Relative to control mice, HA transgenic mice showed lower levels of IL-2 and IFN-γ (Fig. 1b and c); however, IL-10 concentration was higher in transgenic mice (Fig. 1d).

3.2. HA regulates the production of IL-2, IFN-γ, and IL-10 through PGE_2 induction in mice

The regulatory effect of HA on IL-2, IFN-γ, and IL-10 was demonstrated *in vitro* [2]. To verify the effect of HA overexpression *in vivo*, we measured PGE_2 expression levels. As shown in Fig. 2a, PGE_2 concentration in transgenic mice was significantly elevated relative to that observed in wild-type mice. It was demonstrated that HA markedly reduces IL-2 production in phytohemagglutinin-stimulated bovine peripheral blood mononuclear cell cultures *via* a prostaglandin-dependent pathway [4], and we previously reported a similar result in RAW264.7 cells. Here, we determined whether the regulatory role of HA on IL-2, IFN-γ, and IL-10 was related to PGE_2 induction *in vivo*. Following injection of mice with the cyclooxygenase-2 inhibitor NS-398, we observed significant suppression of PGE_2 expression (Fig. 2a) and abrogation of the regulatory effect of HA on IL-2, IFN-γ, and IL-10 (Fig. 2b–d). These results suggested that HA regulated the production of IL-2, IFN-γ, and IL-10 through PGE_2 induction in mice.

3.3. Selective blockage of EP4 receptors prevents HA-induced regulation of cytokine expression

The effects of PGE_2 are mediated by four distinct G protein-coupled EP receptors (EP1–4). To identify the EP receptor subtype(s) responsible for HA-induced regulation of IL-2, IFN-γ, and IL-10 expression, transgenic mice were injected with 10 μg/kg SC-51089 (an EP1 antagonist), 50 mg/kg PF-04418948 (an EP2 antagonist), 20 mg/kg L798106 (an EP3 antagonist), or 50 mg/kg ER-819762 (an EP4 antagonist), and 1-d later, the expression levels of IL-2, IFN-γ, and IL-10 were measured by ELISA. We observed no effects on cytokine expression following treatment with the EP1, EP2, or EP3 antagonists;

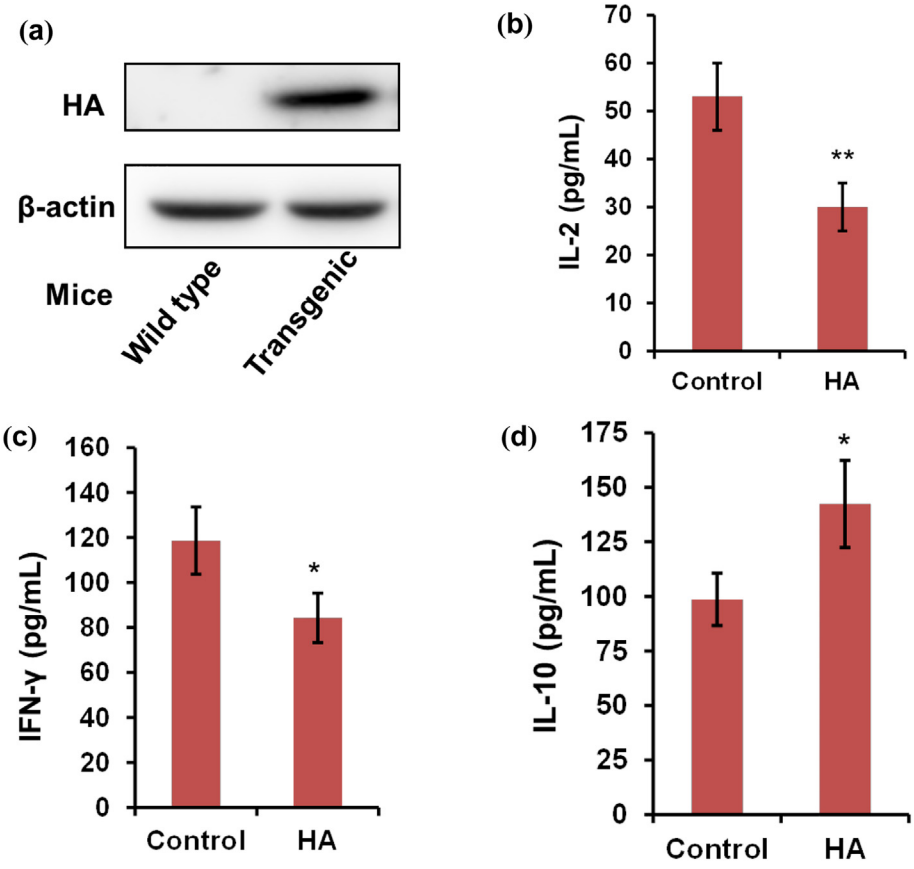

Fig. 1. HA overexpression in mice induced IL-2 and IFN-γ production and downregulated IL-10 secretion. Three HA transgenic mice or negative FVB mice were used in the experiments. Protein was isolated from transgenic or wild type mice back skin, and western blot was performed to detect the HA expression (a). Blood was obtained from the eyeballs of the mice, and the concentrations of IL-2, IFN-γ, and IL-10 were detected by ELISA (b, c, d). Results are representative of three independent experiments. Statistical significance was assessed based on *P*-value. $^{*}P < 0.05$, $^{**}P < 0.01$.

however, treatment with the EP4 antagonist abrogated HA-induced cytokines expression Figs. 3 and 4.

3.4. Overexpression of HA blocks TLR4 signaling

Excessive induction of TLR4 signaling contributes to the pathogenesis of numerous inflammatory diseases, including post-transplantation graft failure [5]. To determine the effect of HA overexpression on TLR4 signaling, we measured lipopolysaccharide (LPS)-induced cytokine expression in RAW264.7 cells. Cells were transduced with Lv-HA or control, and 36-h later, cells were then stimulated with LPS for 4 h. Quantitative RT-PCR and ELISA were performed to measure IL-1β and TNF-α expression, which can be regulated by TLR4. The results suggested that HA overexpression significantly suppressed LPS-induced IL-1β and TNF-α expression *in vitro*.

We subsequently detected a similar result *in vivo*. Wild-type and transgenic mice were injected with LPS, and 24-h later, IL-1β and TNF-α concentrations in serum were evaluated by ELISA. Similar to the *in vitro* results, we observed a significantly induction of IL-1β and TNF-α expression in wild-type mice; however, this induction was inhibited in HA-overexpressing transgenic mice. Therefore, these findings suggested that HA can effectively block LPS-induced TLR4-signal activation.

4. Discussion

Immunological rejection significantly inhibits graft viability during organ transplantation. Despite significant progress in development of immunosuppressive agents, there remain many issues with the method. For example, prevention of allograft rejection using the immunosuppressive agent cyclosporine A is limited by agent toxicity, as they lead to chronic nephrotoxicity [6,7]. Given that parasites are capable of eluding host immunity for extended periods, the investigation of their potential immunosuppressive mechanisms provides new strategies for methods to reduce graft-rejection response.

H. lineatum larvae secrete substances, such as hypodermins A, B, and C, that abrogate host immune defenses. Specifically, HA can suppress host immune response *via* several signaling pathways. HA blocks antigen-induced proliferation of peripheral blood mononuclear cells [8], and can reduce inflammation by cleaving complement component C3 in bovines [9]. Additionally, HA inhibits C3-mediated cytotoxicity by cleaving C3 in rats and humans [9]. Malassagne et al. [10] found that HA prevents hyperacute rejection through C3 degrading and significantly prolongs the viability of grafts in guinea pig heart transplants [10].

In contrast to xenotransplants, acute and chronic rejection of allotransplantations is the major problems that need to be resolved. Cytokines, such as IL-2, IL-10, IFN-γ, and PGE_2, play important roles in acute and chronic rejection. IL-2 and IFN-γ concentrations increase during acute rejection and are highly correlated with rejection severity [11]. Some immunosuppressive agents, such as cyclosporine A, inhibit graft rejection by downregulating IL-2 expression [12]. Several studies have demonstrated that PGE_2 can modulate a variety of immune response, and play an important role in immunosuppression. For example, Auletta et al. [13] demonstrated that PGE_2-expression induction can decrease T cell proliferation, reduce IFN-γ expression level, and attenuate graft-*versus*-host-disease [13]. Our previously study showed that HA downregulates the immune response *in vitro* [2]. Here, although we do not demonstrate that HA is responsible of the immune process altered in mice, we found the similar immunosuppress

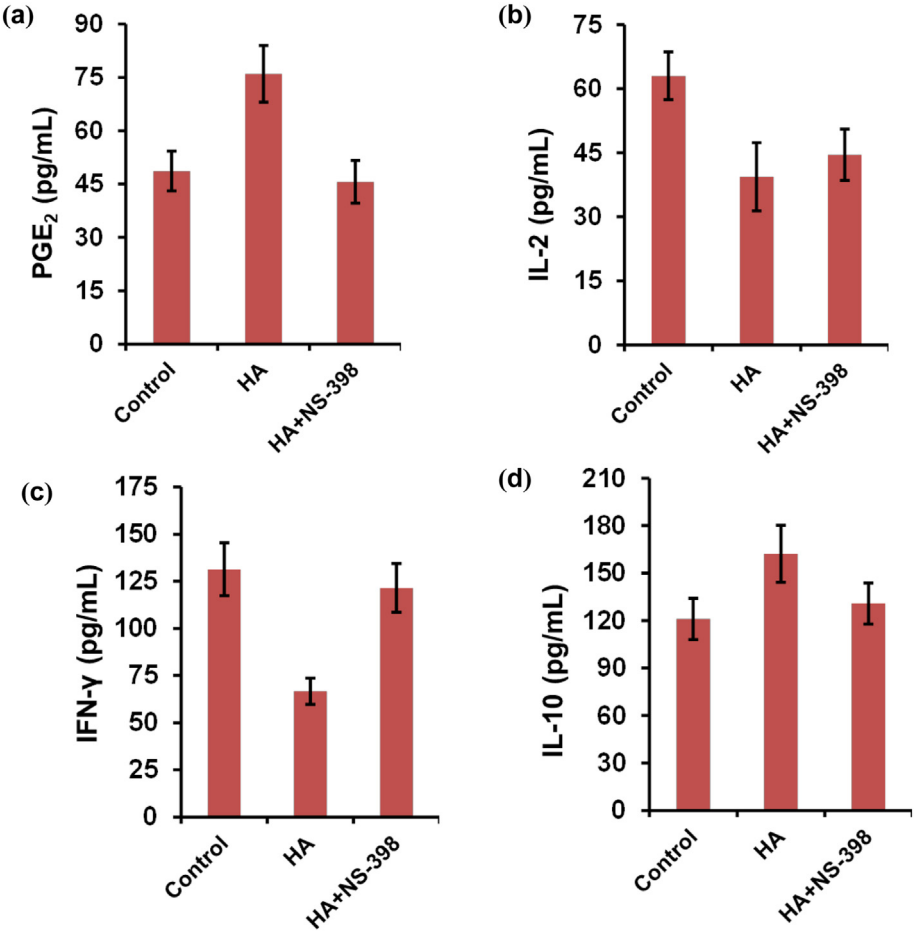

Fig. 2. HA downregulates the production of IL-2 and IFN-γ and promotes IL-10 production. HA transgenic mice were injected intraperitoneally with NS-398 (5 mg/kg), and 1 d later, the concentrations of PGE_2, IL-2, IFN-γ, and IL-10 were evaluated by ELISA. Results are representative of three independent experiments.

role of HA *in vivo*. The reason of the level expression of genes could be any physiological condition in the mice, so the precise regulatory mechanisms need to be investigated further.

TLRs are critical mediators of innate immunity that can be activated by damage-associated molecular patterns and endogenous ligands [14]. Recently, increased TLR4 expression was observed in patients exhibiting acute or chronic rejection as compared with levels observed in control patients [15]. Braudeau et al. [16] investigated renal transplant recipients with chronic rejection and found higher TLR4-mRNA levels in stable grafts [16]. Additionally, Dong et al. [17] demonstrated that blocking excessive TLR4 activation prolongs the viability of islet allografts through the suppressed expression of proinflammatory molecules [17]. Here, we detected the effects of HA overexpression on the TLR4 signaling pathway and found that HA overexpression significantly inhibited LPS-induced TLR4 activation both *in vitro* and *in vivo*. These results revealed a novel mechanism associated with HA

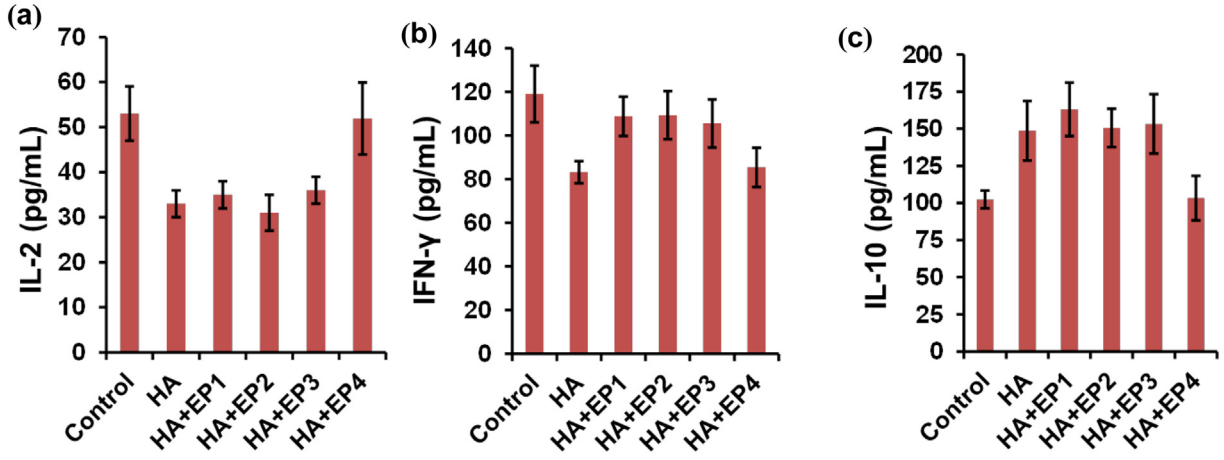

Fig. 3. HA regulates the expression of IL-2, IFN-γ, and IL-10 *via* the PGE_2-EP4 receptor. HA transgenic mice were injected intraperitoneally with SC-51089, PF-04418948, L798106, or ER-819762, and 1-d later, the concentrations of IL-2, IFN-γ, and IL-10 were measured by ELISA. Results are representative of three independent experiments.

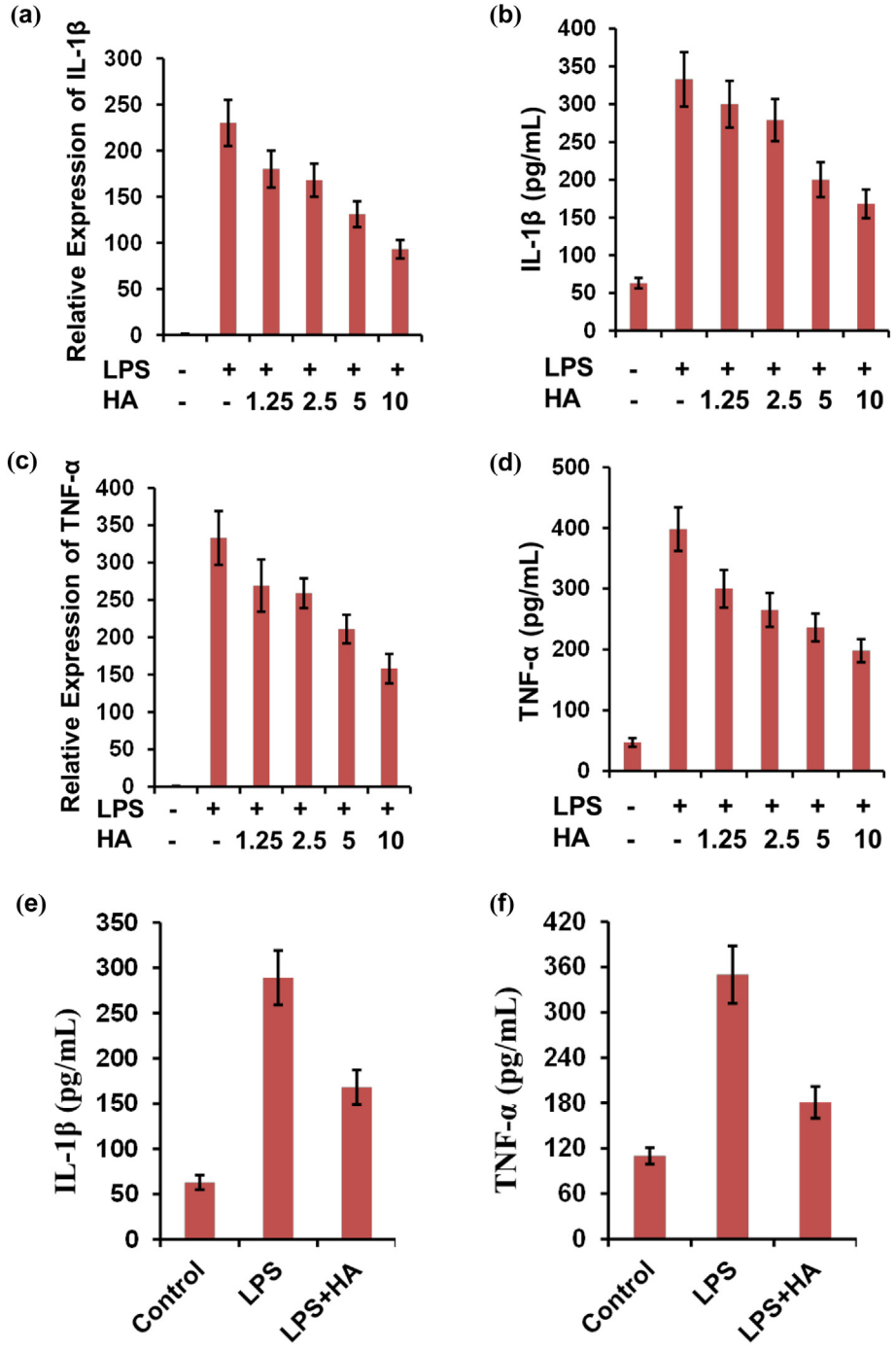

Fig. 4. The effects of HA on LPS-induced TLR4 induction. RAW264.7 cells were seeded into 24-well plates and transduced with different doses (1.25, 2.5, 5, 10 MOI) of a lentiviral vector expressing HA protein or control. After 36 h, cells were continuously exposed to 1 μg/mL of LPS for 18 h. (a–d) Both the mRNA and protein levels of IL-1β and TNF-α were evaluated by quantitative RT-PCR and ELISA. (e–f) To evaluated the effects of HA overexpression *in vivo*, HA transgenic or wild-type mice were injected with LPS (10 μg/kg), and ELISA was performed to measure IL-1β and TNF-α levels in serum.

on immunity regulation. The mechanism associated with HA overexpression as related to suppression of TLR4 signaling was not investigated in this study; however, we speculate that, except for cleaving the C3 protein in mice, HA might cleave other proteins related to TLR. For example, certain viral proteins can cleave immune-related proteins to promote downregulation of host immunity [18]. Furthermore, Chen et al. [3] found that in an HA transgenic skin-allograft mouse model, the viability of HA-expressing grafts was significantly prolonged [3], suggesting that HA-related treatment could be a good option to improve the viability of grafts in human.

Conflict of interest

We declare that we have no conflicts of interest to this work.

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References

[1] Moiré N, Bigot Y, Periquet G, et al. Sequencing and gene expression of hypodermins A, B, C in larval stages of *Hypoderma lineatum*. Mol Biochem Parasitol 1994;66(2):233–40. https://doi.org/10.1016/0166-6851(94)90150-3.

[2] Chen Q, Chen R, Liu J, et al. Hypodermin A, a potential agent for prevention of allogeneic acute rejection. Transpl Immunol 2015;33(3):198–203. https://doi.org/10.1016/j.trim.2015.09.004.

[3] Chen RJ, Wang ZZ, Yuan HH, et al. Hypodermin A improves survival of skin allografts. J Surg Res 2016;203(1):15–21. https://doi.org/10.1016/j.jss.2016.03.037.

[4] Nicolas-Gaulard I, Moire N, Boulard C. Effect of the parasite enzyme, hypodermin A, on bovine lymphocyte proliferation and interleukin-2 production via the prostaglandin pathway. Immunology 1995;85(1):160–5.

[5] Tang AH, Brunn GJ, Cascalho M, et al. Pivotal advance: endogenous pathway to SIRS, sepsis, and related conditions. J Leukoc Biol 2007;82(2):282–5. https://doi.org/10.1189/jlb.1206752.

[6] Longoni B, Boschi E, Demontis GC, et al. Apoptosis and adaptive responses to oxidative stress in human endothelial cells exposed to cyclosporin A correlate with BCL-2 expression levels. FASEB J 2001;15(3):731–40. https://doi.org/10.1096/fj.00-0163com.

[7] Duru M, Nacar A, Yönden Z, et al. Protective effects of N-acetylcysteine on cyclosporine-A-induced nephrotoxicity. Ren Fail 2008;30(4):453–9. https://doi.org/10.1080/08860220801985942.

[8] Baron RW, Weintraub J. Lymphocyte responsiveness in cattle previously infested and uninfested with *Hypoderma lineatum* (de Vill.) and *H. bovis* (L.) (Diptera: Oestridae). Vet Parasitol 1987;24:285–96.

[9] Chen RJ, Hu AK, Yuan HH, et al. The immunosuppression mechanism of hypodermin A on complement. Parasitol Int 2014;63(2):392–6. https://doi.org/10.1016/j.parint.2013.12.016.

[10] Malassagne B, Regimbeau JM, Taboit F, et al. Hypodermin A, a new inhibitor of human complement for the prevention of xenogeneic hyperacute rejection. Xenotransplantation 2003;10(3):267–77. https://doi.org/10.1034/j.1399-3089.2003.02030.x.

[11] Cottrez F, Hurst SD, Coffman RL, et al. T regulatory cells 1 inhibit a Th2-specific response in vivo. J Immunol 2000;165(9):4848–53. https://doi.org/10.4049/jimmunol.165.9.4848.

[12] O'Keefe SJ, Tamura J, Kincaid RL, et al. FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature 1992;357(6380):692–4. https://doi.org/10.1038/357692a0.

[13] Auletta JJ, Eid SK, Wuttisarnwattana P, et al. Human mesenchymal stromal cells attenuate graft-versus-host disease and maintain graft-versus-leukemia activity following experimental allogeneic bone marrow transplantation. Stem Cells 2015;33(2):601–14. https://doi.org/10.1002/stem.1867.

[14] Lehnardt S, Schott E, Trimbuch T, et al. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. J Neurosci 2008;28(10):2320–31. https://doi.org/10.1523/JNEUROSCI.4760-07.2008.

[15] Kwon J, Park J, Lee D, et al. Toll-like receptor expression in patients with renal allograft dysfunction. Transplant Proc 2008;40(10):3479–80. https://doi.org/10.1016/j.transproceed.2008.06.073.

[16] Braudeau C, Ashton-Chess J, Giral M, et al. Contrasted blood and intragraft toll-like receptor 4 mRNA profiles in operational tolerance versus chronic rejection in kidney transplant recipients. Transplantation 2008;86(1):130–6. https://doi.org/10.1097/TP.0b013e31817b8dc5.

[17] Dong H, Zhang Y, Song L, et al. Cell-permeable peptide blocks TLR4 signaling and improves islet allograft survival. Cell Transplant 2016;25(7):1319–29. https://doi.org/10.3727/096368916X690449.

[18] Huang C, Zhang Q, Guo XK, et al. Porcine reproductive and respiratory syndrome virus nonstructural protein 4 antagonizes beta interferon expression by targeting the NF-kappaB essential modulator. J Virol 2014;88(18):10934–45. https://doi.org/10.1128/JVI.01396-14.