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Original Article

Effects of a traditional Chinese medicine, *Longdanxiegan* formula granule, on Toll-like receptor pathway in female guinea pigs with recurrent genital herpes

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ABSTRACT

Objective: The aim of the present study was to investigate the effects of *Longdanxiegan* formula granule (LDXGFG), a Chinese traditional medicine on Toll-like receptor (TLR) pathway in recurrent genital herpes.
Materials and Methods: An experimental recurrent genital herpes model was constructed using herpes guinea pig model. The effect of LDXGFG on expression levels of TLR pathway genes were detected using real-time polymerase chain reaction. Furthermore, the dendritic cells and Langerhans cells were isolated and the TLR pathway genes of these cells were assayed after LDXGFG treatment.
Results: The result suggested two different expression patterns of TLR pathway genes in genital herpes and recurrent genital herpes, including upregulated genes and downregulated genes. TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10 showed a significant decrease while, TLR2, TLR3, and TLR5 increased in genital herpes and recurrent genital herpes guinea pigs. Meanwhile, the downregulated genes in genital herpes and recurrent genital herpes were stimulated by LDXGFG. By contrast, the upregulated genes decreased significantly after LDXGFG treatment. In both dendritic cells and Langerhans cells, the TLR pathway genes exhibited same pattern: the LDXGFG corrected the abnormal expression of TLR pathway genes.
Conclusion: The present results suggest that LDXGFG is an alternative, inexpensive, and lasting-effect medicine for herpes simplex virus 2 infection.

Introduction

Genital herpes is a common disease throughout the world, which is caused by herpes simplex virus (HSV) infections [1]. HSV has been regarded as being able to transmit through mucosal membranes as well as invading into nerve tissues, which results in prolonging infection [2]. Two different kinds of HSV—HSV1 and HSV2—are reported in trigeminal ganglia and lumbosacral ganglia, respectively. Subsequent study suggested that orofacial areas and genital tract also could be infected by HSV. From 2001, HSV2 infection has increased sharply and extended around the globe [3]. Since HSV infections have been widely investigated, the antiviral drugs also develop rapidly. The most common medicines, including acyclovir (ACV), valacyclovir, famciclovir, and penciclovir are used in clinical therapy [4,5]. Meanwhile, these medicines also have defects: (1) all the medicines are too expensive and not economic for using in poor areas; and (2) genital herpes is a prolonged disease and these medicines are not persistent. As a result, new therapy strategy to anti-HSV infection is needed, which would aid to cure this disease, especially in developing areas.

Humoral and cellular immune responses are observed after HSV infection [6–8]. In addition, the depression of cellular immune responses in recurrent genital herpes has been demonstrated in a guinea pig model [9,10]. Meanwhile, the HSV-specific T cells also has been confirmed that plays a crucial role in anti-HSV in the infected organisms [9]. Th1 type CD4+ T cells were raised after HSV infection. In previous studies, it has been confirmed that HSV-specific antibodies are not sufficient to prevent HSV infection and the consequent damages in nervous system [11]. Sin et al [12] demonstrated that interleukin (IL)-12 could be a suitable adjuvant for HSV2 and

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Table 1
The formula of *Longdanxiegan* formula granules in the present study.

Ingredients	Amount (%)
Gentian	14.01
Plantago	14.01
Skullcap	14.01
Gardenia	9.35
Angelica	9.35
Rehmannia	9.35
Alisma	9.35
Radix	9.35
Akebia	5.61
Licorice	5.61
Total	100

stimulate antigen-specific Th1 type CD4+ T cell responses, which depressed the morbidity and mortality of HSV2. In conclusion, proinflammatory cytokines participate in HSV-infected recurrent genital herpes.

In recent years, substantial progress has been made in understanding Toll-like receptor (TLR) pathways involved in viral infected orgasms. HSV1 and HSV2 trigger TLR expression, which was found to be stimulated by cytokines and chemokines [13]. These effects could be triggered by T- and B-cell-mediated immunity. TLR activation leads to lymph node remodeling [14]. Meanwhile, the innate response serves to affect acquired immune response mediated by TLR recognition [15]. Recent research has shown that TLR agonists could mediate in prophylactic or therapeutic devices [16]. Thus, new treatment strategies from the perspective of TLR pathway on HSV infection need to be established.

Chinese medicine provides a new insight in anti-HSV infection and shows superiority in low prices and natural resources. *Longdanxiegan* formula granule (LDXGFG) is one of the most popular drugs for recurrent genital herpes and shows a great effective [17]. Ten different herbaceous plants—gentian, plantago, skullcap, gardenia, angelica, rehmannia, alisma, radix, akebia, and licorice—make up LDXGFG [18]. This mixture formula granule

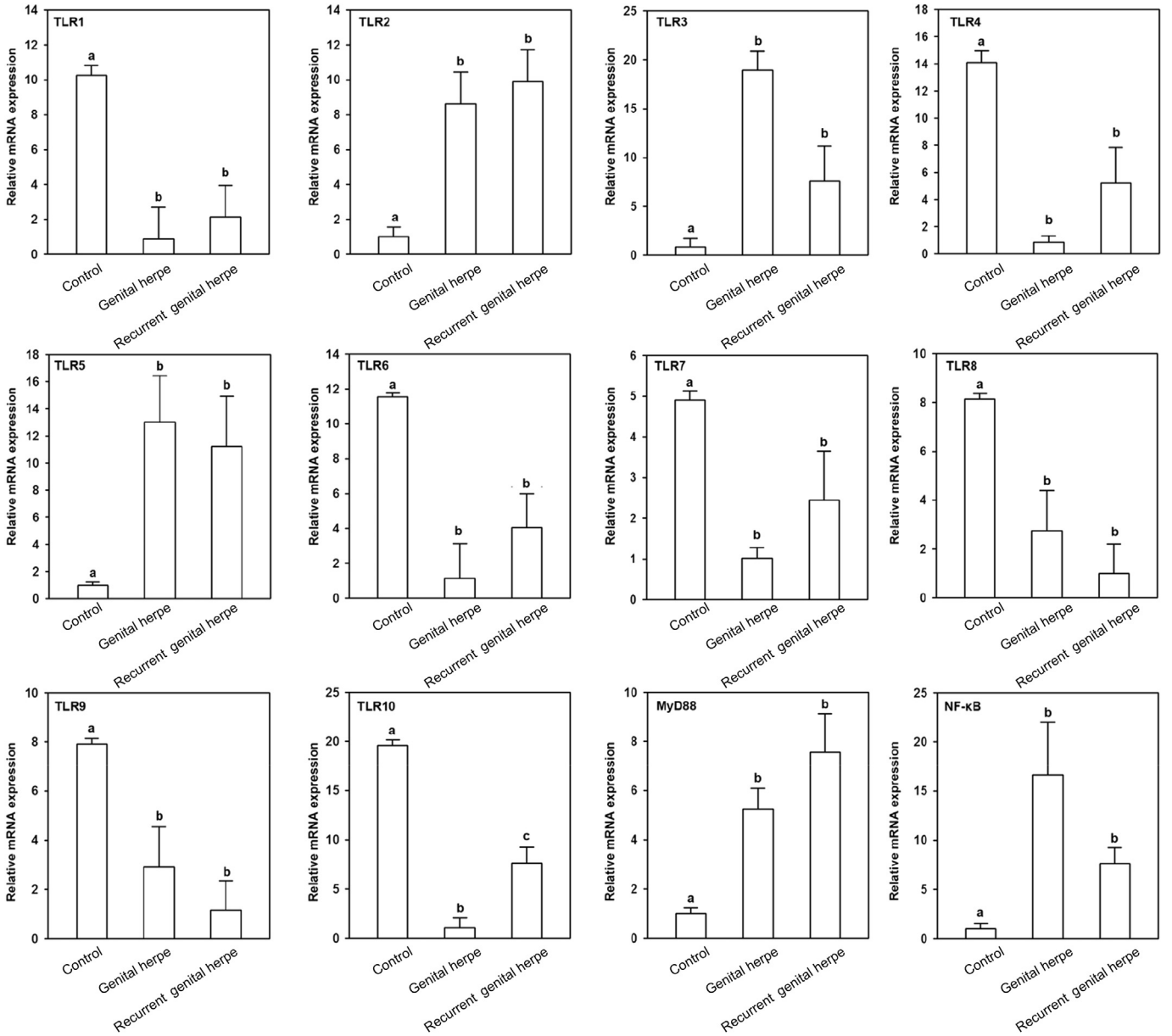

Figure 1. mRNA expression changes of Toll-like receptor (TLR) pathway genes in genital herpes and recurrent genital herpes guinea pig in vulva by real-time polymerase chain reaction. Different letters show significant difference between the control and genital herpes groups ($p < 0.05$).

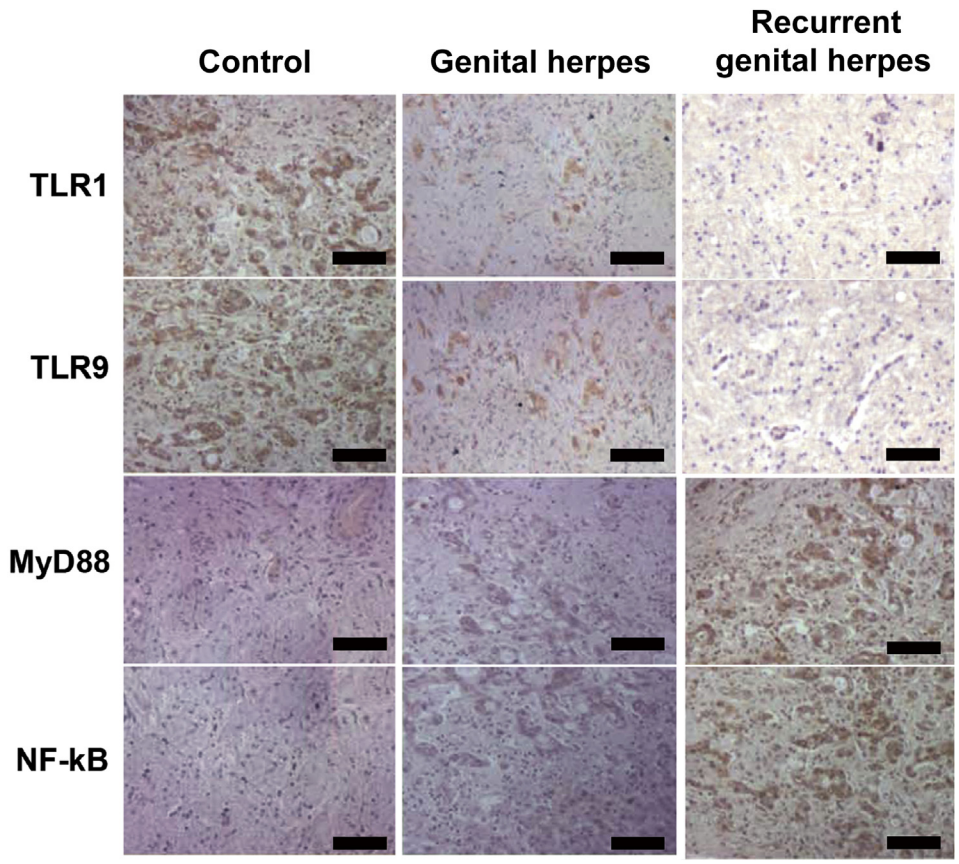

Figure 2. Immunohistochemistry assay of Toll-like receptor (TLR)1, TLR9, myeloid differentiation primary response gene 88 (MyD88), and nuclear factor (NF)-kB in genital herpes and recurrent genital herpes guinea pig. Bar = 50 μm.

promotes immune response. However, information about immune response of cell types needs to be elucidated.

In the present study, we investigate the different expression of TLR pathway genes after infection with HSV2 in female guinea pigs. We also assayed the expression of TLR pathway genes after LDXGFG treatment. Dendritic cells (DCs) and Langerhans cells (LCs) were isolated and the LDXGFG effects on these cells were demonstrated. These results support the idea that LDXGFG can modulate TLR pathway to infectious agents in recurrent genital herpes.

Materials and methods

Animals, virus, and drugs

Female Hartley guinea pigs, age 4 weeks (250 ± 20 g) with viral infection, were obtained from the animal culture center in Hunan University of Chinese Medicine (Chansha, Hunan). HSV2 strains clinically isolated from patients in Hunan University of Chinese Medicine Affiliated Hospital (Changsha, Hunan). By DNA sequencing, we determined that the virus strain is HSV2SAV [19]. ACV was purchased from Sigma–Aldrich (St. Louis, MO, USA). The LDXGFG was purchased from Sanjiu Medical and Pharmaceutical Co., Ltd. (Shenzhen, China). The formula is in Table 1. The studies were strictly approved by the Animal Ethical Committee of Hunan University of Chinese Medicine.

Construction of genital herpes guinea pig model

The guinea pig vulva first was cleaned by physiological saline. After that, each guinea pig was injected of 0.1 mL (1% HSV2SAV) into 3–4 cm deep in the vagina. After 1 week, the genital herpes guinea pig model was confirmed by polymerase chain reaction (PCR) and clinical characterization. Based on the clinical characterization of vaginitis, the disease course of guinea pig was scored from 0 to 6 on a composite scale: 0, no symptom of infection; 1, few small vesicles found in vulva; 2, 10–50% of the vulva area covered by vesicles; 3, 50–100% of the vulva area covered by vesicles; 4, 10–50% of the vulva area was covered by small ulcers; 5, 50–100% of the vulva area covered by severe ulcers; and 6, hind limb paralysis or death. As the following study is long-standing, we used the animals that scored 4 in this study [20]. The recurrent genital herpes model was constructed. By cyclophosphamide injection and 5 minutes UVB (270–320 nm, 7000 μW/cm^2) irradiation five times every day, the clinical symptoms of recurrent genital herpes were observed.

LDXGFG effects on the genital herpes guinea pig model

To understand the effects of LDXGFG on expression of TLR pathway genes genital herpes guinea pig, LDXGFG or ACV feeding was performed. The low, moderate, and high LDXGFG mice were fed with 1 g/kg, 10 g/kg, and 20 g/kg LDXGFG, respectively. The ACV group was fed with 25 mg/kg ACV. For each group, five individuals were maintained. The treatment was performed once a day and the guinea pigs were fed twice a day. The experiment lasted for 30 days. After the experiment, the gene expression levels were detected by real-time PCR.

Separation and differentiation of DCs

The DCs were generated following the previous study [21]. First, peripheral blood mononuclear cells (PBMCs) were isolated from jugular venous blood using lymphocytes separation medium by

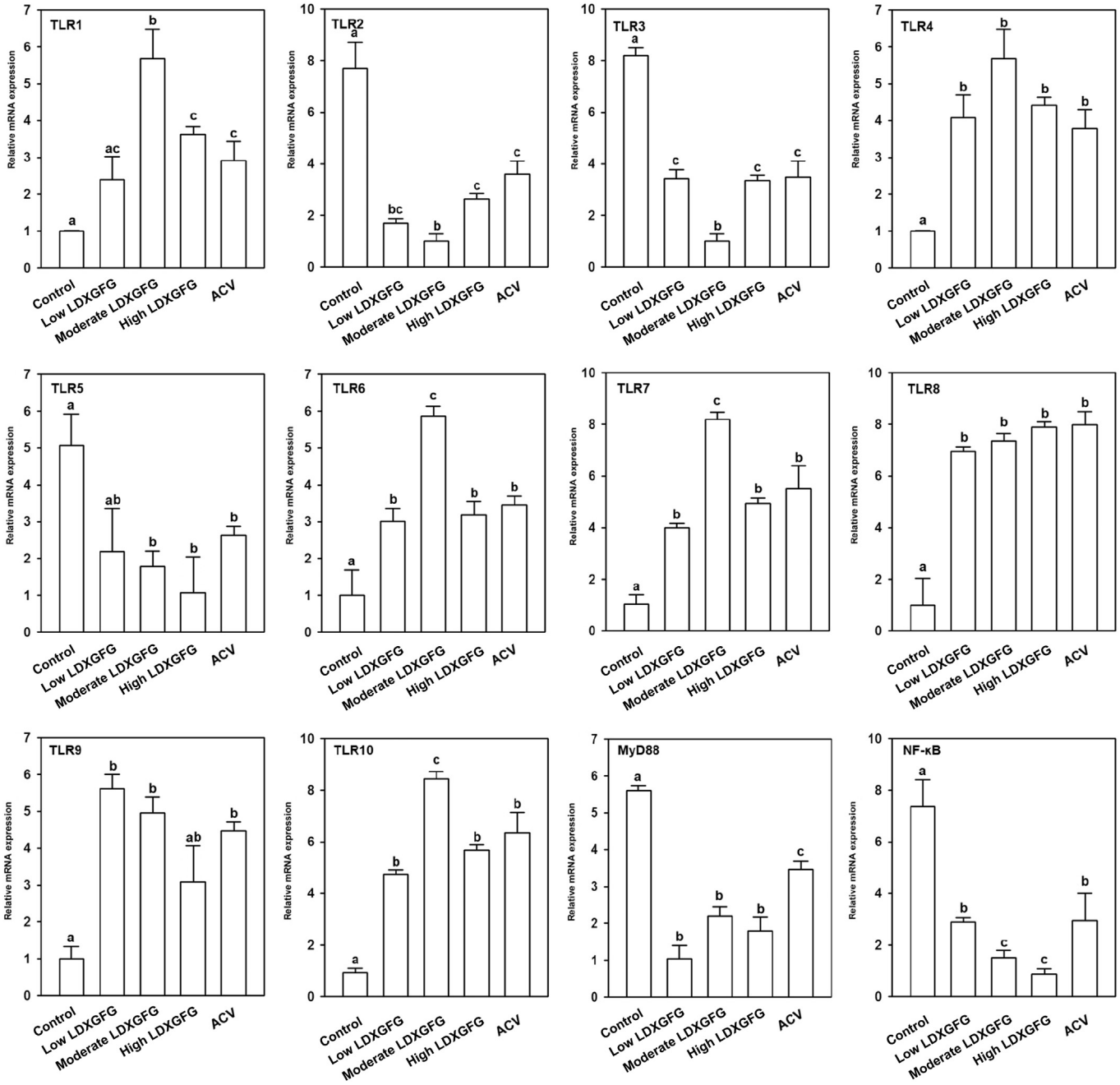

Figure 3. Effects of *Longdanxiegan* formula granule (LDXGFG) on mRNA expression of Toll-like receptor (TLR) pathway genes in genital herpes and recurrent genital herpes guinea pig in vulva by real-time polymerase chain reaction. Different letters show significant difference between the control and genital herpes groups ($p < 0.05$).

Ficoll–Paque PLUS gradient centrifugation (GE Healthcare, Uppsala, Sweden). After 2 hours, the adherent monocytes were cultured for 5 days in GM-CSF (500 U/mL; Gentaur, Brussels, Belgium), 20 ng/mL of IL-4 and 20 ng/mL of tumor necrosis factor-α in serum-free CellGro DC media (CellGenix, Heidelberg, German). After 5 days, the cells were used for further studies. For dosage studies, the DCs were randomly divided into four groups: control (with no LDXGFG treatment); low LDXGFG (0.1nM); moderate LDXGFG (10nM); and high LDXGFG (1000nM). All the groups were treated for 24 hours and then the cells were collected for real-time PCR analysis. For time-dependent study, the cells were divided into seven groups: 0 hours, 2 hours, 4 hours, 8 hours, 16 hours, 24 hours, 48 hours, and 96 hours. The treatment dosage was moderate LDXGFG (10nM). After treatment, the mRNA expression was detected by real-time PCR.

Separation of LCs

The LCs were derived following previous studies [21] and the expression of phenotypic marker were characterized of the LCs. In brief, the cells were first isolated from infected tissues. Then the cells were separated in Ficoll lymphocyte separation medium. After density gradient equilibrium and centrifugation for 30 minutes at 3000 g/min, the cells at intermediate layer were obtained. Subsequently, the cells were incubated by CD1a monoclonal antibody immunomagnetic beads at 4°C for 20 minutes. After washing with phosphate-buffered saline and magnetic cell sorting, the LCs were isolated. The dosage- and time-dependent studies of LDXGFG effect were performed according to the studies in DCs.

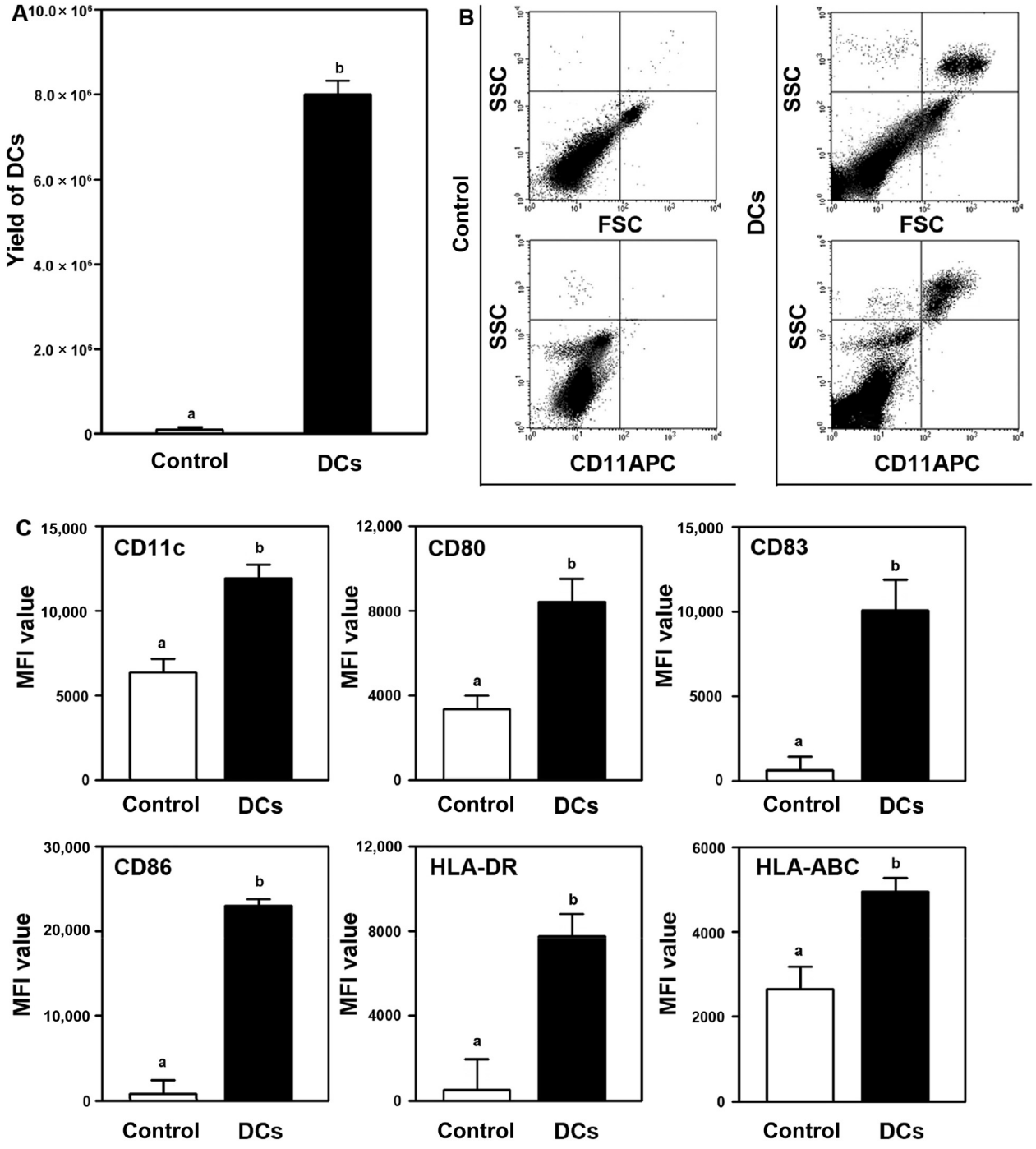

Figure 4. Characteristics of dendritic cells (DCs). (A) Total number of DCs compared with blood monocytes control that were generated from 75 × 10^6 cells. (B) Viability (percentage of FSC and CD11APCs) of DCs. C. Expression levels of DCs compared with blood monocytes control. Different letters show significant difference between the control and genital herpes groups ($p < 0.05$).

Real-time PCR

Once the tissues and cells were collected from the guinea pigs, they were stored at −80°C until used. Total RNA were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The quantity and quality of the RNA were assayed. After digestion with DNase I (Fermentas, Vilnius, Lithuania) to eliminate residual DNA, the RNAs were reverse transcripted to the first strand cDNA by using PrimeScript One Step RT-PCR Kit version 2 (Takara, Dalian, China). The primers of the studied gene were synthesized from previous published sequences [22,23]. The real-time PCR reactions were carried out on Applied Biosystems 7500 Real-Time PCR system. The PCR reaction condition was: 95°C for 3 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 40 seconds. Each sample with the same primers was repeated three times. A melting curve was obtained to confirm if the amplification products were specific. The relative expression was calculated by ABI 7500 software version 2.0.1 (Applied Biosystems, CA, USA) using the $2^{-\Delta\Delta Ct}$ method.

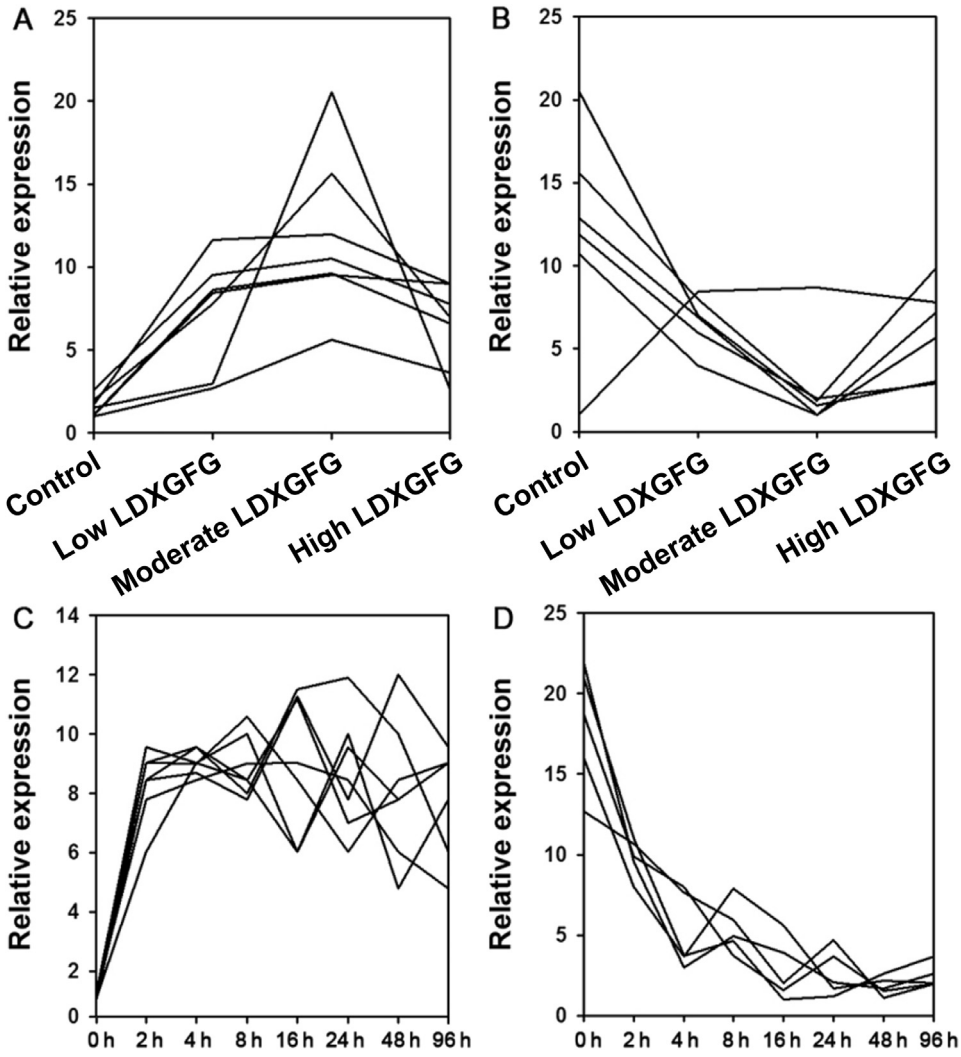

Figure 5. Expression changes of Toll-like receptor (TLR) pathway genes in dendritic cells (DCs) after *Longdanxiegan* formula granule (LDXGFG) treatment. (A) Downregulated genes in genital herpes (TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10) stimulated by LDXGFG in DCs. (B) Upregulated genes in genital herpes [TLR2, TLR3, TLR5, myeloid differentiation primary response gene 88 (MyD88), and nuclear factor (NF)-κB] depressed by LDXGFG in DCs. (C) Time-dependent studies of downregulated genes after LDXGFG treatment in DCs. (D) Time-dependent studies of upregulated genes after LDXGFG treatment in DCs.

Immunohistochemistry

For immunohistochemical assay, 6-μm paraffin sections were first prepared. Then the tissue sections were incubated with 10% H_2O_2 for 30 minutes and blocked with 10% normal calf serum (Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. The sections were incubated with primary antibodies for 24 hours at 4°C. All the primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA; dilution 1:200). The signals were detected using Histostain-Plus Bulk kit (Invitrogen) against rabbit immunoglobulin G. 3,3′-Diaminobenzidine was used to visualize the signals. The sections were observed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and the photos were taken using an Olympus C-5050 digital camera (Olympus, Tokyo, Japan).

Flow cytometry

To analyze the cell activity and distinctive factors, the cells were assayed by flow cytometry. First, the cells were stimulated with 25 ng/mL PMA (Sigma–Aldrich) at 37°C for 4 hours. Then, for the test, FITC-labeled CD11c, CD80, CD83, CD86, HLA-DR and HLA-ABC, and Percp-cy5.5-labeled CD11APC were incubated with the cells for 20 minutes at 4°C. After fixing and permeabilization, the cells were analyzed by an Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA). The calculation was performed with EXPO32 ADC software.

Statistical analysis

The data in the study are expressed as mean ± standard deviation. All the statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to assay the significant differences. A p value < 0.05 was considered statistically significant.

Results

TLR signaling pathway changes in recurrent genital herpes

The levels of TLR signaling pathway genes in recurrent genital herpes guinea pigs were detected by real-time PCR. TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10 decreased significantly after infecting with HSV2SAV as well as in recurrent genital herpes guinea pigs. However, TLR2, TLR3, and TLR5 increased in genital

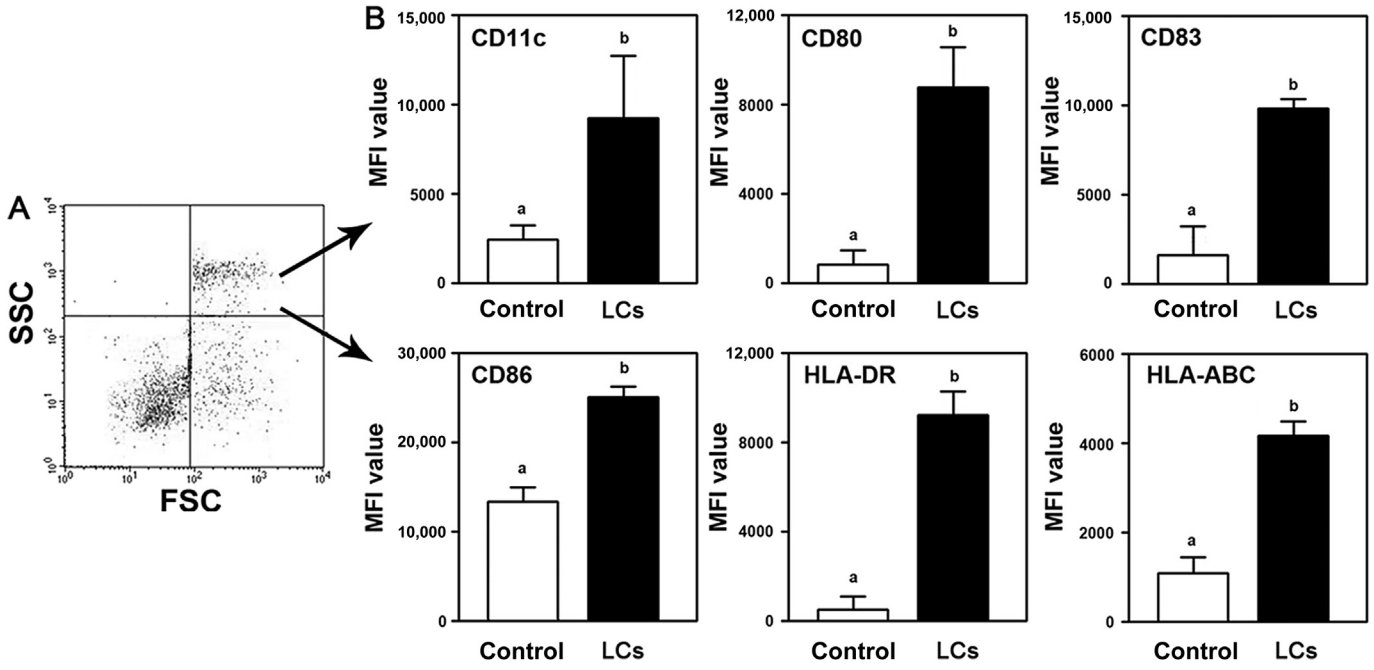

Figure 6. Characteristics of Langerhans cells (LCs). (A) Viability (percentage of FSC) of LCs. (B) Expression levels of LCs compared with unisolated cells control. Different letters show significant difference between the control and genital herpes groups ($p < 0.05$).

herpes and recurrent genital herpes guinea pigs. Similarly, myeloid differentiation primary response gene 88 (MyD88) and nuclear factor (NF)-κB were upregulated in both genital herpes and recurrent genital herpes guinea pigs (Figure 1). The result indicated that the genes could be assigned into two different groups—decreased group (TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10) and increased group (TLR2, TLR3, TLR5, MyD88, and NF-κB)—indicating different expression changes after HSV2 infection. The immunohistochemistry result also indicated the decreased TLR1 and TLR9 as well as increased MyD88 and NF-κB in genital herpes and recurrent genital herpes guinea pigs (Figure 2).

LDXGFG effects on TLR signaling pathway in recurrent genital herpes

The effects of LDXGFG on TLR signaling pathway genes in genital herpes and recurrent genital herpes guinea pigs were determined. For TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10, the levels of genes in pudendum tissues were increased significantly with LDXGFG or ACV feeding (Figure 3). By contrast, TLR2, TLR3, TLR5, MyD88, and NF-κB levels decreased after LDXGFG or ACV feeding (Figure 3). The moderate LDXGFG dosage was the most effective. Thus, it can be concluded that 10 g/kg LDXGFG amount is the optimal dosage for genital herpes guinea pigs. In addition, LDXGFG showed similar effects with ACV in this study (Figure 3). These results indicate different effects of LDXGFG and ACV on the decreased group (TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10) and increased group (TLR2, TLR3, TLR5, MyD88, and NF-κB) genes.

LDXGFG acts on TLR signaling pathway in DCs

We first compared the characteristics of DCs differentiated in culture cells to normal PBMCs as control. Significant differences between control (blood monocytes) and DCs were found both in the yield of DCs (Figure 4A) and their viability (Figure 4B). The FSC/SSC and CD11APC/SSC analysis showed that DCs were larger and more granular than control cells. To determine the capacity of immature DCs to be activated by TLR ligands, we evaluated the expression of costimulatory molecules including CD11c, CD80, CD83, CD86, HLA-DR, and HLA-ABC. All these costimulatory molecules increased significantly in DCs compared to normal PBMCs (Figure 4C).

After determining the effects of LDXGFG on the TLR signaling pathway in recurrent genital herpes, we found that the factors of TLR signaling pathway could divide into two different groups: downregulated genes (TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10) and upregulated genes in genital herpes (TLR2, TLR3, TLR5, MyD88, and NF-κB). Meanwhile, the DCs were treated with LDXGFG. The results suggest that the downregulated genes could be stimulated by LDXGFG (Figure 5A) while the upregulated factors were depressed by LDXGFG (Figure 5B). The moderate LDXGFG had the best effects both in stimulation of the downregulated genes and depression of upregulated genes. Time-dependent study showed that LDXGFG had a long-term effect at least until 96 hours (Figures 5C and 5D).

LDXGFG effects on TLR signaling pathway in LCs

LCs were identified by flow cytometry. This showed that the costimulatory molecules including CD11c, CD80, CD83, CD86, HLA-DR, and HLA-ABC were highly expressed in LCs (Figure 6). The LCs were treated by LDXGFG. All the downregulated genes in genital herpes were stimulated after LDXGFG treatment in low, moderate, and high dosages (Figure 7A) while all the upregulated genes in genital herpes exhibited a decrease after LDXGFG treatment in all the dosage groups (Figure 7B). In the time-dependent study, the stimulation of downregulated genes and the depression of upregulated genes in genital herpes both lasted until 96 hours (Figures 7C and 7D).

Discussion

Several studies show abnormal expression of immune factors mediated in HSV1 and HSV2 infected genital herpes. This research

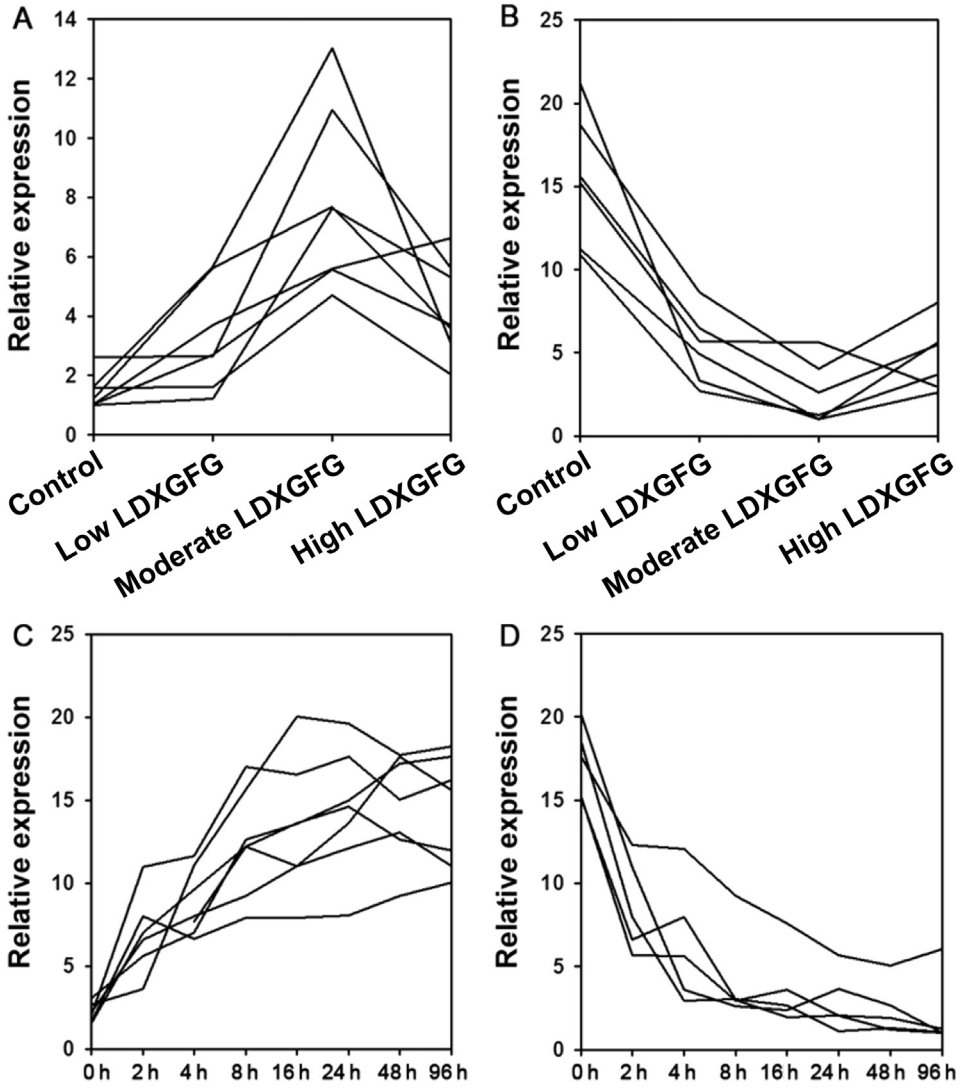

Figure 7. Expression changes of Toll-like receptor (TLR) pathway genes in Langerhans cells (LCs) after *Longdanxiegan* formula granule (LDXGFG) treatment. (A) Downregulated genes in genital herpes (TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10) stimulated by LDXGFG in LCs. (B) Upregulated genes in genital herpes [TLR2, TLR3, TLR5, myeloid differentiation primary response gene 88 (MyD88), and nuclear factor (NF)-κB] depressed by LDXGFG in LCs. (C) Time-dependent studies of downregulated genes after LDXGFG treatment in LCs. (D) Time-dependent studies of upregulated genes after LDXGFG treatment in LCs. Different letters show significant difference between the control and genital herpes groups ($p < 0.05$).

focused on proinflammatory cytokine changes after infection [13,24–26]. Also, the immune expression changes and hormone-triggered reproductive immunity in vertebrates have been studied in detail [27,28]. Several medicines which work against genital herpes have been developed, such as valacyclovir, famciclovir, and penciclovir [29]. These drugs also show effects on regulating the immune system against the virus. However, due to the migration and prolonging attribution of HSV in host, patients after treatment with these drugs usually suffer from recurrent genital herpes. According to our clinical experience, a Chinese medicine, LDXGFG shows a prolonged effect against HSV2 infected recurrent genital herpes [18]. LDXGFG affects expression of several immune factors such as proinflammatory cytokines. However, the mechanisms of LDXGFG on recurrent genital herpes especially the effects TLR pathway factors are still not clear.

First, we showed expression changes of TLR pathway factors in genital herpes and recurrent genital herpes tissues. The results showed the same expression pattern of TLR pathway factors in genital herpes and recurrent genital herpes tissues. The analyzed genes were divided into two groups by the expression changes. TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10 were downregulated and significantly decreased after HSV2 infection, while TLR2, TLR3, TLR5, MyD88, and NF-κB were upregulated groups and significantly increased in genital herpes and recurrent genital herpes tissues. Studies into expression changes of TLR pathway factors after HSV2 infection are rare. Li and colleagues [22] reported that HSV2 stimulated IL-1, IFN-β, TLR4, and TLR9 expression in cervical epithelial cells, which is contradictory to the present finding. This may be due to the different samples between these two studies. We used *in vivo* guinea pigs while the previous study used *in vitro* cervical epithelial cells. However, they concluded that HSV2 depressed TLR4 and TLR9 expression in guinea pigs as we found. TLR pathway factors are enormous which contains several genes. Herein, we assayed 12 genes in this pathway. Thus, in the present study, we initially showed a duality of TLR pathway factors in HSV2 infected genital herpes.

Furthermore, the LDXGFG effects on TLR pathway factors of recurrent genital herpes were analyzed. The present result suggested that TLR1, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10 showed a

significant increase after LDXGFG intake as well as after ACV. ACV, as one of the most common treatments for HSV2 infection, was used in the present experiment. The downregulated genes in genital herpes and recurrent genital herpes were stimulated by LDXGFG, while the upregulated genes were significantly decreased after LDXGFG treatment. In total, the result showed that LDXGFG corrected the abnormal expression of TLR pathway genes, and provided a probable therapy for HSV infection. Meanwhile, the details of expression information on the mechanism and location of TLR pathway genes needs to be elucidated.

DCs are the strongest antigen-presenting cells in mammalian immune systems [26]. The DCs is differentiated from blood monocytes and could be induced by colony stimulating factor, IL-4 and tumor necrosis factor-α. In the present studies, we differentiated the DCs from peripheral blood mononuclear cells. The flow cytometry analysis determined that all the tested costimulatory molecules are highly expressed in DCs. After treatment with LDXGFG, the expression of TLR pathway genes was changed, which was similar to the genes in recurrent genital herpes. The downregulated genes and the upregulated genes in genital herpes were stimulated and depressed by LDXGFG treatment, respectively. In addition, LCs were isolated from infected tissues and the TLR pathway changes were also detected. Similarly, in LCs, the TLR pathway genes exhibited same of LDXGFG correcting the abnormal expression of TLR pathway genes. Here, we found that the effects of LDXGFG last at least 96 hours both in DCs and LCs, which demonstrates the long-term effect of this medicine.

Nowadays, traditional Chinese medicine, as a healthcare medical system provides a different insight into immune response and treatment therapy. LDXGFG, which consists of 10 different ingredients, regulates immune factors, including proinflammatory cytokines and TLR pathway genes. In our present study, we reported the rescue of LDXGFG on abnormal expression of TLR pathway genes after HSV2 infection, which is mediated by DCs in peripheral blood and LCs in infected tissues. Meanwhile, the present study suggests LDXGFG as an alternative, inexpensive, and lasting-effect medicine for curing HSV2 infection caused genital herpes.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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