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Crosstalk between Toll-like receptor 3 and Notch signaling contributes to CD14$^+$ monocytes activity in enterovirus 71 infected hand, foot, and mouth disease

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ABSTRACT

Interaction between Toll-like receptor (TLR) and Notch signaling contributes to inflammatory response in nephropathy and fungicidal infection, however, the role of this crosstalk remains not fully elucidated in enterovirus 71 (EV71)-induced hand, foot, and mouth disease (HFMD). The aim of this study was to investigate the crosstalk between TLR and Notch in inflammatory regulation in EV71 infection. Thirty-seven EV-71-indcued HFMD (16 mild and 21 severe cases) and eleven normal control (NC) were enrolled. CD14$^+$ monocytes were purified, and were stimulated with either TLR3/4 agonists [poly(I: C) or LPS] or Notch signaling inhibitor. TLRs and Notch receptors expression, proinflammatory cytokines production, and important molecules in signaling pathways were measured by real-time PCR, ELISA, and Western blot. TLR3 and TLR4 was significantly elevated in CD14$^+$ monocytes from HFMD patients than NC. Notch1 and Notch2 mRNA was also remarkably increased in CD14$^+$ monocytes from severe HFMD. Poly(I: C) stimulation resulted in robust increase of IL-8, IL-6, and TNF-α by CD14$^+$ monocytes in severe HFMD compared to NC. Activation of Notch1, Notch2, and target genes, Hes1 and Hes5 was also enhanced upon ploy(I: C) treatment. Although inhibition of Notch signaling did not affect TLR3 expression, poly(I: C)-induced inflammatory response was robustly attenuated, which was accompanied by silencing Src phosphorylation in CD14$^+$ monocytes from severe HFMD patients. The current data indicated that crosstalk between TLR3 and Notch signaling modulated CD14$^+$ monocytes function and inflammatory responses in the progression of EV71-induced HFMD.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a common pediatric infectious disease, which is usually caused by enterovirus A species, including enterovirus 71 (EV71) and coxsackievirus A16 [1]. EV71, which belongs to positive single-stranded RNA virus, always leads to severe cases of HFMD with neurological complications and cardiopulmonary failure [2,3]. Moreover, the mechanisms of EV71-induced severe HFMD are still not completely elucidated. EV71 infection could not only present direct cytopathic activity to various cell types (including intestinal epithelial cells [4] and neuronal cytoplasm [5]), but also induce immune disorder in HFMD patients [6], both of which play important roles in the pathogenesis of EV71 associated severe HFMD.

Toll-like receptor (TLR) is pattern recognition receptors, and is commonly expressed on CD14$^+$ monocytes. Activation of TLRs not only leads to the initiation of innate immune response in early host defense to clear invading pathogens, but also results in the expression of cytokines/chemokines to control adaptive immune responses [7]. EV71 infection induced elevated expression of TLR7 and TLR8 in lung and brain tissues in severe HFMD patients [8], and TLR3c.1377T allele was found to be associated with susceptibility to severe EV71-induced HFMD in Chinese children [9]. However, controversy remains as the role of increased TLRs expression in the pathogenesis of EV71 infection. EV71 virus-like particles enhanced the maturation of human monocytes-derived dendritic cells and increased proinflammatory cytokines production *via* TLR4 signaling pathway [10]. EV71 infection also mediated TIR domain-containing adaptor inducing interferon-β (TRIF) cleavage, and this leaded to the inhibition of TLR3-mediated antiviral responses [11]. In contrast, TLR9 knockout mice developed neurological lesion-associated symptoms following EV71 infection, indicating a

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host protective activity of TLR9 in EV71-related infections [12].

Notch signaling pathway is an evolutionally conserved cell signaling system present in most multicellular organisms [13]. Four different Notch receptors, known as Notch1～Notch4, are single-pass transmembrane proteins. Notch signaling regulates immune cells development, and may be involved in HFMD process by affecting the number and status of peripheral lymphocytes [14]. Furthermore, crosstalk between Notch1 signaling and TLR4 contributed to inflammatory response in IgA nephropathy [15] and fungicidal infection [16]. Thus, we hypothesized that crosstalk between Notch signaling and TLRs also play a vital role in EV71-induced severe HFMD. To test this possibility, we screened the expression of Notch receptors, TLRs and proinflammatory cytokines production in HFMD patients, and identified interaction between TLR and Notch in inflammatory regulation in EV71 infection.

2. Patients, materials, and methods

2.1. Subjects

The study protocol was approved by the Ethics Committee of First Hospital of Xinxiang Medical University. A total of 37 children (including 16 of mild HFMD and 21 of severe HFMD) with EV71-induced HFMD were enrolled in the current study. All children were under seven years old, and were hospitalized at the First Hospital of Xinxiang Medical University during outbreaks between March 2016 and March 2017. The diagnoses were made in accordance with the Guidelines for the Diagnosis and Treatment of HFMD (2010 edited version) by the Ministry of Health of the People's Republic of China (http://www.moh.gov.cn/mohyzs/s3586/201004/46884.shtml). EV71 infection was confirmed by detection of EV71 VP1 gene in body fluids (including throat swabs, stools, and cerebrospinal fluids) using EV71 nucleic acid detection kit (Da'An Gene, Guangzhou, Guangdong Province, China). The definitions of mild and severe case were described previously [17]. No children were co-infected with bacteria or affiliated with immune disorders before baseline sampling. For normal control (NC), eleven healthy children matched for age and sex were also enrolled, and these children were followed-up for regular health examinations during the same period. The baseline characteristics of all enrolled subjects were shown in Table 1.

2.2. Cytokine assay

Cytokines expression in the serum was measured using Magnetic Luminex High Performance Assay Multiplex Kit (R&D System, Minneapolis, MN, USA) by Luminex LX-200 Instrument with xPONENT 3.1 (R&D System) following the instructions of manufacturer. The following cytokines were tested, including granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF-α, and vascular endothelial growth factor (VEGF).

Table 1
Baseline characteristics of enrolled subjects.

	NC	Mild HFMD	Severe HFMD
Cases (n)	11	16	21
Gender (male/female)	6/5	11/5	13/8
Age (months)	47.6 ± 10.8	49.1 ± 9.2	38.1 ± 6.9
Fever	No	81.25% (13/16)	100% (21/21)
Rash	No	100% (16/16)	100% (21/21)
WBC ($\times 10^9$/L)	7.17 ± 0.94	8.17 ± 3.01	15.76 ± 5.02
CRP (mg/L)	0.35 ± 0.09	8.16 ± 3.88	24.18 ± 10.39

WBC: white blood cells; CRP: C-reaction protein.

2.3. Peripheral blood mononuclear cell (PBMC) isolation and $CD14^+$ cells purification

PBMC was isolated from peripheral blood by density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA). $CD14^+$ cells were purified using human CD14 Microbeads (Miltenyi, Bergisch Gladbach, Germany) following manufacturer's instructions. The purity of enriched $CD14^+$ cells was > 95% by flow cytometry determination.

2.4. Cell culture

10^5 of purified $CD14^+$ cells were stimulated with TLR3 ligand poly (I: C) (InvivoGen, San Diego, CA, USA; final concentration, 20 μg/mL for 24 h stimulation [18]), TLR4 ligand LPS (InvivoGen; final concentration, 100 ng/mL for 24 h stimulation [19]), or Notch signaling inhibitor DAPT (Selleck Chemicals, Huston, TX, USA; final concentration, 75 μmol/mL for 12 h stimulation [20]), respectively. Cells and supernatants were harvested 24 h post-stimulation for further experiments.

2.5. Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from PBMC using TRIzol reagent (Invitrogen, Carlsbad, CA, China). RNA was reversely transcribed with random hexamers using PrimeScript RT Master Mix (TaKaRa, Dalian, Liaoning Province, China). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) with primers specific for human TLR1-TLR10 [21], Notch1/2, or Hes 1/5 [20]. Related gene expressions were semi-quantified by $\Delta\Delta C_T$ methods using 7500 System Sequence Detection software (Applied Biosystems, Foster, CA, USA).

2.6. Western blot

Cells were lysed for 15 min on ice in 2 × SDS loading buffer, and supernatants were harvested by centrifugation at 13,000 × *g* for 1 min. Total proteins were separated on SDS-PAGE using Mini-protean 3 electrophoresis cell systems (Bio-Rad, Hercules, CA, USA), and were electroblotted onto a PVDF membrane. The membrane was soaked in PBS containing 5% non-fat milk and 0.05% Tween 20, and then incubated overnight in the presence of rabbit anti-TLR3 (Abcam, Cambridge, MA, USA; 1: 1000 dilution), rabbit anti-TLR4 (Abcam; 1: 1000 dilution), rabbit anti-TLR7 (Abcam; 1: 1000 dilution), rabbit anti-TLR9 (Abcam; 1: 1000 dilution), rabbit anti-Src (phospho Y418) (Abcam; 1: 1000 dilution), rabbit anti-IRF3 (phospho S386) (Abcam; 1: 1000 dilution), rabbit anti-MyD88 (Abcam; 1: 1000 dilution), rabbit anti-TRIF (Abcam; 1: 1000 dilution) or anti-GAPDH (Abcam; 1: 2000 dilution). Horseradish peroxidase-conjugated goat anti-rabbit antibody IgG (Abcam; 1: 2000 dilution), which was used for secondary antibodies, was added for additional 2-hour incubation. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz, CA, USA). The densities of the bands were semi-quantified using ImageJ software (Softonic, San Francisco, CA, USA).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The expressions of IL-6, IL-8, and TNF-α in the cultured supernatants were measured using commercial ELISA kits (R&D System) following the instructions of manufacturer.

2.8. Statistical analyses

All data were analyzed using SPSS version19.0 for Windows (SPSS, Chicago, IL, USA), and data were shown as mean ± SD. SNK-*q* test or *t*-test was used for comparison among groups. Paired *t*-test was used for