Original Article
Investigation for the roles of TLR2, TLR9 and Th17/Treg in the pathogenesis of infectious mononucleosis

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Abstract: Infectious mononucleosis (IM) is caused by Epstein-Barr virus (EBV) infection. Toll-like receptor 2 (TLR2) has most recognized ligands, and is possibly direct receptor for anti-EBV. EBV can also activate TLR9 on B cells for inducing anti-viral response. T helper cell 17 (Th17) can promote inflammation by secreting IL-17, and is possibly involved in IM pathogenesis. Regulatory T cell (Treg) is a T cell sub-population with immune suppression. The previous study has indicated insufficient Treg cell immunity for acute IM onset. This study thus aims to investigate the TLR2, TLR9, Th17 and CD4+CD25+Treg cell expression, and to explore its potential role in IM. A total of 98 acute IM children and 76 IM children at recovery period were recruited in our hospital, in parallel with 52 healthy children. The qRT-PCR and western blot assay were used to test mRNA or protein expressions of TLR2 and TLR9, respectively. Flow cytometry was used to test percentage of Th17 and CD4+CD25+Foxp3+T cell in total CD4+T cells. ELISA was employed for detecting serum levels of IL-17, IL-22, IL-19 and TGF-β. TLR2 and TLR9 expression, percentage of Th17/CD4+T cells, Th17/Treg, IL-17 and IL-22 contents were significantly elevated in acute IM patients compared to recovery patients and control. Meanwhile, the Treg/CD4+T cells, IL-10 and TGF-β contents were significantly lower compared to recovery patients and control. Elevation of TLR2 and TLR9, plus higher Th17 cells, IL-17 and IL-22 contents, depressed Treg cell ratio, and lower TGF-β and IL-10 level occurred in acute IM. Therefore, the elevation of TLR2, TLR9 and Th17/Treg imbalance might play a role in the IM pathogenesis.

Keywords: Infectious mononucleosis, toll-like receptor 2, toll-like receptor 9, T help cell 17, regulatory T cell

Introduction

Infectious mononucleosis (IM) is one systemic hyperplasia in mononuclear macrophage system caused by primary infection of Epstein-Barr virus (EBV). It is one common infectious disease in children, and is featured with fever, isthmitis, swelling lymph node, liver/spleen, elevated peripheral lymphocytes, and occurrence of abnormal lymphocytes [1]. IM may affect multiple tissues and organs, and lead to various typical or atypical symptoms, making the clinical diagnosis and treatment relatively difficult. B lymphocyte expresses EBV receptor, and is the primary targeting effective cells after infecting EBV, and is life-long time reserve of virus [2]. After infection with B lymphocytes, EBV can activate T cells to induce anti-viral defense response for clearing EBV-infected B cells [3]. Various studies have been reported the correlation between EBV infection and multiple tumors, including gastric carcinoma [4], colorectal cancer [5], breast cancer [6], Hodgkin lymphoma [7], Burkitt lymphoma [8] and nasopharyngeal carcinoma [9]. Toll-like receptor (TLR) is one type of pattern recognition receptor (PRR) as it can recognize multiple structural component of exogenous pathogen, facilitate transcription, synthesis and secretion of inflammatory factors, and induce anti-infectious immune response. As the first line fighting against infection, PPR recognizes structural domain of pathogens and induce cascade immune response as one important factor. TLR2 might be the most direct PPR in anti-EBV infection, and plays an important role in inducing inflammatory factor release and pathological injury after EBV infection [10]. TLR9 is mainly expressed on B cell surface, and can be activated by one DNA sequence containing special struc-
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The immune system plays a crucial role in the body’s defense against viruses. EBV (Epstein-Barr virus) acts as one ligand of TLR9 to activate and induce the production of anti-viral cytokines. T helper cell 17 (Th17) is one newly identified T cell sub-population that can secrete interleukin-17 (IL-17), and is important for fighting against exogenous infection and body immune response. In 1995, Sakaguchi et al. [12] first reported one T cell sub-population with CD4 and CD25 co-expression, plus immune modulation and suppression functions, and showed their roles in body autoimmune tolerance, therefore, naming it as regulatory T cell (Treg). Forkhead transcription factor 3 (Foxp3) is one member of foxhead transcription factor family, and is specifically expressed in CD4+CD25+Treg cells. Due to its necessary role in maintaining development and function of Treg cells, Foxp3 is commonly accepted as specific marker for identifying CD4+CD25+Treg cells [13]. Previous study [14] has indicated that immune deficiency caused by lower Treg cell number might be one important reason for acute onset of IM. Chiffolean et al. [15] found that EBV could regulate proliferation and biological effects of CD4+CD25+Treg cells by activating TLR9. This study thus investigated TLR2, TLR9, Th17 cells, and CD4+CD25+Treg cells expression in peripheral blood mononuclear cell (PBMC) in IM patients, to explore its potential role in IM pathogenesis.

Materials and methods

Reagent and material

PBMC separation buffer Ficoll-Paque PREMIUM was purchased from GE healthcare (USA). RPMI 1640 culture medium was purchased from Gibco (USA). Flow cytometry antibody for CD4, CD25, Foxp3 and IL-17 were purchased from BD Pharmingen (USA). BCA protein quantification kit was purchased from Boster (China). RIPA lysis buffer was purchased from Beyotime (China). Trizol was purchased from Invitrogen (USA). Reverse transcription kit ReverTra Ace aPCR RT kit and SYBR Green dye were purchased from Toyobo (Japan). Rabbit anti-human TLR2 and TLR9 antibodies were purchased from Santa Cruz (USA). ELISA kit for IL-10, TGF-β, IL-17 and IL-22 were purchased from eBioscience (USA).

Clinical information

A total of 98 acute IM children (52 males and 46 females, aging from 2.6 to 11.8 years, average age = 5.4 ± 3.7 years) diagnosed in the Central Hospital of Xianyang from January 2014 to December 2015 were recruited. All included patients fitted IM diagnostic criteria [16]: (1) Fitter any three of these clinical symptoms: fever, isthmatis, tonsillitis, and swelling of lymph node or liver/spleen; (2) Positive for EBV DNA by PCR; (3) Abnormal lymphocyte ≥ 10% in peripheral blood, or absolute value ≥ 1 × 10^9/L; (4) Positive for specific serology assay, with elevated titer for VCA-IgM and IgG antibody; (5) Positive of serum heterophile agglutination reaction. All patients had no IM before, nor had the use of glucocorticoid hormone or cytotoxic drug, or immune modulating drug. All patients showed fever for more than 3 days. There were 82 cases of isthmatis, 56 cases of tonsillitis, 73 cases of skin rash, 54 cases of eyelid edema, 66 cases of lymph node swelling, 58 cases of liver swelling, and 27 cases of spleen swelling. All patients received anti-viral treatment using acyclovir or Ganciclovir for 2 weeks. Partial patients received intramuscular injection of interferon for 3~5 days to potentiate anti-viral treatment. Symptomatic supporting treatment was also applied. For those with liver dysfunction, protective measures including vitamin C, diammonium glycyrrhizinate and glucurone were given. Recovery period refers to acute IM patients after treatment and disappearance of clinical symptoms for more than 1 month. There were 36 males and 40 females in recovery group, aging between 2.8~13.2 years (average age = 6.0 ± 6.6 years). Another cohort of 52 healthy children (28 males and 24 females, aging between 2.5 and 12.4 years, average age = 5.8 ± 4.2 years) were recruited as control group from clinic of our hospital. No significant difference regarding age or sex existed among three groups (P>0.05).

Treg and Th17 assay

Fasted blood was collected from all patients and placed in heparin. PBMC was separated by Ficoll-Paque PREMIUM via density gradient centrifugation. Cells were washed in PBS twice. 100 μl PBMC suspension was added with 5 μl FITC-CD4, PE-CD25 and APV-Foxp3 antibody, in parallel with isotype controlled antibody. After
dark incubation for 15–20 min at room temperature, supernatant was discarded for PBS rinsing. Cells were re-suspended in 0.5 ml PBS, and were measured on Beckman FC500 MCL flow cytometry apparatus. The ratio of CD4+CD25+Foxp3+T cell among all CD4+T cells were evaluated in this study.

Th17 cells were detected in the following steps: PBMC were re-suspended in RPMI1640 medium containing 10% FBS. PMA (final concentration 50 ng/ml), Ionomycin (final concentration 1 μg/ml) and Monensin (final concentration 1.7 μg/ml) were sequentially added, followed by 5 h incubation at 37°C with 5% CO₂. 100 μl PBMC suspensions were mixed with 0.5 ml fixation buffer for 20 min room temperature incubation, followed by 350 g centrifugation for 5 min. The supernatant was discarded, with the addition of 2 ml membrane rupture buffer for 20 min room temperature incubation. The mixture was then centrifuged at 350 g twice, and was re-suspended in 100 μl membrane rupture buffer. Total of 20 μl Alexa Fluor 647 mouse IgG1, κ isotype control/CD4 FITC or IL-17 Alexa Fluor 647/CD4 FITC antibody was added for 30 min dark incubation at room temperature. After rinsing in membrane rupture twice, cells were re-suspended in 0.5 ml staining buffer, and were loaded for analyzing Th17 ratio in CD4+T cells of peripheral blood.

qRT-PCR for gene expression

Total RNA was extracted from cells by Trizol method. In brief, PBMC were mixed with 1 ml Trizol. Following vigorous vortex, 200 μl chloroform was added. The supernatant was saved after 15 min incubation. RNA was precipitated by isopropanol, and was rinsed in 1 ml 70% ethanol in centrifugation. RNA precipitation was solved in DEPC treated water for measuring concentration and purity. cDNA was synthesized by ReverTra Ace qPCR RT Kit in a 10 μl system including 1 μg total RNA, 2 μl RT buffer (5 ×), 1 μl oligo dT + random primer mix, 1 μl RT enzyme mix, 1 μl RNase inhibitor, and ddH₂O. The reaction conditions were: 37°C for 15 min, 56°C for 3 min, followed by 98°C 5 min. cDNA products were kept at -20°C fridge. Using cDNA as the template, PCR amplification was performed under the direction of DNA polymerase using primers (TLR2PF: 5’-ATGCC GCTCT ACTCC GTGAG-3’; TLR2PR: 5’-GGATG CGGTT GGAGG ACA-3’; β-actinPF: 5’-GAACC CTAAG GCCAA C-3’; β-actinPR: 5’-TGTCA CGCAC GATT CC-3’; TLR9PF: 5’-CTGCC TTCCT ACCCT GTGAG-3’; TLR9PR: 5’-GGATG CGGTT GGAGG ACA-3’; β-actinPF: 5’-GAACC CTAAG GCCAA C-3’; β-actinPR: 5’-TGTCA CGCAC GATT CC-3’; In a PCR system with 10 μl total volume, we added 5.0 μl 2 × SYBR Green Mixture, 1.0 μl forward/reverse primer (at 2.5 μm/l), 1 μl cDNA, and 3.0 μl ddH₂O. PCR conditions were: 95°C for 15 s, 60°C for 30 s and 74°C for 30 s. The reaction was performed on ABI7500 fluorescent quantitative PCR cycler for 40 cycles to collect fluorescent data.

Blotting

RIPA buffer was used to extract total proteins from PBMC. Protein concentration was determined by BCA kit. Proteins were denatured by 5 min boiling in four time volume of 5 × loading buffer. 50 μg protein samples were separated by 8% SDS-PAGE (40 V for 300 min), and were transferred to PVDF membrane (300 mA for 100 min). The membrane was blocked in 5% defatted milk powder for 1 h, followed by primary antibody (anti-TLR2 at 1:400, anti-TLR9 at 1:400, or anti-beta-actin at 1:1000) incubation at 4°C overnight. By PBST washing (3 min × 5 times), HRP-labeled secondary antibody (1: 5000 dilution) was added for 1 h incubation. After PBST rinsing for three times (5 min each), ECL reagent was added for 2 min dark incubation. The membrane was then exposure in dark. Quantity One image analysis software (BioRad, US) was used to analyze relative grey density of bands.

Serum cytokine assay

Total of 5 ml fasted peripheral blood samples were collected. After 4°C overnight incubation, serum was separated at 4000 r/min for 5 min, and was kept in -80°C fridge. ELISA was used to test cytokine content in serum. In brief, 100 μl antibody was used to pre-coat 96-well plate at 4°C overnight incubation. The plate was then washed for three times in wash buffer. 200 μl ELISA/ELISPOT diluent was added into each well for 60 min room temperature incubation, followed by rinsing in wash buffer. 100 μl culture supernatant or 2-fold diluted standards in ELISA/ELISPOT dilution was added into each well, followed by room temperature for 2 h. Wash buffer was added for 4 times of rinsing. 100 μl detecting antibody was added into each well for 60 min room temperature incubation. After wash buffer rinsing (4 times), 100 μl
Avidin-HRP was added for 30 min room temperature incubation, followed by 6 times of wash buffer. 100 μl TMB reaction substrate was added for 15 min incubation at room temperature. The reaction was stopped by 50 μl quenching buffer, and was measured in a microplate reader at 450 nm wavelength.

Statistical analysis

SPSS18.0 was used to record all data for statistical analysis. Measurement data were presented as mean ± standard deviation (SD).

Table 1. Comparison of Treg, Th17 and Th17/Treg in all people

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Treg/CD4⁺T (%)</th>
<th>Th17/CD4⁺T (%)</th>
<th>Th17/Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute IM</td>
<td>98</td>
<td>2.15 ± 0.98*</td>
<td>2.89 ± 0.96*</td>
<td>1.16 ± 0.43*</td>
</tr>
<tr>
<td>Recovery IM</td>
<td>76</td>
<td>4.57 ± 1.76*,#</td>
<td>1.46 ± 0.57*,#</td>
<td>0.45 ± 0.18*,#</td>
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<tr>
<td>Healthy control</td>
<td>52</td>
<td>6.89 ± 2.03</td>
<td>0.92 ± 0.32</td>
<td>0.17 ± 0.08</td>
</tr>
<tr>
<td>F value</td>
<td></td>
<td>165.225</td>
<td>149.934</td>
<td>215.998</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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</tbody>
</table>

Note: *, P<0.05 compared to control group; #, P<0.05 compared to acute patients.

One-way analysis of variance (ANOVA) was employed for comparison among multiple groups, followed by LSD test in paired comparison. Enumeration data were presented in percentage, and were analyzed by chi-square test. A statistical significance was defined when P<0.05.

Results

Significantly elevated TLR2 and TLR9 expression in acute IM patients

qRT-PCR results showed significantly elevated TLR2, TLR9 mRNA levels in PBMC from acute IM group compared to that of recovery group, which had remarkably higher TLR2 and TLR9 mRNA compared to healthy control group (Figure 1A, 1B). Western blotting results showed remarkably higher TLR2 and TLR9 protein expression levels in PBMC of acute IM patients compared to IM recovery group, in which TLR2 and TLR9 protein expression level was higher than control group (Figure 1C).
Elevated Th17 cell and decreased Treg cells during acute IM

CD4⁺CD25⁺Fopx3⁺Treg/CD4⁺T cell ratio was significantly lower in acute IM patients compared to recovery patients and healthy control people. Treg/CD4⁺T cell ratio in recovery IM patients was significantly lower than healthy control people. Th17/CD4⁺T cell ratio and Th17/Treg ratio were significantly higher in acute IM patients compared to recovery IM patients and healthy control people. IM recovery patients also had remarkably higher Th17/CD4⁺T cell ratio and Th17/Treg ratio than healthy control group (Table 1). Representative flow cytometry results for CD4⁺CD25⁺Fopx3⁺Treg cell and CD4⁺IL-17⁺Th17 cell were shown in Figure 2A, 2B.

Increased inflammatory factor and decreased anti-inflammatory factor in acute IM

ELISA showed significantly elevated contents of pro-inflammatory factors IL-17 and IL-22 in peripheral blood of acute IM patients compared to recovery patients or healthy control people. Meanwhile, anti-inflammatory factors IL-10 and TGF-β contents were significantly lower (Figure 3). Recovery IM patients had higher IL-17 and IL-22, plus lower IL-10 and TGF-β levels than those of control group (Figure 3).

Discussion

IM is one syndrome caused by acute EBV infection, and is one acute or sub-acute multiple organ systemic disease [17]. After entering into
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human body, EBV firstly attacks target B lymphocytes expressing EBV specific receptor CD21 [2]. EBV infection causes B lymphocyte proliferation and activation, transforming them into immortal B lymphocytes, and making it acquiring persistent proliferation, replication and spreading. EBV codes for latent membrane protein 1 (LMP1) and LMP2A, both of which are persistent stimulating signal molecules inducing B cell activation and proliferation [18]. Over-proliferation and viral spreading of EBV-infected B cells can initiate cell immunity featured with CD8+ cytotoxic T cell (CTL) production. Activation of CTL significantly accelerate the process for destructing and clearance EBV-infected B cells, and producing a series of clinical manifestations including fever, isthmitis, swelling of lymph node and liver/spleen [19]. Although alternation of body immune function plays a critical role in IM pathogenesis, inhibiting B cell proliferation and activation, and clearance of EBV, it can also cause immune dysfunction of children and dys-regulation of regulator mechanisms.

Toll-like receptor is one trans-membrane protein that can effectively recognize conserved structural molecules of exogenous microbes, activate body to generate immune cell response, and initiate non-specific immunity and specific immunity. TLR2 is one Toll-like receptor that recognizes most types of ligands among all TLRs. It can participate in immune response against multiple Herpes viruses including EBV. Gaudreault et al. [10] found that TLR2 might be the most direct PRR for anti-EBV infection, and plays a critical role in inducing inflammatory factor release and pathological injury after EBV infection. TLR9 is mainly expressed on B lymphocyte surface, and can be activated by one type of DNA sequence containing special structure of virus, thus inducing B lymphocyte proliferation and activation. Zauner et al. [11] found the activation of TLR9 on B lymphocytes involves in the anti-viral immune response via various mechanisms. LaRosa et al. [20] found elevated viral titer and mortality by TLR9 deficiency, indicating the important role of TLR9 in anti-viral immune response. T helper cell 17 (Th17) is one newly discovered T cell sub-population that can secrete interleukin 17 (IL-17), and has important implication in autoimmune disease and body defense response. Th17 cells mainly secrete inflammatory factors including IL-17, IL-22 and IL-23 to exert anti-inflammatory function, and are correlated with autoimmune and pathological immune inflammatory response. Broderick et al. [21] found significant difference of Th17 cell related inflammatory factors between acute and recovery IM patients after EBV infection, indicating the possible involvement of Th17 cellular function in IM pathogenesis. As one immune modulatory cell, Treg cell mainly inhibit T cell activation and proliferation via secreting anti-inflammatory factors TGF-β and IL-10, thus playing an important role in immune suppression, maintaining auto immune homeostasis, and preventing autoimmune disease [22]. Previous study [14] attributed decreased Treg cell number and related immune suppression function insufficiency as one important reason for acute pathogenesis of IM. Liu et al. [23] found that TLR2 played an important role in regulating Th17/Treg ratio after viral infection. Various studies have demonstrated the regulatory role of TLR2 and TLR9 on proliferation and function of CD4+CD25+Treg cell [15, 24, 25]. This study thus quantified TLR2, TLR9, Th17 cells and CD4+CD25+Treg cells expression in IM children, in an attempt to investigate their potential roles in IM pathogenesis.

Test results showed significantly elevated TLR2 and TLR9 expression, Th17 cell ratio, and Th17/Treg ratio in PBMC of acute IM patients, plus decreased CD4+CD25+Foxp3+Treg cell ratio, elevated Th17 cell related inflammatory factor IL-17 and IL-22, and down-regulation of Treg cell related anti-inflammatory factor IL-10 and TGF-β. Wingate et al. [14] showed significantly lower CD4+CD25+Treg cell number in peripheral blood of acute IM patients, which is consistent with
our results. Recovery IM patients had decreased TLR2/TLR9 expression, Th17 cell ratio and Th17/Treg ratio to different extents, accompanied with increased CD4⁺CD25⁺Foxp3⁺Treg cell ratio, decreased inflammatory factor IL-17 and IL-22, and elevated anti-inflammatory factor IL-10 and TGF-β, but still having significant difference compared to control group. Results showed that during acute phase of IM, up-regulation of TLR2 and TLR9 benefited the recognition of invaded EBV, thus causing body anti-viral immune response. Lowered CD4⁺CD25⁺Foxp3⁺Treg cell number and immune suppression during acute phase of IM may benefit amplification of EBV lysate cycling protein reaction by T cells, facilitating abundantly proliferation of EBV specific cytotoxic CD8⁺T cells, thus enhancing the killing of EBV by B cells, and accelerating viral clearance process. Rahal et al. found the injection of certain titer of virus in mouse tail veins significantly up-regulated contents of Th17 cell related factors IL-17 and IL-23 in peripheral blood [26], indicating the role of Th17 cells in response EBV infection induced immunity. Ohta et al. [27] found significantly increased Th17 cell number in peripheral blood of active EBV infecting people, indicating the possible participation of Th17 expression enhancement in acute pathogenesis of IM. These results fitted those reported by Rahal et al. [26] and Ohta et al. [27]. During recovery period, CD4⁺CD25⁺Foxp3⁺Treg cell ratio was elevated, and was re-initiated for its immune suppression function, inhibiting over-activation of effector T cells and abundantly release of inflammatory factor to certain extents, thus protecting body from injury by overshooting of immune reaction. Various studies have shown that TLR2 and TLR9 ligands could induce activation and proliferation of mouse CD4⁺CD25⁺Foxp3⁺cells, but weakened Treg immune suppression function [15, 24, 25]. Therefore, up-regulation of TLR2 and TLR9 could further weaken immune suppression potency of CD4⁺CD25⁺Treg cells, and potentiate host anti-viral immune response and EBV clearance, although detailed mechanisms require further elaborations.

Conclusion

TLR2 and TLR9 are significantly up-regulated in acute IM patients, which also had elevated Th17 cell ratio, and related inflammatory factors IL-17 and IL-22. However, the Treg cell ratio and related anti-inflammatory factors IL-10 and TGF-β contents are all remarkably decreased. Up-regulation of TLR2 and TLR9, plus Th17/Treg imbalance thus plays a role in IM pathogenesis.

Disclosure of conflict of interest

None.

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