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TLR9 played a more important role than TLR2 in the combination of maltose-binding protein and BCG-induced Th1 activation

Weihua Ni[1], Fang Wang[1], Guomu Liu, Nannan Zhang, Hongyan Yuan, Jing Jie, Guixiang Tai*

Department of Immunology, Norman Bethune College of Medicine, Jilin University, Changchun, China

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ABSTRACT

Our previous study demonstrated that maltose-binding protein (MBP) combined with BCG induced synergistic mouse Th1 activation *in vivo*. Here, to explore the mechanism of MBP combined with BCG on Th1 activation, mouse purified $CD4^+$ T cells were stimulated with MBP and BCG in *vitro*. The results showed that MBP combined with BCG synergistically increased IFN-γ production, accompanied with the upregulation of TLR2/9 expressions, suggesting that TLR2/9 were involved in the combination-induced Th1 activation. Next, TLR2 antibodies and TLR9 inhibitor were used to further analyze the effects of TLRs in Th1 activation. Results showed TLR2 antibody partly decreased MBP combined with BCG-induced IFN-γ production, MyD88 expression and IκB phosphorylation, indicating that TLR2-mediated MyD88-dependent pathway was involved in the MBP combined with BCG-induced Th1 activation. Moreover, MBP combined with BCG-induced Th1 activation was completely abrogated by TLR9 inhibitor, suggesting that TLR9-mediated MyD88-dependent pathway played a more important role than TLR2 in the combination-induced Th1 activation. Further study showed that TLR9 inhibitor downregulated TLR2 expression, suggesting that TLR9 signaling regulated TLR2 activation to favor Th1 resonse induced by MBP combined with BCG. Collectively, we demonstrated for the first time that the cross-talk of TLR2 and TLR9 triggered Th1 activation collaboratively and our findings provided valuable information about designing more effective adjuvant for cancer therapy.

1. Introduction

Maltose-binding protein (MBP), a component of the maltose transport system of *Escherichia coli*, has been commonly thought to have minimal bioactivity (Choi et al., 2004; Riggs, 2000). However, our previous studies found that MBP induces the activation of mouse macrophages, NK and T helper 1 (Th1) cells, which confirmed the potent immune-enhancing activities of MBP (Ni et al., 2014; Zhang et al., 2011; Wang et al., 2015). Remarkably, we also found that immunization of MBP combined with BCG synergistically induced a strong Th1 response and inhibited the growth of Lewis lung carcinoma (Zhao et al., 2011). However, the underlying mechanism still has not been characterized. In recent studies, we found that MBP directly induces Th1 activation and M1 polarization *via* toll-like receptor 2 (TLR2) (Ni et al., 2014; Wang et al., 2015). Bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, is used as an effective adjuvant to activate TLR2/9 in cancer immunotherapy (Gil et al., 2014; Peter et al., 2015). So, we presumed that both TLR2 and TLR9 might be involved in the MBP combined with BCG-induced Th1 activation.

It has been reported that TLR2 functionally expresses on mouse CD4+ T cells surface, while TLR9 localizes in intracellular vesicles (Kullberg and Mihai, 2014). Both TLR2 and TLR9 utilize MyD88 to transduce signaling (Kullberg and Mihai, 2014). Some studies have shown that TLR2 agonist, P3CSK4 can induce IFN-γ secretion of mouse $CD4^+$ T cells *in vitro* combined with TCR stimulation (Kawai and Akira, 2011). Some studies of TLR9 have shown that oligodeoxynucleotides (ODNs), TLR9-associated ligand, resulted in Th1 activation *via* MyD88-dependent pathway. Recent studies on the interactions between different TLRs ligands indicated that the cross-talk results in either suppressing or synergizing a particular immune response. For example, the synergistic interaction between TLR2 and TLR9 produces stronger Th1-biased immune response *via* MyD88-dependent pathway (Andre et al., 2005; Kawai and Akira, 2011). Treatment of murine macrophages with a combi-

* Corresponding author at: Department of Immunology, Norman Bethune college of Medicine, Jilin University, 126 Xinmin Street, Changchun, 130021, China.
E-mail address: taiguixiang@163.com (G. Tai).
[1] These authors contributed equally to this work.

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nation of TLR2, TLR4 and TLR9 agonists synergize to produce nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) (Hao et al., 2013). Therefore, we paid particular attention to the effect of the cross-talk between TLR2 and TLR9 on regulating Th1 activation in this paper.

Here, we demonstrated that the combination of MBP and BCG synergistically induced Th1 activation and TLR2/9 favored this process. More importantly, we found that TLR9 could upregulate the activation of TLR2 signaling in Th1 response. The results from this study confirmed the key role of TLR9 in Th1 activation induced by the cross-talk between TLR2/9, and provided valuable information for the design of more effective adjuvant for cancer therapy.

2. Materials and methods

2.1. Mice

C57BL/6 mice, six to eight weeks of age, were purchased from the Norman Bethune Medical School of Jilin University, and maintained under specific pathogen-free conditions. The experimental manipulation of mice was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the approval of the Scientific Investigation Board of Science & Technology of Jilin Province.

2.2. Reagents

BCG strain Pasteur was purchased from the Chengdu Institute of Biological Products Co. Ltd. The pMAL-c2 and amylose resin were purchased from New England Biolabs. E-TOXATE kit was purchased from Sigma. The $CD4^+$ T cell mouse lymphocytes enrichment kit was purchased from Becton Dickinson. IFN-γ and IL-4 ELISA kit were purchased from eBioscience. TRIzol was obtained from Invitrogen. Anti-mouse CD3 monoclonal antibody was purchased from Sungene Biotech. M-MLV reverse transcriptase and oligo (dT) primers were purchased from Promega. Anti-TLR2 antibodies used for blocking was obtained from Biolegend. TLR9 inhibitor, SAT05f (5′-CCTCCTCCTCCTCCTCCTCCTCCT-3′) was designed by Sangon Biotech. Polymyxin B (LPS-binding antibiotic), anti-GAPDH, anti-IκB, anti-p-IκB and anti-TLR2/4/9 antibodies used for Western blot were all purchased from Cell signaling technology. Anti-MyD88 antibody was purchased from Abcam. Cell culture medium and reagents were purchased from Gibco. The mouse lymphocyte separation medium was obtained from Dakewe Biotech.

2.3. MBP preparation

MBP used in this paper was obtained from an *E. coli* strain (DH-5α) carried MBP expression vector pMAL-c2 (New England Biolabs, USA), which encodes MBP preceded by methionine, with the final four amino acids replaced by 23 residues encoded by the polylinker of pMAL-c2. MBP proteins were purified by affinity chromatography on amylose resin as previously described (Zhao et al., 2011). Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels in the Mini-Protean system. Endotoxin was removed with DeToxi gel and quantified using an E-TOXATE kit according to the manufacturer's instructions. The quantity of endotoxin in MBP was less than 0.005 EU/mg.

2.4. $CD4^+$ T cells isolation and culture

$CD4^+$ T cells were purified with magnetic beads by negative selection according to the manufacturer's protocol. Briefly, C57BL/6 mouse splenic mononuclear cells were isolated with mouse lymphocyte separation medium, and then $CD4^+$ T cell was purified from the splenic mononuclear cells using the $CD4^+$ T cell mouse lymphocytes enrichment negative selection kit containing CD8, CD11b, CD45R/B220, CD49b and TER-119 antibodies to remove $CD8^+$ T cells, phagocytes, B cells, NK cells and erythrocytes, respectively. The purity of $CD4^+$ T cell population was >98% assessed by flow cytometry using CD3/CD4 antibodies. The cell viability was evaluated over 90% by Trypan blue staining.

The purified $CD4^+$ T cells were stimulated with plate-bound anti-CD3 antibody (5 μg/ml) over night, and then stimulated with soluble anti-CD28 antibody (2 μg/ml) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin for 48 h.

Table 1
Primer sequences and reaction parameters used for qRT-PCR analysis.

Gene	Primer
β-Actin	Forward:5′-TTCTTTGCAGCTCCTTGG-3′ Reverse: 5′-TTCTGACCCATTCCCACC-3′
TLR2	Forward:5′-GCTTCGTTGTTCCCTGTGTT-3′ Reverse: 5′-AGTGGTTGTCGCCTGCTT-3′
TLR4	Forward:5′-TTCACCTCTGCCTTCACTACA-3′ Reverse: 5′-ACACTACCACAATAACCTTCCG-3′
IL-4	Forward:5′- TGACGGCACAGAGCTATTGATG-3′ Reverse: 5′- AGCACCTTGGAAGCCCTACAGA-3′
IFN-γ	Forward:5′-TCTGAGACAATGAACGCTAC-3′ Reverse: 5′- CTTCCACATCTATGCCACTT-3′

2.5. Activated $CD4^+$ T cells stimulation

Activated $CD4^+$ T cells were cultured with medium (control), MBP (10 μg/ml), BCG (4×10^4 CFU/ml), or the combination of MBP and BCG for 48 h to detect cytokine production or for 2 h to detect mRNA or protein expression. For endotoxin analysis, the cells were cultured with medium (control), MBP (10 μg/ml) and LPS (5 μg/ml) with or without polymyxin B (2 μg/ml) for 48 h. The mouse IgG was used as the isotype control.

2.6. ELISA

Activated $CD4^+$ T cells (1×10^6) were treated with or without anti-TLR2 antibody (10 μg/ml) or TLR9 inhibitor (2 μg/ml) for 2 h, and then stimulated with MBP, BCG or the combination for 48 h. Culture supernatants were harvested to detect IFN-γ and IL-4 production by ELISA according to the manufacturer's protocol.

2.7. qRT-PCR

Activated $CD4^+$ T cells were treated with or without anti-TLR2 antibody or TLR9 inhibitor for 2 h, and then stimulated with MBP, BCG and the combination for 2 h. IFN-γ, IL-4, TLR2/9 mRNA expressions were detected by qRT-PCR. RNA was isolated with TRIzol. Total RNA was converted to cDNA using M-MLV reverse transcriptase and oligo (dT) primers following the manufacturer's protocol. β-Actin was used as internal control gene. The primer sequences and reaction parameters were shown in Table 1. Calculations were performed using the $2^{-\Delta\Delta CT}$ method.

2.8. Western blot

Activated $CD4^+$ T cells were treated as described in Section qRT-PCR. TLR2/9, MyD88 expression and IκB phosphorylation were detected by Western blot. Cell lysate was prepared with RIPA lysis buffer in the presence of protease inhibitor leupeptin and phosphatase inhibitor. Protein concentration was obtained by bicinchoninic acid protein detection system. Proteins were sepa-