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Recombinant *E.coli* LLO/OVA Induces Murine BMDCs Maturation *via* TLR4 and NOD1 Receptor and Promotes Specific Cytotoxic T Cell Immunity[1]

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Objective To explore the immune stimulation effect of recombinant *E.coli* LLO/OVA on mice bone marrow-derived dendritic cells (BMDCs) and T lymphocytes *in vitro*. **Methods** After BMDCs stimulated by *E.coli* LLO/OVA, their Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) receptor signalling pathway were examined by superarray hybridization; and the priming effect of the vaccine activated BMDCs on $CD4^+$T and $CD8^+$T was determined by [3H]thymidine uptake and ELISA, the tumor cytotoxic effect of activated $CD8^+$T cells was determined by cytotoxic assay. **Results** After BMDCs were activated by *E. coli* LLO/OVA *via* TLR4, NOD1 receptor and NF-κB signalling pathway, the expression of their surface molecules including MHC class Ⅰ, MHC class Ⅱ, CD40, CD80 and CD86 significantly up-regulated; the secretion of IL-12 and IFN-γ increased also. The mature BMDCs stimulated the allergic $CD4^+$T and $CD8^+$T cells proliferation and their IL-2 and IFN-γ secretion, and the activated $CD8^+$T cells effectively killed B16-OVA melanoma cells and RMA-S/OVA lymphoma cells *in vitro*. **Conclusion** *E.coli* LLO/OVA is effective in inducing BMDCs maturation *via* activating TLR4 and NOD1 receptor signalling pathway and promoting specific anti-tumor T cell immunity *in vitro*.

Key words: Bone marrow-derived dendritic cells, BMDCs; Recombinant *Escherichia coli;* Toll-like receptor (TLR); Nucleotide-binding oligomerization domain (NOD); $CD8^+$T cells

INTRODUCTION

Bacteria vaccine encoding tumor antigens evokes significant anti-tumor immune response in preclinical experiments[1]. However, the mechanism remains unclear. Non-pathogenic *Escherichia coli*, as a vector encoding tumor antigen, successfully suppresses the growth and metastasis of the tumor in murine models[2]. Listeriolysin O (LLO), the major virulent factor of *L. Monocytogenes*, has been reported effective to induce dendritic cells maturation and the acquired T-cell immunity of the host[3]. Our previous study revealed that subcutaneous injection of recombinant *E.coli* LLO/OVA carrying LLO and tumor antigen model ovalbumin (OVA) provokes a more potent anti-tumor effect than *E.coli* OVA in B16-OVA melanoma burden mice[4]. As T lymphocytes play a major role in rejecting malignant tumor, and the proliferation and the function of T lymphocytes crucially depend on the interaction between T lymphocytes and dendritic cells (DCs), tumor antigen epitopes presentation, co-stimulatory molecules expression and cytokines secretion of DCs play key roles. Some reports have revealed that DCs induce innate immune and modified acquired immune by recognizing bacteria ingredient through pattern recognition receptors (PPR). Toll-like receptors (TLRs) are members of PPRs. TLRs belong to the family from TLR1 to TLR13, most of which are located on the cytoplasm membrane, and each TLR recognizes specific molecule. For example, TLR2 recognizes lipopeptides of G^-bacteria, while TLR4 recognizes lipopolysaccharides (LPS) of *E.coli*. TLR and Myd88/NF-κB signaling pathway activation induce the DC maturation, such as high expression of MHC class Ⅱ and co-stimulation molecules, and enhance DCs' ability to stimulate T cells. TLR4 activation promotes interleukin (IL) 12 secretion of DCs and provokes anti-tumor immune response in mice[5-8]. Other than TLRs, nucleotide-binding oligomerization domain (NOD) receptor, such as NOD1 (Card4) and NOD2 receptors

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induces cytokines secretion and provokes innate immune response after DC sensing intracellular pathogens; in addition, the synergistic activation of NOD receptors and TLRs evokes Th1 cells generation[9-10]. Since the components of *E.coli* LLO/OVA are supposed to possibly activate BMDCs *via* TLR, NOD receptor, and NF-κB signalling pathway, we have explored the TLR and NOD receptor signalling pathway by supperarray hybridization and their mature station by flow cytometry after BMDCs are stimulated by *E.coli* LLO/OVA *in vitro*. The priming effect of the vaccine activated BMDCs on $CD4^+$T and $CD8^+$T is determined by [^{3}H]thymidine uptaking and ELISA, while the tumor cytotoxic effect of the activated $CD8^+$T cells is determined by cytotoxic assay. This study has demonstrated that *E.coli* LLO/OVA is effective to induce BMDCs maturation *via* TLR4 and NOD1 receptor signalling pathway and promote specific anti-tumor T cell immunity *in vitro*.

MATERIAL AND METHODS

Murine BMDCs Culture and Recombinant E.coli Stimulation

6-8 weeks-old female C57BL/6 mice were obtained from Harlan Breeders (Oxfordshire, UK) and kept in a germ-free environment. Bone marrow cells were isolated from femurs and tibia of the mice, and the cells were cultured at 37 ℃, 5%CO_2 in RPMI 1640 medium (Cancer research UK) supplemented with 10% FCS, and recombinant murine GM-CSF (6ng/mL, Peprotech, UK) and antibiotics. On day 4 the nonadherent cells were removed and adherent cells were cultured in a new complete medium. On day 7 suspending BMDCs were harvested. Before stimulation of BMDCs, *E.coli* LLO/OVA, and *E.coli* OVA were fixed with 0.5% paraformaldehyde/PBS at room temperature for 30 min and washed 3 times with endotoxin free PBS buffer. BMDCs were stimulated with *E. coli* LLO/OVA or *E. coli* OVA at BMDCs: *E.coli* ≈ 1:5 in polypropylene tubes for 1 h. After washing with warm endotoxin free PBS Buffer 3 times, BMDCs were cultured in RPMI medium with 10% FCS till 2, 4, 6, 8, and 24 h, and then were harvested for RNA isolation and flow cytometry.

Superarray Hybridization

High quality total RNA of BMDCs was isolated using a combination of the Trizol protocol (Invitrogen) and RNeasy column (Qiagen) according to the manufacturer's instruction. 3 μg of total RNA samples were reverse transcribed, and then the biotinlabeled cRNA were synthesized with Truelabeling-AMP™ Linear RNA Amplification Kit (Superarray, USA). 6 μg of biotinlabeled cRNA samples were hybridized with Oligo GEArray® mouse NF-κB signalling pathway membranes respectively for 18 h in a hybridization Oven (Techne Hybridiser HB-1D) according the protocol. The hybridized membranes were exposed to Kodak film after chemiluminescent staining, and the films were scanned on a Canon scanner. The hybridization signals were analyzed using GEArray Expression Analysis Suite online (http://GEASuite.supperarray.com).

RT-PCR Assay

2 μg of high quality total RNA samples of *E.coli* LLO/OVA or *E.coli* OVA stimulated BMDCs at 2, 4, 6, and 8 h were reverse transcribed with Taq Man Reverse Transcription Agent Applied Biosystem (Roche). PCR were performed using specific primers [Tlr4 (439 bp) sense 5'-GGAAGGACTATGTGATG TGACC-3', antisense 5'-GCTCTTCTAGACCCA-TGAAATTGG-3', Nod1 (469 bp) sense 5'-CTTG CATTCAATGGCATCTC-3', antisense 5'-ACATCG GTGTGCACTGTGGA-3', Gapdh (496bp) sense 5'-TGATGGGTGTGAACC-ACGAG-3', antisense 5'-TCAGTGTAGCCCAAGATGCC-3']. The conditio ns for amplification were as follows: initial denature at 94 ℃ for 5 min, then denature at 94 ℃ for 45 s, anneal at 55 ℃ for 45 s, extend at 72 ℃ for 45 s for 30 cycles, and final extension at 72 ℃ for 5 min. The PCR products were analyzed on 1.5% agarose gels.

Flow Cytometry

The BMDCs' surface molecules staining was performed using the following anti-murine mAbs, FITC conjugated anti-H2K (MHC class Ⅰ), anti-IAb (MHC class Ⅱ), anti-CD40, anti-CD80, and anti-CD86 (BD Phamingen). The BMDCs were incubated with saturating concentration of each mAb for 30 min at 4 ℃. After BMDCs were washed twice, their fluorescence intensity was determined by flow cytometry (FACScalibur, BD Biosciences).

ELISA Assay

After stimulation by *E. coli* LLO/OVA or *E. coli* OVA 1 h, 2×10^6/mL BMDCs were washed with warm endotoxin free PBS and cultured in RPMI for 24 h, then the supernatant were harvested. In co-culture assay of BMDCs and $CD4^+$T or $CD8^+$T cell, the supernatant were collected at 72 h. The concentration of IL-2, IL-12, and IFN-γ were determined by ELISA according the manure of Quantikine® ELISA Kits(R & D, USA).

Proliferation Assay

The spleenocytes of *E.coli* LLO/OVA or *E.coli* OVA vaccinated C57BL/6 mice (4mice/groupe) on day 1, 4, and 8 were harvested, then $CD4^+$T and CD8a ($CD8^+$T) cells were magnetically sorted (MACS, Miltenyi Biotech) on day 9.1×10^4, 5×10^4, 10×10^4 recombinant *E.coli* LLO/OVA or *E.coli* OVA activated BMDCs were co-cultured with allergic 1×10^5 $CD4^+$T or $CD8^+$T cells in RPMI 1 640 medium with anti-CD3 (0.5 μg/mL) respectively for 54 h in U-bottom 96 well plates at 37 ℃ and 5% CO_2, then 1Ci/well [^{3}H]thymidine was added in the medium for 18 h and the absorption of [^{3}H] thymidine was determined.

Cytotoxicity Assay

The effecters were $CD8^+$T cells which were co-cultured with *E.coli* LLO/OVA or *E.coli* OVA stimulated BMDCs for 3 days. The targets were ^{51}Cr-labeled B16-OVA melanoma cells and RMA-S/OVA lymphoma cells. The effecters mixed with targets at various ratios for 4 h at 37 ℃. Then OVA specific tumor cell lyses were analyzed in standard ^{51}Cr-released assays. Specific killing was calculated as [(experimental release-spontaneous release)/ (maximum release – spontaneous release)] ×100.

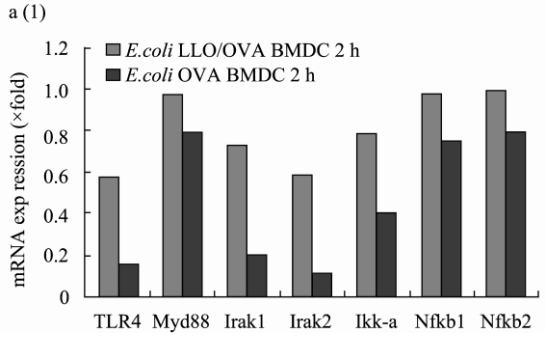
Statistical Analysis

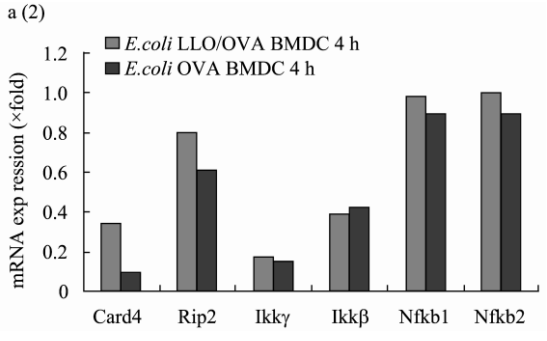
The concentration of interleukins, the absorption of [^{3}H] thymidine and the OVA specific killing rates were expressed as $\bar{x}\pm s$. All statistic analysis was conducted using a student *t*-test of SAS 8.1 software. $P<0.05$ was set as the level of significance.

RESULTS

BMDCs Activated by E. coli LLO/OVA via TLR4 and NOD1 (Card4) Receptor Signalling Pathway

At first, we examined the activation signalling pathway of BMDCs by supperarray hybridization. After BMDCs were stimulated by *E.coli* LLO/OVA for 2 h, the mRNA of Tlr4, Myd88, Irak1, Irak2, IKK-α, Nfkb1, and Nfkb2, significantly up-regulated; but BMDCs primed by *E.coli* OVA showed that the mRNA of Tlr4, Irak1, Irak2 and IKK-α were slightly up-regulated, and the transcription level of Nfκb1 and Nfκb2 was slightly lower than the BMDCs which were primed by *E.coli* LLO/OVA. After BMDCs were primed by *E.coli* LLO/OVA for 4 h, mRNA of Nod1(Card4), Rip2, IKK-γ, IKK-β, Nfκb1, and Nfκb2 were up-regulated; but BMDCs primed by *E.coli* OVA showed lower- level mRNA transcription of Nod1(Card4) and Rip2. After BMDCs were primed by *E.coli* LLO/OVA for 8 h the mRNA of Nod1(Card4), Rip2, IKK-γ, IKK-β, Nfκb1, and Nfκb2 were continuously up-regulated, and the mRNA level of Nod1(Card4) and IKK-β was slightly higher than that of BMDCs primed by *E.coli* OVA (Fig. 1a). The RT-PCR results showed that after BMDCs were activated by *E.coli* LLO/OVA, their TLR4 transcription was significantly up-regulated at 2 h and continued to 4 h, but after BMDCs were activated by *E.coli* OVA, their TLR4 transcription was only up-regulated at 2 h. After BMDCs were activated by *E.coli* LLO/OVA, their Nod1 (Card4) transcription was up-regulated from 4 h to 8 h, but after BMDCs were activated by *E.coli* OVA, their Nod1(Card4) transcription was only up-regulated at 6 h (Fig. 1b).





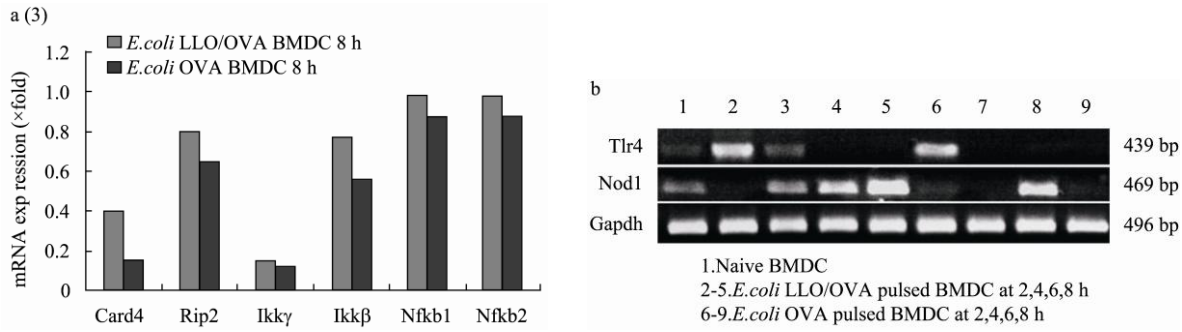


FIG. 1. (a) Superarray hybridyzation of NF-κB signalling pathway. Columns represented the gene transcription of BMDCs stimulated by *E. coli* LLO/OVA or *E. coli* OVA at 2, 4, and 8 h. (b) The mRNA expression of Tlr4 and Nod1 (Card4). Tlr4 and Nod1 (Card4) transcription levels of *E. coli* LLO/OVA or *E. coli* OVA stimulated BMDCs were determined by RT-PCR at 2, 4, 6, and 8 h.

BMDCs Pulsed by E. coli LLO/OVA Expressed Surface Molecules and Secreted Cytokines

We observed the maturation status of the vaccine stimulated BMDCs. After having been primed by *E.coli* LLO/OVA 24 h, BMDCs expressed significantly higher level of CD40, CD80, CD86, MHC class Ⅰ (H2K) and MHC class Ⅱ (IAb) than naive BMDCs, but there was no significantly different expression of these molecules between *E.coli* LLO/OVA stimulated BMDCs and *E.coli* OVA stimulated BMDCs (Table 1). The ELISA results showed that both vaccines stimulated BMDCs secreted high level of IL-12 in the supernatant at 24 h; however, the *E.coli* LLO/OVA stimulated BMDCs secreted higher level of IFN-γ than that of *E.coli* OVA stimulated BMDCs. (P<0.05, Fig. 2).

TABLE 1

The Surface Molecules Expression of BMDCs (%)

	CD40	CD80	CD86	IAb	H2K
E.coli LLO/OVA Pulsed BMDCs	69.52	84.08	74.10	84.80	81.52
E.coli OVA Pulsed BMDCs	69.58	83.39	76.45	84.61	78.21
Naive BMDCs	50.06	80.03	70.28	80.04	63.90

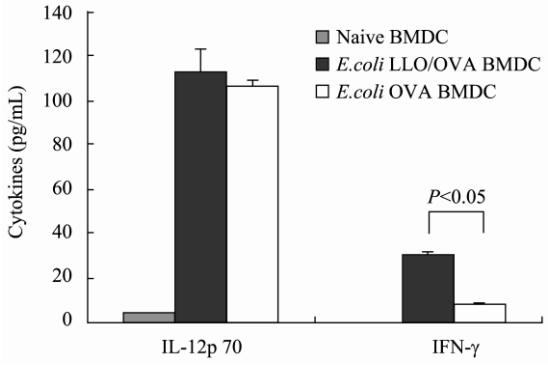


FIG. 2. The concentration of IL-12 and IFN-γ. After BMDCs were stimulated by *E.coli* LLO/OVA and *E.coli* OVA respectively 24 h, the level of IL-12 and IFN-γ in the supernatant was determined by ELISA. Value represent the means of three experiments±SD. Statistical analysis was performed compared to the level of IFN-γ in the supernatant of *E.coli* OVA stimulated BMDCs (P<0.05).

Activated BMDCs Promoted Proliferation and IL-2 Secretion of CD4$^+$T Cells

In order to further investigate the effect of *E.coli* LLO/OVA pulsed BMDCs on priming CD4$^+$ T cells, we co-cultured the BMDCs and CD4$^+$T cells. The result showed that *E.coli* LLO/OVA stimulated BMDCs promoted [^{3}H] thymidine uptake of CD4$^+$T cells, but there were no difference between *E.coli* LLO/OVA allergic CD4$^+$T cells and *E.coli* OVA allergic CD4$^+$T cells(figure 3a); However, IL-2 concentration in the supernatant of the *E.coli* LLO/OVA allergic CD4$^+$T cells was significantly higher than that of *E.coli* OVA allergic CD4$^+$T cells and naive CD4$^+$T cells (P<0.05, P<0.01, Fig. 3b).

Activated BMDCs Promoted Proliferation and IFN-γ Secretion of CD8$^+$T Cells

We observed the priming role of *E.coli* LLO/OVA stimulated BMDCs on CD8$^+$T cells. The result showed that [^{3}H] thymidine uptake of CD8$^+$T cells co-cultured with *E.coli* LLO/OVA pulsed BMDCs was significantly higher than that CD8$^+$T cells co-cultured with *E.coli* OVA pulsed BMDCs, especial CD8$^+$T cell : BMDC at 1:0.5 and 1:1 ($^*P$<0.05, Fig. 4a); in addition, the *E.coli* LLO/OVA

allergic CD8$^+$T cells secreted significantly higher level of IFN-γ than that of *E.coli* OVA allergic CD8$^+$ T cells and naive CD8$^+$T cells (P<0.05, P<0.001, Fig. 4b).

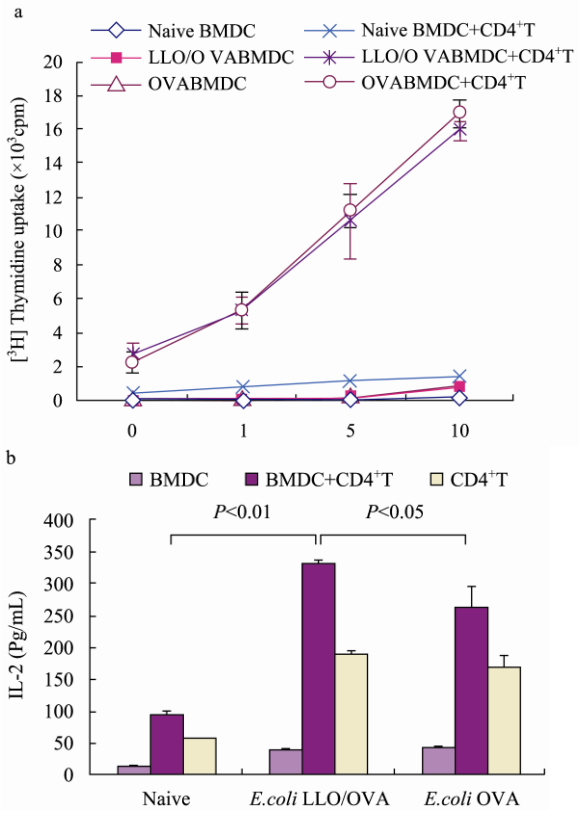


FIG. 3. The proliferation and IL-2 secretion of CD4$^+$T cells. *E.coli* LLO/OVA or *E.coli* OVA vaccinated mice on day 1, 4, and 8. (a) The spleenocytes were harvested on day 9, CD4$^+$T cells were magnetically sorted and collected, then 1×10^4, 5×10^4, 10×10^4 *E.coli* LLO/OVA or *E.coli* OVA activated BMDCs co-cultured with 1×10^5 CD4$^+$T cells for 54 h. [^{3}H] thymidine was added in the medium for 18 h, and the absorption of [^{3}H] thymidine was determined. (b) The level of IL-2 in the supernatant of co-cultured CD4$^+$T cells and BMDCs for 3 days was determined by ELISA. Statistical analyses was performed compared with IL-2 concentration in the supernatant of each group (P<0.05, P<0.01).

E.coli LLO/OVA Allergic CD8$^+$T Cells Effectively Killed tumor Cells

Finally, we examined the cytotoxic effect of *E.coli* LLO/OVA allergic CD8$^+$T cells. The result showed that CD8$^+$T cells co-cultured with *E.coli* LLO/OVA activated BMDCs were significantly more potent in killing B16-OVA melanoma cells (figure 5a) and RMA-S/OVA lymphoma cells (Fig. 5b) than *E.coli* OVA allergic CD8$^+$T cells (P< 0.05).

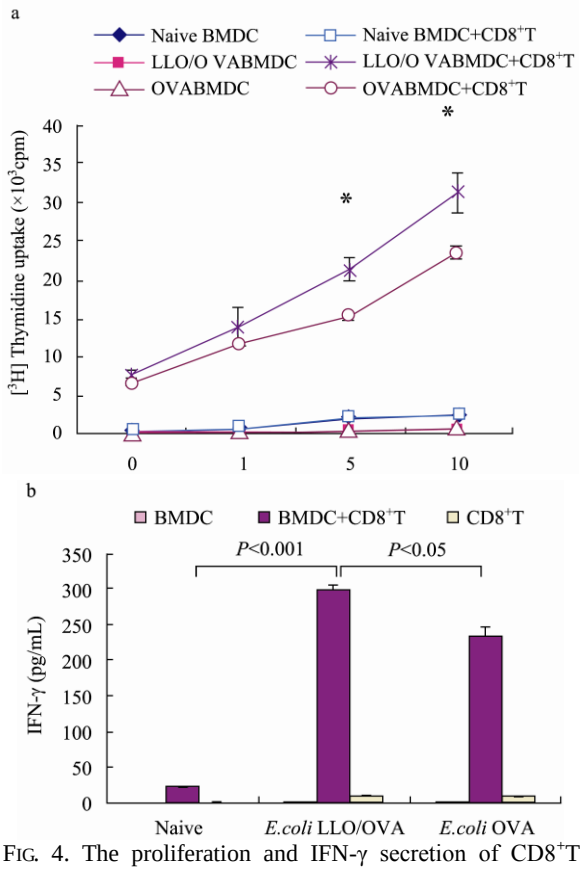
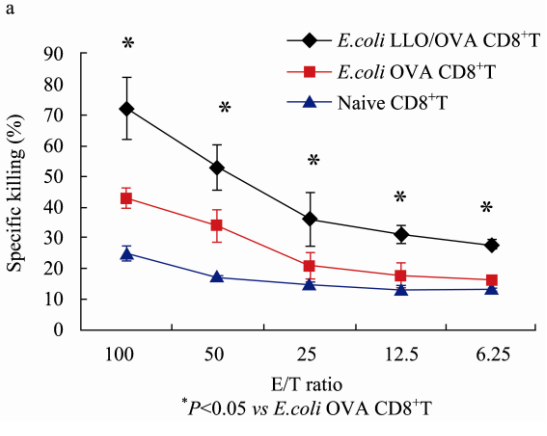


FIG. 4. The proliferation and IFN-γ secretion of CD8$^+$T cells. Mice were respectively vaccinated by *E.coli* LLO/OVA and *E.coli* OVA on day 1, 4, and 8. (a) The spleenocytes were harvested on day 9, CD8$^+$T cells were magnetically sorted and collected, then 1×10^4, 5×10^4, 10×10^4 *E.coli* LLO/OVA or *E.coli* OVA activated BMDCs were co-cultured with 1×10^5 CD8$^+$T cells for 54 h. [^{3}H] thymidine was added in the medium for 18h, and the absorption of [^{3}H] thymidine was determined. (b) The level of IFN-γ in the supernatant of co-cultured CD8$^+$T cells and BMDCs for 3 days was determined by ELISA. Statistical analysis was performed compared with the level of IFN-γ in the supernatant of each group (P<0.001, P<0.05).



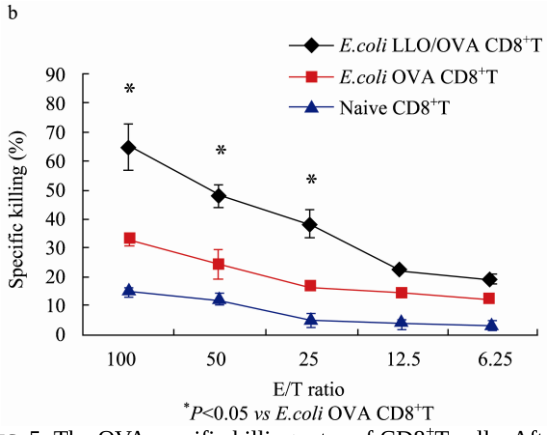


FIG. 5. The OVA specific killing rates of $CD8^+$T cells. After having been co-cultured with *E.coli* LLO/OVA or *E.coli* OVA activated BMDCs, $CD8^+$T cells were mixed with ^{51}Cr labeled B16-OVA melanoma cells (a) and RMA-S/OVA cells. (b) at various ratio for 4 h. The average killing rates of B16-OVA melanoma cells and RMA-S/OVA cells were compared respectively ($^*P<0.05$).

DISCUSSION

Bacteria vaccine not only plays a role in presenting tumor antigen but also promotes the host immunity by its ingredient. This study showed that after stimulation by *E.coli* LLO/OVA, the high level gene expression of TLR4, NOD1 receptor, and NF-κB signalling pathway molecules of BMDCs were induced, and then the BMDCs matured. In early phase, recombinant *E.coli* LLO/OVA evoked from BMDCs a transient and strong TLR4 mRNA transcription, and the mRNA transcription of Myd88, Irak1, Irak2, IKK-α, Nfκb1 and Nfκb2 was also evoked. This result was coincident with the report that DC was activated by TLR4 and Myd88/NF-κB signalling pathway[5]. The RT-PCR result also showed that TLR4 mRNA transcription was significantly up-regulated after BMDCs were stimulated by *E. coli* LLO/OVA for 2 h, and then low level TLR4 mRNA transcription continued for 4 h; however, the TLR4 mRNA transcription was up-regulated only after BMDCs were stimulated by *E.coli* OVA to 2 h. Although LPS of *E.coli* vaccine might induce the activation of TLR4 and NF-κB signalling pathway, longer activation of TLR4 was observed in *E. coli* LLO/OVA stimulated BMDCs than in *E. coli* OVA stimulated BMDCs in this study, suggesting that immune adjuvant LLO might play a role in promoting the activation of TLR4. A report demonstrated that the component of *E.coli* might activate the intestinal epithelial cells by Nod1/NF-κB signalling pathway[11]. In this study, Nod1 (card4) mRNA transcription of BMDCs was up-regulated after they were pulsed by *E.coli* LLO/OVA for 4 h and 8 h, the time frame was longer than that in *E.coli* OVA pulsed BMDCs. This phenomenon suggested that LLO might trigger the phagosome breakdown, facilitate the vaccine component releasing into BMDCs' cytoplasm and stimulate NOD1 activation. The high expression of co-stimulatory molecules CD40, CD80, and CD86 demonstrated that *E.coli* LLO/OVA was effective in stimulating BMDCs maturation; meanwhile, high level of Th1 type cytokines IL-12 and IFN-γ secretion also confirmed that. IL-12 was a key cytokine that promoted Th0 differentiating to Th1 cells and induced anti-tumor immunity of the host[13]. IFN-γ promoted DC mature by autocrine and induced innate immunity of the host. In addition, IFN-γ primed acquired immunity of the host through up-regulating tumor cells' MHC molecules and antigen presentation, and synergistically induced $CD8^+$T differentiating to CTL with IL-12[14-15].

As $CD4^+$T cells play a key role in $CD8^+$T cells differentiation and proliferation, we observed the immune promoting effect of *E.coli* LLO/OVA activated BMDCs on $CD4^+$T cells and the result showed that $CD4^+$T cells proliferated and secreted high level of IL-2 after co-culture with *E.coli* LLO/OVA activated BMDCs for 3 days. IL-2 plays an important role not only in promoting T cells' clone proliferationbut also in antigen cross-presentation to $CD8^+$T cells[16]. As DC could cross-present tumor antigen to $CD8^+$T, we co-cultured $CD8^+$T cells with *E.coli* LLO/OVA activated BMDCs for 3 days. The resulted showed that *E.coli* LLO/OVA stimulated BMDCs significantly promoted the proliferation and IFN-γ secretion of $CD8^+$T cells; in contrast, *E.coli* OVA activated BMDCs promoted the proliferation and IFN-γ secretion of $CD8^+$T cells at lower level. This difference might be associated with LLO promoting tumor antigen. OVA escaped into the cytoplasm of BMDCs and be presented to $CD8^+$T cells by MHC class I molecule. Pozzi also observed that DC stimulated $CD8^+$T cells' proliferation and CTL differentiation[17]. In another study, we demonstrated that *E.coli* LLO/OVA vaccination suppressed the negative regulation effect of $CD4^+CD25^+$Treg in C57BL/6 mice[18], the $CD4^+CD25^-$T cells of spleen significantly promoted $CD8^+$T cells' proliferation *in vitro*, and more OVA-specific $CD8^+$T cells proliferated in spleen of *E.coli* LLO/OVA vaccinated mice than that of *E.coli* OVA vaccinated mice (the data not be showed). The further cytotoxic effect assay showed that *E.coli* LLO/OVA activated $CD8^+$T cells were significantly more effective in killing B16-OVA melanoma cells and RMA-S/OVA lymphoma cells than *E.coli* OVA

activated $CD8^+$T cells. This result supported that *E.coli* LLO/OVA were highly effective in stimulating the proliferation and promoting the cytotoxic effect of CTL.

This results from this study concludes that *E. coli* LLO/OVA is effective in activating BMDCs via TLR4 and Nod1 signalling pathway, and in promoting specific anti-tumor T cell immunity.

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