Original Research

Corynebacterium pyruviciproducens, as an immune modulator, can promote the activity of macrophages and up-regulate antibody response to particulate antigen

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Abstract

Corynebacterium pyruviciproducens **is a newly discovered Corynebacterium species with no known pathogenic components such as diphtheria toxin and tuberculostearic acid, and it has similar biological properties to *Propionibacterium acnes*, but its role of immunoregulation is drawing people's attention. In this work, based on the role of macrophages in removal of pathogenic bacteria as a primary scavenger and particulate antigen-presenting cell, the stimulation of macrophages by *C. pyruviciproducens* was analyzed through detecting the levels of cytokine secretion and expression of membrane molecules, and the effect of *C. pyruviciproducens* in promoting antibody response to sheep red blood cells (SRBC) *in vivo* was detected. *In vitro*, *C. pyruviciproducens* led to a sharp release of interleukin-6 and tumour necrosis factor-α and encouraged the activation of macrophages including enhanced expressions of MHC-II, CD40, CD80 and CD86. *In vivo*, it enhanced the humoral immune response against SRBC, a particulate antigen. These observations suggest that *C. pyruviciproducens*, as an immunoregulator, can promote the host humoral immune response to pathogenic microorganisms by regulating macrophage function.**

Keywords: *C. pyruviciproducens*, *P. acnes*, immunomodulator, adjuvant, macrophage

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Introduction

Corynebacterium pyruviciproducens is a newly discovered Corynebacterium and characterized by the metabolic product, pyruvic acid, with no known pathogenicity.[1] Compared with *Propionibacterium acnes*, the traditional bacterial immunoadjuvant,[2,3] our previous studies demonstrated that this bacterium showed a great effect on activating dendritic cells (DCs) and promoting immune response against soluble antigens.[4] In the last decade, it has been reported that most adjuvants derived from bacterial components regulate the immune system by activating toll-like receptors (TLRs), so called TLR-dependent adjuvants.[5,6] TLRs act as microbial sensors and are expressed by DCs and other leukocytes.[7] De Gregorio[8] emphasizes that the activation of DCs plays a central role in the mechanism of vaccine adjuvants, but other accessory cells like macrophages, monocytes and mast cells should not be ignored, and are now poorly characterized. As is well known, macrophages, as important immunological cells, exhibit a broad range of immunoregulatory activities and arise in response to a variety of different stimuli, especially bacteria or other particulate antigens.[9] Importantly, most TLRs are expressed by macrophages; it is necessary for any bacteria to induce the host immune defense against infection.[10]

To expand the evidence of *C. pyruviciproducens* as an immunoregulator, there are still a lot of experiments that need to be performed, to investigate its activation to other immunocytes, its non-specificity on various pathogens, its own effective components and so on. In the present study, first we focused on the role of *C. pyruviciproducens* in the activation and maturation of macrophages. Second, we investigated the ability of *C. pyruviciproducens* to enhance the immunogenicity of sheep red blood cells (SRBC), a typical particulate antigen. Finally, the effect of *C. pyruviciproducens* on inducing adaptive immunity was further evaluated *in vivo*.

Materials and methods

Bacterial strains and culture

C. pyruviciproducens (CCUG 57046, obtained from Olive View Medicine Center, University of California, Los Angeles, CA, USA) was cultured in LB medium with 5% new-born calf serum (NCS) (Sigma, St Louis, MO, USA) at 37°C for three days, then washed with saline to remove contaminants of bacterial culture origin. Collected bacteria were resuspended in saline and killed by 62°C for four hours; then 10 μL bacterial suspension was inoculated into a TSA blood plate to check that there were no residual live bacteria. *P. acnes* (ATCC 6919, obtained from Guangdong Culture Collection Center, Guangdong, China) was treated by the same way. The bacterial suspension was centrifuged to collect the bacteria. The air-dried bacteria were stored at −70°C in the refrigerator.

RAW264.7 and peritoneal macrophages

The RAW264.7 cell line (kept by our laboratory) was cultured in RPMI1640 medium containing 10% NCS (Sigma) at 37°C, 5% CO_2 atmosphere. Peritoneal macrophages were derived from BALB/c mice; in brief, the abdominal cavity was washed by phosphate buffer saline repeatedly to get the mixed cell suspension, then red blood cells (RBCs) were destroyed by RBC lysis buffer and the remaining cells were adjusted to 1×10^6/mL and cultured at 37°C, 5% CO_2 atmosphere in RPMI1640 medium containing 10% NCS. After 24 h, the cells adhered to the bottom were kept by removal of suspension. F4/80 was detected by flow cytometry (Becton Dickinson, Sparks, MD, USA), which testified that more than 80% of the adherent cells were mononuclear macrophages.

Quantitative realtime polymerase chain reaction for detecting interleukin-6, tumour necrosis factor-α, gata 3 and T-bet mRNA

RAW264.7 cells or peritoneal macrophages were cultured in media containing 100/500/1000 g/mL heat-killed *C. pyruviciproducens* or *P. acnes* for two hours, and then interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) mRNA were detected. Spleen cells from SRBC-immunized mice were used to analyze *gata 3* and *T-bet* mRNA using quantitative realtime polymerase chain reaction (qRT-PCR). Total RNAs were isolated with Trizol (Invitrogen, Carlsbad, CA, USA) from cells and complementary (c) DNA was reversely transcribed using a RT-PCR reagent kit (Toyobo, Osaka, Japan).[11]

qRT-PCR was performed using a SYBR Green1 qRT-PCR kit (BIOER, Shanghai, China). Gene expression was normalized against the reference gene β-actin. All sequences of primers are shown in Table 1.

Table 1 Genes determined by qRT-PCR

Genes	Primers (5′–3′)	Length (bp)	Tm (°C)
β-Actin	For: TGGAATCCTGTGGCATCCATGAAAC Rev: TAAAACGCAGCTCAGTAACAGTCCG	262	60
TNF-α	For: AACTAGTGGTGCCAGCCGAT Rev: CTTCACAGAGCAATGACTCC	334	58
IL-6	For: GGCCTTCCCTACTTCACAAG Rev: ATTTCCACGATTTCCCAGAG	126	58
T-bet	For: ATGCCAGGGAACCGCTTAT Rev: CAGATGCGTACATGGACTCAAA	364	63
gata 3	For: CTGTGGGCTGTACTACAAGCTTCA Rev: ACCCATGGCGGTGACCATGC	309	60

qRT-PCR, quantitative realtime polymerase chain reaction; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6

Measurement of IL-6, IL-4, TNF-α and TNF-γ by enzyme-linked immunosorbent assay

The suspensions of RAW264.7 cell and peritoneal macrophages stimulated by *C. pyruviciproducens* or *P. acnes* were used to analyze IL-6 and TNF-α by enzyme-linked immunosorbent assay (ELISA). The sera levels of IL-4 and interferon-γ (IFN-γ) from SRBC-immunized mice were measured by ELISA. All of these detections were performed following the manufacturer's protocols (R&D, Minneapolis, MN, USA).[12]

Cell proliferation determined by 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide

The proliferation of RAW264.7 cells or peritoneal macrophages stimulated by *C. pyruviciproducens* or *P. acnes* was detected using 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumromide solution (MTT) (Y-S Biotechnology, Shanghai, China). Briefly, the cells were seeded into a 96-well flat-bottom microtiter plate (3000 cells/well) in 200 μL medium with 200 μg/mL *P. acnes* or *C. pyruviciproducens*. The plates were incubated at 37°C in a humid atmosphere with 5% CO_2 for one to four days, respectively, and 20 μL 5 mg/mL MTT was added to each well and incubated for another 4 h; the untransformed MTT was removed carefully by pipetting and 150 μL dimethyl sulfoxide was added. Ten minutes later, the optical density (OD) was determined in an ELISA reader at 570 nm and the value of optical density reflected the number of living cells.

Flow cytometry for detecting the expressions of MHC II and co-stimulatory molecules on macrophages

RAW264.7 cells or peritoneal macrophages were stimulated by killed *C. pyruviciproducens* or *P. acnes* (200 μg/mL) for 24 h. The cells were collected and the expression levels of MHC II and co-stimulatory molecules (CD40/80/86) were analyzed by flow cytometry, as per our previous description.[4]

Immunization and detection of antibody secretory level

10% SRBC (SINRY, Hangzhou, China) were injected intraperitoneally into mice (BALB/c × A/J, 6–8 weeks, female), combined with killed *C. pyruviciproducens* or *P. acnes* in saline (500 μg/500 μL/animal). Five days later, the spleen cells were harvested from the SRBC-immunized mice and used to analyze the secretion ability of antibody to SRBC. There were several groups as follows: saline only (control group), SRBC alone (SRBC immune group), SRBC plus

P. acnes (PA regulation group) and SRBC plus *C. pyruviciproducens* (CP regulation group).

The suspensions of SRBC-immunized spleen cells were adjusted to 1×10^7/mL after washing twice by PBS. One milliliter suspension was mixed with 1 mL complement (1:30 diluted *Cavia cobaya*'s sera) and 1 mL 0.2% SRBC. The mixture was incubated at 37°C for one hour, and the supernatant was obtained by centrifugation at 1000 rpm for 10 min and used to detect the absorption value at 313 nm by a 722 type spectrophotometer (XINMAO, Tianjin, China). The absorption value reflected the level of antibody to SRBC and indirectly represented the amount of antibody-forming cells.

CD4-positive T-cell frequency analysis

The SRBC-immunized spleen cells were incubated in RPMI1640 containing 10% NCS with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, MO, USA), 1 μg/mL ionomycin (Alexis, Laussen, Switzerland) and 2 ug/mL monensin (Alexis) at 5% CO_2 atmospheric, 37°C for five hours. Then, the IL-4- or IFN-γ-producing cells in CD4 + T lymphocytes (Th2 or Th1 cells) were determined by intracellular staining method using anti-mouse IL-4-APC or anti-mouse IFN-γ-APC (eBioscience, San Diego, CA, USA), and analyzed by flow cytometry as previously described[4].

Results

C. pyruviciproducens induced RAW264.7 and peritoneal macrophages to secrete inflammatory cytokine

The levels of inflammatory factors produced by *C. pyruviciproducens*-stimulated macrophages was assessed. The results showed that the *C. pyruviciproducens* induced the secretions of IL-6 and TNF-α in a dose-dependent manner in RAW264.7, a macrophage cell line, both at mRNA (Figures 1a and b) and protein level (Figures 2a and b). The production of IL-6 and TNF-α was already obvious under 100 μg/mL *C. pyruviciproducens* stimulation and reached a maximum level when 500 μg/mL *C. pyruviciproducens* was used. *P. acnes* also showed such

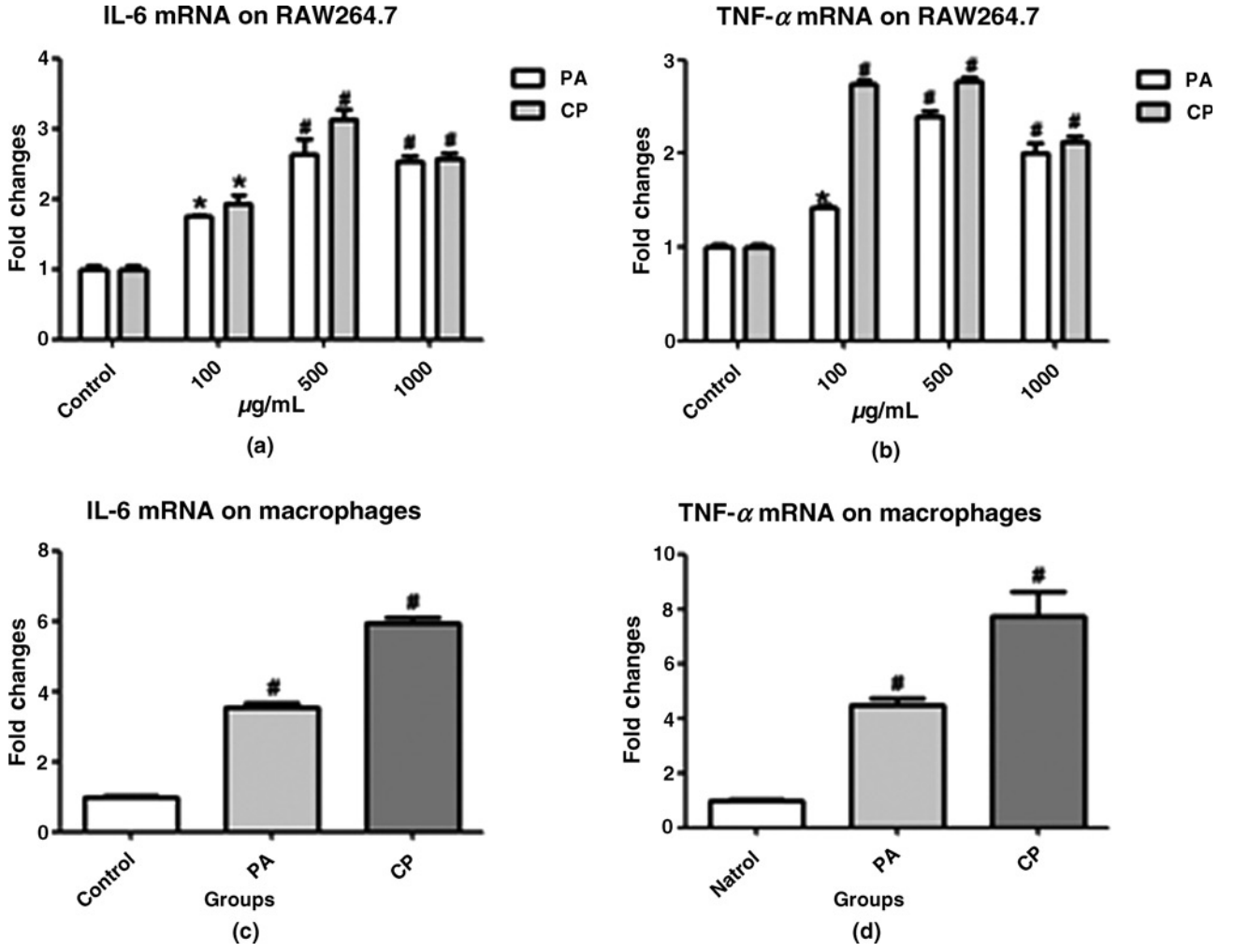

Figure 1 The mRNA levels of inflammatory factors expressed by RAW264.7 and peritoneal macrophages stimulated with *C. pyruviciproducens*. RAW264.7 (a, b) were stimulated by CP/PA at 100/500/1000 μg/mL for two hours, and peritoneal macrophages were stimulated by CP/PA at 500 μg/mL. Total RNA was isolated for analysis of IL-6 mRNA (a, c) and TNF-α mRNA (c, d) by qRT-PCR, and fold changes were on the basis of β-actin. Differences between 100/500/1000 μg/mL and control groups were analyzed by Student's *t*-test, $^{*}P < 0.05$, $^{\#}P < 0.01$. Data shown are representative of at least three independent experiments with $n = 3$. CP, *C. pyruviciproducens*; PA, *P. acnes*; qRT-PCR, quantitative realtime polymerase chain reaction; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6

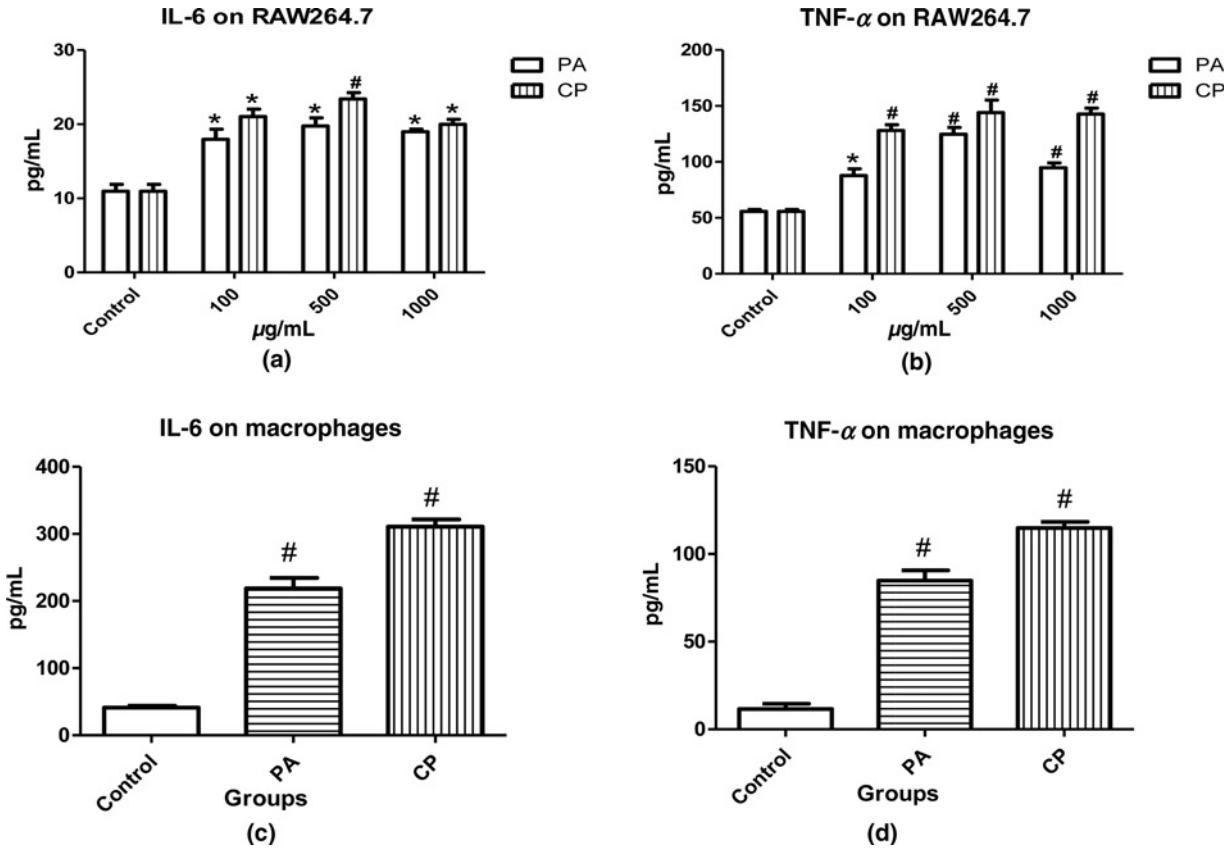

Figure 2 *C. pyruviciproducens* stimulation induced RAW264.7 and peritoneal macrophages to secrete inflammatory cytokines. RAW264.7 (a, b) were stimulated by CP/PA at 100/500/1000 μg/mL for 24 h, and peritoneal macrophages at 500 μg/mL. Supernatants were harvested for analysis of IL-6 (a, c) and TNF-α (c, d) by ELISA. Differences between 100/500/1000 μg/mL and control groups were analyzed by Student's *t*-test, $^{*}P < 0.05$, $^{\#}P < 0.01$. Data shown are representative of at least three independent experiments and $n = 3$. CP, *C. pyruviciproducens*; PA, *P. acnes*; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ELISA, enzyme-linked immunosorbent assay

biphasic effects, but whose strength was weaker than *C. pyruviciproducens*' (Figures 1a and b and 2a and b). The dominance of *C. pyruviciproducens* was testified further on primary peritoneal macrophages at the optimal dose of 500 μg/mL (Figures 1c and d and 2c and d).

Increased cell proliferation stimulated by *C. pyruviciproducens*

Effect of *C. pyruviciproducens* on RAW264.7 proliferation was evaluated by the MTT method. The ability of proliferation in RAW264.7 or peritoneal macrophages stimulated by *C. pyruviciproducens* was significantly enhanced for the first three days (Figure 3). A similar result was found when *P. acnes* was used as a stimulus.

C. pyruviciproducens stimulation induced macrophage maturation

The function of *C. pyruviciproducens* on inducing macrophage activation was judged by the expression of MHC II and co-stimulatory factors (CD40/80/86). Data in Figures 4 and 5 revealed that both *C. pyruviciproducens* and *P. acnes* could boost the levels of these surface molecules, yet they showed different tendencies particularly. *C. pyruviciproducens* displayed a remarkable contribution to CD86 on the cell line RAW264.7 (Figure 4), as well as MHC II and CD86 on primary peritoneal macrophages (Figure 5). Simultaneously, *P. acnes* increased the expression of CD80 on both RAW264.7 (Figure 4) and peritoneal macrophages (Figure 5).

C. pyruviciproducens promoted antibody production

In this experiment, SRBC were used as an antigenic stimulus because of their particulate antigen properties like bacteria, and the hemolysin from SRBC dissolution could be observed by spectrophotometric detection. The contribution of *C. pyruviciproducens*' stimulation to the antibody response against SRBC was measured by modified hemolytic plaque test. Data in Figure 6 demonstrated that the spleen cells from CP-immunized mice could induce more severe SRBC hemolysis, which illustrated that *C. pyruviciproducens* played an important role in promoting antibody production against SRBC.

C. pyruviciproducens stimulated Th2-skewed humoral response in SRBC-immunized mice

The analysis of immune states on SRBC-immunized mice was further done through various experiments. The results showed that gata 3 (a Th2-specific transcription factor)

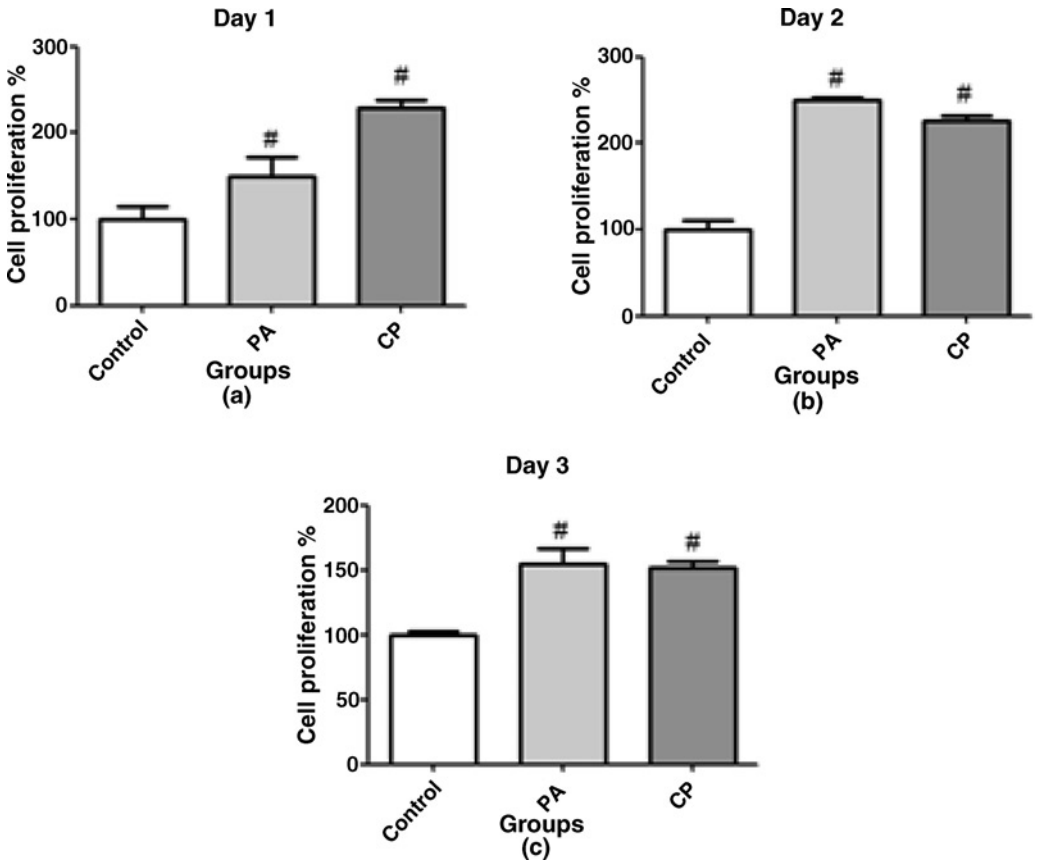

Figure 3 Proliferation effect of RAW264.7 stimulated by *C. pyruviciproducens*. Proliferation of RAW264.7 co-cultured with 200 μg/mL PA/CP was detected using MTT on days 1–3 (a, b, c). Cell proliferation percentage of RAW264.7 was calculated on the basis of untreated group (control) at the same time. The difference between control and PA/CP groups was determined by Student's *t*-test, $^{*}P < 0.05$, $^{\#}P < 0.01$. Data shown are representative of at least three independent experiments. CP, *C. pyruviciproducens*; PA, *P. acnes*; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumromide

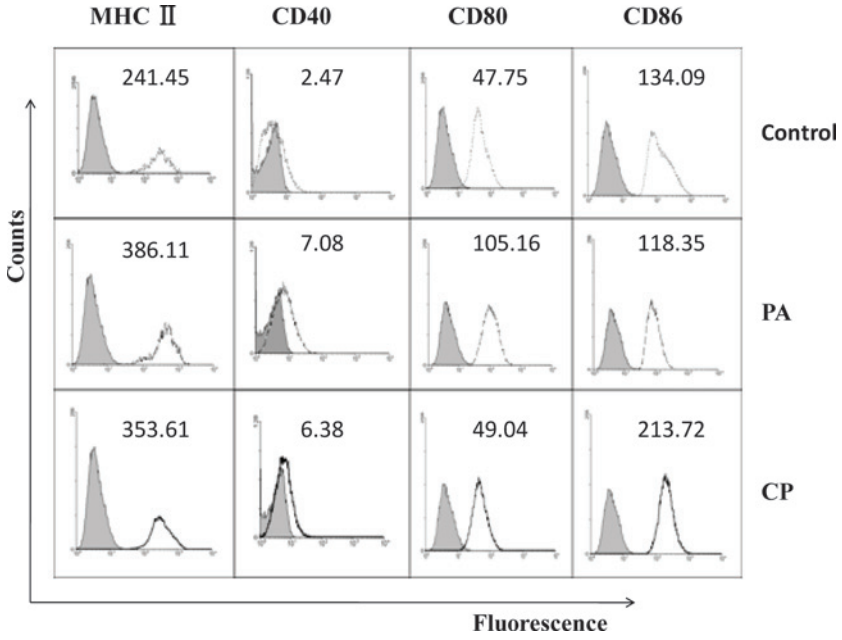

Figure 4 *C. pyruviciproducens* stimulated RAW264.7 cell maturation. RAW264.7 was cultured with 200 μg/mL PA/CP and medium only (control) for 24 h, and then MHC II, CD40, CD80 and CD86 were analyzed by flow cytometry. The graphs show the expression levels of membrane molecules involving cell maturation on RAW264.7 stimulated by PA/CP, as determined by increased mean fluorescence intensity. Data shown are representative of three independent experiments. CP, *C. pyruviciproducens*; PA, *P. acnes*

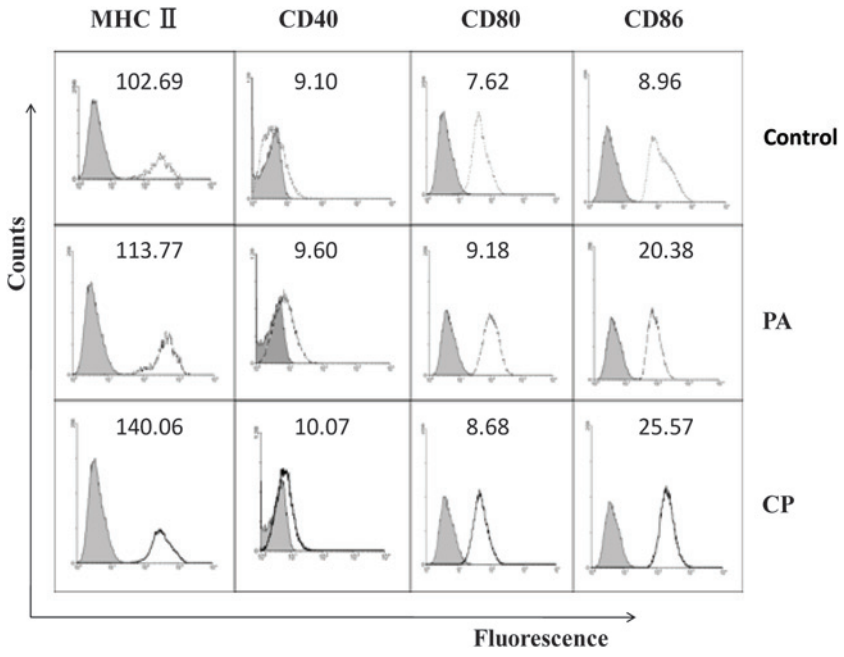

Figure 5 *C. pyruviciproducens* induced peritoneal macrophage maturation. Peritoneal macrophages were cultured with 200 μg/mL PA/CP and medium only (control) for 24 h, and then the expression levels of MHC II, CD40, CD80 and CD86 were analyzed by flow cytometry. Graphs are presented to show the maturation state of macrophages stimulated by CP or PA, as determined by increased mean fluorescence intensity. Data shown are representative of three independent experiments. CP, *C. pyruviciproducens*; PA, *P. acnes*

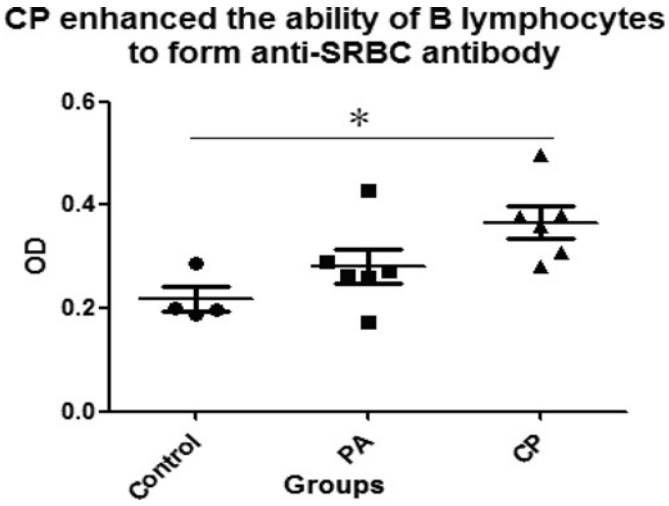

Figure 6 *C. pyruviciproducens* promoted the production of antibody to SRBC. Spleen cells from SRBC-immunized mice were used to analyze the ability to produce SRBC-specific antibody by a spectrophotometric method. Absorbance (OD) was monitored at the wavelength of 313 nm. These data are representative of two independent experiments ($n = 6$) with similar results. Differences between control and PA/CP groups were analyzed by Student's *t*-test, $^{*}P < 0.05$. CP, *C. pyruviciproducens*; PA, *P. acnes*; SRBC, sheep red blood cell; OD, optical density

was enhanced in lymphocytes of spleen tissues from SRBC plus *C. pyruviciproducens* treated mice, accompanied by a decrease in T-bet (a Th1-specific transcription factor) (Figure 7). *P. acnes* stimulation showed a similar tendency (Figure 7a). Flow cytometry results demonstrated the increasing percentage of IL-4-producing cells (Th2) in CD4 + T-cells from SRBC plus *C. pyruviciproducens* treated mice (Figure 8a), and it was significant (Figure 8c, $P < 0.05$). On the contrary, the number of IFN-γ-producing cells (Th1) was decreased (Figure 8b and d). In addition, increased IL-4 (Th2-specific cytokine) was also found in sera from SRBC plus *C. pyruviciproducens*-immunized mice (Figure 9a), but the expression level of IFN-γ was no significant change (Figure 9b).

Discussion

Macrophages are abundant in diverse tissues and organs, where they can function as immune effectors, immune regulators, tissue remodelers or quiescent scavengers. Some external stimuli can cause macrophages to undergo a dramatic and coordinated change in the expression of multiple gene products, changing the functional capacity of the cell. The diversity of environments surrounding macrophages in different tissues is responsible for an equally diverse constellation of macrophage phenotypes in the host. Experimentally, there are some well-defined stimuli that cause macrophages to adopt distinct polarized 'activation' phenotypes.[13] The longest recognized is classical macrophage activation (now called M1 activation), which is induced by IFN-γ plus either TNF-α or molecules that induce TNF-α (e.g. lipopolysaccharide or peptidoglycan), and is characterized by enhanced microbicidal effector activity. 'Alternative activation', now renamed M2a activation, results from IL-4 exposure and has been associated with tissue remodeling and inhibition of inflammation. Antigen stimulation can also lead to M2b and M2c macrophage activation; the M2b reflects a role in antigen presentation leading toward Th2 differentiation.[14]

In this work, *C. pyruviciproducens* as well as *P. acnes* could not induce immature macrophages to form some

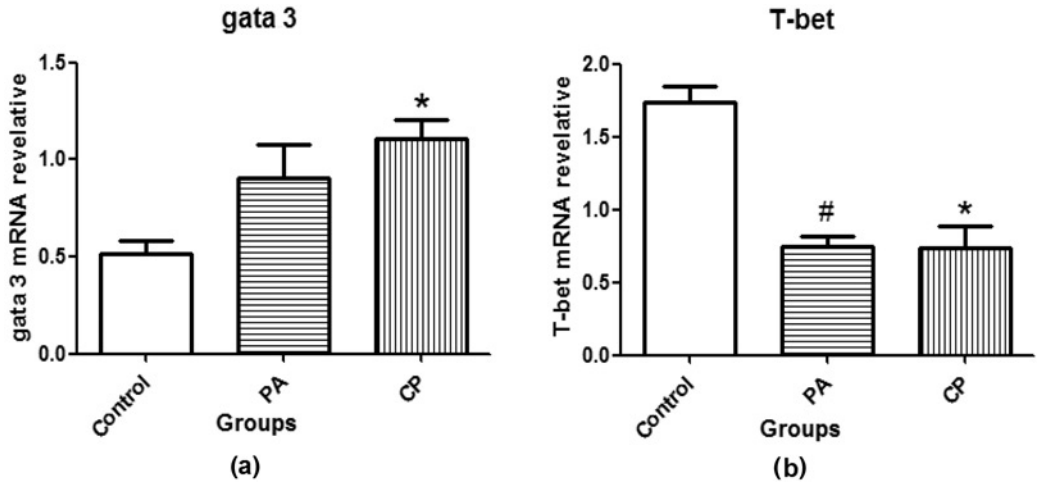

Figure 7 The mRNA expression of gata 3 and T-bet in spleen cells from SRBC-immunized mice. Spleen tissues from PA/CP treated mice were collected to analyze the mRNA expression levels of gata 3 (a) and T-bet (b), and the differences between PA/CP and control groups were analyzed by Student's *t*-test. The data shown are representative of two independent experiments ($n = 6$) with similar results. $^{*}P < 0.05$, $^{\#}P < 0.01$. CP, *C. pyruviciproducens*; PA, *P. acnes*; SRBC, sheep red blood cell

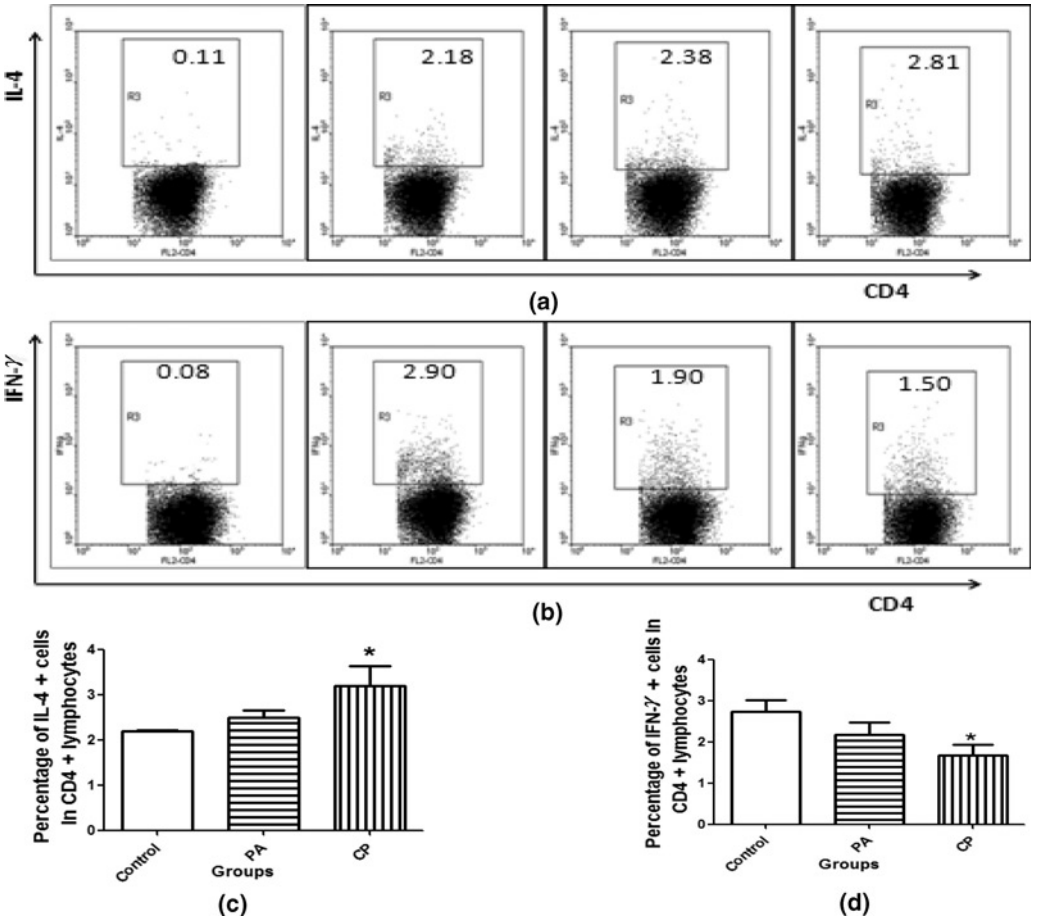

Figure 8 Percentage of IL-4+/IFN+ Th cells in spleen from SRBC-immunized mice. Spleen cells from SRBC-immunized mice were obtained to determine the percentage of IL-4-producing (a) or IFN-γ-producing (b) cells in CD4 + T-cells by flow cytometry. Statistical analysis was also done between PA/CP and control groups (c, d). The data are representative of two independent experiments results ($n = 6$), $^{*}P < 0.05$. CP, *C. pyruviciproducens*; PA, *P. acnes*; SRBC, sheep red blood cell; IL-4, interleukin-4; IFN-γ, interferon-γ

specific subtypes *in vitro*. However, compared with *P. acnes*, the activation of macrophages caused by *C. pyruviciproducens in vitro* was characterized by the strong release of cytokines and a high level of CD86. In addition, *C. pyruviciproducens* led to an obvious Th2-skewed immune response *in vivo*. All of that reflected the diversity of bacteria in the structures and relationships with the host.

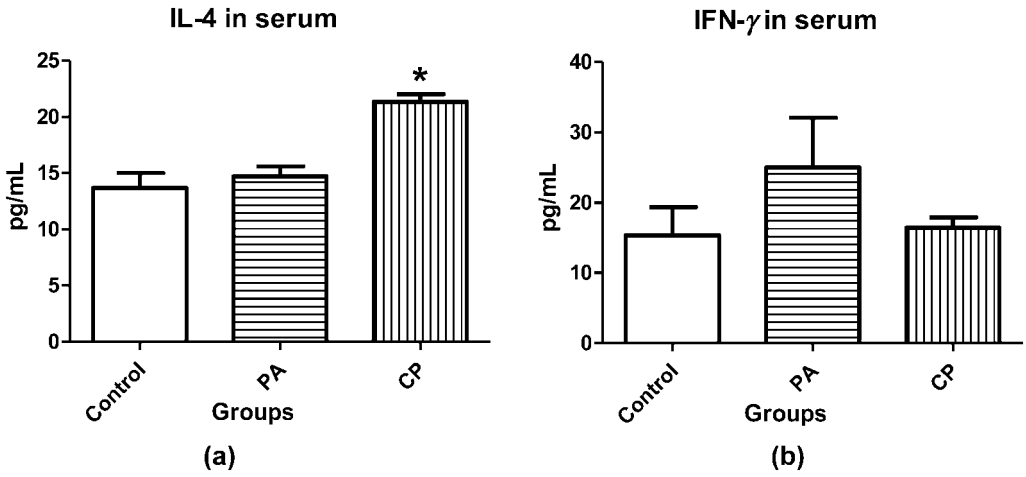

Figure 9 IL-4 and IFN-γ cytokines in sera of SRBC-immunized mice detected by ELISA. IL-4 (a) and INF-γ (b) in sera from PA/CP treated mice were detected by ELISA. Differences between PA/CP and control groups were analyzed by Student's *t*-test, $^{*}P < 0.05$. The data are representative of two independent experiments ($n = 6$). CP, *C. pyruviciproducens*; PA, *P. acnes*; ELISA, enzyme-linked immunosorbent assay; SRBC, sheep red blood cell; IL-4, interleukin-4; IFN-γ, interferon-γ

IL-6 and TNF-α are very important inflammatory factors. As Park reported,[15] IL-6 is capable of controlling the mature states through IL-6-gp130-STAT3 and also affects the differentiation of myeloid lineages, including macrophages and DCs.[16] Furthermore, IL-6 contributes more to Th2 polarization through an up-regulation of nuclear factor of activated T-cells, a transcription factor regulating IL-4 transcription.[17,18] TNF-α, as a typical proinflammatory factor, produces a marked effect on inducing other cytokine secretion, like IL-1 and IL-6.[19] Thus, it was found that *C. pyruviciproducens* promoted macrophages to release IL-6 and TNF-α in this work, which favored the activation of macrophages, and also facilitated the whole immune response.

Our data also showed that *C. pyruviciproducens* was very capable of significantly promoting proliferation of macrophages. Undoubtedly, it was of benefit to increase the number of macrophages at the local injection sites to uptake, process and present antigen, and also it was essential for an effective vaccine adjuvant.[4,20] Besides, we found that *C. pyruviciproducens* induced the maturation and differentiation of macrophages with high levels of MHC II and co-stimulatory factors. It indicated that *C. pyruviciproducens* stimulation could promote the ability to present antigen and the steady interaction with T lymphocytes, since the macrophage itself is a key professional antigen presenting cell.[21,22] *In vivo* experiments, the antibody response to SRBC was much stronger in *C. pyruviciproducens* regulation mice than that in the *P. acnes* regulation group. The level of antibody to SRBC indirectly represented the amount of antibody-forming cells in spleen. In addition, the investigation of immune states of the mice was consistent with the high antibody level, with augmented Th2 percentage in spleen cells and high IL-4 in serum samples; the immunized mice exhibited a Th2 polarized state.[23,24] On the basis of all the data in this work, the regulation of *C. pyruviciproducens* on macrophage activation could be summarized as follows: first, in *in vitro* experiments, it was confirmed that *C. pyruviciproducens* stimulation boosted a greater sharp release of cytokines, like IL-4, IL-6, IFN-γ and TNF-α, which not only led to M1 activation (IFN-γ and TNF-α should be involved) but also induced M2a activation or alternative activation (IL-4 should be involved). M1 activation might contribute to enhance microbicidal effector activity, and M2a could shift to M2b under the external granule antigen (SRBC) stimulation and IL-6 presence. Second, these macrophages stimulated by *C. pyruviciproducens* became growingly capable to uptake and present the particulate antigen SRBC following increased expressions of MHC II and co-stimulatory factors.[25,26] Third, *C. pyruviciproducens* stimulation showed a stronger power to induce more IL-6 release and higher level of CD86 on macrophages, and both of them were observed to favor Th2 response.[27–29]

In a previous study, we have demonstrated that *C. pyruviciproducens* promoted the antigen presentation of DCs and enhanced the production of ovalbumin (the soluble antigen) specific antibody.[4] In this work, it was further confirmed that *C. pyruviciproducens* could polarize various macrophage activation phenotypes and definitely augment the Th2 humoral response to particulate antigen SRBC, which is beneficial for resisting extracellular pathogens such as various bacteria and almost all the helminthes. It shows the application prospect of *C. pyruviciproducens* as an immune adjuvant or bacterial immunomodulator.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. JT, QH, SW, ZS, DZ and PS conducted the experiments; SX, XH and QS supplied critical animals (BALB/c); and QH and HX wrote the manuscript. QH is the co-first author.

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