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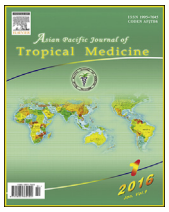

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Effect of vitamin D3 on maturation and antigen-presenting function of dendritic cells treated with *Mycobacterium tuberculosis*

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ABSTRACT

Objective: To investigate the phenotypic characteristics and functional capability differences of mouse bone marrow-derived dendritic cells after stimulation with *Mycobacterium tuberculosis* in the presence or absence of vitamin D3.

Methods: Mouse bone marrow-derived cells were cultured with GM-CSF (20 ng/mL). Then, one was added with 100 nmol/L of 25(OH)D3, while the other did not. On day 6, 5 μg/mL of BCG was added to stimulate the cells for 24 h. On day 7, suspension cells were harvested for phenotypic and functional analyses.

Results: The percentages of CD86 dendritic cells (DCs) in the control group and 25(OH)D3 group were 66.97% ± 8.29% and 52.18% ± 8.52%, respectively; the mean fluorescence intensities of MHC-II in the control group and 25(OH)D3 group were 1 102.16 ± 371.02 and 681.62 ± 292.71. The expression levels of MHC- II and CD86 on the surface of the DCs in 25(OH)D3 group were significantly lower than those of the control group. The ability of the DCs to stimulate proliferation of T-lymphocytes was also significantly lower than that of the control group.

Conclusions: These findings suggest that 25(OH)D3 modulates the immune response by affecting the maturation and function of DCs in *Mycobacterium tuberculosis* period.

1. Introduction

Tuberculosis is a globally common disease which severely threatens human health. According to statistics, more than one million people died from tuberculosis in some years [1]. 25(OH)D3 is an important active compound of vitamin D3. It is a good index of detecting the level of vitamin D3. In 1940s, 25(OH)D3 was used to treat tuberculosis because it had a calcification effect on sites of tuberculosis disease. But with the developments and applications of anti-tuberculosis drugs, the use of 25(OH)D3 in tuberculosis therapy decreased generally [2,3]. However, problems gradually emerged in the process of using anti-tuberculosis drugs. For instance, many patients represented drug resistant tuberculosis [4,5]. Traditional anti-tuberculosis drugs do not show satisfactory efficacy. Therefore, we rethink that whether we should reuse 25(OH)D3 to treat tuberculosis and whether we should continuously explore its action mechanism. At present, although there are lots of reports on treating tuberculosis, there is no agreement. Some reports claimed that 25(OH)D3 could not only treat tuberculosis and improve clinical efficacy, but also improve the imaging manifestations of tuberculosis patients [6]. However, some other reports argued that 25(OH)D3 had bare effects on improving clinical efficacy of tuberculosis patients [7]. Thus, the effects of 25(OH)D3 on treating tuberculosis needs further explorations.

There were researches showing that 25(OH)D3 had participated in the occurrence of the body tuberculosis immunity [8,9]. Not long ago, literature researches revealed that 25(OH)D3 influenced the innate immune mechanism of tuberculosis [10,11]. But there are no studies about the concrete mechanism of 25(OH)D3 in tuberculosis acquired immune. Dendritic cells (DCs) play an important role in the process of initiating tuberculosis acquired immune [12–14]. Researches *in vitro* and *in vivo* showed that the phagocytosis function of DCs decreased and its expressions of superficial co-stimulatory molecules and antigen-presenting molecules increased after phagocytizing mycobacterium *Mycobacterium tuberculosis*, which meant that those cells tended to be mature. When DCs are mature, the expressions of their superficial molecules increase, such as MHC-II, MHC-I, CD80, CD86 and so on [15,16]. In addition, the latest research showed that when antigen

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ESAT-6 is recognized by DCs, cell immunity response $CD4^+$ T initiates and starts to work [17]. So, there have been many reports about the effects of 25(OH)D3 in the maturity of CDs [18], but there are fewer researches about the influential mechanism of 25(OH)D3 in DCs when *Mycobacterium tuberculosis* infects.

Hence, the main purpose of the study is to investigate the functional capability differences of mouse bone marrow-derived dendritic cells after stimulation with *Mycobacterium tuberculosis* in the presence or absence of vitamin D3.

2. Materials and methods

2.1. In vitro cultivation of DCs

The experimental methods were acquired from adjusted Inaba. The main processes were as follows: 4–8 week-old male mice without specific pathogen were provided by Laboratory Animal Center of Third Military Medical University. After breaking necks to death, bilateral femurs and tibias were taken out and soaked in 70% of alcohol for 2 min. After that, femurs and tibias were taken out from that and soaked in uncompleted PRMI-1640-cultured medium. The process should keep aseptic strictly. Then both ends of the femurs and tibias were cut and the bone marrow cells were washed out by sterile syringe which had absorbed the uncompleted PRMI-1640-cultured medium. The bone marrow cells were centrifuged with speed of 1 200 rpm for 8 min after blowing and beating evenly. Then, they were cultivated in the completed PRMI-1640-cultured medium after discarding the supernatants and suspended again. According to a density of 2.5×10^5/mL, they were inoculated 24-pore plates (0.5 mL a pore) after adding 20 ng/mL of rmGM-CSF cytokines into them. Then, 100 nmol/L of 25(OH)D3 were added into group 25(OH)D3. The control group did not. On the next day, 0.4 mL cultured medium were suctioned out from the 24-pore plates and supplemented completed PRMI-1640-cultured medium with 20 ng/mL of rmGM-CSF. On the fourth day, 0.25 mL cultured medium were suctioned out; cells were collected by centrifugation and suspended again in 24-pore plates by 0.25 mL fresh completed PRMI-1640-cultured medium with 20 ng/mL of rmGM-CSF. On the sixth day, cells were collected and suspended by centrifugation according the methods of day four and were made to be mature by stimulation of 5 μg/mL of BCG after resuspension. On day seven, the mature DCs were collected and invert microscope was used to observe the morphologies of DCs at all states.

2.2. Analysis and identification of the flow cytometric phenotype of DCs

DCs which had cultivated to the last day were centrifuged with the speed of 800 rpm/min for 10 min; the supernatants were discarded again; then, PBS was used to wash them to make sure the density of cells was 5×10^5/mL. Next, 0.5 μL of FITC-labelled CD11C, CD86, CD80 and MHC-II were respectively added into them and used to make isotype control. After mixing evenly, they were placed in 4 °C dark environment for 30 min; then, they were taken out, washed and tested by flow cytometry finally.

2.3. Mixed lymphocytes reaction

After putting those mice to death, their spleens were taken out immediately. Fewer RPMI 1640 cultured medium were added into it and monocytes were separated and then mixed lymphocytes were separated. It then became allogeneic mixed T-lymphocytes after being cultivated in the 37 °C incubator with 5% CO_2 for 4 h. According to the four cells proportions 1:200, 1:100, 1:50 and 1:20, mature DCs were used to make mixed-lymphocytes reaction with $CD4^+$ T in 96-pores plate. There were three complex pores of each group to cultivate for 96 h and 37kBq 3H2TdR was added to cultivate for 16 h according to the standard of 1 μ Ci a pore. The value of cpm was detected by liquid scintillation counter.

2.4. Lacks of mice model building and the detections of $CD4^+$ T cells and $CD8^+$ T cells of 25(OH)D3

BALB/C mice were divided randomly into 25(OH)D3 and the control groups. Mice in 25(OH)D3 group were fed with normal fodders with vitamin D and given lights in the day and no lights at nights. Mice in the control group, however, were fed with fodders without vitamin D, covered with blackout fabric and given yellow light (without ultraviolet ray) in the day and no lights at nights. Mice of both groups were fed for 12 weeks. Ten of them were randomly taken out and 0.05 mg of BCG was injected into tail vein of then. Then, they were taken back to the previous environment to be continuously fed for 6 weeks. After that, they were broken necks to death to make lymphocytes suspension; 360 μL of a lymphocytes suspensions were absorbed and placed in two EP tubes respectively with 18 μL of each tube. Rabbit antimouse CD3e-PE and CD4-FITC were added into one tube. In the other tube, rabbit antimouse CD3e-PE and CD8-FITC were added into it, which was the control group. Both were analyzed by flow cytometer.

2.5. Statistical analysis

SPSS17.0 was used to make statistical analysis. Mean ± standard deviation was used to represented expression quantity of DCs' surface molecules, mean fluorescent intensity and the level of T-cells' multiplication. *t*-Test was used to make intergroup comparison. Percentage form was used to show the positive rate of DCs' surface molecules.

3. Results

3.1. Morphology of DCs

After two days of cultivation, fewer short and small dendritic protrusions could be observed on some DCs' surface; after six days of cultivation, it could be observed that the number of dendritic protrusions increased and there were agglomerate cell colonies in some parts. There were many longer and mature DCs releasing

Table 1
Expressions of CD11C, MHC-II, CD80 and CD86 between the two groups (%).

Groups	CD11C	MHC-II	CD80	CD86
Control group	83.99 ± 3.20	87.62 ± 3.23	85.28 ± 7.39	66.97 ± 8.29
Group 25 (OH)D3	81.13 ± 5.18	84.99 ± 3.17	85.11 ± 8.02	52.18 ± 8.52
t	1.485	1.838	0.049	3.934
P	0.155	0.083	0.961	0.001

Table 2
Average fluorescence intensities of CD11C, MHC-II, CD80 and CD86 between the two groups.

Groups	CD11C	MHC-II	CD80	CD86
Control group	445.19 ± 129.82	1102.16 ± 371.02	350.11 ± 101.18	149.18 ± 29.13
Group 25(OH)D3	444.21 ± 109.28	681.62 ± 292.71	341.01 ± 113.27	139.15 ± 25.91
t	0.018	2.814	0.189	0.814
P	0.986	0.011	0.852	0.427

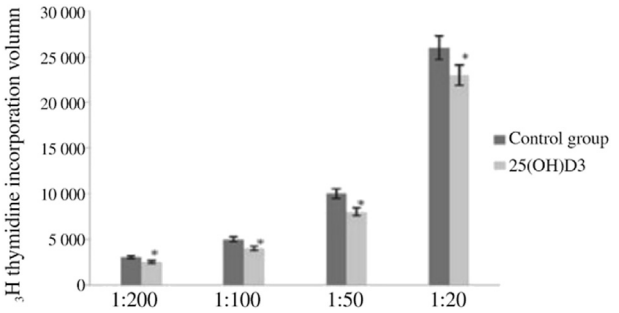

Figure 1. Multiplication levels of $CD4^+$ T cells of the two groups were compared. *$P < 0.05$.

nearly those cell colonies. On day seven, the volumes of those DCs increased and protrusions became more and longer.

3.2. Phenotypic analysis of DCs

DCs which were separated from mice's bone marrows were cultured by rmGM-CSF; 5 μg/ml of BCG were added to stimulate their maturity on the sixth day. Flow cytometry was applied to test the expressions of their surface molecules. The averages of the positive rates of CD11C, MHC-II, CD86 and CD80 were 89.73%, 94.17%, 69.12% and 92.16%; the positive rates and purity of DCs were pretty low. It indicated that the differentiated and mature abilities of separated-cultured DCs were comparatively ideal and could be used for the subsequent experiences and researches.

3.3. Inhibited DCs from mature by 25(OH)D3

The expression of CD86 of the control group was significantly higher than that of the 25(OH)D3. ($P < 0.05$) (Table 1). Among them, the average of fluorescence intensity of MHC-II in the control group was significantly higher than that in 25(OH)D3 ($P < 0.05$) (Table 2).

3.4. Inhibited antigen presenting function of DCs by 25(OH)D3

Compared with group 25(OH)D3, the DCs of the control group significantly improved the multiplication level of $CD4^+$ T cells (Figure 1).

Table 3
Effect of 25(OH)D3 in the distribution of $CD4^+$ T and $CD8^+$ T.

Groups	$CD4^+$ T(%)	$CD8^+$ T(%)	$CD4^+$ T/$CD8^+$ T
Control group	27.11 ± 0.50	13.12 ± 0.59	2.07 ± 0.11
Group 25(OH)D3	25.31 ± 0.32	11.50 ± 0.55	2.20 ± 0.13
t	9.588	6.351	−2.414
P	0.000	0.000	0.027

3.5. Effect of 25(OH)D3 in distribution of $CD4^+$T and $CD8^+$T

The results showed that the accounts for $CD4^+$ T cells of the control group were significantly higher than those of 25(OH)D3 ($P < 0.05$). The values of $CD4^+$ T/$CD8^+$ T in 25(OH)D3 were significantly higher than those in the control group ($P < 0.05$) (Table 3).

4. Discussion

Former studies showed that 25(OH)D3 deficiency had a close relation with tuberculosis susceptibility [19]. However, there are few researches on genic polymorphism and tuberculosis susceptibility of vitamin D receptor *Taq*I and *Fok*I and there are still some disagreements [20–22]. Therefore, a further study of the action mechanism of vitamin D3 in tuberculosis therapy is very important.

Immunity in tuberculosis includes innate immunity and acquired immune. Many studies have reported that 25(OH)D3 influences the concrete mechanism of its innate immunity [23]. Nevertheless, studies about the effect of 25(OH)D3 in tuberculosis acquired immune are not deep-going enough. In the procedure of T cells activation by tuberculosis infection, DCs play a very important role there. Studies have shown that 100 nmol/L is in the normal range of physiological concentration of 25(OH)D3. Deficiency of vitamin D3 refers to the lack of 25(OH)D3, so studies on the effects of 25(OH)D3 in DCs remain extremely important [3]. In this study, we mainly investigate the changes of DCs' phenotypes and functions after those DCs become mature by the stimulation of 5 μg/mL of BCG with the presence of 100 nmol/L of 25(OH)D3.

Compared with the control group, we found that the positive rate of CD86 of DCs' surfaces in group 25(OH)D3 significantly decreased, while the rate of CD11C, CD80 and MHC-II in the two groups showed no obvious differences. Besides, the average fluorescence intensity of MHC-II in the control group was significantly higher than in group 25(OH)D3. CD11C is an important tagged molecule of the detection of DCs, while CD80, CD86 and MHC-II are superficial co-stimulatory molecules in the procedure of differentiation of DCs. The data of the study showed that 25(OH)D3 deceased the expressions of MHC-II and CD86. There were researches showing that the decreases of MHC-II and CD86 were common features of functional damages of DCs in all kinds of 25(OH)D3 models [24]. This study data also manifested that 25(OH)D3 could not only inhibit DCs from maturity, but also weaken its antigen-presenting capacity.

What's more, BCG was used to stimulate the two groups in the study. Compared with the control group, we found that the percentages of $CD4^+$ T and $CD8^+$ T cells in group 25(OH)D3 significantly decreased, while the ratio of $CD4^+$ T/$CD8^+$ T increased obviously. These data indicated that lack of 25(OH)D3

could promote BCG to produce a stronger immune response in bodies, which consistently matched with the *in vitro* experiment that 25(OH)D3 could weaken the antigen-presenting capacity of DCs and inhibit the T cells from proliferation. The decrease of the ratio of $CD4^+$ T/$CD8^+$ T in the control group was a main manifestation of immune damage in tuberculosis patients.

In conclusion, it is found in this study that 100 nmol/L of 25(OH)D3 decreases the expressions of DCs' superficial co-stimulatory molecules MHC-II and CD86 so as to weaken the antigen-presenting capacity of DCs and inhibit the T cells from proliferation. Further researches about the effects of 100 nmol/L of 25(OH)D3 on *in vivo* DCs of patients infected by *Mycobacterium tuberculosis* are conductive to the achievement of a new tuberculosis therapy project.

Conflict of interest statement

We declare that we have no conflict of interest.

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