Evaluation of the Immunomodulatory Effect of Curdlan on Maturation and Function of Mouse Spleen-Derived Dendritic Cells

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ABSTRACT

Background: T helper 1 and T helper 17 cells play important roles in immunity against foreign invaders. Differentiation of these Th subsets is affected by state of maturation and cytokines that are produced by dendritic cells (DCs). Curdlan is a linear (1→3)-β-glucan and has shown activity against tumors and infectious agents. **Objective:** This study aims to investigate whether curdlan plays its role through affecting the maturation and cytokine production by DCs. **Methods:** DCs were isolated from the spleen of BALB/c mice by MACS method. After an overnight culture of DCs in the presence of curdlan, the expression levels of CD40, CD86, and MHC-II molecules were determined by flow cytometry. The production of cytokines involved in Th1 and Th17 cell differentiation (IL-12 and IL-6, respectively) was also evaluated by ELISA. Lipopolysaccharide (LPS) treated and untreated cells were considered as positive and negative controls, respectively. **Results:** The results of this study did not show a significant difference in the levels of surface expression of CD40 (p=0.82), CD86 (p=0.79), and MHC class II (p=0.84) molecules upon exposure to curdlan. However, LPS increased the intensity of CD40 expression on dendritic cells (p=0.04). In addition, it was revealed that curdlan-exposed DCs are not able to produce a significant amount of IL-6 and IL-12 cytokines. Conversely, LPS-treated DCs were able to make a significant amount of IL-12 (p=0.005). **Conclusion:** The results of the present study suggest that curdlan has no effect on Th1 or Th17 differentiation while LPS may induce Th1 deviation by induction of CD40 expression and IL-12 production.

Soltani MH, et al. Iran J Immunol. 2012; 9:168-74.

Keywords: Dendritic Cells, IL-12, IL-6, CD40, CD86

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INTRODUCTION

Curdlan is structurally the simplest β-1,3-D-glucan with no glycosyl side chains (1). This glucan is widely distributed in fungal cell walls, and higher plants. Safety of curdlan has widely been evaluated in both *in vitro* and *in vivo* studies. In addition, it has been approved as an inert dietary fiber for use in Korea, Taiwan, and Japan (2). It has also been registered in the United States as a food additive. Similar to other β-1,3-glucans, curdlan has both medical and pharmacological potentials. Moreover, curdlan has been believed to induce anti-coagulant, anti-bacterial, anti-fungal, anti-viral, and wound repair activities as well as the protection against radiation (3,4).

The cells of the innate immune system, such as dendritic cells (DCs), detect and respond to pathogens through the expression of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), Nod-like receptors, and Dectin-1(5,6). Dectin-1 is a type II transmembrane protein of C-type lectin family. Although it was originally identified as a DC-specific C-type lectin, Dectin-1 was also detected in macrophages and neutrophils as well as on a subset of B and T lymphocytes (7). This PRR binds to β-1,3-glucans, such as curdlan, on the cell wall of the fungi and some bacteria (8), thereby activating DCs (9). This activation results in the production of cytokines which eventually modulates the type of T cell responses and functions.

Through interaction with DCs, $CD4^+$ T cells can differentiate into a variety of effector and regulatory subsets, including classical Th1 and Th2 cells, regulatory T cells (Treg), and Th17 cells. It has been shown that the nature of the cytokines produced by DCs in response to various ligands determines the type of Th cell response. The differentiation of Th1 or Th17 cells is determined by exposing the T cells to IL-12 or transforming growth factor-β (TGF-β) + IL-6, respectively (10,11). These cytokines affect the newly primed $CD4^+$ T cells to induce the expression of the transcription factor T-bet which promotes Th1 responses (12) or RORγt, a transcription factor required for the differentiation of Th17 cells (13).

Activation of Dectin-1 by curdlan promotes signaling through the Syk kinase which leads to the production of reactive oxygen species and activation of MAP kinases, NF-kB, and NFAT (14,15). It has been reported that immature bone marrow derived-DCs are able to be activated selectively via the Dectin-1/Syk pathway and acquire effector functions through up-regulation of co-stimulatory molecules, such as CD86, CD80, and CD40, and also production of TNF-α, IL-6, and IL-23 (16,17). These activated DCs can also prime and induce potent cytotoxic T-lymphocyte (CTL) responses (18,19). Recently, it has also been reported that DCs activated via Dectin-1 can change Treg cells into IL-17 producing cells (20).

Considering the effect of Dectin-1 on immature bone marrow derived-DCs (18) as well as its ability in induction of Th17 cells (20), the aim of the present study was to determine the effect of curdlan on maturation and the production of Th1- or Th17-deviating cytokines (IL-12 and IL-6, respectively) by spleen derived-DCs.

MATERIALS AND METHODS

Animals. Eight to 10 week-old inbred female BALB/c mice were purchased from Pasteur Institute of Iran (Tehran, Iran). The mice were kept under conditions of hygiene, temperature, and humidity with 12 h light: 12 h darkness cycle. The authors of the

present study followed the ethical guidelines set by the Institute for Animal experimentation all through the process of the research.

Enrichment of Spleen-Derived DCs. A DC-enriched population was prepared from the mice spleen by magnetic-activated cell separation (MACS) method, with minor modifications. Briefly, after cervical dislocation, the spleens were separated from BALB/c mice under aseptic conditions. Tissues were cut into small slices, suspended in 5–10 ml RPMI-1640 (Gibco, UK) containing collagenase D (1 mg/ml; Roche, Germany) and DNase (0.02 mg/ml; Roche, Germany), and then digested for 30 min at 37°C in a 5% CO2 incubator. To disrupt cell aggregations or DC–T cell complexes, EDTA (5 mM, pH 7.2) was added at the end of incubation period and the cell suspension was pipeted several times. Afterwards, the undigested stromal fragments were removed by passing the suspension through a stainless steel sieve. The cell suspension was washed twice with phosphate-buffered saline (PBS) containing 5 mM EDTA at 4°C, and centrifuged at 300g for 10 min. The pellet was immediately resuspended in 2–3 ml RPMI, added slowly on 2 ml Nycodenz 13% (w/v), d =1.068 (Axis-Shield, Norway), and centrifuged at 600g for 15 min at 4°C. Low-density cells were recovered from the interface, washed twice with RPMI, and then conventional ($CD11^+$) and plasmacytoid ($mPDCA\text{-}1^+$) DCs were positively selected by sorting with anti-CD11c and anti-mPDCA-1 antibodies conjugated magnetic beads (Miltenyi Biotec). Spleen DC purity was checked by flow cytometry, and was routinely found to be over 90%.

Treatment of DCs with Curdlan. To evaluate the effect of curdlan (Sigma-Aldrich) on the maturation and function of DCs, different concentrations of curdlan were added to the overnight culture of DCs obtained from BALB/c mice. For this purpose, cells were divided into three groups. During the overnight culture, the first group was pulsed with curdlan (10, 100, and 500 μg/ml), while the second and the third groups were used as positive (cultured with 250 ng/ml LPS) and negative controls, respectively. DCs were cultured in RPMI 1640 containing 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and GM-CSF (40 ng/ml). After incubation time of 14–16 h, the non-adherent cells were collected and analyzed using flow cytometry (BD, Partec BD FACS Calibour).

Flow Cytometric Analysis. In order to evaluate the purity of the isolated DCs and the effect of curdlan on the expression of DC surface markers, DCs were stained for their phenotypic markers using monoclonal antibodies. In brief, DCs were treated on ice with 5% normal mouse serum for 15 minutes and then with hamster anti-mouse CD11c antibody (Pharmingen, Australia) diluted to 1 $\mu g/10^6$ cells in PBS containing 2% FCS (PBS-FCS) for 30 min at 4°C. After washing twice, the cells were stained with FITC-conjugated monoclonal antibodies against MHC-II, CD86, or CD40 (Pharmingen, Australia) for the next 30 min. Then, the cells were washed twice, resuspended in 0.5 ml cold PBS-FCS, and retained on ice until analyzed with a flowcytometer (BD FACS Calibour). Appropriate isotype controls were used for staining.

Cytokine Analysis. Cell-free supernatants collected from DC cultures were tested for IL-6 and IL-12 levels by ELISA assays. ELISA was conducted using paired Abs and standards (eBioscience Systems) according to the manufacturer's recommendations. The amount of a particular cytokine was determined using an appropriate cytokine-specific standard curve. The sensitivities of ELISA for IL-6 and IL-12 were 4 pg/ml and 6 pg/ml, respectively.

Statistical Analysis. Statistical significance was determined by a two-tailed unpaired t-test with Graphpad Prism (GraphPad Software).

RESULTS

Effects of Curdlan and LPS on Maturation of Spleen-Derived DCs. To evaluate the maturation of DCs, various concentrations of curdlan (10, 100, 500 µg/ml) and LPS (250 ng/ml) were added to 1×10^5 DCs and cultured overnight. The results of flow cytometric analysis using double staining of CD11c and MHC-II, CD86 or CD40 on curdlan-treated DCs (Figures 1A and 2) showed no significant effect on splenic DCs in terms of the expression levels of CD86, CD40, and MHC-II (120/8 ± 35.96, 20.45 ± 2.23 and 44.58 ± 16.8, respectively), compared to the negative controls (107.8 ± 31.41, 19.73 ± 2.09 and 39.95 ± .15, respectively). However, as shown in figures 1A and 2, LPS increased the intensity of CD40 on DCs compared to the negative controls (27.48 ± 2.20 and 19.73 ± 2.09, respectively; p=0.04).

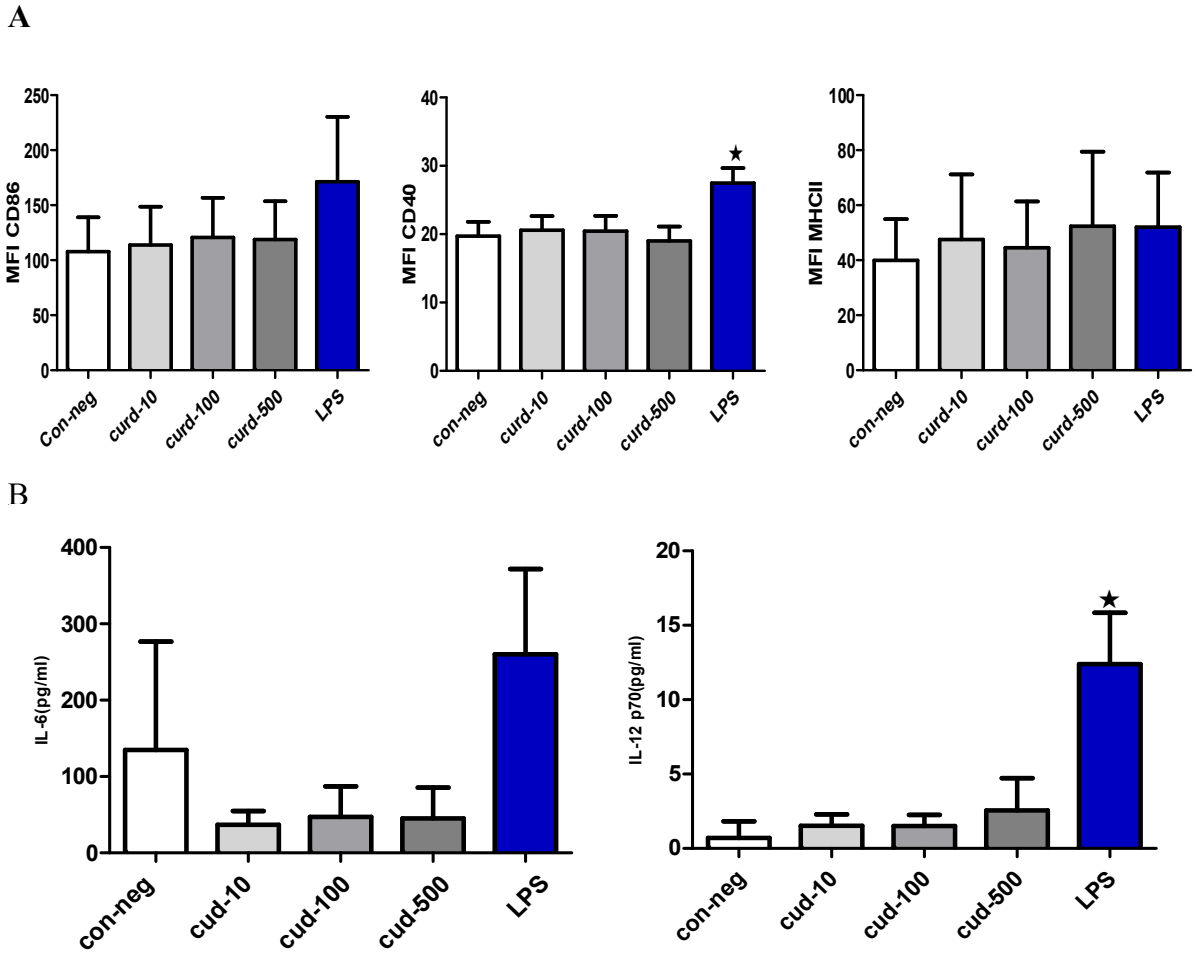

Figure 1. DCs were activated with Curdlan and LPS for 18h and upregulation of surface markers and secretion of cytokines were determined. **A**. Bar graph depicts the mean fluorescence intensity [MFI] of CD40, CD86 and MHCII on DCs after activation. The figure shows mean ± S.E. of 4 such experiments. **B**. Bar graphs depict the pg/ml level of IL-6 and IL-12p70 secreted by DCs in response to the stimuli. *Significant (p<0.05).

Effects of Curdlan and LPS on Cytokine Production by Splenic DCs. As demonstrated in figure 1B, cytokine assays in the supernatant of cultured DCs showed that in comparison to untreated DCs, curdlan exposure has no effect on the production of IL-6 (134.8 ± 70.98pg/ml and 47.25 ± 19.98pg/ml, respectively; p=0.28) and IL-12 (0.70 ± 0.64pg/ml and 1.50 ± 0.43pg/ml, respectively; p=0.36). However, LPS treatment induced a significant increase in IL-12 production compared to the negative control (12.40 ± 1.98pg/ml and 0.70 ± 0.64 pg/ml, respectively; p=0.005).

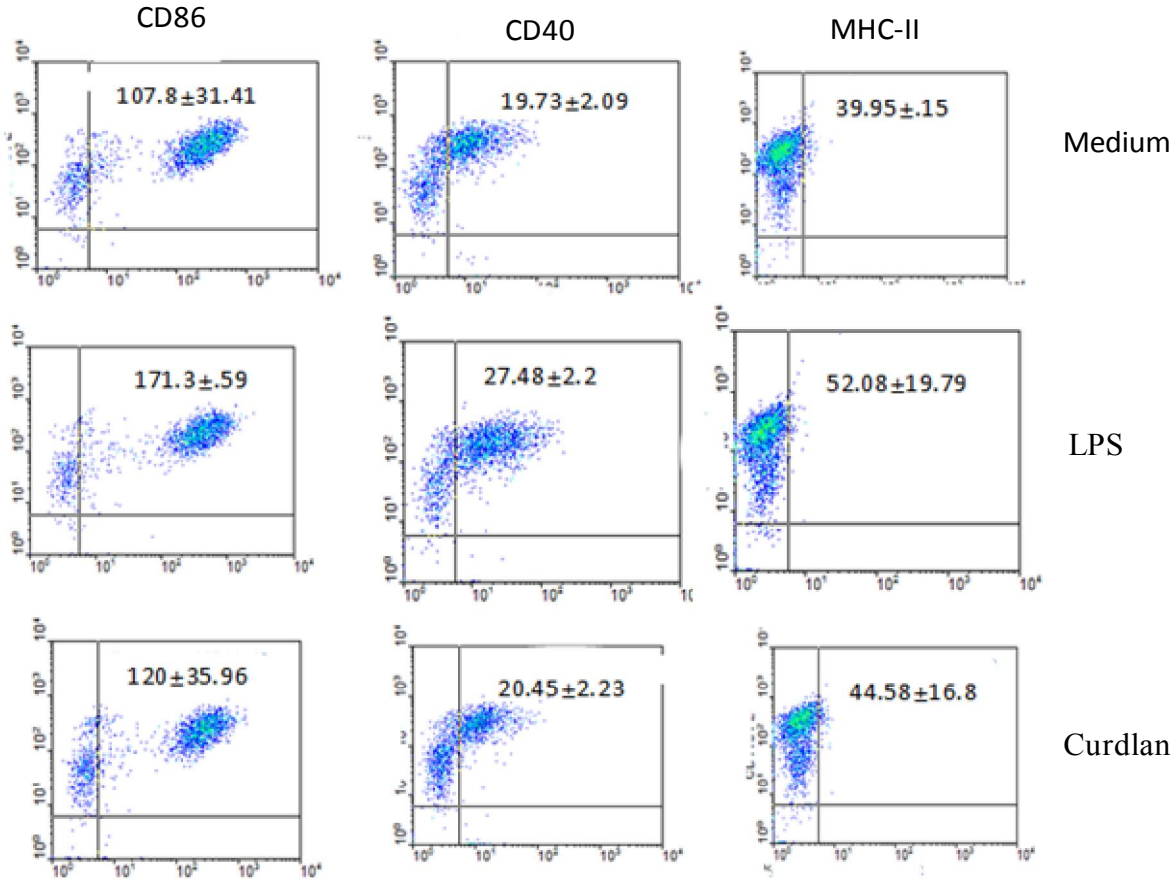

Figure 2. Effects of Curdlan on surface phenotype of splenic DCs. DCs were purified from spleen of BALB/c mice and cultured overnight in the presence of 100 µg/ml Curdlan, 250 ng/ml of LPS or in their absence (using the medium alone). The surface phenotype of DCs was analyzed by two-color flow cytometry. The dot-plots show representative data out of four independent experiments. Data presented in the upper right show the mean fluorescence intensity [MFI] of double-positive cells ± SD.

DISCUSSION

Since DCs are major regulators of the immune response determining the choice between tolerance and immunity, and considering that there have been few studies conducted on the effect of curdlan on dendritic cell maturity and activity, the following research was an attempt to study if maturation of splenic-derived DCs can be affected by curdlan. In

order to achieve this goal, expressions of CD86, CD40 and MHCII molecules were evaluated through flow cytometry. Also, the ability of splenic-derived DCs in producing IL-6 and IL-12 were also investigated.

In previous studies, it was indicated that immature bone marrow derived-DCs that are activated selectively via Dectin-1/Syk pathway acquire effector functions through up-regulation of co-stimulatory molecules such as CD86, CD80, and CD40 and also through production of TNF-α, IL-6, and IL-23 (16,17). These activated DCs can also prime and induce strong cytotoxic T-lymphocyte (CTL) responses (18). Recently, it has also been reported that DCs activated through Dectin-1 can change Treg cells into TH17 cells (20). Since the effect of curdlan on isolated DCs from various organs might be different, in the present study the effect of this beta glucan on the maturation of splenic-derived DCs were studied. The results indicated that the exposure of splenic DCs to curdlan had no effect on the expression of CD40, CD80 and MHCII molecules, while treatment with LPS significantly increased the expression of CD40 molecule on DCs compared to the untreated DCs (27.48 ± 2.20 vs. 19.73 ± 2.09; p=0.04). In this respect, Iezzi et al. also investigated the effect of curdlan and zymosan on the expression of CD40, CD86, and MHCII molecule on splenic DCs, and showed that these stimuli significantly increase the above mentioned molecules (21). In contrast to Iezzi et al. report and in agreement with our findings, a study conducted by Karumuthil-Melethil et al. revealed that zymosan has no effect on CD40, CD86, and MHCII expression by splenic DCs (22). At present the cause of these conflicting results are not known and due to limited studies on the effect of curdlan on the maturation of splenic-derived DCs and the lack of agreement in the results, further studies are needed.

The results of the present study also revealed that stimulation of splenic-derived DCs by curdlan did not induce the production of significant amounts of IL-6 and IL-12. However, a significant increases in IL-12 production was induced by LPS compared to the untreated controls (12.40 ± 1.98 pg/ml and 0.70 ± 0.64 pg/ml, respectively; p=0.005). It has been reported that Th1 and Th17 are produced in the presence of an intense stimulation (21) and appropriate cytokine microenvironment. In fact, IL-12 is one of the important cytokines for the differentiation of Th1 cells (23) while TH17 cells are differentiated in the presence IL-6 and TGF-β (10). Since, LPS could drastically increase CD40 expression (p=0.04) and IL-12 production (p=0.005), it can be concluded that LPS is able to provide necessary signal strength as well as required cytokines for Th1 deviation. However, due to the fact that curdlan neither increases CD40 expression nor induces IL-6 and IL-12 production by splenic-derived DCs, it seems that curdlan does not have a great role in the differentiation of Th1 or Th17 cells. However, the effect of cytokines such as IL-1β, IL-23 and TGF-β on deviation of Th0 cells towards the Th1 and Th17 is not indisputable and further studies on the effect of curdlan on these cytokines are suggested.

In summary, the results of the present study suggest that curdlan has no effect on maturation as well as the induction of IL-6 and IL-12 production by the spleen-derived DCs. Therefore, curdlan has no effect on Th1 or Th17 differentiation, while LPS may induce Th1 deviation by induction of CD40 expression and IL-12 production by the spleen-derived DCs.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Farzaneh Taki and Mr. Maysam Bagheri from Autoimmune Diseases Research Center for their technical helps and Dr. Nasrin Shokrpour and Ms. Afsaneh Keivanshekouh at Center for Development of Clinical Research of Nemazee Hospital for editorial assistance. The present article was extracted from the thesis written by Mr. Soltani to achieve his MSc degree in Immunology and was financially supported by Shiraz University of Medical Sciences grants No 90-5540.

REFERENCES

1 Harada T, Misaki A, Saito H. Curdlan: a bacterial gel-forming beta-1,3-glucan. Arch Biochem Biophys. 1968;124:292-8.
2 Spicer EJ, Goldenthal EI, Ikeda T. A toxicological assessment of curdlan. Food Chem Toxicol. 1999;37:455-79.
3 Demleitner S, Kraus J, Franz G. Synthesis and antitumour activity of derivatives of curdlan and lichenan branched at C-6. Carbohydr Res. 1992;226:239-46.
4 Chen J, Seviour R. Medicinal importance of fungal beta-(1-->3), (1-->6)-glucans. Mycol Res. 2007;111:635-52.
5 Manicassamy S, Pulendran B. Modulation of adaptive immunity with Toll-like receptors. Semin Immunol. 2009;21:185-93.
6 Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140:805-20.
7 Willment JA, Marshall AS, Reid DM, Williams DL, Wong SY, Gordon S, et al. The human beta-glucan receptor is widely expressed and functionally equivalent to murine Dectin-1 on primary cells. Eur J Immunol. 2005;35:1539-47.
8 Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L, et al. Dectin-1 is a major beta-glucan receptor on macrophages. J Exp Med. 2002;196:407-12.
9 Kennedy AD, Willment JA, Dorward DW, Williams DL, Brown GD, DeLeo FR. Dectin-1 promotes fungicidal activity of human neutrophils. Eur J Immunol. 2007;37:467-78.
10 Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. Eur J Immunol. 2010;40:1830-5.
11 Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med. 1997;185:817-24.
12 Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100:655-69.
13 Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126:1121-33.
14 Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood. 2005;106:2543-50.
15 Goodridge HS, Simmons RM, Underhill DM. Dectin-1 stimulation by Candida albicans yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. J Immunol. 2007;178:3107-15.
16 LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol. 2007;8:630-8.
17 Yoshitomi H, Sakaguchi N, Kobayashi K, Brown GD, Tagami T, Sakihama T, et al. A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. J Exp Med. 2005;201:949-60.
18 Leibundgut-Landmann S, Osorio F, Brown GD, Reis e Sousa C. Stimulation of dendritic cells via the dectin-1/Syk pathway allows priming of cytotoxic T-cell responses. Blood. 2008;112:4971-80.
19 Agrawal S, Gupta S, Agrawal A. Human dendritic cells activated via dectin-1 are efficient at priming Th17, cytotoxic CD8 T and B cell responses. PLoS One. 2010;5:e13418.
20 Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G, et al. DC activated via dectin-1 convert Treg into IL-17 producers. Eur J Immunol. 2008;38:3274-81.
21 Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4+ T cells. Proc Natl Acad Sci U S A. 2009;106:876-81.
22 Karumuthil-Melethil S, Perez N, Li R, Vasu C. Induction of innate immune response through TLR2 and dectin 1 prevents type 1 diabetes. J Immunol. 2008;181:8323-34.
23 Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5:987-95.