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# The effect of microorganisms on cytokine secretion from bone marrow dendritic cells

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**Aim:** Dendritic cells (DCs) are potent antigen-presenting cells crucial for initiation of cell-mediated immune responses to foreign antigens. They also contribute to the innate resistance against microbial pathogens. The present study examined the effect of microorganisms such as *Escherichia coli (E. coli)*, *Serratia marcescens (S. marcescens)*, *Methicillin-resistant Staphylococcus aureus (MRSA)*, coagulase negative staphylococci (CoNS), and *Candida albicans (C. albicans)* on cytokine secretion in bone marrow-dendritic cells (BM-DCs).

**Materials and methods:** The levels of cytokines in culture supernatants of the BM-DC were determined by enzyme-linked immunosorbent assay.

**Results:** DCs stimulated by microorganisms produced large amounts of TNF-α, IL-1β, IL-6, and IL-10. The levels of IFN-γ, IL-2, and IL-4 did not increase in the samples with DCs stimulated by microorganisms compared to those of the samples including only DCs. The levels of TNF-α increased remarkably in the DCs stimulated with *C. albicans*.

**Conclusion:** Our results indicated that gram-negative bacteria tended to induce higher levels of cytokines from DCs than the gram-positive bacteria induced and gram-negative bacteria caused a predominantly inflammatory cytokine response. These results may contribute to the understanding of the role of DC in the mechanisms to prevent infections.

**Key words:** Dendritic cells, microorganisms, cytokines

## Kemik iliği kökenli dendritik hücrelerden sitokin salınımına mikroorganizmaların etkisi

**Amaç:** Dendritik hücreler (DH), yabancı antijenlere hücresel immün yanıtın başlamasında önemli rol oynayan antijen sunucu hücrelerdir. Aynı zamanda, mikrobiyal patojenlere karşı doğal dirençte de rol oynarlar. Bu çalışmanın amacı; *Escherichia coli (E. coli), Serratia marcescens (S. marcescens), Metisiline dirençli Staphylococcus aureus (MRSA)*, koagülaz negatif stafilokok (CoNS), ve *Candida albicans (C. albicans)* gibi mikroorganizmaların kemik iliği kökenli dendritik hücrelerden sitokin salınımı üzerine etkinliğini araştırmaktır.

**Yöntem ve gereç:** Kemik iliği kökenli dendritik hücre kültürü süpernatanlarında sitokin düzeyleri ELISA yöntemi ile araştırıldı.

**Bulgular:** Mikroorganizmayla uyarılan DH'ler bol miktarda TNF-α, IL-1β, IL-6 ve IL-10 üretti. Mikroorganizma ile uyarılan DH'lerde IFN-γ, IL-2 ve IL-4 düzeyleri, sadece DH içerenlerle kıyaslandığında herhangi bir artış göstermedi. *C. albicans* ile uyarılan DH'lerde TNF-α düzeyleri anlamlı olarak arttı.

**Sonuç:** Çalışmamızın sonuçları; DH'den sitokin salınımı üzerine, gram negatif bakterilerin gram pozitif bakterilere oranla daha yüksek oranda etkili olduğunu ve inflamatuvar sitokin yanıtının oluştuğunu göstermiştir. Bu sonuçlar, DH'in enfeksiyonları önleme mekanizmalarındaki rolünün anlaşılmasına yardımcı olabilir.

**Anahtar sözcükler:** Dendritik hücre, mikroorganizma, sitokin

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## Introduction

Dendritic cells (DCs) are crucial to the presentation of peptides and proteins to T and B lymphocytes and are widely recognized as the most potent antigen presenting cells (APCs) (1). They are critical for the induction of T cell responses resulting in cell-mediated immunity (CMI). DCs also induce humoral immunity by presentation of antigen to B cells. DCs play an important role in both innate and adaptive immune responses (2). DC precursors originate in the bone marrow, enter the blood, and reside in nonlymphoid tissues.

The immature DCs differentiate into mature DCs as they migrate to tissue-draining secondary lymphoid organs, where they efficiently present antigen to T cells (3). DCs are responsible for detecting microbes and presenting their antigenic structures to T cells (4). After the recognition of infectious agents, resting immature DCs undergo a maturation process that leads to an increase in their capacity for antigen processing and presentation (5). Their ability to prime T cells, to regulate the Th1/Th2 cell responses, and to contribute to the inflammatory response largely depends on the up-regulation of costimulatory and adhesion molecules and the secretion of inflammatory cytokines (2). DCs are a major source of many cytokines, namely, IL-1 (Interleukin-1), IL-6, IL-7, IL-12, and IL-15, all of which are important in the elicitation of a primary immune response when they come in contact with pathogenic bacteria (6,7). Cytokines play an important role in regulating host immune responses against infections (7,8).

Since the type of immune response induced is vital to fight the infection, the type of cytokine secretion by DCs is important for the induction of immunity to microbes (9). DC may determine whether antigen encounter results in non-responsiveness or the generation of T helper1 (Th1) and Th2 responses (Th2) (10). The Th1 response is characterized by the production of cytokines such as IL-2, IL-12, interferon-gamma (IFN-γ), and tumor necrosis factor beta (TNF-β), and enhanced resistance against reinfection. The Th2 response, which produces IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, is linked with the development of chronic disease. Th1 cytokines primarily activate cellular immune responses, whereas Th2 cytokines primarily stimulate humoral immune responses (11). However, it is generally accepted that a critical balance of both Th1 and Th2 cytokines is necessary to develop protective immunity (3).

When DCs are activated, they stimulate naïve T cells, B cells, or other immune cells, and regulate the antigen or pathogen-specific adaptive immune response. In recognition of their primary function as immunostimulatory cells, DC-based immunotherapy is being tested for treatment of cancer in clinical trials. A similar immunotherapeutic approach has been successfully demonstrated in animals against infectious diseases and as well as in animal models of organ transplantation (3,12). Moreover, it has been reported that bacterial immunomodulators can initiate DC maturation and they may be used for production of DC-based vaccines (13).

The aim of the present study was to evaluate the effects of various microorganisms on bone marrow DCs (BM-DCs), in terms of proinflammatory and antiinflammmatory cytokines release. To determine whether BM-DCs are capable of responding to microorganisms, we assessed cytokine production after stimulation of gram-negative bacterial strain, gram-positive strains, and *Candida albicans* (*C. albicans*). A better understanding of the interaction between DC and infections may contribute to the understanding of the role DC plays in the mechanisms to prevent infections.

## Materials and methods

### *Mice*

Female Swiss Albino mice, 4 to 12 weeks of age (obtained from Gazi University School of Medicine, Experimental Animal Research Center) were used throughout the study. The animal experiments were conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals' of the Ethical Committee of Gazi University School of Medicine.

### *Microorganisms*

Isolates of the microbial species, *Escherichia coli* (*E. coli*) and *C. albicans*, were obtained from the American Type Culture Collection (ATCC) (ATCC 25922, ATCC 10230 respectively). Strains of methicillin-resistant *Staphylococcus aureus* (MRSA),

coagulase-negative staphylococci (CoNS), and *Serratia marcescens* (*S. marcescens*) were isolated from clinical specimens. All the microorganisms were cultured aerobically at 37 °C for 24 h. At the end of the incubation, microorganisms were harvested by centrifugation, washed 3 times in phosphate-buffered saline (PBS), and adjusted at a concentration of $10^8$ CFU/mL (colony forming unit) in PBS. The strains were inactivated by exposure to UV light for 15 min to inhibit uncontrolled microbial growth, which was confirmed by negative viable counting, and then stored at –70 °C.

***Bone marrow preparation***

Mice were sacrificed by cervical dislocation. Femurs and tibiae of mice were removed and purified from the surrounding muscle tissue by rubbing with paper tissues. Thereafter intact bones were left in 70% ethanol for 2-5 min for disinfection and washed with PBS. Then both ends were cut with scissors and the marrow was flushed with PBS using a syringe with a 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting and washed in PBS (14).

***Bone marrow cell culture with GM-CSF***

The principle method for generating BM-DC with GM-CSF was adapted from previous publications (12). Cell culture medium (Complet RPMI) was RPMI-1640 (Sigma, Germany) supplemented with penicillin (100 U/mL, Biochrom AG, Germany), streptomycin (100 µg/mL, Biochrom AG, Germany), L-glutamin (2 mM, Biochrom AG, Germany), 2-mercaptoethanol (50 µM, Fluka Switzerland), and 10% heat-inactivated FCS (Biochrom AG, Germany). On day 0 BM leukocytes were seeded at $2 \times 10^6$ per 100 mm dish in 10 mL of complete RPMI containing 200 U/mL (=20 ng/mL) rmGM-CSF ($5 \times 10^6$ U/mg; Peprotech, USA). Culture medium was replaced partially every 3 days and fresh cytokine (200 U/mL rmGM-CSF) was added. For complete maturation at the day 10, non-adherent cells were collected by gentle pipetting, centrifuged at 300 ×*g* for 5 min at RT, and resuspended with 10 mL of fresh complete RPMI in a 100 mm tissue culture plastic dish containing 100 U/mL rmGM-CSF and lipopolysaccharide (LPS, Sigma, Germany) at 1 µg/mL. Cells were then cultured for 1 or 2 more days. Cultured cells were washed once and an aliquot volume mixed 1:1 in Trypan Blue solution (Sigma, Germany). DCs were counted as viable under the microscope in a Neubauer chamber and the number was adjusted to $5 \times 10^6$ cells/mL.

***Cell separation and flow cytometry***

$CD11c^+$ DCs were purified through magnetic cell sorting (MACS) by using positive selection according to the manufacturer's protocol (Miltenyi, USA). Briefly, the cells were incubated with magnetic microbeads conjugated with monoclonal anti-mouse CD11c antibodies in MACS buffer for 15 min at 4 °C. After this, the cells were run through a MACS column (Miltenyi) in a magnetic field. The column was then removed from the magnet, and the positive cells were flushed out. Then $CD11c^+$ DCs were analyzed further by flow cytometer (Coulter, USA).

***Preparation of cell culture***

One milliliter of BM-DCs ($10^6$) was delivered per well. One milliliter of 5 different UV-killed microorganisms ($10^6$ CFU/mL) such as *E. coli*, *S. marcescens*, MRSA, CoNS, and *C. albicans* were added to the wells. Thus, DCs were stimulated by UV-killed microorganisms. Control culture flasks did not contain microorganisms. They only contained medium and DCs. Each sample was studied in triplicate. Control culture plates and culture plates containing UV-killed microorganisms were incubated for 24 h in 5% $CO_2$ at 37 °C. At the end of this period, the contents of the culture plates were transferred into tubes and centrifuged. The culture supernatants were removed and stored at –70 °C until used in the enzyme-linked immunosorbent assay (ELISA).

***Cytokine secretion***

Levels of cytokines such as TNF-α, IFN-γ, IL-1β, IL-2, IL-4, and IL-6 (minimum detectable dose; 1.7 pg/mL for TNF-α, <4 pg/mL for IFN-γ, 1 pg/mL for IL-1β, 4 pg/mL for IL-2, <2 pg/mL for IL-4, <2 pg/mL for IL-6) were determined by specific ELISA techniques according to the manufacturer's instructions (Biosource, California, USA). The concentration of cytokines was determined spectrophotometrically. The absorbance was read at 450 nm (BioTek, USA). We constructed a standard curve using cytokine standards. The cytokine concentrations for unknown samples were calculated according to the standard curve.

### *Statistical methods*

The results were analyzed using one-way analysis of variance (ANOVA). The Bonferroni test was used as post hoc analysis. $P < 0.05$ was considered significant.

## Results

### *Analysis of DCs*

Analysis of cells by flow cytometry on a plot of CD11c versus side scatter showed that more than 94.7% of the purified cells expressed $CD11c^+$ (Figure 1).

### *The levels of cytokines*

We found that in vitro infection of mouse DCs with UV-inactivated microorganisms leads to the production of TNF-α, IL-1β, IL-6, and IL-10 (Figure 2). The other cytokines IFN-γ, IL-2, and IL-4 did not have any increase in the samples with DCs stimulated by UV-inactivated microorganisms compared to those of the DCs non-stimulated by UV-inactivated microorganisms.

In our study, maximum levels of IL-1β were observed after stimulation of DCs with LPS. In addition, DCs produced significantly high levels of IL-1β after stimulation with *E. coli* and *C. albicans* ($P < 0.05$). The production of IL-1β in DCs stimulated by *S. marcescens*, MRSA, and CoNS did not differ significantly from those of samples including only DCs ($P > 0.05$).

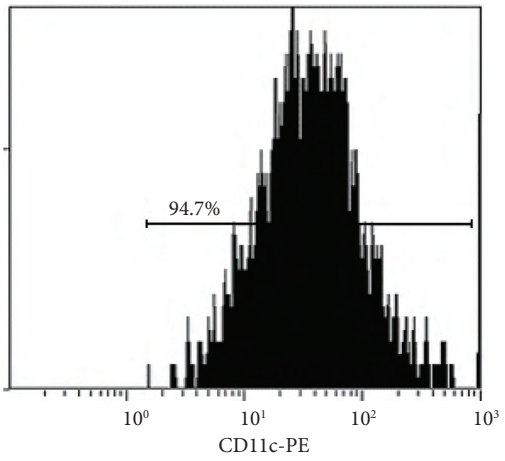


Figure 1. Flow cytometric analysis of CD11c (+) DCs.

The stimulation of DCs with gram-negative bacteria and gram-positive bacteria caused a more remarkable increase in the IL-6 levels compared to the samples including only DCs ($P < 0.05$), but the gram-negative strains tended to induce higher levels of IL-6 from DCs than the gram-positive bacteria induced, also the difference was statistically significant ($P < 0.05$).

We observed that the levels of IL-10 increased more significantly especially in the DCs stimulated by *E. coli*. Moreover, the increase was significantly higher than those of the DCs stimulated by LPS. DCs stimulated with *S. marcescens* and *C. albicans* caused significant increases in IL-10 levels ($P < 0.05$). After stimulation with gram-positive bacteria, the levels of IL-10 did not differ significantly from those of samples that contained only DCs ($P > 0.05$).

Although DCs stimulated with LPS, gram-negative and gram-positive bacteria triggered a significant increase in the levels of TNF-α compared to those of the specimens that contained only DCs, the levels of TNF-α increased remarkably in the DCs stimulated by *C. albicans* ($P < 0.05$).

## Discussion

DCs are potent antigen-presenting cells crucial for initiation of cell-mediated immune responses to foreign antigens. They also contribute to the innate resistance against microbial pathogens (2). DCs are activated by various microorganisms and their products (15). After the recognition of infectious agents, resting immature DCs undergo a maturation process that leads to an expression of cytokines that are crucial for both the activation and control of innate and adaptive immune responses (5).

In our study, stimulation of DCs with microorganisms induced the secretion of IL-1β, IL-6, IL-10, and TNF-α. We found that the cytokine response of the DCs is profoundly influenced by the nature of the pathogen. Some pathogens, such as *C. albicans*, mainly induced TNF-α, whereas others, such as *E. coli* and *S. marcescens*, induced IL-6 and IL-10. It is suggested that the nature of the pathogen determines the response of the dendritic cell, and cytokine response of the DCs is profoundly influenced by the nature of the pathogen. Scott et al. (16) also reported that dendritic cells are plastic in

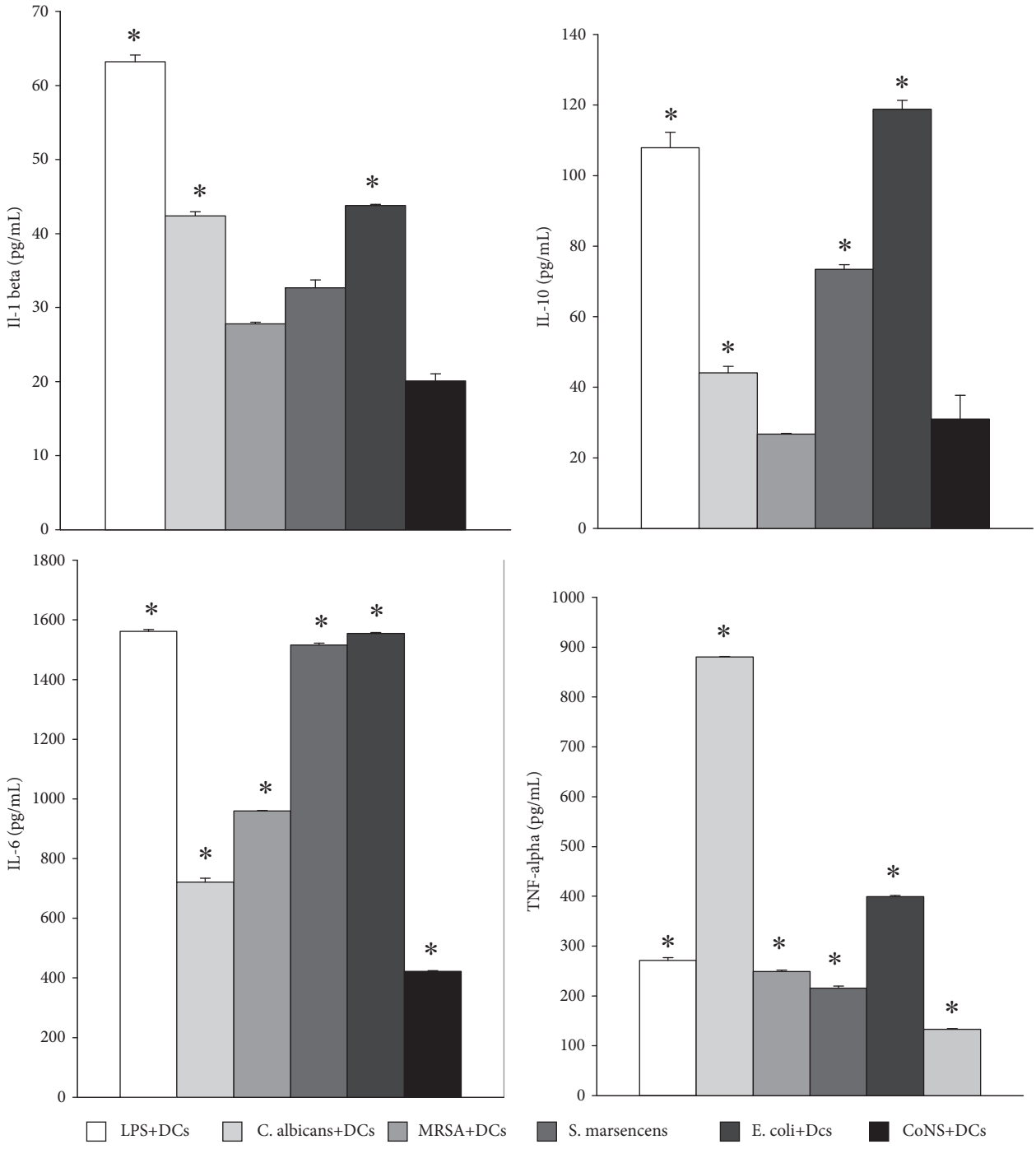


Figure 2. Cytokines production from DCs after stimulation with the microorganisms. Values represented are mean ±SEM. Asterisks indicate significant differences from samples that contained only DCs: $P < 0.05$.

their response to microbial stimuli and that the nature of the pathogen dictates the response of the dendritic cell.

In the present study we found that although DCs stimulated with *C. albicans* caused significant increases in the levels of IL-1β, IL-6, and IL-10, the

highest production was observed in the levels of TNF-α. Netea et al. (17) also observed that DC produced significant amounts of TNF-α after stimulation with *C. albicans* blastoconidia or hyphae. Therefore, we suggested that the cell wall components of *C. albicans* stimulate DCs and induce the release of proinflammatory cytokines such as TNF-α, IL-6. Pietralla et al. (18) demonstrated that the mannoproteins from *C. albicans* (MP65) stimulated DC maturation by increasing costimulatory molecules and induced the release of TNF-α (19). Moreover, it was shown that DCs are involved in the immunoregulatory events manifested during a vaginal Candida infection (19).

Our study also revealed that DCs secreted large amounts of TNF-α, IL-6, and IL-10 in response to gram-negative bacterial strains such as *E. coli* and *S. marcescens*. However, DCs did not produce IL-1β and IL-10 in response to gram-positive bacterial strains such as MRSA and CoNS. Karlsson et al. (4) also indicated that DCs were practically unresponsive to gram-positive bacterial strains, such as *Lactobacillus plantarum* and *Bifidobacterium adolescentis***.**

In the present study, we found that gram-negative bacteria are more potent inducers of cytokine secretion in DCs than gram-positive bacteria. This is possibly related to the strong proinflammatory effect of LPS. The mainly increased cytokines in DCs stimulated by gram-negative bacteria were proinflammatory cytokine such as IL-1β, IL-6, and TNF-α. Karlsson et al. (4) also found that DCs secreted large amounts of TNF-α and IL-6 in response to gram-negative bacterial strains such as *E. coli* and *Veillonella parvula*. Frieling et al. (20) also stated that gram-negative bacteria induced significantly higher levels of proinflammatory cytokine production than gram-positive bacteria. Hence, we also suggested that this situation leads to a bit more acute and fulminant clinical infection compared to infection with gram-positive bacteria.

Since Th1 effector cells produce largely proinflammatory cytokines, it is considered that DCs may trigger predominantly Th1 type cytokine response associated with cell-mediated immunity (21). Pietilä et al. (7) also showed that DCs contribute to stimulating Th1 adaptive immune responses to Salmonella. As Th1 response enhances resistance against reinfection, we suggest that the interactions of DCs with pathogenic microorganisms may help the induction of protective immunity (22).

Furthermore, we also found that DC activation by microorganisms upregulates the synthesis of antiinflammatory cytokines, such as IL-10, in addition to proinflammatory cytokines. Proinflammatory cytokines are thought to play a major role in chronic inflammation, whereas antiinflammatory cytokines inhibit the production of the proinflammatory cytokines (23). Therefore, we propose that the balance between these 2 groups of cytokines may lead to new approaches based on DCs to control infection. All findings suggest that, following stimulation with microorganisms, DCs may be involved in early inflammatory processes, until the adaptive immune response is launched, and initiate a microorganism specific Th1 immune response by producing cytokines (17).

Although Granucci et al. (5) reported that DCs produce IL-2 after microbial challenge, we did not observe any significant changes in the levels of IL-2. Our failure to detect a change in the levels of some cytokines, particularly IL-2, could be due to the fact that production of cytokines is transient at early time points after the microbial encounter.

We think that our study is different from other studies on the basis that it enables us to determine the secretion of proinflammatory and antiinflammatory cytokines in DCs stimulated by various microorganisms including gram-negative bacteria, gram-positive bacteria, and yeast.

In conclusion, DCs stimulated by microorganisms can produce immunoregulatory cytokines and they play a key role in the host defense against infections. A better understanding of the interaction between DC and pathogenic microorganisms will not only offer insights into pathogenesis but will also provide new strategies in immunotherapies. These findings may help the development of DC vaccines against infection agents.

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