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Dendritic Cells post-Maturation Are Reprogrammed with Heightened IFN- γ and IL-10

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Abstract

Mature Dendritic cells (mDCs) undergo “exhaustion” in producing cytokines. Nevertheless, whether this “exhaustion” of mDCs is selective to certain cytokines, or whether mDC have specific cytokine-producing profiles has yet to be defined. Herein, we investigated the cytokine production in vitro by immature DCs (iDCs) and LPS-induced mDCs. Compared to iDCs, mDCs produced comparable levels of IL-6 and TNF- α . Strikingly, mDCs produced significantly higher IFN- γ and IL-10. IL-12 production of mDCs was suppressed. Kinetic studies of the responses of iDCs and mDCs to LPS or CD40L showed that mDCs acquired progressively heightened activity in producing IFN- γ and IL-10. TNF- α , IL-6-producing capability of mDCs was maintained. Nevertheless, IL-12 production by mDCs was not recovered at any time point. Mature DCs were potent in priming both Th1 and Th2 cells. In conclusion, upon maturation, DCs are reprogrammed with a distinct cytokine-secreting profile, which may play an important role in regulating T cell functions.

Keywords

Dendritic cells; immature/mature; Th1/Th2 cells; cytokines

INTRODUCTION

T cell response induced by dendritic cells (DCs) is determined, to some extent, by the cytokines secreted by DCs. Upon activation, DCs produce various cytokines including pro-inflammatory cytokines, such as IL-12, IL-6 and TNF- α , as well as anti-inflammatory cytokines, such as IL-10 (1–3). It is well known that IL-12 promotes Th1 (1) and IL-10 facilitates Th2 (2) and Tr1 differentiation (3,4). Recently, IL-6 was demonstrated to play an important role in initiating Th2 differentiation (5,6). It was also demonstrated that IL-6 secreted by DCs could inhibit the suppressive function of regulatory T cells (7,8).

Mature DCs (mDCs) are the most potent antigen-presenting cells and actively stimulate T cells via DC-T cell interaction (1,2,9). Phenotypic characteristics, such as high-level expression of MHC and B7 co-stimulatory molecules on mDCs are important for activating T cells (1,2,10), the cytokines from DCs during DC-T cell interaction are also important for directing T cell differentiation (1–3). It was described that human monocyte-derived mDCs lost their function in producing cytokines such as IL-10 and IL-12 (11,12), which has been designated as an “exhaustion” (11,13). Those DCs predominantly induce Th2 cell differentiation (11). This

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concept has led to a recently proposed strategy of using short-term stimulated DCs which maintain IL-12-producing activity for cancer immunotherapy (14,15). Nevertheless, whether this “exhaustion” state of mDCs is permanent or reversible is yet unclear. It is also unknown whether this “exhaustion” is selective for certain cytokines. The answer to these questions will provide an important insight into the nature of mDC-T cell interaction and T cell responses induced by mDCs.

The evidence shown in this study indicates that DCs post-maturation are not exhausted, but reprogrammed with a distinct cytokine-secreting profile, especially the acquired heightened IFN- γ and IL-10 production in response to LPS or CD40L stimulation. Post maturation DCs are potent in stimulating T cell proliferation and inducing differentiation of both Th1 and Th2 cells.

MATERIALS AND METHODS

Mice

8-12-w-old Balb/cByJ (H-2d) were purchased from specific pathogen-free Mouse Colony at Department of Pathology, Immunology, Laboratory Medicine, University of Florida. DO11.10 TCR-transgenic mice specific for OVA₃₂₃₋₃₃₉ were purchased from Jackson Laboratory.

Dendritic Cell culture

Bone marrow cells were obtained from femurs and tibias of mice. Two million of bone marrow cells after RBC lysis were cultured in 2 ml culture RPMI1640-10%FCS in the presence of GM-CSF (10 ng/ml) and IL-4 (5 ng/ml) (PeproTech, Cherry Hill, NJ) in one well of 12-well plate. Half of the media volume was changed every other day. On day 5, 0.5 μ g/ml of LPS (Sigma, St. Louis, MO) was added to part of the cultures for 24 h to make mDCs.

Immature DCs and mDCs were labeled with CD11c-microbeads (Miltenyi Biotech) and isolated by magnetic cell sorting. The purity of CD11c⁺ cells was around 95%. Immature DCs or mDCs were maintained in one well of 12-well plate with 2 ml of media containing GM-CSF (10 ng/ml) for 2 days. Then, the DCs (5×10^4) above were stimulated with LPS (0.5 μ g/ml) in 200 μ l of RPMI1640-10%FCS for 24 h. The supernatants were harvested and stored at -20°C for cytokine assay at later time.

For kinetic studies, iDCs or mDCs (5×10^4) were stimulated with LPS (0.5 μ g/ml) or CD40L (1 μ g/ml) (R & D systems, Inc) for 24 h in 200 μ l of media containing GM-CSF (10 ng/ml) in one well of 96-well plate at different time points (day 0–3). Day 0 was defined as a time point right after primary LPS stimulation for making mDCs. GM-CSF was added to the cultures to keep DCs survival. The supernatants were harvested and stored at -20°C for cytokine assay at later time.

To exclude the possibility of NK cell contamination in the purified DCs for this study, we stained the cells from the DC culture before and after CD11c⁺ cell purification using anti-CD11c-APC (HL3) and anti-mouse NK-T/NK cell antigen (U5A2-13)(BD-PharMingen). The Stained cells were analyzed by flow cytometry.

Isolation of CD4⁺ T cells from mouse spleen

CD4⁺ spleen T cells were prepared by negative selection using CD4⁺ T cell enrichment cocktail from StemCell Technologies Inc (Vancouver, Canada) according to the instructions from manufacturer. The purity of CD4⁺ T cells was consistently around 95%.

MLR

The stimulators in MLR were Balb/c mouse bone marrow-derived iDCs, semi-mDCs having been treated with 0.5 μ g LPS for 6 h, and mDCs having been treated with LPS for 24 h and rested for 2 days. Different cell concentrations of DCs were cultured with 1×10^5 CD4⁺ T cells from DO11.10 mice in each well of a U-bottom 96-well plate in 200 μ l RPMI1640-10% FCS in the presence 5 μ g/ml of OVA₃₂₃₋₃₃₉ for 6 days. [³H]-thymidine (1 μ Ci) was added to each well for the last 16 h. The cells were harvested using PHD cell harvester. Thymidine incorporation was determined by scintillation counting. All experiments were performed in triplicate incubations.

For CD4⁺ T cell priming, Balb/c DCs (1×10^4) mentioned above were incubated with CD4⁺ T cells (1×10^5) from DO11.10 mice in each well of a U-bottom 96-well plate in 200 μ l RPMI1640-10%FCS in the presence of 5 μ g/ml of OVA₃₂₃₋₃₃₉ for 5 days. The supernatants in the above cultures were harvested and stored at -20 °C for cytokine assay at later time.

Cytokine assay by Luminex

The cytokines produced by DCs and CD4⁺ T cells were measured by Luminex using Beadlyte Mouse Multi-Cytokine Detection System 2 (Upstate, Lake Placid, NY) according to the protocol from the manufacturer.

Real-time PCR

Purified iDCs and mDCs which had been treated with LPS (0.5 g/ml) for 24 h and then rested for 2 days were stimulated with LPS (0.5 g/ml) for 8 h and 20 h. Total RNA from DCs was extracted using Qiagen's RNeasy mini kit following manufacturer's suggestions (Qiagen, Valencia, CA). Total RNA was reverse transcribed using TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in duplicate by real-time PCR using Taqman Gene expression assay kits meant for GAPDH, IFN- α , IL-10 and IL-12 (Applied Biosystems, Foster City, CA). The mRNA level of each target molecule was normalized relative to GAPDH mRNA expression. Data were presented as the fold increase relative to iDCs, mDCs, iDC at 8h for IL-10, IL-12, and IFN- γ expression, respectively.

RESULTS AND DISCUSSION

Previous report demonstrated that LPS-stimulated mDCs lost their response to stimulation for cytokine production (11). To access whether this unresponsiveness of mDCs is permanent or temporary, mouse bone marrow-derived DCs developed with GM-CSF and IL-4 were stimulated with LPS for 24 h, then maintained in media containing GM-CSF for additional 2 days. GM-CSF has been shown to be a critical survival factor for DCs (16), and was therefore used in this experiment. The iDCs and mDCs that have been rested for 2 days post LPS stimulation were stimulated with LPS for 24 h. The results of the cytokine assay for the above cultures shown in Figure 1A demonstrated that upon LPS stimulation, mDCs produced significantly higher level of IL-10 and IFN- γ in contrast to iDCs. mDCs and iDCs secreted comparable level of IL-6 and TNF- α . Consistent with the previous report (11,12), mDC largely lost IL-12 production. We examined gene expression of IL-10, IL-12 and IFN- γ using real-time PCR. The results confirm the protein assay results (Fig 1B), and suggest that cytokine production may be regulated through regulation of gene transcription. The data shown above suggest that after 2 days rest, mDCs may be partially recovered for further cytokine production. However, the different cytokine-producing profile of mDCs from iDCs suggests that after maturation, DCs are reprogrammed with a distinct cytokine-producing profile. To exclude a possibility of contamination of NK cells that might produce large amount of IFN- γ , we measured NK cells by staining the cultured cells before and after DC purification. We found that there was almost no NK cell contamination in DCs after magnetic cell sorting using CD11c-

microbeads. Surprisingly, almost all CD11c⁺ DCs expressed NK cell antigen (Fig 1C), which is of interest and needs to be further investigated.

To determine whether mDCs undergo kinetic change in cytokine production, we conducted kinetic studies for mDCs and iDCs using LPS stimulation at different time points (day 0–3). We found that mDCs produced higher IL-10 and IFN- γ than iDCs upon LPS stimulation even at day 0. Immature DCs produced low level of these two cytokines in response to LPS stimulation at any time point. However, mDCs produced progressively heightened IL-10 and IFN- γ with time (Fig 2). Although IFN- γ is well known as the signature cytokine of T and NK cells, recent studies demonstrated that DCs also produced fair amount of IFN- γ (17–20). Most of these studies showed that IFN- γ production by DCs was in IL-12- or IL-18-dependent manner in which activation of STAT4 (20) and induction of T-bet (19) may be involved. To our knowledge, the data shown in the current study provides the first evidence that DCs acquire heightened IFN- γ - and IL-10-producing capability post maturation. As shown in previous reports (11,12) and data of ours (Fig 1), mDCs lose the capability of producing IL-12. It appears therefore, IL-12 does not play a critical role in induction of IFN- γ for mDCs. Furthermore, even if iDCs produced higher level of IL-12 upon LPS stimulation, they only produced low level of IFN- γ (Fig 1), suggesting that autocrine IL-12 may not be necessary for DCs to make IFN- γ in our culture system. It is well known that mDCs are highly effective in priming Th1 cells. Although it has been suggested the potent Th1-priming activity of mDCs is possibly through other cytokines or certain B7 and TNF family members (10,21,22), the definitive factors are still unclear. The feature of mDCs in producing high level of IFN- γ could play a role in facilitating Th1 differentiation. In addition, IFN- γ -producing capability gained by mDCs may play a role in promoting the recovery of DCs from LPS stimulation (23).

Kinetic observation of TNF- α production demonstrated that mDCs undergo a transient reduced responsiveness to the following stimulation in production of TNF- α and recovered later (Fig 2 and 3). Enhanced IFN- γ production by mDCs may play a role in this recovery (23). TNF- α is an important pro-inflammatory cytokine, and involved in many pathological processes (24). During mDC-T interaction, high level TNF- α produced by mDCs may play an important role in regulating T cell differentiation, particularly in Th1 cell development (25).

It is of interest that mDCs maintain high-level IL-6 production (Fig 1–3). IL-6 is a pleiotropic cytokines. Pasare et al (7) reported a toll pathway-dependent blockade of CD4⁺CD25⁺ T cell-mediated suppression by DCs was through IL-6 from DCs. Fehervari et al (8) reported that mDCs reversed Treg's suppression partially through IL-6. Recent evidence also shows that early IL-6-induced signal through initiating c-Maf expression, along with TCR-initiated signals is important for initiating polarization of Th2 cells (6). Thus, high amount of IL-6 production by mDCs may exert important functions on regulation of T cell responses. On the other hand, Hegde et al (26) reported that IL-6 produced by DCs acted as an anti-inflammatory cytokine partially through inducing production of IL-10. Whether enhanced IL-10 production by mDCs in the current study is through autocrine IL-6 needs to be further investigated. Thus, high level of IL-6 produced by mDCs may play an important role in orchestrating immune responses via acting on both DCs and T cells.

To mount effective adaptive immunity, it is required that DCs as antigen-presenting cells mature through activation by microbial stimuli or other environmental factors before interacting with antigen-specific T cells. Therefore, the properties of mDCs, particularly the cytokine-secreting profile during mDC-T cell interaction would be more important in determining T cell differentiation (13). One of the most important cross-talks between DCs and T cells is the CD40-CD40L ligation during which DCs are further stimulated by CD40L on T cells and secrete cytokines. Consequently, T cell responses are regulated. To mimic this immunological process, we investigated the kinetic cytokine production by mDCs in the

presence of soluble CD40L. At the same time, we applied LPS as a control to determine whether CD40L stimulation behaves the same way as LPS. The results shown in Fig 3 demonstrated that upon CD40L stimulation, mDCs had the pattern of cytokine production similar to that induced by LPS stimulation. However, iDCs had significantly lower response in production of all cytokines investigated in response to CD40L than LPS stimulation, perhaps because of lower expression of CD40 on iDCs. With regard to mDCs, compared with LPS, CD40L stimulated comparable level of IFN- γ and IL-6, but lower IL-10 and TNF- α (Fig 3). CD40L stimulated very low or even undetectable level of IL-12 in both iDCs and mDCs (data not shown). It is obvious that DCs matured by LPS maintain cytokine-producing activity in response to not only LPS, but other activation factors, such as CD40L. High level of IFN- γ and IL-6 produced by mDCs under stimulation of CD40L suggests that these cytokines may play important roles in regulating Th1 and Th2 responses during DC-T cell interaction.

It has been demonstrated semi-mDCs by short-term stimulation (<8h) maintain IL-12-producing activity and predominantly prime Th1 cells (11–13). It is therefore, suggested that semi-mDCs would be more useful for cancer immunotherapy (14,15). Thus, we finally compared the potency of Balb/c bone marrow-derived iDCs, semi-mDCs which had been treated with LPS for 6 h and mDCs which had been treated with LPS for 24 h and rested for 2 days in stimulation of DO11.10 mouse CD4⁺ T cells in the presence of OVA_{323–339}. From the T cell proliferation in MLR, it was noted that mDCs were the most potent stimulator of CD4⁺ cells, i.e. T cell proliferation induced by mDCs was dramatically higher than that induced by semi-mDCs and iDCs (Fig 4A). As expected, the T cell proliferation induced by iDCs was the lowest. Although semi-mDCs were less potent than mDCs, they were more potent than iDCs in stimulation of T cells (Fig 4A). In addition, we found that mDCs were most active in priming both Th1 and Th2 cells (Fig 4B), which was inconsistent with the results as previously described (11,12). The recovery of some cytokine production and acquired heightened IFN- γ and IL-10 by mDCs (Fig 1–3) could have contributed to this difference. Also, mouse DCs in the current study and human DCs as previously reported (11,12) may behave differently. This issue is being under investigated.

In conclusion, mDCs remain response to the following stimulation in cytokine production. However, the cytokine-producing profile is altered. The initial IFN- γ production by mDCs during DC-T cell interaction may play an important role in initiating Th1 differentiation, while increased IL-6 and IL-10 may facilitate Th2 polarization as well as regulate regulatory T cell function.

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Fig. 1. Cytokine production by iDCs and mDCs

A, Balb/c mouse bone marrow cells were cultured in media containing 10%FCS, GM-CSF and IL-4 for 5 days. Thereafter, 0.5 μ g/ml LPS was added to part of the culture for 24 h. CD11c⁺ DCs were purified using CD11c-microbeads by magnetic cell sorting. The purified cells were plated in media containing GM-CSF for additional 2 days. Immature and mDCs (5×10^4) were stimulated with LPS (0.5 μ g/ml) for 24 h in 200 μ l media in 96-well plate. Each incubation was in duplicate. IL-6, -10, -12, TNF- α and IFN- γ in the supernatants were measured by Luminex. Black and gray bar represents iDCs and mDCs, respectively. The similar results were obtained from five independent experiments and C57BL/6 mouse strain as well. **B**, DCs were prepared as described above. Immature DCs or mDCs were stimulated with LPS (0.5 μ g/ml) for 8h and 20 h. The gene expressions of IL-10, IL-12 and IFN- γ were detected by Taqman Gene expression assay kits. The data were presented as increase-fold relative to the expression of IL-10, IL-12, and IFN- γ induced by 8 h LPS-stimulation for iDCs, mDCs, iDCs, respectively. One out of two independently performed experiments is shown. **C**, NK cells and DC purity of the cultured cells before and after CD11c⁺ cell purification.

Fig. 2. Kinetic cytokine production by iDCs and mDCs

Immature DCs and mDCs were prepared as described in Fig 1 legend. Immature DCs and mDCs (5×10^4) were maintained in 200 μ l of media containing GM-CSF (10 ng/ml) and stimulated with LPS (0.5 μ g/ml) at different time points (day 0–3) for 24 h. Day 0 is defined as the time point right after primary LPS stimulation without rest. Each incubation was in duplicate. The production of IL-6, -10, -12, TNF- α and IFN- γ by DCs was measured by Luminex. Representative data of at least four separate experiments are shown.

Fig. 3. Comparison of kinetic cytokine production by iDCs and mDCs induced by LPS and CD40L Immature DCs and mDCs were prepared as described in Fig 1 legend. Immature DCs and mDCs (5×10^4) were maintained in 200 μ l of media containing GM-CSF (10 ng/ml) and stimulated with LPS (0.5 μ g/ml) or CD40L (1 μ g/ml) at different time points (day 0–3) for 24 h. Each incubation was in duplicate. The cytokine production by DCs was measured by Luminex. Data from one of three separately performed experiments are shown.

Fig. 4. T cell stimulatory activity of iDCs, semi-mDCs and mDCs

A, Different concentrations of Balb/c bone marrow-derived DCs including iDCs, semi-mDCs having been treated with LPS for 6 h and mDCs having been treated with LPS for 24 h and rested for 2 days were incubated with 1×10^5 CD4⁺ T cells from DO11.10 mice in the presence of 5 μ g/ml OVA₃₂₃₋₃₃₉ in each well of U-bottom 96-well plate in 200 μ l media for 6 days. [³H]-thymidine (1 μ Ci/well) was added into each well and incubated for the last 16 h. All experiments were performed in triplicates. The cells were harvested using a PHD cell harvester. The incorporation of [³H]-thymidine was determined by scintillation counting. **B**, The above different DCs (1×10^4) were separately incubated with 1×10^5 CD4⁺ T cells from DO11.10 mice in the presence of 5 μ g/ml OVA₃₂₃₋₃₃₉ in each well of U-bottom 96-well plate in 200 μ l media for 5 days. T cell cytokines, IL-4, IL-10 and IFN- γ in the supernatants were measured by Luminex. The similar results were obtained from two independently performed experiments.