Interferon γ Stimulates Cellular Maturation of Dendritic Cell Line DC2.4 Leading to Induction of Efficient Cytotoxic T Cell Responses and Antitumor Immunity

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Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) for the initiation of antigen (Ag)-specific immune responses. In most studies, mature DCs are generated from bone marrow cells or peripheral monocytes; in either case, the harvested cells are then cultured in medium containing recombinant GM-CSF, IL-4 and TNF-α for 7-10 days and stimulated with lipopolysaccharide (LPS). However, this approach is time-consuming and expensive. There is another less cost approach of using immobilized DC cell lines, which can easily grow in the medium. A disadvantage with the immobilized DC cell lines, however, is that they are immature DCs and lack expression of MHC class II and costimulatory CD40 and CD80 molecules. This, therefore, limits their capacity for inducing efficient antitumor immunity. In the current study, we investigated the possible efficacy of various stimuli (IL-1β, IFN-γ, TNF-α, CpG and LPS) in converting the immature dendritic cell line DC2.4 to mature DCs. Our findings were quite interesting since we demonstrated for the first time that IFN-γ was able to stimulate the maturation of DC2.4 cells. The IFN-γ-activated ovalbumin (OVA)-pulsed DC2.4 cells have capacity to upregulate MHC class II, CD40, CD80 and CCR7, and to more efficiently stimulate in vitro and in vivo OVA-specific CD8+ T cell responses and antitumor immunity. Therefore, IFN-γ-activated immortal DC2.4 cells may prove to be useful in the study of DC biology and antitumor immunity. Cellular & Molecular Immunology. 2007;4(2):105-111.

Key Words: IFN-γ, DC2.4 line, DC maturation, CTL, antitumor immunity

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) for the initiation of antigen (Ag)-specific immune responses. They migrate as precursors from the bone marrow into various organs, where they usually reside in an immature state. Within internal organs, they can efficiently capture and process Ag (1). The microenvironment wherein DCs acquire their Ag determines their potential immunogenicity. For example, at sites of bacterial infection, DCs are activated by “danger signals”, including endogenous (e.g., IL-1β, tumor necrosis factor alpha [TNF-α], interferon gamma [IFN-γ], CD40L) and exogenous (e.g., lipopolysaccharide [LPS] and CpG) mediators, and undergo a programmed maturational response wherein they upregulate MHC class II and costimulatory molecules important for activation of T cells (1). Upon activation, they undergo a differentiation process that results in decreased Ag-processing capacity, enhanced expression of major histocompatibility complex (MHC) class I and class II, costimulatory and adhesion molecules, and upregulated chemokine receptor CCR7. These cells then migrate to secondary lymphoid tissues to interact with and activate naïve T cells.

Based on the above-mentioned properties of DCs, namely i) the potent Ag-presenting ability of immature DCs and ii) the particular capacity of mature DCs to initiate T cell responses, an important avenue of investigation is the potential function of DCs in modulating immune mechanisms such as the induction of tumor immunity. Many reports have shown that DCs pulsed with tumor-derived MHC class I-restricted peptides or tumor lysates can induce significant cytotoxic T lymphocyte (CTL)-dependent antitumor immune responses in vitro as well as in vivo (2). DCs loaded with tumour Ags have...
become the centre piece of clinical trials testing active immunotherapy strategies. Important variables include the source of DCs, the choice of antigens, the method of antigen loading and the route and timing of administration (3, 4). A variable that recently has been receiving particular attention is the maturation status of DCs. This is due to observations from in vitro studies and animal models demonstrating that mature DCs induce more potent antigen-specific T cell responses than their immature counterparts (5, 6). Furthermore, preliminary observations in human studies suggest that immature DCs might actually down-regulate antigen-specific T-cell responses (7, 8).

The immune system can recognize microbes through Toll-like receptors (TLRs), which constitute an evolutionarily conserved family of receptors expressing on a variety of innate immune cells, including DCs (9-11). TLRs have broad specificity for conserved molecular patterns shared by large groups of pathogens (e.g., LPS in Gram-negative bacteria, bacterial CpG DNA). For example, LPS from E. coli signals through TLR4 (12), while TLR9 recognizes certain types of CpG-rich DNA (13). Maturation of DCs can be triggered by multiple stimuli, including proinflammatory cytokines such as IL-1β, IFN-γ, and TNF-α, unmethylated CpG oligonucleotides, poly(I:C) and CD40 ligand (14-16). LPS is another potent inducer of DC maturation, and it also facilitates the production of inflammatory cytokines and immunologically important cell-surface molecules by DCs and enhances their Ag presentation (17).

In the present study, we investigated the putative effect of various stimulatory agents (IL-1β, IFN-γ, TNF-α, CpG and LPS) on maturation induction of an immature DC cell line DC2.4. We found that IFN-γ signaling was able to induce cellular maturation of DC2.4 cells leading to stimulation of efficient CD8+ T lymphocyte responses and antitumor immunity.

Materials and Methods

Reagents and animals

Ovalbumin (OVA) and LPS were obtained from Sigma (St. Louis, MO). Recombinant mouse IL-1β, TNF-α and human IFN-γ were obtained from R&D Systems (Minneapolis, MN). Unmethylated CpG oligonucleotide CpG-ODN1826 was purchased from Qiagen (Alameda, CA). Biotin-conjugated anti-mouse H-2Kb, la-b, CD11c, CD40, CD54, CD80, CC chemokine receptor 7 (CCR7), TLR4 and TLR9 antibodies (Abs) were purchased from PharMingen (San Diego, CA). Phycoerythrin (PE)-labeled H-2Kb/OVA257-264 tetramer and FITC-labeled rat anti-mouse CD8 (Clone: KT15) antibody were obtained from Beckman Coulter (San Diego, CA). The anti-OVA peptide major histocompatibility complex (pMHC) I Ab (18) and the mouse dendritic cell line DC2.4 (19) were obtained from K. Rock (Dana-Farber Cancer Institute, Boston, Mass). The mouse OVA-specific B16 melanoma cell line BL6-10OVA was generated in our laboratory (20). Female C57BL/6 (H-2Kb) and BALB/c (H-2Kd) mice were obtained from Charles River Laboratories (St. Laurent, Quebec, Canada). The OVA-specific T cell receptor (TCR) transgenic OT I mice were obtained from Jackson Laboratory (Bar Harbor, MA). All mice were housed in the animal facility of the Saskatoon Cancer Center. All animal experiments were carried out according to the guidelines of the Canadian Council for Animal Care.

Cell culture

DC2.4 cells were cultured in RPMI 1640 medium supplemented with L-glutamine (2 mM), streptomycin (100 μg/ml), and penicillin (100 U/ml), non-essential amino acid (100 μM), 2-mercaptoethanol (2-ME, 50 μM) and 10% fetal calf serum (FCS). To assess the potential effect of various stimulants on DC2.4 cell maturation, DC2.4 cells were also cultured in the above medium with recombinant IL-1β (20 ng/ml), IFN-γ (20 ng/ml), TNF-α (20 ng/ml), LPS (1 μg/ml), and CpG (5 ng/ml), respectively, for 24 h, and then analyzed by flow cytometry. DC2.4 cells cultured in the above medium with i) OVA (0.5 mg/ml) and ii) OVA (0.5 mg/ml) plus IFN-γ (20 ng/ml) were referred to as DC2.4OVA and DC2.4IFNOVA, respectively.

Immuno phenotypic analyses

For phenotypic analysis, DCs were stained for 30 min on ice with biotin-conjugated Abs specific for H-2Kb, la-b, CD11c, CD40, CD54, CD80, CCR7 and pMHC I (each, 5 μg/ml), washed three times with phosphate-buffered saline (PBS), and then incubated for an additional 30 min on ice with FITC-conjugated streptavidin. After three more washes with PBS, the cells were analyzed by flow cytometry. Isotype-matched monoclonal antibodies were used as controls.

Quantitation of cytokine secretion

The DC2.4 culture supernatants were measured for expression of mouse IL-6, IL-12, IFN-γ and TNF-α. Quantitation of secreted cytokines was done in an enzyme-linked immuno-sorbent assay (ELISA) using the respective cytokine ELISA kits (BD Biosciences, San Diego, CA). The results were normalized to the known standard curves.

In vivo DC2.4 cell migration assay

DC2.4 or DC2.4IFNγ were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE) (0.5 μM, Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) at 37°C for 20 min, and washed twice with PBS, resuspended in 10 ml warm media with fetal bovine serum and incubated at 37°C for 30 min, and washed three times with PBS. CFSE-labeled DC2.4 or DC2.4IFNγ (1 × 106 cells per mouse) was s.c. injected into the hind footpads of C57BL/6 mice. One day later, the draining lymph node cells from the recipients were harvested and analyzed by flow cytometry.

In vitro T cell proliferation assay

Naïve CD8+ T cells were prepared from splenocytes of wild-type BALB/c and transgenic OT I mice, enriched by
In vivo T cell proliferation assay
For in vivo T cell proliferation assays, naïve C57BL/6 mice were i.v. immunized with DC2.4_OVA and DC2.4_IFN/OVA (1 × 10^6 cells per mouse), respectively. Blood was taken from the tail of mice 6 days after the immunization. The blood samples were incubated with 10 μl PE-conjugated H-2K^b/ OVA257-264 tetramer and 1 μl FITC-conjugated anti-CD8 Ab for 30 min at room temperature. The erythrocytes were then lysed using lysis/fixated buffer. The cells were washed and analyzed by flow cytometry. In another set of experiments, naïve OT I CD8^+ T cells (2 × 10^6 cells per mouse) were labeled with CFSE (1.5 μM) and i.v. injected into C57BL/6 mice. One day later, each mouse was s.c. immunized with DC2.4_OVA or DC2.4_IFN/OVA (1 × 10^6 cells per mouse). Three days after the immunization, the draining lymph node cells from the recipients were harvested and analyzed by flow cytometry.

Results

IFN-γ signaling stimulates DC2.4 cellular maturation
To assess the maturation status of the DC2.4 cell line, DC2.4 cells were subjected to flow cytometric analysis. As shown in Figure 1, DC2.4 cells displayed expression of MHC class I (K^b), CD11c (DC marker) and CD54, but a low amount of CD80 and no expression of MHC class II, CD40 and CCR7 molecules, indicating that they are an immature DC cell line. The degree of differentiation or maturation of DCs largely determines their functional capabilities. Immature DCs have maximal Ag processing capabilities while mature DCs expressing enhanced levels of MHC class II (Ia^b) and costimulatory molecules are more effective at T cell sensitization (22). To stimulate DC2.4 maturation, we...
incubated the DC2.4 cell line in a culture medium containing various stimuli (IL-1β, TNF-α, IFN-γ, CpG and LPS) for 24 h. After culturing, the DC2.4 cells were analyzed by flow cytometry. The immature phenotype of DC2.4 cells cultured in presence of IL-1β, TNF-α, CpG and LPS remained unchanged (data not shown). However, DC2.4 cells cultured in the presence of IFN-γ displayed upregulation of MHC class II, CD40, CD80 and CCR7 (Figure 1), indicating that IFN-γ-stimulated DC2.4 cells and converted them into mature DCs. In addition, IFN-γ-treated and OVA-pulsed DC2.4 cells also expressed pMHC I complexes. Over and above the phenotypic characteristics, IFN-γ-stimulated DC2.4 cells also upregulated expression of the cytokines IL-6, IL-12, IFN-γ and TNF-α (Figure 1B).

**IFN-γ-stimulated DC2.4 induce enhanced T cell proliferation in vitro**

DCs are potent stimulators of primary MLRs and can induce the proliferation of allogeneic CD8+ T cells *in vitro*. Just as stimulation of antigen-specific T cell responses by DCs is much influenced by their maturation status, so too is stimulation of primary MLRs. As shown in Figure 2 (left panel), the mature DC2.4IFN induced stronger allogeneic CD8+ T cell proliferative responses *in vitro* than the immature DC2.4 cells. We also compared the efficacy of the 2 respective DC2.4 populations in stimulating autologous OT I CD8+ T cells. As shown in Figure 2 (right panel), the mature OVA-pulsed DC2.4IFNOVA cells induced stronger autologous OT I CD8+ T cell proliferative responses *in vitro* than the immature OVA-pulsed DC2.4OVA cells, while the DC2.4IFN cells induced essentially no proliferative responses of CD8+ T cells.

**IFN-γ-stimulated DC2.4 are capable of migrating to the draining lymph nodes**

To investigate the *in vivo* migratory capacity, DC2.4 and DC2.4IFN cells were labeled with the fluorescent dye CFSE, then injected s.c. into the hind footpad of mice. One day later, draining lymph nodes were isolated and the total number of CFSE+ cells was measured by flow cytometric analysis. As shown in Figure 3A, DC2.4IFN displayed a stronger migratory capacity compared with DC2.4, indicating that DC2.4 maturation promotes their capacity for migration into regional lymph nodes.

**IFN-γ-stimulated DC2.4IFNOVA induce T cell proliferation in vivo**

We then assessed the ability of DC2.4OVA and DC2.4IFNOVA to induce *in vivo* proliferation of CD8+ T cells, respectively. We first *i.v.* injected C57BL/6 mice with CFSE-labeled CD8+ T cells derived from transgenic OT I mice. One day later, the mice were *s.c.* immunized with DC2.4OVA and DC2.4IFNOVA, respectively. As shown in Figure 3B, the draining lymph node CD8+ T cells from mice with *s.c.* immunization of DC2.4IFNOVA had undergone as many as three cycles of division, whereas there was no evidence of proliferation in the draining lymph node cells from mice with *s.c.* immunization of DC2.4OVA, indicating that DC2.4IFNOVA are capable of migrating to the draining lymph nodes and stimulating proliferation of transferred CFSE-CD8+ T cells. To assess whether DC2.4IFNOVA, can stimulate host OVA-specific CD8+ T cell responses, C57BL/6 mice were *i.v.* immunized with DC2.4OVA and DC2.4IFNOVA, respectively. Blood samples were collected for double staining for PE-conjugated H-2Kb/OVA257-264 tetramer and CD8. The frequency of OVA-specific CD8+ T cells in the total peripheral CD8+ T cell population was determined. As shown in Figure 3C, DC2.4IFNOVA stimulated 3.56%
and 0.25% OVA-specific CD8^+ T cell responses in vivo, respectively, indicating that DC2.4_{IFN/OVA} was more effective at activating host OVA-specific CD8^+ T cell proliferation.

**Figure 3.** Functional characterization of IFN-γ-treated DC2.4 cell line. (A) DC2.4 migration to lymph nodes. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled DC2.4 or DC2.4_{IFN} were s.c. injected into C57BL/6 mice. One day later, the draining lymph node cells were analyzed by flow cytometry. (B) CFSE-labeled naïve OT I CD8^+ T cells were i.v. injected into C57BL/6 mice. One day later, each mouse was s.c. immunized with DC2.4_{OVA} or DC2.4_{IFN/OVA}. Three days after the immunization, the draining lymph node cells from the recipients were harvested and analyzed by flow cytometry. (C) In tetramer staining assay, C57BL/6 mice were i.v. immunized with irradiated DC2.4_{IFN/OVA}, DC2.4_{OVA}, and phosphate buffered saline (PBS), respectively. Six days after the immunization, the tail blood samples of immunized mice were stained with phycoerythrin (PE)-tetramer and FITC-CD8 Ab, then analyzed by flow cytometry. The mean value in each panel represents the percentage of tetramer-positive CD8^+ T cells versus the total CD8^+ T cell populations. The value in parenthesis represents the SD. One experiment of two is shown.

**Figure 4.** Vaccination of mice with IFN-γ-treated and ovalbumin (OVA)-pulsed mature DC2.4_{IFN/OVA}. C57BL/6 mice (n = 10) were vaccinated with OVA-pulsed DC2.4_{IFN/OVA} and DC2.4_{OVA}, and then challenged s.c. 10 days later with 3 × 10^5 BL6-10OVA tumor cells. Control mice were treated with PBS. The survival time of each mouse was monitored daily. *, p < 0.05 versus cohorts immunized with DC2.4_{OVA} (Log rank test). The animal experiment was repeated once and similar results were obtained.

**Discussion**

DCs are one of the most potent APCs for induction of antitumor immune responses and, as such, have been recognized as potentially important tools for cancer vaccine strategies (23, 24). To examine whether mature DC2.4_{IFN/OVA} cells could induce effective antitumor immunity in vivo, we vaccinated mice with OVA-pulsed DC2.4 from each population, and after 10 days challenged the animals with BL6-10OVA tumor cells. Challenge of mice with 3 × 10^5 BL6-10OVA tumor cells was invariably lethal within 3 weeks of implantation. On the other hand, prior vaccination of the mice with OVA-pulsed DC2.4_{OVA} was sufficient to provide moderate protection against challenge tumor growth (2/10 protected at 10 weeks). However, vaccination with IFN-γ-treated OVA-pulsed mature DC2.4_{IFN/OVA} protected most mice (6/10) from tumor cell challenge for up to 10 weeks (Figure 4), which is significantly more efficient than mice immunized with DC2.4_{OVA} (p < 0.05), indicating that DC2.4_{IFN/OVA} vaccine induces strong antitumor immunity.  

**DC2.4_{IFN/OVA} strongly induce protective tumor-specific immunity**

TLRs, a family of evolutionarily conserved receptors, which are type 1 integral membrane glycoprotein, play a crucial role in early host defense against pathogens (25-27). The signal pathway activated by TLR stimulation (including TIR domain-containing adaptors) involves IL-1 receptor-associated kinases, and TNF receptor-associated factor 6 (TRAF6) (28). TLR stimulation results in the activation of nuclear factor (NF)-κB and MAP kinases, leading to production of...
proinflammatory cytokines. LPS from *E. coli* signals through TLR4 (12), while TLR9 recognizes CpG-rich DNA (13). In this study, we demonstrated that LPS and CpG were unable to stimulate DC2.4 cell maturation, because the immature DC2.4 did not express TLR4 and TLR9 (data not shown), which were expressed on immature DCs freshly prepared from mouse bone marrow cells (29). The reason of loss of TLR4 and TLR9 expression in immature DC2.4 cell line is unclear.

Interestingly, we also demonstrated that IFN-γ signaling was able to stimulate cellular maturation of DC2.4 cell line. Although the innate immune response triggered by TLRs is necessary for host defense against pathogens, a type 1 T helper response (such as Th1 cytokine IFN-γ) may also contribute to the host defense. Responses to IFN-α/β and IFN-γ are vital for both innate and adaptive immunity (30, 31). IFN signaling involves a variety of trans- and cis-acting factors and is mediated through DNA motifs, designated the IFN-stimulated response element and IFN-γ-activated sequence, found in promoters of IFN-inducible genes (32). These genes play prominent roles in the control of growth, differentiation and activation of the immune system. More recently, it has been demonstrated that the IRF-8/IFN consensus sequence-binding protein is involved in TLR signaling and contributes to the cross-talk between TLR and IFN-γ signaling pathways (33).

The capacity of mature DCs to migrate into T-cell areas of lymph nodes for induction of a primary immune response is a key factor in initiating immunity (34). Recent studies have demonstrated that chemokines play a critical role in DC migration. The migratory capability of DCs is dictated by the change of DC responsiveness to various chemokines during their development and maturation. Immature DCs respond to macrophage inflammatory protein (MIP)-3α, regulated on activation, normal T expressed and secreted protein (RANTES) and MIP-1α via chemokine receptor CCR1, CCR5 and CCR6, whereas mature DCs respond to MIP-3β and CCR7. This, for example, is critically important in the migration of DCs from intestinal lamina propria to mesenteric lymph nodes (35). The down-regulation of receptors for the inflammatory cytokines and upregulation of CCR7 receptor for MIP-3β that is expressed in secondary lymphoid organs such as lymph nodes allows mature DCs to leave the sites of inflammation and to migrate to lymph nodes to activate T lymphocytes. In this study, we also demonstrated that, in addition to upregulation of MHC II, CD40 and CD80 expression, IFN-γ signaling was also able to upregulate CCR7 expression of DC2.4 cells. IFN-γ-treated and OVA-pulsed DC2.4 cells after s.c. vaccination were able to migrate to draining lymph nodes and stimulate OVA-specific CD8 T cell proliferation there, leading to induction of efficient antitumor immunity.

All in all, this study provides an alternative method of producing DCs which is cheaper and less time-consuming, i.e., the utilization of immobilized DC lines (36). This contrasts with most other studies in which mature DCs were generated from either bone marrow cells or peripheral monocytes cultured in medium containing recombinant GM-CSF, IL-4 and TNF-α for 7-10 days and stimulated with LPS (37, 38). However, despite the cost advantaged of immobilized DC lines, it has to be recognized that the DCs are all immature cells with no expression of MHC class II and costimulatory CD40 and CD80 molecules. This, therefore, limits their capacity for inducing efficient antitumor immunity. With the latter consideration in mind, the current study went on to demonstrate, for the first time, that IFN-γ signaling stimulates cellular maturation of DC2.4 cell line. The IFN-γ-activated DC2.4 cells upregulate the expression of MHC class II, CD40, CD80 and CCR7. Furthermore, they have the capacity to more efficiently stimulate *in vitro* and *in vivo* CD8 T cell responses and antitumor immunity. Therefore, IFN-γ-activated immortal DC2.4 cells may be useful in study of DC biology and antitumor immunity.

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