Enhancement of antitumor effect using dendritic cells activated with natural killer cells in the presence of Toll-like receptor agonist

Thanh Nhan Nguyen Pham\textsuperscript{1,2,6}, Cheol Yi Hong\textsuperscript{1}, Jung-Joon Min\textsuperscript{3,6}, Joon-Haeng Rhee\textsuperscript{4,6}, Truc-Anh Thi Nguyen\textsuperscript{1,2,6}, Byoung Chul Park\textsuperscript{7}, Deok-Hwan Yang\textsuperscript{1,2}, Young-Kyu Park\textsuperscript{1,5}, Hyeong-Rok Kim\textsuperscript{5,6,8}, Ik-Joo Chung\textsuperscript{1,2,6}, Hyeoung-Joon Kim\textsuperscript{2} and Je-Jung Lee\textsuperscript{1,2,6,8}

\textsuperscript{1}Research Center for Cancer Immunotherapy Chonnam National University Hwasun Hospital Jeonnam 519-809, Korea
\textsuperscript{2}Department of Hematology-Oncology
\textsuperscript{3}Department of Nuclear Medicine
\textsuperscript{4}Department of Microbiology
\textsuperscript{5}Department of Surgery
Chonnam National University Medical School
Gwangju 501-757, Korea
\textsuperscript{6}The Brain Korea 21 Project Center for Biomedical Human Resources at Chonnam National University
Gwangju 500-809, Korea
\textsuperscript{7}Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea
\textsuperscript{8}Corresponding author: Tel, 82-61-379-7638; Fax, 82-61-379-7628; E-mail, drjejung@chonnam.ac.kr

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Abbreviations: BM, bone marrow; CD40L, CD40 ligand; CTL, cytotoxic T lymphocyte; DC, dendritic cell; MACS, magnetic activated cell sorter; MFI, mean fluorescence intensity; MLR, mixed lymphocyte reaction; NK cell, natural killer cell; ROI, region of interest; STAT, signal transducers and activators of transcription; TLR, Toll-like receptor

Abstract

Dendritic cells (DCs) play a role in natural killer (NK) cell activation, while NK cells are also able to activate and mature DCs. Toll-like receptors (TLRs) on the surface of DCs and NK cells induce the maturation and activation of these cells when engaged with their cognate ligand. We investigated to generate potent DCs by maturation with NK cells in the presence of TLR agonist in vitro and tested the efficacy of these DC vaccinations in mouse colon cancer model. The optimal ratios of DCs versus NK cells were 1:1 to 1:2. Immature DCs were mature with NK cells in the presence of lipopolysaccharide, which is TLR4 agonist, and further addition of IL-2 induced phenotypically and functionally mature bone marrow-derived DCs. These potent DCs exhibited not only high expression of several costimulatory molecules and high production of IL-12p40 and IL-12p70, but also high allogeneic T cells stimulatory capacity, and the induction of the high activities to generate tumor-specific CTLs. Consistently, vaccination with these DCs efficiently inhibited CT-26 tumor growth in mouse colon cancer model when compared to other vaccination strategies. Interestingly, combination therapy of these DC-based vaccines and with low-dose cyclophosphamide showed dramatic inhibition effects of tumor growth. These results suggest that the DCs maturated with NK cells in the presence of TLR agonist are potent inducer of antitumor immune responses in mouse model and may provide a new source of DC-based vaccines for the development of immunotherapy against colon cancer.

Keywords: cancer vaccines; chemotherapy; dendritic cells; immunotherapy; natural killer cells; Toll-like receptor

Introduction

Dendritic cells (DCs)-based vaccines continue to be considered an attractive tool for cancer immunotherapy (Ridgway, 2003; Cranmer et al., 2004). Toll-like receptors (TLRs) are pattern-recognition receptors that trigger innate immune responses as well as the adaptive immune system by the induction of DC recruitment and maturation (Akira and Hemmi, 2003; Schuurhuis et al., 2006). Recently, TLRs have been implicated in pathogen recognition by NK cells. Human and murine NK cells constitutively express functional TLRs, produce IFN-\(\gamma\), and induce cytotoxic activities in response to TLR ligands (Chalifour et al., 2004; Schmidt et al., 2004; Sivori et al., 2004; Lauzon et al., 2006). In addition, although NK cells can be directly activated by TLRs, monocytes play an essential role in the activation of effector functions.
Figure 1. DC characteristics in maturation with NK cells. (A) Optimal ratio of DC versus NK cell. Immature DCs (iDCs) were co-cultured with NK cells at various ratios from 1:1 to 1:10. Culture supernatants were analyzed for IL-12p70 production. DCs matured with NK cells at a ratio of 1:2 produced higher level of IL-12p70 than other ratios (*, P < 0.05). The data from a representative of three independent experiments are shown. (B) Comparison of phenotype of mature DCs. iDCs were co-cultured with NK cells at ratio of 1:2 in the presence of LPS and/or IL-2. The expressions of I-Ad, CD40, CD80 and CD86 were analyzed by flow cytometry. DCNK+LPS+IL-2 were expressed the highest levels of these molecules. Shown are representative histograms demonstrating the expression of markers (shaded) compared to isotype controls (black line). (C) Bar graphs showed the mean % expression (+SD) from five independent experiments. *, P < 0.05 compared to DC LPS. There were no significant effects of tumor antigens (TA) on the expressions of surface molecules on DCs.

(TLR-induced stimulation of both DCs and NK cells plays a crucial role in inducing NK cells to activate immature DCs (iDCs) and to facilitate their maturation. NK-DC interactions have been shown to be important for optimal immune cell expansion and activation during viral infection in vivo (Andrews et al., 2003). In vitro interaction between NK cells and maturing DCs results in activation and cytokine production of both cell types (Osada et al., 2001; Ferlazzo et al., 2002; Gerosa et al., 2002; Moretta, 2002; Piccioli et al., 2002). The enhancement of IFN-γ production, cytotoxicity, CD69 expression and proliferation of NK cells in vitro by activated DCs has been reported (Osada et al., 2001; Ferlazzo et al., 2002; Gerosa et al., 2002; Moretta, 2002; Piccioli et al., 2002). Reversely, the DCs can be activated or matured by interactions with NK cells, resulted in increase cytokine production, co-stimulatory molecules expression or ability to stimulate T-cell. NK cells might profoundly influence DC function by lysis immature DCs and secrete cytokines such as IFN-γ, TNF-α, and GM-CSF, promoting the maturation of DCs (Ferlazzo et al., 2002; Moretta, 2002; Cooper et al., 2004). The activation of NK cells by IL-2 are potent...
DC activators, both alone or in synergy with inflammatory stimuli, such as lipopolysaccharide (LPS) (Gerosa et al., 2002; Piccioli et al., 2002). Activated NK cells could boost ongoing adaptive responses by producing IFN-γ, which promotes the Th1 polarization of antigen-specific T cells. In addition, NK cells may boost the activation and the T-cell stimulatory capacity of mature DCs. Activation of human DCs by NK cells results in the differentiation to a DC capable of inducing a more efficient Th1-type and CTL response (Mailliard et al., 2003; Kalinski et al., 2005).

Recent studies have shown that chemotherapeutic agents increase the efficacy of active or adoptive antitumor immunotherapies through beneficial immunomodulatory effects (Ghiringhelli et al., 2004; Mihalyo et al., 2004). Among them, cyclophosphamide (CPM) may eliminate the activities of tumor-induced suppressor T cells in tumor-bearing hosts (North, 1982) and induce the production of immunostimulatory cytokines, such as type I interferon (Proietti et al., 1998). In addition, low-dose cyclophosphamide treatment has been shown to down-regulate suppressor T cells and to decrease the production of TGF-β and IL-10 while inducing a Th2/Th1 shift in the cytokine profile (Berd et al., 1984; Matar et al., 2000, 2002).

The purpose of this study was to identify a new source of DC vaccines against colon cancer. To investigate the role of NK cells in DC maturation and cytokine production, DCs were co-cultured with NK cells at a ratio of 1:2 in the presence of indicated stimulation reagents. (A) All DCs further stimulated with CD40L-transfected J558 cells for IL-12p70 production. DC_NK+LPS+IL-2 showed significantly higher production of IL-12p70 than other DCs (*, $P < 0.05$). (B) Culture supernatants during maturation with NK cells were analyzed for IFN-γ production. IFN-γ production from DC_NK+LPS+IL-2 group was significantly higher than other groups (*, $P < 0.05$). IL-12p40 (C) and IL-12p70 (D) productions during maturation of DCs loaded with (gray bar) or without (black bar) tumor antigens (TA). Loading of tumor antigens onto DCs suppressed the production of IL-12p70 (*, $P < 0.05$), but not IL-12p40. The differences in suppression were not eliminated between the DC_NK+LPS+IL-2 group and other groups (*, $P < 0.05$). The data are shown as the mean (pg/ml) (± S.D) of triplicate cultures from two representative data sets from four independent experiments.
Figure 3. The allostimulatory capacity of DCs. Splenocytes (A) or CD90+ T cells (B) (50,000 cells/well) obtained from allogeneic mice were stimulated with graded doses of irradiated DCs for 5 days, and [3H]-methylthymidine was added 18 h before measurement of proliferative response. The stimulatory capacity of DCNK+LPS+IL-2 was significantly higher in splenocytes and T cells than other DCs (*, $P < 0.05$). Data shown are the mean cpm ($\pm$ S.D) of triplicate cultures from three independent experiments.

when engaged with TLR agonists, we used the nature of DC-NK cell interaction to induce the function of DCs to enhance their anti-tumor responses. Here we demonstrate that functionally potent DCs can be generated by co-culture with NK cells as helper cells in the presence of TLR agonist, and exert strong anti-tumor activity as a cancer vaccine.

Results

NK cells enhance maturation of DCs in the presence of TLR agonist

In the interaction between DCs and NK cells, the ratio of DCs and NK cells is important (Gerosa et al., 2002; Piccioli et al., 2002). To determine the optimal ratio of DCs versus NK cells, we co-cultured iDCs with NK cells at various ratios (1:1, 1:2, 1:5, and 1:10). The levels of CD40, CD80, CD86 and I-A$^d$ expression on mature DCs were markedly increased at the ratios of 1:1 to 1:2, and IL-12p70 was significantly increased at the ratio of 1:2 compared to the other ratios (Figure 1A and Supplemental data Figure S1). There were no significant differences in the production of IL-12p40 and IL-10 among all ratios (data not shown). Therefore, we chose the ratio of 1:2 for the subsequent experiments.

BM cells harvested on day 7 were typically and phenotypically differentiated to iDCs; they expressed intermediate levels of I-A$^d$, CD80 and CD86 and low levels of CD40. Mature DCs were generated by co-culture with the NK cells in the presence of the TLR4 agonist (DCNK+LPS), and markedly increased the expression of I-A$^d$, CD80, CD86 and CD40 (Figure 1B). The addition of IL-2 to DCNK+LPS (DCNK+LPS+IL-2) further increased the expression of these surface molecules. This pattern of the surface molecules indicated that mature BM-derived DCs were generated by co-cultured with NK cells in the presence of TLR agonist. Tumor antigen-pulsed DCs also showed the typical phenotype of mature DCs, suggesting that the antigen-loading strategy did not affect the maturation of DCs (data not shown). The comparison of five independent experiments showed the highest levels of surface marker expression on DCNK+LPS+IL-2 (Figure 1C).

DCs interaction with NK cells profoundly enhance cytokine production

To determine whether co-culture of iDCs with NK cells in the presence of TLR agonist could enhance cytokine production and Th1-promotion, we measured the levels of IFN-γ, IL-12p40 and IL-12p70 production during DC maturation and after subsequent stimulation of the DCs with CD40L-transfected J558 cells. Co-culture of iDCs with NK cells in the presence of TLR agonist (DCNK+LPS) profoundly enhanced the ability of maturing DCs to produce IL-12p40 and IL-12p70 during maturation, as well as IL-12p70 in response to subsequent CD40L stimulation as compared to LPS-primed DCs (DCLPS) (Figure 2A and Supplemental data...
Figure 4. Tumor antigen uptake of DCs and tumor specific IFN-γ secreting CTLs. (A) DCs were loaded with PKH26-labeled UVB-irradiated CT26 cells at a ratio of 2:1. CD11c+ mature DCs, identified by H-2k or CD80 expression, were analyzed for the uptake of PKH26 labeled tumor cells. DC NK+LPS and DCNK+LPS+IL-2 showed more efficiently take up irradiated tumor cells compared to DC LPS. The data from a single representative experiment are shown. (B) Autologous T cells were stimulated with irradiated-pulsed or unpulsed DCs. The CTLs were co-cultured with target cells (CT26 cells and CT26-pulsed-DCs), and were subjected to the IFN-γ release ELISPOT assay. The CTLs stimulated with the pulsed-DC NK+LPS+IL-2 demonstrated significantly higher IFN-γ-secreting cells against target cells than other CTLs (*, P < 0.05). A few IFN-γ secreting cells were produced when YAC-1 was used as target cells. The data are shown as the mean numbers of IFN-γ-secreting cells (± S.D) of triplicate culture from a representative data from three independent experiments.

Figure S2). Moreover, the productions of these cytokines were significantly increased in the presence of the NK cell-activating cytokine, IL-2 (Figure 2A and Supplemental Data Figure S2). Reciprocally, NK cells co-cultured with iDCs in the presence of TLR agonist and IL-2 (DCNK+LPS+IL-2) showed significantly increased production of IFN-γ compared to the other DCs (Figure 2B). These results were compatible with the hypothesis that DCs play a role in the activation of NK cells, while NK cells are also involved in the activation and maturation of DCs.

The ingestion of dying cells by APCs can markedly influence the enhancement or suppression of immune responses (Savill et al., 2002). To evaluate the effect of apoptotic tumor cells on the differentially mature DCs, we measured the levels of IL-12p40 and IL-12p70 production in tumor loaded DCs. Consistent with the immuno-suppressed status of tumor cell-pulsed DCs resulted in decrease the production of Th-1-polarizing cytokines (Savill et al., 2002), the level of IL-12p70, but not IL-12p40, were significantly suppressed as compared to unpulsed DCs (Figures 2C and 2D).

Maturation of DCs with NK cells in the presence of TLR agonist enhance the T cell stimulatory capacity

To evaluate the ability of mouse DC maturation with NK cells in the presence of TLR agonist to stimulate T cells, we performed an allogeneic MLR assay with both splenocytes and T cells. DCNK+LPS showed a potent allogeneic T cell stimulatory capacity in a dose-dependent manner based on the increasing number of DCs. The addition of IL-2 during the maturation of DCs with NK cells and TLR agonist (DCNK+LPS+IL-2) further augmented the MLR activity of these DCs (Figures 3A and 3B).

Maturation of DCs with NK cells in the presence of TLR agonist enhanced the efficiency of tumor antigen uptake and their ability to generate tumor-specific CTLs

To examine the ability to take up tumor antigens, we compared the level of PKH26-labeled-irradiated CT26 cell which can be taken up by DCs. DCNK+LPS showed a more efficient uptake of UV-irradiated tumor cells as compared to the DCs that were
Figure 5. DC vaccinations in mouse colon cancer model. CT-26 cells ($5 \times 10^5$/mouse) were injected subcutaneously into the right flank of BALB/c mice. (A) Tumor-bearing mice were vaccinated subcutaneously on day 3, 7, 11, and 15 with PBS (○), NK cells (●), pulsed-DC LPS (Δ), pulsed-DCNK+LPS (□), and pulsed-DC NK+LPS+IL-2 (◆). The mice vaccinated with DC LPS, DCNK+LPS, and DC NK+LPS+IL-2 significantly inhibited the tumor growths compared to NK cells and PBS control (*, $P < 0.05$ on day 15, 21, and 29). The DC NK+LPS+IL-2 group showed the highest inhibition of tumor growth with a significant prolonged survival rate (C). Each vaccination group was evaluated for survival rate. Experiments consisted of 10 mice per group. (B) Tumor-bearing mice in combination therapy group (DC NK+LPS+IL-2 + CPM) were intraperitoneally treated with 50 mg/kg of cyclophosphamide (CPM) on day 6 and then vaccinated subcutaneously with DCs on day 9, 13, 17, and 21. PBS group (○), cyclophosphamide alone (□), pulsed-DCNK+LPS+IL-2 (◆), and DCNK+LPS+IL-2 + CPM (■) were used for vaccination. Combination therapy of DCNK+LPS+IL-2 vaccine with low dose of cyclophosphamide resulted in markedly inhibited tumor growth (*, $P < 0.05$ on days 35 and 40) and prolonged survival rate (D). Each vaccination group was evaluated for survival rate every 3-4 days. Experiments consisted of 7 mice per group.

We examined the CTL responses of BALB/c purified T cells induced by mature DCs pulsed with or without apoptotic CT-26 tumor cells. Spleen T cells stimulated by tumor antigen-pulsed DCs were evaluated by CTL responses against CT26 target cell lines or CT26-pulsed-DCs. The CTLs stimulated by pulsed-DCs produced more IFN-γ-secreting cells than those stimulated by unpulsed DCs or unprimed T cells ($P < 0.05$). The CTLs that were generated by pulsed-DCNK+LPS+IL-2 produced more IFN-γ-secreting cells than other CTLs ($P < 0.05$). To evaluate the NK cell-mediated IFN-γ production, the NK cell-sensitive YAC-1 cell line was used as target cells for CTLs. These CTLs induced a few number of IFN-γ-secreting cells against the YAC-1 cells, indicating CTL-mediated responses rather than NK cell-mediated responses (Figure 4B).
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DC maturation with NK cells in the presence of TLR agonist shows more potent antitumor effect in vivo

In this study, NK cells with a TLR agonist and IL-2 was the most effective combination for DC maturation to enhance the immune response in vitro. Therefore, we assessed whether these DCs enhanced antitumor immune responses in an animal model. All tumor-bearing mice vaccinated with PBS or NK cells did not inhibit rapid tumor growth that led to sacrifice within three weeks. By contrast, the tumor bearing mice vaccinated with DC_{LPS}, DC_{NK+LPS}, and DC_{NK+LPS+IL-2} significantly inhibited tumor growth compared to those with NK cells and PBS (P < 0.05 on Days 15, 21, and 29 vs. PBS or NK cell). The DC_{NK+LPS+IL-2} group showed the highest inhibition of tumor growth (P < 0.05 on Days 21 and 29 vs. pulsed-DC_{LPS} or pulsed-DC_{NK+LPS}) (Figure 5A) and a significant prolonged survival rate (Figure 5C). The survival of mice vaccinated with DC_{NK+LPS+IL-2} was significantly improved as compared to the pulsed-DC_{LPS} or pulsed-DC_{NK+LPS} vaccinated groups (35.2 days ± 2.9 vs. 31.2 days ± 3.1 and 28.8 days ± 3.1).

To examine the possible immunopotentiating effect to enhance the efficacy of DC_{NK+LPS+IL-2} vaccine, we assessed combination therapy of DC vaccine with immunomodulatory drug, cyclophosphamide. BALB/c mice were inoculated with CT-26 colon cancer cells on day 0, followed by treatment with a low-dose of cyclophosphamide on day 6, and then vaccination with DC vaccine at a 4 day interval from day 9. The treatment with cyclophosphamide alone had little effect on the inhibition of tumor growth (Figure 5B). The vaccination with DC_{NK+LPS+IL-2} alone showed a significantly inhibition of tumor growth and a significant prolonged of survival as compared to groups treated with PBS control or cyclophosphamide alone (P < 0.05). Treatment with a low-dose cyclophosphamide following with DC_{NK+LPS+IL-2} vaccination resulted in a more marked tumor inhibition markedly inhibited tumor growth, likely as tumor eradicated (Figure 5B) (P < 0.05). The survival of mice receiving the combination therapy was significantly prolonged compared to mice receiving the DC vaccine alone (Figure 5D).

To validate the therapeutic efficacy of the DCs, using a molecular imaging tool, the mice were injected with CT26-Fluc and evaluated luciferase signals emitted from the tumor cells using the IVIS machine. On days 12, 17, 22, and 27, the signals from the CT26-Fluc cells were detected in two mice per group. The CT26-Fluc-bearng mice vaccinated with DC_{LPS}, DC_{NK+LPS} and DC_{NK+LPS+IL-2} showed significant inhibition of tumor growth based on the lower of bioluminescence signals as compared to those vaccinated with the NK cells and PBS control (Figure 6A). Mice vaccinated with DC_{NK+LPS+IL-2} displayed the lowest luciferase bioluminescence signals among the DC vaccinated groups, indica-
DC vaccine increases the number of IFN-γ secreting lymphocytes. The number of IFN-γ secreting lymphocytes in spleens of mice treated with PBS, NK cell, DC LPS, DCNK+LPS and DC NK+LPS+IL-2 (n = 10) were counted in an IFN-γ ELISPOT assay. Vaccination with DC LPS and DCNK+LPS caused an increase in IFN-γ secreting lymphocytes, while the vaccination of DC NK+LPS+IL-2 led to a more dramatic increase in IFN-γ secreting lymphocytes (*P < 0.05). The results are expressed as mean numbers of IFN-γ-secreting cells (± S.D.).

Discussion

The presence of cross-talk between DCs and NK cells is well known in the context of immune responses to infectious agents and tumors (Ferlazzo et al., 2002; Gerosa et al., 2002; Moretta, 2002; Piccioli et al., 2002). Previous studies have shown that NK cells activated with TLR ligands result in the increased production of IFN-γ (Chalifour et al., 2004; Schmidt et al., 2004; Sivori et al., 2004; Hart et al., 2005; Lauzon et al., 2006), which may be a crucial factor for enhancing DC maturation in vivo or in vitro (Granucci et al., 2004). In addition, DCs have been shown to secrete IL-12, which is important for IFN-γ production by NK cells in murine viral infection (Orange et al., 1996). Here, we demonstrated that mouse BM-derived DCs could be potentially matured with NK cells in the presence of LPS. Reciprocally, NK cells might be activated by DCs in the presence of LPS. Compared with other DCs in this study, DCNK+LPS+IL-2 group showed higher expression of several costimulatory molecules and 1.5-2 times greater secretion of IL-12. Nanogram quantities of IL-12 were produced by these DCs, and these concentrations of IL-12 were sufficient to increase IFN-γ production by NK cells. Our results suggest one possible model in which LPS directly activates both NK cells and DCs, and the interaction between these two cell types during stimulation further enhances their activation.

The goal of immunotherapy is to induce or up-regulate T cell-mediated tumor-specific immune responses. Although high T cells stimulatory capacity and tumor antigen uptake were observed in the DC LPS group, the DCNK+LPS+IL-2 group induced much more effective and functional T cell responses, based on the allogeneic responses and tumor-specific CTL responses, which were manifested by their superior induction of T cell proliferation, high ability to take up tumor material and higher induction of IFN-γ-secreting cells. The data from this study suggest that fully mature DCs are generated with NK cells in the presence of TLR agonist and IL-2 and could be potentially useful for immunotherapy against cancer.

The suppressive effects of tumor cells during DC generation have been explained previously by the ability of the tumor microenvironment to suppress DC differentiation (Savill et al., 2002; Yu et al., 2007). The suppression is due to the activation of STAT3 and the production of immunosuppressive factors, such as vascular endothelial growth factor, IL-10, and IL-6 in the tumor microenvironment. Although we did not observe significant inhibitory effects of tumor cells during DC maturation in DC phenotype and IL-12p40 production, IL-12p70 production was significantly declined. Therefore, the inhibitory effects of tumor cells may play some
role in DC differentiation. Further studies to provide evidence for this process are needed. Prior studies have shown that STAT3 blocking or inhibition of MAPK activity enhance the immune-mediated anti-tumor effects of DCs (Nefedova et al., 2005; Wang et al., 2006).

A crucial factor for the successful generation of the potent DCs in our study was the ratio of the DCs:NK cells. The interaction of immature DCs with activated NK cells results in either maturation or cell death. The determination between death and maturation when DCs were exposed to activated NK cells was mainly related with the ratio of the DCs:NK cells (Gerosa et al., 2002; Piccioli et al., 2002). When DC:NK cell ratios were > 1:5, inhibition of DC function was the dominant feature of the DC interaction with activated NK cells due to direct killing of immature DCs (Piccioli et al., 2002). Indeed, both DC maturation and cytokine production (TNF-α and IL-12) observed at an equal ratio (≤ 1:2) were abrogated when the number of NK cells were increased (Piccioli et al., 2002). Similarly, our results indicated that the optimal ratio was 1:1 to 1:2, which showed higher expression of several molecules and higher production of cytokines in comparison to other ratios.

Recently, several DC-based vaccines using known tumor antigens in mouse colon cancer models have been reported (Jack et al., 2007; Yamaguchi et al., 2007; Shortman et al., 2009). In our study, tumor apoptotic cells were used to load on DCs to generate CTLs in tumor bearing mice. We demonstrated that DCs (DCNK+LPS+IL-2) vaccine effectively enhanced antitumor effects in mouse colon cancer model. IFN-γ ELISPOT assays revealed that tumor antigen-pulsed DCs resulted in the superior induction of tumor-specific CTLs. These DC vaccines induced antitumor responses against CT26 colon carcinoma and inhibited tumor growth in the tumor-bearing mice. In addition, IFN-γ ELISPOT assay with splenocytes from each group of vaccinated mice reveal that our DC vaccine resulted in the strong tumor specific CTL-mediated responses against CT-26 tumor cells rather than NK cell-mediated response. Therefore, DCNK+LPS+IL-2 could be used as an excellent DC vaccine against colon cancer.

IL-2-activated human NK cells can trigger maturation of immature DCs by up-regulation of co-stimulatory molecule, CD86, resulting in enhancement of T cell stimulatory capacity of DCs (Gerosa et al., 2002). However, IL-2-activated NK cells did not induce more maturation of DCs than LPS-activated NK cells in our study (data not shown). In addition, IL-2 does not act directly on DC-inducing maturation (data not shown). TLR is expressed in both NK cells and DCs, and TLR ligands play a role in the activation of NK cells as well as maturation of iDCs. Interestingly, our data showed that NK cells were efficiently activated by DCs in the presence of LPS and IL-2. Consistently, the effects of NK cells in generation of potent DCs were optimized in the presence of LPS and IL-2. Therefore, we suggest the activation events occurring in co-culture of NK cells and DCs in the presence of LPS and IL-2 as followed. First, NK cells start to activate by IL-2 and then by LPS or both, whereas DCs start to activate by LPS to release abundant IL-12. Second, NK cells exposed to IL-12 are further activated and can kill iDCs, thus favoring the survival of mature DCs. Third, the activated NK cells produce TNF-α (data not shown) to promote further maturation of DCs and production of IFN-γ, which increase the production of IL-12p70 and induce Th1 immune responses. Finally, the inhibitory effects of DCNK+LPS+IL-2 vaccines against tumors were dramatically enhanced through this positive feedback loop.

In animal colon cancer models, DC-based vaccines have been reported to induce significant antitumor responses (Jack et al., 2007; Yamaguchi et al., 2007). However, the methods to improve the efficacy of DC-based vaccines should be investigated to enhance the effect of vaccination. In this study, we investigated the combination effect of low-dose chemotherapy and potent DCNK+LPS+IL-2 vaccines because cyclophosphamide is frequently used to enhance or augment the antitumor effects in the field of immunotherapy against cancer (North, 1982; Ghiringhelli et al., 2004; Mihalyo et al., 2004; Young et al., 2006). The possible effect of cyclophosphamide to enhance the antitumor efficacy of DC vaccine may be due to the increasing proportion of IFN-γ secreting lymphocytes in combination with the suppressing proportion of CD4+CD25+FoxP3+ regulatory T (Treg) cells in tumor-bearing mice (Liu et al., 2007). The results of a clinical trial using allogeneic DC vaccine combined with low-dose cyclophosphamide has revealed that the combination therapy could induce stronger antitumor response compared with DC vaccine alone (Holtl et al., 2005). Consistently, our results showed that a single administration of low-dose cyclophosphamide before the first DC vaccination augmented the antitumor effects of DC vaccine to eradicate tumor completely and consequently prolonged the survival of vaccinated mice.

In conclusion, the results of our study suggest that potent DCs can be generated by NK cells in the presence of TLR agonist and IL-2, and used to effectively induce tumor specific CTLs that exert an anti-tumor immune response.
Methods

Mice and tumor cell lines
Six- to eight-week-old female BALB/c (H-2^d) mice were purchased from Orient Bio (Iksan, South Korea), and were maintained in specific pathogen-free conditions. All animal care, experiments and euthanasia were performed in accordance with protocols approved by the Chonnam National University Animal Research Committee. The murine BALB/c-derived colon carcinoma cell lines, CT26 and CT26-Fluc (CT26 carrying Fluc gene), were kindly provided by Dr. Min JJ (Chonnam National University, Korea) and YAC-1 was purchased from American Type Culture Collection (Rockville, MD). The CD40 ligand (CD40L)-transfected J558 cell line was kindly provided by Dr. Kalinski (University of Pittsburgh). All cell lines were maintained in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% FBS (Gibco-BRL) and 1% penicillin/streptomycin.

Generation of BM-derived DCs

BALB/c BM-derived immature DCs were generated as described previously (Lutz et al., 1999). Briefly, BM was harvested from the femurs and tibiae of mice and cultured in RPMI-1640 (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL) and 1% penicillin/streptomycin, in the presence of 5 ng/ml recombinant murine (rm) GM-CSF (R&D Systems, Minneapolis, MN) and 5 ng/ml rmIL-4 (R&D Systems). On day 3, fresh complete medium containing cytokines was added to the adherent cells. On day 7 of culture, non-adherent and loosely adherent cells were harvested and used for the experiments as immature DCs.

NK cell purification

Mouse NK cells were positively isolated from splenocytes of BALB/c mice. Briefly, the spleen was removed under sterile conditions; tissue was dissociated and then filtered through a 40-μm nylon cell strainer (BD Biosciences-Labware). Erythrocytes were removed using 0.83% (w/v) NH_4Cl red blood cells lysis buffer. NK cells were isolated by positive selection using the magnetic activated cell sorter (MACS; Miltenyi Biotec). The responder T cells (2×10^5) were co-cultured with graded doses (3×10^5 to 2×10^6) of irradiated DC_LPS, DC_NK+LPS, and DC_NK+LPS+IL-2.

Maturation of DCs

Immature DCs were cultured at 1×10^6 cells/well in 24-well plates (BD Biosciences-Labware). For DC maturation using DC-NK interaction, immature DCs (1×10^6 cells) were directly co-cultured with purified NK cells (2×10^5 cells) in the presence of 1 μg/ml lipopolysaccharides (LPS) from Escherichia coli (Sigma-Aldrich, St. Louis, MO) and/or 5 ng/ml mIL-2 (R&D Systems) for 2 days in the following three subgroups: LPS alone (DC_LPS), NK cells in the presence of LPS (DC_NK+LPS) or NK cells in the presence of LPS and IL-2 (DC_NK+LPS+IL-2). Before use for analysis, mature DCs were harvested and washed three times to remove all extra reagents.

Phenotypic analysis of DCs

Fluorescence-activated cell sorting (FACS) analysis was performed using monoclonal antibodies (mAb) against murine MHC class II (I-A^d)-PE, CD40-PE, CD80-FITC and CD86-PE (PharMingen, San Diego, CA). Isotype matched controls were run in parallel. Cell debris was eliminated by forward and side scatter gating. The samples were acquired on a FACS Aria cell sorter (Becton Dickinson, San Jose, CA) and the data were analyzed with WinMDI Version 2.9 (Bio-Soft Net).

Mouse IFN-γ, IL-12p40 and IL-12p70 production

Measurements of mouse IFN-γ, IL-12p40 and IL-12p70 cytokines during maturation of DCs were determined by BD OptEIA™ ELISA Set (BD Bioscience). In addition, mature DCs were plated in 96-well plates at 2×10^4 cells/well and stimulated with CD40L-transfected J558 cells (5×10^4 cells/well), which mimic the interaction with CD40L-expressing Th cells, for 24 h to secrete IL-12p70 upon the follow-up activation.

Generation of apoptotic CT26 cells for loading on DCs

Apoptotic CT26 cells were induced by high-dose UVB irradiation (120 mJ/cm^2) (International light, Newburyport, MA) followed by overnight culture in RPMI-1640. The apoptotic irradiated-CT26 cells were confirmed using Annexin V-FITC Apoptosis Detection Kit (BD Bioscience) and pulsed with immature DCs at 8 h after maturation at a ratio of 2:1 (DCs: apoptotic cells).

To measure tumor antigen uptake by DCs, CD11c^-immature DCs were isolated by MACS separation (Miltenyi Biotec). CT26 cells were stained with PKH26 (Sigma-Aldrich) before UVB irradiation. After maturation, tumor-loaded, mature CD11c^-CD80^+ DCs were analyzed by flow cytometry for the expression of PKH26.

Allogeneic mixed lymphocyte reaction

Allogeneic mouse T cells were positively isolated from splenocytes of B16F10 mice using positive selection with a MACS system (Miltenyi Biotec). The responder T cells (2×10^5/well) were co-cultured with graded doses (3×10^5 to 2×10^6) of irradiated DC_LPS, DC_NK+LPS, and DC_NK+LPS+IL-2. On day 5, the cultured cells were pulsed with 1 μCi of [H]-methylthymidine per well during the last 18 h of culture, and then analyzed in a liquid scintillation counter (Beckman, Fullerton, CA). The results were expressed as the mean cpm±SD of triplicate cultures. Unprimed T cells were used as a control.

In vitro CTL generation and IFN-γ ELISPOT Assay

Autologous mouse T cells (1×10^6 cells) isolated from splenocytes using MACS system (Miltenyi Biotec) were co-cultured as effector cells at a ratio of 5:1 with either tumor-pulsed or unpulsed DC_LPS, DC_NK+LPS or DC_NK+LPS+IL-2 (2×10^4 cells) in 2 ml of medium (RPMI 1640: AIM-V=1:1) containing 10% FBS (Gibco-BRL) and 1% penicillin-strep-
tomycin (Gibco-BRL) supplemented with 20 U/ml rmIL-2 (R&D Systems). The medium was replenished with cytokines every 3 days. On day 7, the CTL lines were harvested and used for ELISPOT assay using a mouse IFN-γ ELISPOT Set (BD Bioscience). The frequency of antigen-specific CTL lines was analyzed using the CT26 tumor cell line and mature DCs pulsed with CT26 apoptotic tumor cells as targets cells. The spots were counted with an ImmunoSpot Reader (Cellular Technology Ltd, Ohio). The data were presented as the mean ± SD of IFN-γ secreting cell per well of triplicate samples. CTL cells alone were used as the control.

**In vivo animal vaccination**

In original schedule to investigate the efficacy of our generated potent DCs, five vaccination groups were used: (1) pulsed-DCPs, (2) pulsed-DCNK, (3) pulsed-DCNK/LPS, (4) NK cells and (5) PBS control. Each vaccine (1 × 10^6 cells/mouse) was administrated subcutaneously at the left flank in a volume of 0.1 ml of PBS at a 4 day interval on days 3, 7, 11 and 15 after subcutaneous inoculation of CT26 tumor cells (5 × 10^5 cells/mouse) on the right flank of BALB/c mice in a volume of 0.1 ml on day 0.

For combine with low-dose chemotherapy, four vaccination groups were used: (1) cyclophosphamide alone, (2) pulsed-DCNK/LPS, alone, (3) pulsed-DCNK/LPS/L2, (4) NK cells and (5) PBS control. Each vaccine (1 × 10^6 cells/mouse) was administered subcutaneously at the left flank in a volume of 0.1 ml of PBS at a 4 day interval on days 3, 7, 11 and 15 after subcutaneous inoculation of CT26 tumor cells (5 × 10^5 cells/mouse) on the right flank of BALB/c mice in a volume of 0.1 ml on day 0.

For in vivo molecular imaging, 2 mice of each group in original schedule were inoculated with CT26-Fluc alone, (2) pulsed-DCNK/LPS alone, (3) pulsed-DCNK/LPS/L2 combination with cyclophosphamide and (4) PBS control. Firstly, to determine the optimal low-doses of cyclophosphamide, a dose range between 50 to 400 mg/kg was tested. The dose of 50 mg/kg was showed to low cytotoxic to CT26 tumor cells compared with other doses. CT26 tumor cells (5 × 10^5 cells/mouse) were inoculated on the right flank of BALB/c mice in a volume of 0.1 ml on day 0. A single low-dose of cyclophosphamide (50 mg/kg) was injected intraperitoneally on day 6 preceding the first DCs vaccination in indicated mice. DC vaccine (1 × 10^6 cells/mouse) was administered subcutaneously at a 4 day interval on days 9, 13, 17 and 21.

To assess the anti-tumor efficacy of vaccinated mice, three perpendicular dimensions (length, width, and height) of each tumor were measured individually every 3-4 days with a vernier caliper, and the tumor volume was calculated using the formula for the standard volume of an ellipsoid as follows: \[ V = \frac{4}{3} \pi \times (\text{length} \times \text{width} \times \text{height})/8. \] To assess the survival prolongation of vaccinated mice, the mice were euthanized when the tumor volume reached 2.5 cm in diameter, which was considered as a death due to the size of tumor.

For in vivo molecular imaging, 2 mice of each group in original schedule were inoculated with CT26-Fluc alone. Imaging was performed 12 days after tumor inoculation, when the bioluminescent signal from CT26-Fluc could be detected. The bioluminescence expression of CT26-Fluc was detected by intraperitoneal injection of 100 μl (7.5 mg/mL) of D-luciferin (Caliper, Hopkinton, MA) using the IVIS100 system (Caliper) equipped with a cooled charged couple detector camera. The results were analyzed using the Living Image software v. 2.25 (Caliper). A region of interest (ROI) was selected manually over the signal intensity. The area of the ROI was kept constant, and the intensity was recorded as maximum within a ROI.

**Tumor antigen specific CTLs of vaccinated mice**

Splenocytes (1 × 10^6 cells) isolated from vaccinated mice on 7 days after the last immunization (day 22) were added to 24-well plates and, then, restimulated with irradiated CT-26 cells (5 × 10^5 cells) for 5 days in RPMI-1640 (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL) and 1% penicillin/streptomycin. After restimulation, the splenocytes were collected and tested for detection of the tumor antigen-specific cytotoxic T lymphocytes using a mouse IFN-γ ELISPOT Set (BD Bioscience). The CT-26 tumor cell line and NK-sensitive YAC-1 cell line were used as targets cells.

**Statistical analysis**

The Mann-Whitney U test was performed for statistical significance of nonparametric differences among groups. Survival of the vaccinated mice was analyzed using Kaplan-Meier method with SPSS 13.0 program software and compared using log-rank test. \( P \) values < 0.05 were considered statistically significant.

**Supplemental data**

Supplemental Data include two figures and can be found with this article online at http://e-enmm.or.kr/article/article_files/SP-42-6-01.pdf.

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