

Dendritic Cell Maturation with CpG for Tumor Immunotherapy

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ABSTRACT

Background: Bacterial DNA has immunostimulatory effects on different types of immune cells such as dendritic cells (DCs). Application of DCs as a cellular adjuvant represents a promising approach in the immunotherapy of infectious disease and cancers. **Objectives:** To investigate the effect of tumor antigen pulsed DCs in the presence of CpG-1826 in treatment of a murine model of cancer. **Methods:** WEHI-164 cells (Balb/c derived fibrosarcoma cell line) were injected subcutaneously in the right flank of mice. Bone marrow cells were cultured in the presence of GM-CSF and IL-4. After 5 days, tumor lysate, CpG-1826, and oligodeoxynucleosides, as control, were added to the culture media and incubated for 2 days. Cytokine production in DCs culture media was measured by ELISA. Then DCs were injected subcutaneously around the tumor site in the right flank of mice. Tumor growth rate was monitored in case and control groups. Two weeks after DCs immunotherapy, cytotoxic assay was conducted using various amounts of effector (splenic T cells) and target cells (WEHI-164 or CT26) for 6 h. **Results:** Immunotherapy with DCs treated with CpG led to a significant increase in the activity of cytotoxic T cells and decreased tumor growth in immunized mice. In the control group which received DCs without CpG treatment, no change in cytotoxic activity and tumor growth rate was detected. **Conclusion:** The current study suggests that specific anti tumor immune responses can be induced by DCs matured with CpG and proposes CpG usage in DCs targeted clinical strategies.

Keywords: Dendritic cell, Immunotherapy, Cancer, CpG Oligonucleotide

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INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system (1). They express high levels of MHC class I and II molecules and a broad spectrum of adhesion and co-stimulatory molecules (2). DC-based vaccines are viewed as promising method for immunotherapy of cancers. Large numbers of DCs can be generated in vitro by culture of plastic adherent peripheral blood mononuclear cells with GM-CSF and IL-4 (3,4). Such DCs are very efficient in antigen uptake by endocytosis but are unstable and immature, because the expression of co-stimulatory molecules is low and stimulation of T cells is suboptimal (5). To obtain fully matured DC (mDCs) additional signals are required. In peripheral tissues, DC maturation is triggered by pro-inflammatory signals or pathogen cell wall.

In general, DC maturation state is defined by certain phenotypes, most notably expression of CD80 and CD83, and cytokine production, IL-12 and IL-18. These cytokines play a critical role in the polarization of CD4⁺ T cells into TH1 cells (6). Thus mDCs have the full ability to activate CD4⁺ TH1 cells, essential in priming CD8⁺ cytotoxic T lymphocytes (7).

CpG is a dinucleotide containing cytosine (C) and guanine (G). Bacterial DNA and synthetic oligodeoxynucleosides (ODN) containing CpG motifs have been shown to activate the immune system, via production of a wide variety of Th1-promoting cytokines, such as IL-12, IFN- γ , TNF- α , and IL-6 (1-4). As a strong immunostimulatory agent, CpG ODN is able to protect effectively against bacterial, viral, and parasitic infections (5-7). CpG ODN also enhances the anti-tumor efficacy of monoclonal antibodies or cancer vaccines when used as immune adjuvants in animal tumor models (8,9). The administration of CpG ODN alone is also capable of triggering potent anti-tumor immune responses against various experimental tumors, including lymphoma, leukemia, melanoma, colon carcinoma, glioma, cervical cancer, and neuroblastoma (8,10-14).

In the present study we investigated the effect of tumor antigen pulsed DCs in the presence of CpG-1826 in treatment of a murine model of cancer.

MATERIALS AND METHODS

Animals and Cell Lines. Six- to ten-week-old female Balb/c mice were purchased from Institute Pasteur of Iran. Balb/c derived fibrosarcoma (WEHI-164) and colon carcinoma (CT26) cell lines were implemented.

Cell lines were maintained in a medium of RPMI 1640 (Sigma), 10% heat inactivated fetal bovine serum (Gibco), penicillin and streptomycin (100 μ g/ml), and L-glutamine (2 mM).

Preparation of Tumor Lysate. Tumors were obtained surgically from tumor-bearing mice. Cell lysates were generated by repeated freeze-thaw cycles (liquid nitrogen and 37°C water bath). After centrifugation (10 min, 900g), supernatants were collected and passed through a 0.2- μ m pore filter. Protein concentration of the lysate was determined by Lowry method.

Oligonucleotides. ODNs including CpG ODN 1826 (TCC ATG ACG TTC CTG ACG TT) and non-CpG control ODN (TCC AGG ACT TTC CTC AGG TT) were obtained from Alpha DNA Company (Montreal, Canada).

Bone Marrow–Derived DCs. Bone marrow cells were obtained from the femur and tibia of female Balb/c mice. Red blood cells were removed by lysing. Following washing with PBS, cells were plated in RPMI plus 10% FCS, 10 ng/ml GM-CSF, and 10 ng/ml IL-4. On day 3 non-adherent granulocytes and T and B cells were gently removed and fresh media were added. On day 5 loosely adherent proliferating DC aggregates were dislodged and re-plated in fresh media supplemented with 100µg tumor lysate, 10µg CpG 1826, and control ODN. Non-adherent mDCs were harvested on day 7 of the culture.

Flow Cytometry. DC phenotypes were determined using FACS analysis system. FITC-conjugated mAbs against CD86, CD80, CD40, and PE-conjugated anti-CD11c were implemented (PharMingen, USA). The isotype matched control mAbs were also used.

Cytokine Production by DCs. Supernatants of DCs' culture were collected for measurement of IL-12 and IL-18 by commercially available ELISA kits (Bender, Medsystems, Germany).

Tumor Challenge and Treatment. To generate tumor, mice were injected subcutaneously in the right flank with 0.1 mL of a single-cell suspension containing 5×10^6 WEHI-164 cells. Treatment applied after 7 days by injection of 10^6 DC around the tumor. Tumor growth rate was monitored in different groups by digital collis.

Cytotoxicity Assay. Two weeks after immunization, spleen cells were separated from treated mice as effector cells. Tumor cell lines (WEHI-164 and CT26) were used as target cells. Cytotoxic activity was measured by LDH cytotoxicity detection kit (Roche Applied Science, Germany). Following washing the effector and target cells with the assay medium (RPMI1640 with 1% BSA), effector cells were co-cultured with target cells in a 96-well round bottom plate for 6 h at 37 °C, plates were centrifuged, and supernatants were transferred to another flat-bottom ELISA plate. 100 µl of LDH detection mixture was added to each well and incubated for 30 minutes at room temperature. Absorbance was measured by an ELISA reader at 490 nm. The percentage of cell mediated cytotoxicity was determined by the following equation: cytotoxicity (%) = [(experimental release- background)-(spontaneous target release-background)-(spontaneous effector release-background)] / (maximum target release – spontaneous target release) x100%.

Statistical Analyses. Comparison of means was performed by two-tailed Student *t* test. *P*-values less than 0.05 were considered significant.

RESULTS

CpG-1826 Induction of DC Maturation. To investigate the effect of CpG ODN 1826 on the phenotypes and maturation of DCs, bone marrow (BM) cells were cultured in the presence of CpG. The expressions of MHC-II and co-stimulatory molecules (CMs) were analyzed by flow cytometry. As shown in figure 1, all DCs expressed mouse BM-derived DC specific marker CD11c ($P>0.05$). CpG-DC expressed high levels of surface CMs.

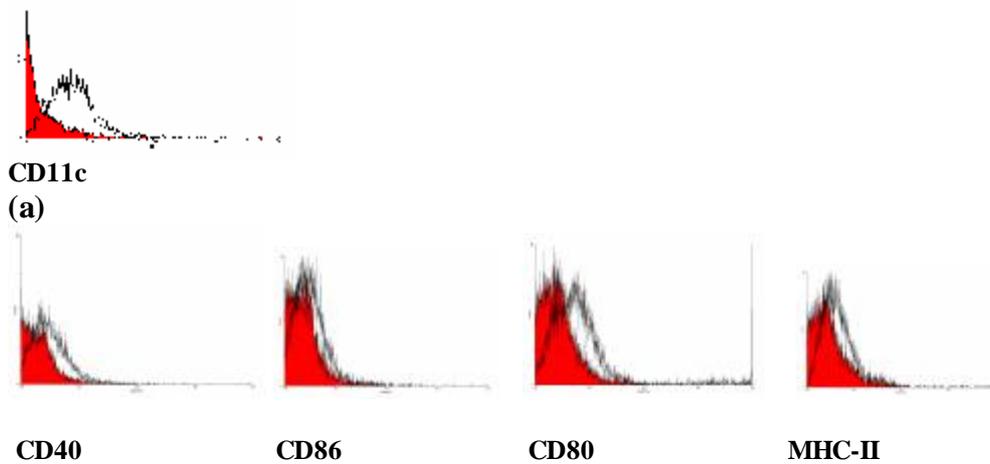


Figure 1. (a) Expression of CD11c by DCs. (b) Effect of CpG on expression of MHC II and co-stimulatory molecules by DCs.

IL-12 and IL-18 Production by DCs. mDCs were analyzed for IL-12 and IL-18 production, an indicator for TH1-polarizing capacity. CpG-induced mDCs produced high levels of IL-12 and IL-18 (Fig. 2). These cytokine profiles suggest a tendency toward induction of TH1 response. Control ODN-induced mDCs and immature DC also produced IL-12 and IL-18, albeit at lower levels.

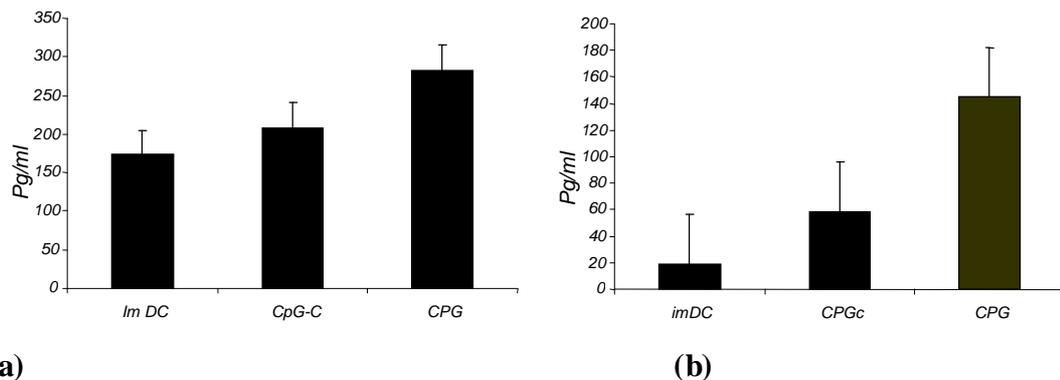


Figure 2. CpG-ODN 1826 stimulated cytokine production in DCs. Supernatants of DC cultures treated with CpG-ODN 1826, control CpG-ODN 1982, or Immature DC were assayed for IL-12 ($p < 0.01$) [a] and IL-18 ($p < 0.01$) [b] by ELISA.

Effect of Matured DCs with CpG 1826 on Cytotoxicity. Spleen cells were obtained 14 days after DC injection. Cytotoxic activity was determined against WEHI-164 tumor cells with CT26 cells as a control target. CpG-DCs demonstrated considerable levels of specific cytotoxicity against WEHI-164 targets. Cytotoxic T lymphocyte (CTL) activity was significantly ($P < 0.01$) higher than that of the control groups treated with control ODN-DC or PBS. Furthermore, we demonstrated that the cytotoxicity was specific for WEHI-164 tumor cells because there was little cytotoxic effect on CT26 tumor cells (Figure 3).

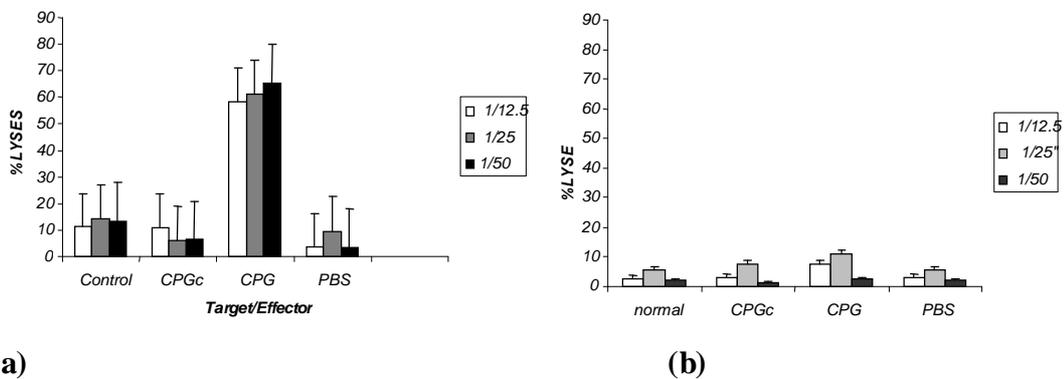


Figure 3. Tumor-specific CTL response to WEHI cell line (a) and CT26 (b) in immunized Balb/c mice. Spleen cells were collected 14 days after treatment and used as effector cells in the CTL assay.

Immunotherapy with CpG Matured DCs Prolonged the Survival of Tumor-bearing Mice. Tumor-bearing mice were treated with CpG or control ODN mDCs or with PBS seven days after tumor challenge. Tumor growth rate was monitored in different groups and survival was determined. As shown in figure 4, treatment with CpG-DC significantly improved the survival compared with control ODN-DC or PBS ($P < 0.01$).

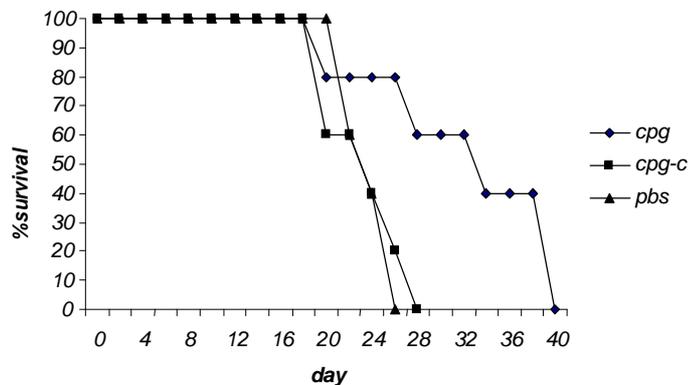


Figure 4. Effect of CpG-DC therapy on the survival of tumor-bearing mice.

DISCUSSION

DCs vaccination is a promising approach for cancer treatment, a number of studies evaluating its efficacy have been published. It is now clear that the development of effective anti-tumor vaccine protocols requires further optimization and refinement, a process greatly facilitated by appropriate animal models.

Maturation of DCs can be induced by various adjuvants including mycolic acid, lipoarabinomannan, LPS, lipoteichoic acid, bacterial DNA, and double-stranded RNA. These adjuvants are specifically recognized by invariant receptors such as Toll Like Receptors (TLR) (15). Unmethylated CpG oligodeoxynucleosides (CpG

ODN), recognized by TLR-9 were proven to have anti-tumor effects via maturation of DCs (16).

Using a murine tumor model, we evaluated DC maturation in the presence of CpG-1826 and demonstrated that the extent of DC maturation, reflected by density of co-stimulatory and MHC II molecules, was correlated with the induction of strong CTL and anti-tumor immune response *in vivo*.

CpG ODN 1826 is known as a strong immune activator, inducing protective and curative Th1 responses against infections and tumors *in vivo*. The immunostimulatory effects of CpG ODN 1826 are dependent on the CpG motifs within ODNs (17-19). Our results show that peri-tumoral injection of matured DCs with CpG ODN 1826 as a single therapeutic agent suppressed the growth of implanted fibrosarcoma in mice, but the control ODN was ineffective. Our findings are consistent with the previous observations from several tumor models treated with CpG ODN 1826 (19-21).

The induction of Th1 response is related to the capacity of DCs to produce IL-12. This cytokine is known as a major Th1-promoting factor and is essential in generating Th1-biased cells from naive precursors (22). IL-18, an additional member of IL-1 cytokine family, is also produced by DCs and cells of monocytes/macrophage lineage. IL-18 was shown to have synergy with IL-12 in the induction of IFN- γ production by Th1 cells (23). In the present study we showed that CpG matured DCs produce high amounts of IL-12 and IL-18, therefore CpG can stimulate DCs to shift the T cells toward a type1 response.

Several studies demonstrated that tumor-specific CD8+ CTL constitute an important effector arm of the anti-tumor immune response (24,25). We showed that DC generated in the presence of CpG, induces strong CTL response against our tumor model.

To conclude, administration of CpG-matured DCs to the tumor site is able to induce a detectable CTL-mediated anti-tumor immune response, leading to retarded tumor growth and prolonged survival of the tumor-bearing mice. This approach is applicable in generating DC-based anti-tumor vaccines useful in clinical trials of human neoplasms.

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REFERENCES

- 1 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol.* 1991;9:271-96.
- 2 Freudenthal PS, Steinman RM. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc Natl Acad Sci U S A.* 1990;87:7698-702.
- 3 Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B et al. Proliferating dendritic cell progenitors in human blood. *J Exp Med.* 1994;180:83-93.
- 4 Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 1994;179:1109-18.
- 5 Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med.* 1995;182:389-400.
- 6 Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity.* 2003;19:641-4.
- 7 Wang RF. The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. *Trends Immunol.* 2001;22:269-76.

- 8 Wooldridge JE, Ballas Z, Krieg AM, Weiner GJ. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood*. 1997;89:2994-8.
- 9 Davila E, Celis E. Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. *J Immunol*. 2000;165:539-47.
- 10 Blazar BR, Krieg AM, Taylor PA. Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potent stimulators of antileukemia responses in naive and bone marrow transplant recipients. *Blood*. 2001;98:1217-25.
- 11 Sharma S, Karakousis CP, Takita H, Shin K, Brooks SP. Intra-tumoral injection of CpG results in the inhibition of tumor growth in murine Colon-26 and B-16 tumors. *Biotechnol Lett*. 2003;25:149-53.
- 12 Carpentier AF, Xie J, Mokhtari K, Delattre JY. Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. *Clin Cancer Res*. 2000;6:2469-73.
- 13 Baines J, Celis E. Immune-mediated tumor regression induced by CpG-containing oligodeoxynucleotides. *Clin Cancer Res*. 2003;9:2693-700.
- 14 Carpentier AF, Chen L, Maltonti F, Delattre JY. Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. *Cancer Res*. 1999;59:5429-32.
- 15 Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol*. 2001;2:675-80.
- 16 Weiner GJ. CpG DNA in cancer immunotherapy. *Curr Top Microbiol Immunol*. 2000;247:157-70.
- 17 Walker PS, Scharton-Kersten T, Krieg AM, Love-Homan L, Rowton ED, Udey MC et al. Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-gamma-dependent mechanisms. *Proc Natl Acad Sci U S A*. 1999;96:6970-5.
- 18 Gramzinski RA, Doolan DL, Sedegah M, Davis HL, Krieg AM, Hoffman SL. Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. *Infect Immun*. 2001;69:1643-9.
- 19 Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, Waldschmidt M et al. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. *J Immunol*. 2001;167:4878-86.
- 20 Sharma S, Karakousis CP, Takita H, Shin K, Brooks SP. Intra-tumoral injection of CpG results in the inhibition of tumor growth in murine Colon-26 and B-16 tumors. *Biotechnol Lett*. 2003;25:149-53.
- 21 Heckelsmiller K, Rall K, Beck S, Schlamp A, Seiderer J, Jahrsdorfer B et al. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J Immunol*. 2002;169:3892-9.
- 22 Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell*. 2001;106:263-6.
- 23 Kumar S, McDonnell PC, Lehr R, Tierney L, Tzimas MN, Griswold DE et al. Identification and initial characterization of four novel members of the interleukin-1 family. *J Biol Chem*. 2000;275:10308-14.
- 24 Knight SC, Hunt R, Dore C, Medawar PB. Influence of dendritic cells on tumor growth. *Proc Natl Acad Sci U S A*. 1985;82:4495-7.
- 25 Greenberg PD. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv Immunol*. 1991;49:281-355.