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Nanodiamond and Nanoplatinum Liquid, DPV576, Activates Human Monocyte-derived Dendritic Cells *In Vitro*

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Abstract. *Background: The influence of nanoparticles on the immune system is poorly understood. It was recently shown that exposure to a mixture of nanodiamond (ND)- and nanoplatinum (NP)-coated material (DPV576-C) activates murine T-cells. This study examined the role of a dispersed aqueous mixture of ND/NP (DPV576) in activating human dendritic cells (DCs) in vitro. Materials and Methods: Human monocyte-derived DCs were treated with DPV576 at various concentrations (50, 100 and 200 μg/ml) for 24 hours in vitro. Activation of DCs was determined by assessing the expression of co-stimulatory and maturation markers (CD80, CD83, CD86, HLADR), production of cytokines, and induction of proliferation of naïve CD4 T-cells. Expression of co-stimulatory molecules and cell proliferation were analysed by flow cytometry and cytokine secretion by ELISA. Results: DPV576 treatment of DCs resulted in: (i) increased CD83 and CD86 expression on DCs, (ii) up-regulation in the levels of DC-secreted cytokines IL-6, TNF and IL-10, and (iii) increased ability to induce proliferation in CD4$^+$ T-cells which is associated with increased expression of T-cell activation marker CD25. Conclusion: Solution containing ND/NP (DPV576) activated human DCs and DCs-driven CD4 naive T-cell proliferation in vitro, which may be useful in boosting immune responses in cancer treatment.*

Nanomaterials have recently become the focus of research for their use in biomedical fields including, but not limited to, cancer treatment and diagnosis (1, 2). Polymeric micelles, ceramic nanoparticles, viral-derived capsid nanoparticles, and silica nanoparticles are all promising candidates for biological applications. Recently, a mixture of two nanomaterials, nanodiamond (ND) and nanoplatinum (NP), was introduced and studied for their immune modulatory effect.

NDs were first synthesized approximately 50 years ago *via* a detonation synthesis by explosive decomposition of highly explosive mixtures in a non-oxidizing medium. In this type of synthesis, the explosive dictates the detonation characteristics and the composition of the detonation products. NDs synthesized *via* this route have a chemically inert diamond core and a shell containing reactive functional group (3). Recently, dispersed aqueous solutions of ND have been synthesized. The availability of these newly produced aqueous dispersed forms facilitates their possible use in nanomedicine and biorelated studies (4). Current studies regarding the medical properties of NDs in animals and humans with cancer are motivated by positive results, observed in both *in vitro* drug delivery and cell targeting experiements (5, 6). In addition, metal nanoparticles for biotechnological applications are increasingly becoming a focus of research. In particular, NP particles have recently emerged as a new candidate for use in bionanotechnology. Recent studies have shown that the physicochemical properties of NP make it suitable for the medical treatment of oxidative stress diseases (7), photoactivated anticancer therapy (8) and targeted controlled release cancer drug delivery systems (9).

Although ND/NP are promising candidates for medical purposes, the effect of these nanoparticles on the immune system is poorly understood. It was recently shown that aged mice exposed to ND/NP (DPV576-C)-coated garments exhibit increased T lymphocyte responses *ex vivo* (10). The present study was undertaken to examine the ability of the ND/NP liquid mixture (DPV576) to activate human dendritic cells (DCs), and the DC-directed T-cell response in an *in vitro* culture model. This study is focused on DCs because these cells are the most active antigen-presenting cells capable of activating naive T-cells, and initiating antigen-specific immune responses against pathogens (11). These cells are capable of processing both exogenous and endogenous antigens, and present peptides in the context of either MHC class I or II molecules.

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Table I. *Characteristics of nanoparticles in the DPV576 solution.*

Type of nanoparticle	Concentration (ng/ml)	Mean particle diameter (nm)	Shape
Platinum	225	20	Round
Diamond	15000	120	Oval

Materials and Methods

DPV576 liquid. A mixture of ND and NP solution known as DPV576 was used. Table I shows details regarding particle size, shape, and composition in the DPV576 solution. DPV576 was supplied by Venex Co, Ltd, Kanagawa, Japan.

Isolation and culture of monocyte-derived DCs. Monocyte-derived dendritic cells (MDDCs) were prepared essentially as described elsewhere (12, 13). Briefly, peripheral blood mononuclear cells (PBMCs) from nine normal healthy donors (approved by the Institutional Review Board (IRB), Charles Drew University) were separated over Ficoll-hypaque density gradient centrifugation. Cells were allowed to adhere to culture plates for 2 hours. Non-adherent cells were removed. The resulting monocytes were cultured for 6 days under a humidified atmosphere of 5% CO_2 at 37˚C in RPMI- 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, human granulocyte-macrophage colony-stimulating factor (GM-CSF) at 50 ng/ml (Peptrotech, Rocky Hill, NJ, USA) and 10 ng/ml recombinant human IL-4 (Peprotech). Half of the medium was replaced every 2 days with fresh medium and MDDCs ($CD1a^+CD14^-HLA\text{-}DR^+CD11c^+$) were collected after 6 days. The purity of the MDDCs obtained was >95%. These immature DCs were pulsed with 1 μg/ml *Escherichia coli* LPS (E.LPS) or DPV576.

DC phenotype. The expression of cell surface markers was determined by flow cytometry. Briefly, gated $CD14^-CD11c^+HLA\text{-}DR^+$ DCs were analyzed for the expression of CD80, CD83, CD86 and HLADR (BD Pharmingen, San Diego, CA, USA).

Cytokine production by DCs. Immature DCs were incubated with LPS (1 μg/ml) or 50,100 and 200 μg/ml DPV576 for 24 hours. Supernatants were collected and stored at –70˚C until analyzed. Cytokines TNF-α, IL-6, and IL-10 in the supernatants were measured by specific ELISA kits (BD Pharmingen) as *per* the manufacturer's protocol.

DC stimulation of T-cell cultures. DCs were stimulated with LPS and DPV576 for 24 h as described above. After washing, 2×10^4 MDDCs were cultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled 1×10^5 purified naïve CD4 T-cells (negatively selected using a magnetic bead-based kit from Stem Cell Technology, Vancouver, BC, Canada) for 5 days. The purity of the CD4 naïve T-cells obtained ranged from 93 to 97% as determined by CD4+/CD45 RA+ staining. Proliferation of T-cells was measured by dilution of CFSE dye (13).

Statistics. All experiments were repeated with samples from nine individual donors. Probability that the mean values of two experimental groups were identical was tested by the two-tailed *t*-test for paired samples. The level of significance was set at $p<0.05$.

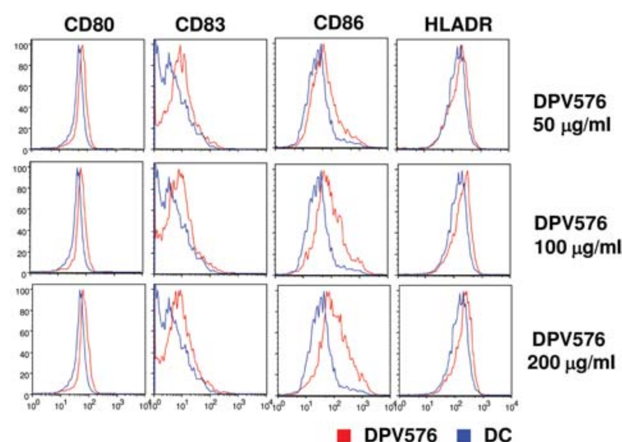

Figure 1. *Effect of DPV576 on DC activation. Monocyte-derived DCs were treated with DPV576 at a concentration of 50, 100 and 200 μg/ml for 24 hours. Expression of cell surface markers CD80, CD83, CD86 and HLADR was determined by flow cytometry and is shown as the mean fluorescent intensity (MFI). Results are shown from one representative donor among nine donors.*

Results

Immature DCs from nine individual donors were treated with NP/ND liquid (DPV576) or with LPS as a positive control and the following parameters were investigated: DC activation, cytokine production and the ability of DCs to stimulate T-cell proliferation.

DC activation. The ability of DPV576 to cause DC maturation was examined. The levels of DC cell-surface co-stimulation and maturation markers, including CD80, CD83, CD86 and HLADR post treatment with DPV576 was examined by flow cytometry. Data in Figure 1 show that the expression of maturation markers CD80 and HLADR were slightly increased post treatment with DPV576. However, the expression of CD83 and CD86 was significantly increased in a dose-dependent fashion. The activating ability of DPV576 was detected at a low concentration of 50 μg/ml and was maximized at 200 μg/ml. The level of up-regulation was comparable to that obtained with LPS in earlier studies.

Cytokine production. The levels of several cytokines (IL-6, TNF and IL-10) secreted by the DCs were measured post exposure to DPV576. Data in Figure 2 show DPV576 had the ability to activate DCs to secrete cytokines. This resulted in the up-regulation of IL-6 production that was significantly increased ($p<0.05$) at a low concentration of 50 μg/ml and continued to increase with higher concentrations. DPV576

also induced DCs to secrete TNF production that was detected on treatment at a low concentration of 50 μg/ml and a 15-fold increase was observed at a concentration of 200 μg/ml (p<0.05) as compared to control. Finally, a 5-fold increase in up-regulation of IL-10 production was detected post exposure of DCs at a concentration of 200 μg/ml, as compared to control (p<0.05).

T-Cell proliferation. The ability of DPV576-treated DCs to activate $CD4^+$ T-cell proliferation was examined. Data in Figure 3 show that DPV576-treated DCs induced an increased proliferation of $CD4^+$ cells as compared to untreated DCs. The activating effect followed a dose-dependent pattern. DC- $CD4^+$ T-cell proliferation alone was 32.5%, that increased to 47.4% post treatment with DPV576 at a low concentration of 50 μg/ml, and was further increased to 52% at 200 μg/ml, representing an almost 1.6-fold increase in proliferation.

Expression of activation marker CD25. Data depicted in Figure 4 show that T-cells co-cultured with DPV576-treated DCs exhibited a significant increase in the expression of activation marker CD25. DPV576 at a concentration of 100-200 μg/ml caused a 1.6-fold increase in the expression of CD25 as compared to DC-CD4 cells alone (p<0.05).

Discussion

Previous research from this group has demonstrated that a dual-component ultra-dispersed mixture of ND/NP coated onto fabrics (DPV576-C) represents a unique nano-based system for use as a biological response modifier (10). Aged mice exposed to DPV576-C-coated garment showed increased proportions of splenic $CD4^+$ and $CD8^+$ T-cells and their activation markers, CD25 and CD69 (10). A characteristic feature of cutaneous DCs and T-cells is their migration in and out of the skin (14,15). Therefore, activation of cutaneous DCs may be reflected in other organs. The focus of the current study was to determine whether the ND/NP mixture dispersed in an aqueous solution has the capacity to activate DCs in the co-culture human DC model. These nanoparticles were able to activate human DCs and the activated DCs increased $CD4^+$ T-cell proliferation and expression of their activation marker, CD25.

DCs are potent antigen-presenting cells that possess the ability to stimulate naïve T-cells. Immature DCs are characterized by high endocytic activity and low T-cell activation potential (11). These DCs phagocytose pathogens and subsequently degrade their proteins into small pieces. Upon maturation, DCs present the protein fragments to T-cells. At the same time, they up-regulate cell-surface receptors that act as co-receptors in T-cell activation, such as CD80 (B7.1), CD86 (B7.2), CD83 and CD40, and greatly

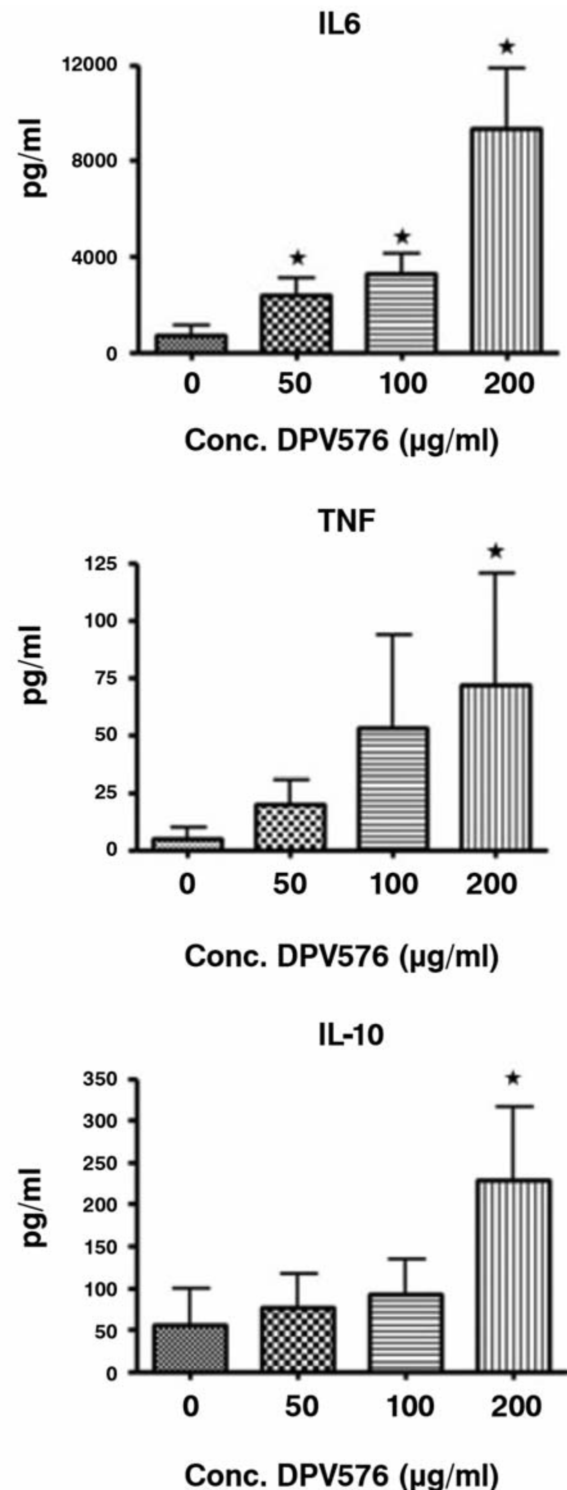

Figure 2. *Effect of DPV576 on cytokine production secreted by DCs. Immature DCs were incubated with LPS (1 μg/ml) or 50, 100 and 200 μg/ml DPV576 for 24 hours. Supernatants were collected and cytokines IL-6, TNF and IL-10 were measured by specific ELISA kits. Boxes and error bars represent the mean and SD (standard deviation) from 9 individual experiments, respectively. *p<0.05, as compared to control untreated cells.*

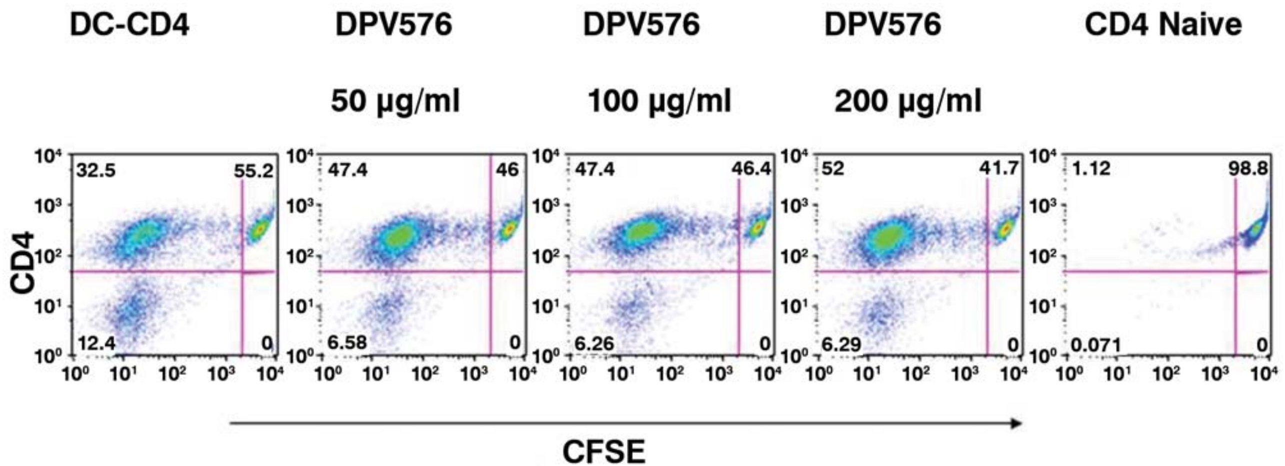

Figure 3. *Effect of DPV576 on CD4+ T-cell proliferation. DCs were stimulated with LPS and DPV576 for 24 hours as described in the Materials and Methods. After washing, DCs were cultured with CFSE-labeled T-cells for 5 days. Proliferation of T-cells was measured by dilution of CFSE dye. Results are shown from one representative donor among nine donors.*

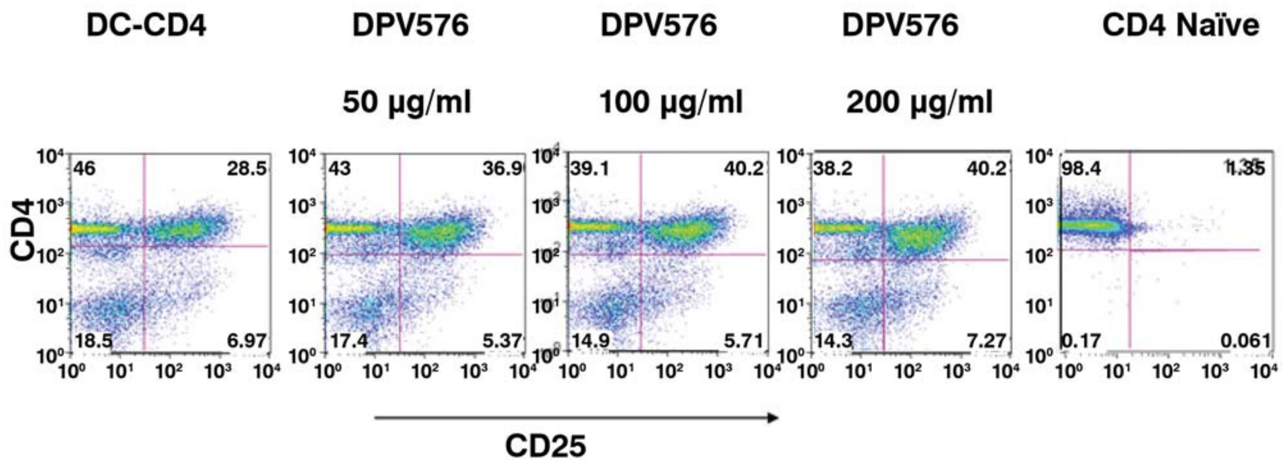

Figure 4. *Action of DPV576 on the percentage of CD4+ T-cell activation marker CD25. DCs were stimulated with LPS and DPV576 for 24 hours as described in the Materials and Methods. Proliferation of CD4+ T-cells expressing CD25 was measured by flow cytometry. Results are shown from one representative donor among nine donorrs.*

enhance their ability to activate T-cells. Activated T-cells later differentiate into helper, cytotoxic and regulatory T-cell subsets and DCs play a crucial role in T-cell differentiation *via* cell–cell contact and secretion of cytokines.

This study showed that ND/NP liquid (DPV576) activates DCs and induces their maturation as indicated by the increased expression of costimulatory and maturation markers and cytokine production. In addition, DPV576-activated DCs were more efficient in activating naïve T-cell proliferation and T-cell maturation. These findings are in agreement with previous studies regarding DC maturation using various nanopartcles (16-20). These studies reported that DCs can be activated by carbon magnetic nanoparticles, proteoliposome, monophosphoryl lipid A containing copolymer, poly(gamma-glutamic acid) nanoparticles and polystyrene beads.

The mechanism(s) by which a NP/ND dispersed aqueous solution activates DCs are not known. Two alternative hypotheses can be proposed to account for activation of DCs by nanoparticles. First, it is possible that DCs phagocytose nanoparticles, and the internalized particles subsequently trigger activation of DCs. Previous studies have shown that DCs have the capacity to uptake particles with diameters of up to 2300 nm (21). The mean particle diameter of NP is 20 nm with a round shape, and ND is 120 nm with an oval shape.

These characteristics make them easy targets for uptake by DCs, since the size and the shape of nanoparticles appear to be critical for their uptake by DCs, and their modulation of DC activation and maturation (18, 22, 23). Secondly, an NP/ND dispersed aqueous solution may also induce activation of DCs *via* a cell-surface interaction with pattern recognition receptors. Specifically, DC surface receptors have been shown to interact with nanoparticles. Recent studies have demonstrated that ND particles surround the DC cell borders, and are attached to neurite extensions (24). There are several receptors expressed by DCs, including pattern recognition receptors and C-type lectin family (25, 26).

In conclusion, this study showed that co-culturing human DCs with an NP/ND dispersed aqueous solution results in DC activation, which may have potential for its use in DC-based cancer vaccine strategies.

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