in vivo *24*: 141-146 (2010)

Modulation of Aged Murine T Lymphocytes *In Vivo* by DPV576-C, a Nanodiamond- and Nanoplatinum-coated Material

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Abstract. *Background: Nanotechnology is rapidly emerging in biomedical applications, including cancer therapy. Here, a mixture of ultra dispersed nanodiamond and nanoplatinum was coated onto fabrics in the form of a cloth (DPV576-C). The role of DPV576-C in modulating T lymphocytes of aged mice was examined. Materials and Methods: C57BL/6 mice were treated with DPV576-C as a lining in a mouse house for 1 month. Splenic cells were analyzed for $CD4^+$ and $CD8^+$ T-cells and NK activity using flow cytometry. Results: DPV576-C-treated aged mice showed an: (1) increase in the percentages of $CD4^+$ and $CD8^+$ T-cells and their activation markers, CD25 and CD69, over untreated aged mice; (2) enhancement of NK activity; and (3) absence of adverse side effects as determined histopathologically. Conclusion: The enhancement of lymphocytes by DPV576-C may be useful for patients suffering from immune dysfunction.*

In the last decade, there has been increasing interest in using nanomaterials for biomedical applications including but not limited to disease treatment and diagnosis. Research into rational delivery and targeting of therapeutic and diagnostic agents is at the forefront of cancer research (1-5). Common nanomaterials under investigation include polymeric micelles, polymeric and ceramic nanoparticles, viral-derived capsid nanoparticles, liposomes, albumin nanoparticles, and silica nanoparticles. In the current study, we introduce a new nanomaterial that we have been investigating recently in our laboratory. This nanomaterial is onto fabrics called DPV576 cloth (DPV576-C). DPV576-C was examined for its ability to modulate the T lymphocytes of aged mice.

T-cells are pivotal mediators of host defense against uncontrolled cancer growth (6, 7). Although most murine models used for cancer research use young mice, cancer is primarily a disease of aging individuals. In the U.S., more than 50% of cancer diagnoses are made after the age of sixty-five (8). Concurrently, aging is associated with numerous alterations in immune function, resulting in a diminished capacity of the aged immune system to respond to infections or vaccinations (9, 10). We therefore wanted to assess age-related changes in T-cells with respect to percentage and activation *in vivo* and to determine how these alterations could be corrected by DPV576-C treatment.

The major function of the T lymphocytes is to protect the host against infections, cancers, and autoimmune diseases. T lymphocytes play a central role in cell mediated immunity (11, 12), and there is increasing evidence that T-cells are able to control tumor growth and survival in cancer patients (13, 14). In addition, natural killer (NK) cells have been shown to play a critical role in surveillance against the development of cancer (15-18). Selecting aged mice as a model for studying the immune modulatory effect of DPV576-C is advantageous because aging is associated with dysfunction of T lymphocytes, therefore changes in immune response due to treatment can be easily detected. Aging has been demonstrated to affect T-cells as manifested by a decline in T-cell number, proliferation and activation as mice age, which may affect cancer incidence and life span (19-22). Additionally, aging adversely affects the cytotoxic activity of NK cells (23, 24), which is greatly associated with an increased incidence of neoplasia as the mice age (25-28).

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Key Words: DPV576-C, nanodiamond, CD4+ cells, CD8+ cells, *in vivo*.

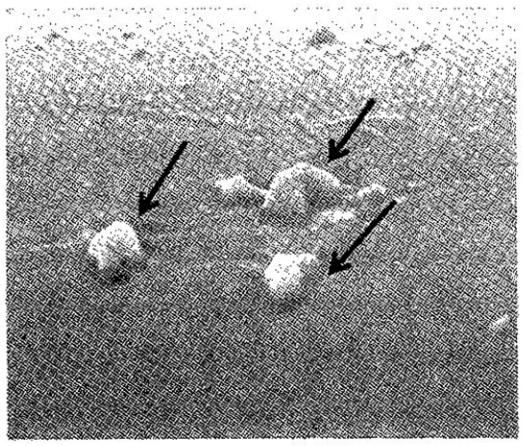

Figure 1. *An illustration of fiber coated with DPV576. Fibers coated with DPV576 were examined by SEM at 30,000x magnification. Arrows indicate the nanoparticles protruding from the surface of the fibers.*

Materials and Methods

Animals. Female C57BL/6 mice were used in the current study. The animals were two months old, purchased from Harlan Laboratories (Chicago, IL), housed five per cage, and permitted free access to water and food. Animals were accommodated until ages of 3 months old (young mice) or 13 months old (aged mice) before experiments began. This study was approved by the Institution Animal Care and Use Committee (IACUC) at Charles Drew University of Medicine and Science.

Complete medium (CM). RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, and 100 μg/ml streptomycin and 100 U penicillin was used to maintain cell cultures.

Tumor cell line. Target cells for use in NK activity assays were the YAC-1 cell line, a Maloney leukemia virus-induced T-cell lymphoma of A/Sn mouse origin. Cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were maintained in CM at a starting density of 3×10^5 cells /ml.

DPV576 cloth (DPV576-C). A nanodiamond (ND) and nanoplatinum (NP) solution was coated onto a cloth called DPV576-C. In preparation of the DPV576, the ND and NP solution was adsorbed strongly to two pieces of fabric that were sewn together, such that ND and NP were adhered to both the outer cover and the inside of the fabric. Table I shows details regarding particle size and density on the fabric, and Figure 1 shows how ND and NP adhere to fabric. DPV576-C was supplied by Venex Company (Japan) and used as lining in a mouse house that was changed once per week for a duration of one month.

Experimental design. Mice were randomly divided into 2 groups: (1) mice receiving DPV576-C, and (2) control mice that had mouse–house lined with fabrics without ND and NP. At the end of the treatment period (one month), animals were euthanized, and spleens were removed and examined for: (1) the percentage of T-cell subsets, $CD4^+$ and $CD8^+$ T-cells, and their activation markers, CD25 and CD69; and (2) NK cell activity. In addition, different organs were examined for pathology.

Table I. *Particle size and density on the fabric.*

	Particles/cm^3 inside fabric	Particles/cm^3 outer cover of fabric	Particle diameter
NP	1.16×10^4	1.35×10^4	10-20 nm
ND	3.9×10^6	6.0×10^{11}	100-200 nm

Preparation of splenic cells. DPV576-C-treated mice and control mice were euthanized, spleens were removed, teased in CM, and contaminating erythrocytes were lysed with distilled water for 20 seconds at room temperature (25°C). Single cell suspensions were washed once with Hanks balanced salt solution (HBSS), and cells were resuspended in CM to a concentration of 1×10^7 cells/ml. Cells were counted using a hemocytometer and a light microscope.

Staining of cells. The phenotype and percentages of $CD4^+$ and $CD8^+$ T-cells were determined by flow cytometry by staining the total splenic cells with specific antibodies (eBioscience, San Diego, CA, USA) as previously described (29). Briefly, 5×10^5 splenic cells were stained using antibodies against the CD4- and CD8-activation markers, CD25 and CD69.

Determination of NK cell function. NK cell-mediated cytotoxicity was determined by a non-radioactive cytotoxicity assay kit (ACT1, Cell Technology Inc., Mountain View, CA) using flow cytometry according to the manufacturer's instructions. Briefly, murine tumor cells, YAC-1, were used as target cells (1×10^4 cells) and were labeled with the cell tracking dye CFSE (laser emission, FL1) and were then cultured with murine splenic lymphocytes (2.5×10^5 cells) from DPV576-C-treated and control mice at effector:target ratio of 12.5:1. After a 6 hour incubation at 37°C, live dead stain 7AAD or propidium iodide (PI, Laser emission FL3 channel) was added to measure cell death. 7AAD and PI only enter membrane-compromised cells and bind to DNA and thus stain dead cells. For each sample, data from 10,000 cells were collected by FACScan flow cytometer and analyzed. During analysis, an electronic gate was placed on CFSE-labeled target cells and the number of dead cells (7AAD or PI positive cells) was determined.

Biosafety of DPV576-C. DPV576-C-treated mice were examined for adverse side effects using two methods: (1) monitoring animal behavior and (2) performing histopathological analysis.

Animal behavior. Experimental animals were examined daily for adverse side effects by assessing the changes in the normal feeding/drinking cycles and life activity patterns for the entire treatment period.

Histopathological analysis. Experimental animals were killed at 4 weeks. Eight different organs were excised and tissues from brain, liver, heart, lung, stomach, small intestine, spleen and skin were fixed in 10% formalin and processed overnight. 3-5 μm paraffin embedded sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope. Histopathological changes were compared with those of control untreated animals.

Statistical analysis. Statistical significance was determined by the Student's *t*-test. Differences were considered significant at the $p<0.05$ level.

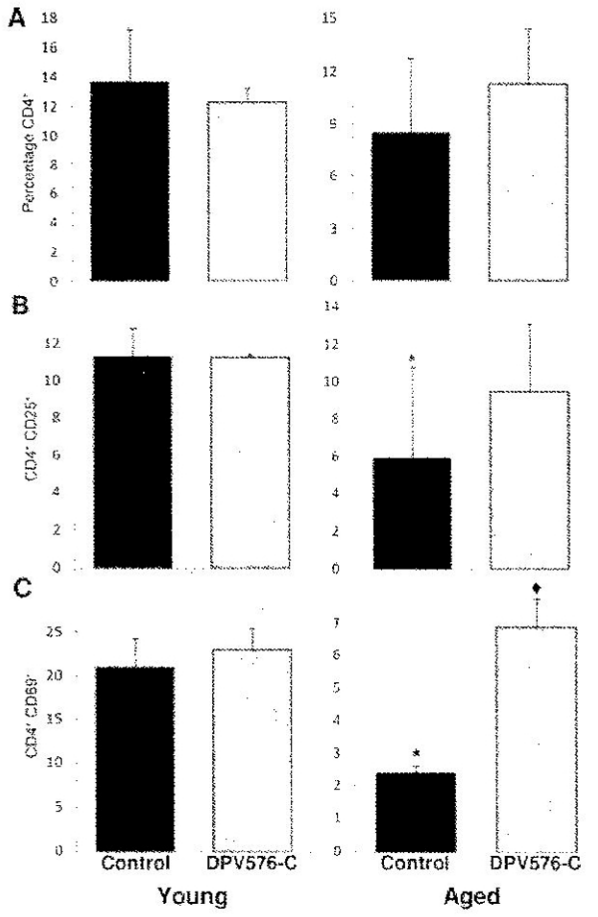

Figure 2. *Action of DPV576-C on the percentage of CD4+ T-cells and CD4+ activation markers CD25 and CD69 in young and aged mice. Mice were treated with DPV576-C for 4 weeks. Spleens were used to examine the percentage of (A) CD4+ T-cells, (B) CD4+CD25+ and (C) CD4+CD69+. Data represent the mean±SD of at least 5 mice in each group (DPV576-C-treated group and control group). *p<0.05, as compared to young control untreated mice, ♦p<0.05 as compared to aged control untreated mice.*

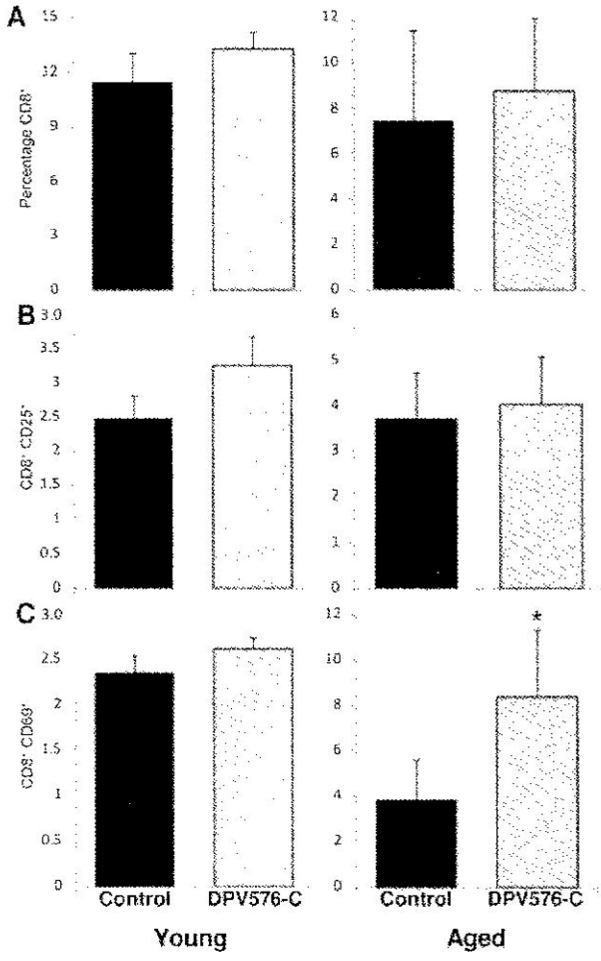

Figure 3. *Action of DPV576-C on the percentage of CD8+ T-cells and CD8+ activation markers CD25 and CD69 in young and aged mice. Mice were treated with DPV576-C for 4 weeks. Spleens were used to examine the percentage of (A) CD8+ T-cells, (B) CD8+CD25+ and (C) CD8+CD69+. Data represent the mean±SD of at least 5 mice in each group (DPV576-C-treated group and control group). *p<0.05, as compared to aged control untreated mice.*

Results

1. Age associated changes in CD4+ and CD8+ T-cells. Age-related changes in the percentage of $CD4^+$ and $CD8^+$ T-cell subtypes and their activation markers were determined in young (3 months) and aged (13 months) mice by flow cytometry. Data depicted in Figures 2 and 3 show that the percentages of $CD4^+$ and $CD8^+$ T-cells decline with increased age. In addition, as mice age, there is a significant decrease in the expression of $CD4^+/CD25^+$ and $CD4^+/CD69^+$ T-cells ($p<0.05$) and a decrease in $CD8^+/CD69^+$ T-cells.

2. Effect of DPV576-C on the percentages of T-cell subtypes and their activation markers. The ability of DPV576-C to repair the age-dependent defects in the percentage of $CD4^+$ and $CD8^+$ T-cell subtypes and the expression of their CD25 and CD69 activation markers was examined.

a) Percentage of CD4+ T-cells and CD25 and CD69 activation markers. Data in Figure 2A show that treatment of aged mice with DPV576-C causes an increase in the percentage of $CD4^+$ T-cells. Data in Figure 2B show that DPV576-C-treated aged mice demonstrate a slight increase in the percentages of $CD25^+$ T-cells, and significant up-regulation in the expression of CD69 in $CD4^+$ T-cells ($p<0.05$) was noticed

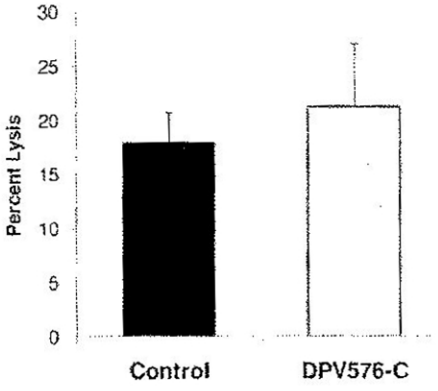

Figure 4. *Action of DPV576-C on NK cell activity in aged mice. NK activity in mice was examined 4 weeks after treatment with DPV576-C. Activity of NK cells was measured in spleens at an effector:target ratio of 12.5:1. Data represent mean±SD of 3 experiments.*

post-treatment with DPV576-C in aged mice (Figure 2C). Young mice showed no changes in the percentage of $CD4^+$ T-cells or expression of CD25 and CD69 activation markers in $CD4^+$ T-cells after treatment with DPV576-C (Figure 2A-C).

b) Percentage of $CD8^+$ T-cells and CD25 and CD69 activation markers. The percentage of $CD8^+$ T-cells and the expression of activation markers CD25 and CD69 in DPV576-C-treated mice are shown in Figure 3. DPV576-C-treated young mice did not show significant changes in the percentages of $CD8^+$ T-cells or the activation markers CD25 and CD69 as compared to control, untreated young mice. However, treatment with DPV576-C in aged mice caused an increase in the percentage of $CD8^+$ T-cells (Figure 3A) and their expression of CD25 (Figure 3B) and a significant increase in expression of CD69 ($p<0.05$) (Figure 3C).

c) NK Cells. DPV576-C-treated aged mice were examined for activity of NK cells. The data depicted in Figure 4 show that there is elevated NK cell activity post treatment with DPV576-C as compared to control mice.

3. Biosafety. We examined whether DPV576-C exerted any adverse side effects *in vivo* using the aged mice model treated with DPV576-C for 1 month. Adverse side effects were assessed in these animals in 2 different ways: a) animal behavior, and b) histopathological examination.

a) Animal behavior. Daily examinations of mice showed that the treatment with DPV576-C resulted in no adverse side effects as indicated by normal feeding/drinking and life activity patterns during the treatment period.

b) Histopathology. DPV576-C-treated mice were sacrificed at 1 month and 8 different organs were examined for pathology. Data in Figure 5 show that the histopathology of liver, brain, lung, and spleen were within the normal limits of control mice. Similar results were observed with other organs, including the heart, stomach, small intestine, and skin (data not shown).

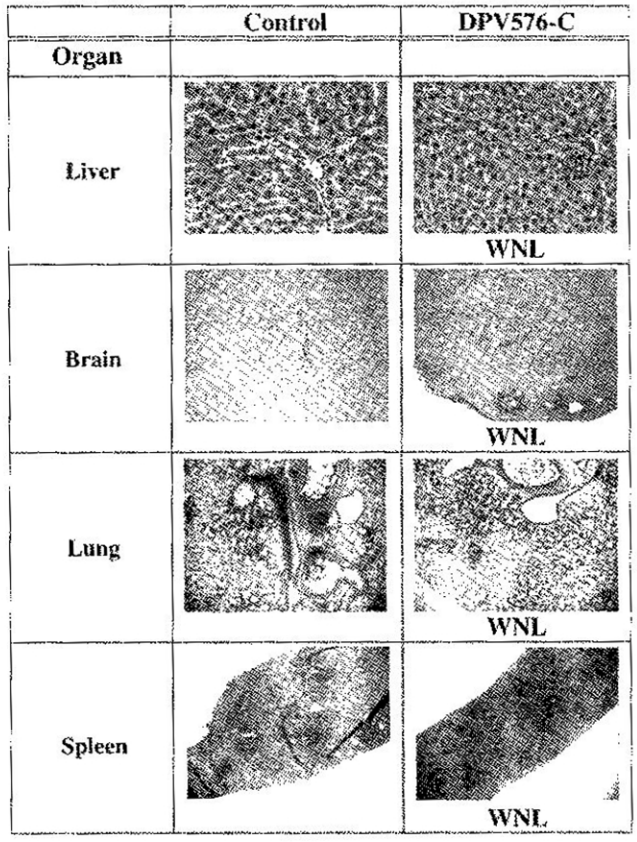

Figure 5. *Histopathology of DPV576-C-treated aged mice. Mice (13 months old) were treated with DPV576-C daily. At 4 weeks post treatment, animals were euthanized and different organs were harvested for histopathology examination, stained with H&E. Results indicate that pathology induced by DPV576-C is within normal limits. WNL=within normal limits.*

Discussion

Recently, nanotechnology has been rapidly emerging in biomedical applications including cancer therapy (2, 3, 30, 31). Recent studies revealed that carbon nanomaterials possess several characteristics that make them attractive for various biomedical applications. These characteristics include the non-cytotoxic nature of nanodiamond (ND), their unique, strong and stable photoluminescence, their small size, and their large specific surface area (32).

T-cells are pivotal mediators of host defense against uncontrolled cancer growth (6, 7). In the current study, a decrease in the percentage of $CD4^+$ T-cells and expression of CD69 and CD25 was observed in aged mice as compared with young mice. This observation is in accordance with others who have shown that aging is associated with functional defects in $CD4^+$ T-cells in humans and mice (33-36). The decline in $CD4^+$ T-cells was attributed to alterations in both the glycosylation of the T-cell surface molecules and the cytoskeleton (33). In this study, we showed that DPV576-C treatment partially counteracts

age-associated decline in the percentage of $CD4^+$ T-cells. In addition, treatment with DPV576-C resulted in an increase in the expression of $CD4^+$ T-cell activation markers, CD25 and CD69 cells. $CD4^+CD25^+$ cells exhibit regulatory/suppressive activity, play a crucial role in maintaining self-tolerance (37-40) and preventing development of autoimmunity (37, 38), and may also be useful in regulating the immune responses to tumors (41-44). CD69 is expressed on recently activated T-cells and may represent a surrogate marker of an ongoing immune response (45). A recent study has showed that tumor-infiltrating $CD4^+CD69^+$ T-cells may also have a positive impact on survival of patients with head and neck squamous cell carcinoma (46).

$CD8^+$ T-cells are critical mediators of protective immunity against cancer. $CD8^+$ cytotoxic T lymphocytes (CTLs) infiltrate solid tumors, recognize tumor antigens, and kill tumor cells (47). In the current study, a decline in the percentage of $CD8^+$ T-cells was noticed as mice aged, an observation that is in agreement with previous work by others (48). DPV576-C treatment caused an increase in the percentage of $CD8^+$ T-cells as well as expression of their activation markers, CD69 and CD25.

The mechanism by which DPV576-C induces immune modulation is not fully understood, but it may involve dorsal root ganglion (DRG) neurons. These cells bring sensory information into the central nervous system (CNS). Our recent studies revealed that treatment with a solution of ND and NP affects the Ca^{2+} release from DRG neurons *in vitro* and expression of nociceptive P2X3 receptors *in vivo* (our unpublished data), suggesting that DPV576-C may affect the DRGs, which in turn influence immune responses. A body of compelling evidence for the existence of bidirectional neural-immune interactions has accumulated as exemplified by findings showing that alteration in the function of the immune system is induced by lesions in the CNS (49, 50), and may also operate through the sympathetic nervous system (SNS) (51, 52). The sympathetic fibers (SFs) innervate all tissues including skin, release norepinephrine (NE) and acetylcholine as their main neurotransmitters, and can modulate immune function as well as the skin immune system (53). In addition, nerve fibers terminate in close contact with lymphocytes in the lymphoid organs (54). Moreover, DPV576-C may directly interact with dendritic cells of the skin, thus influencing T lymphocytes.

Toxicity of these nanomaterials is of great importance for the safety and success of these materials in treating cancer patients, and is currently being studied across the globe. We are not aware of any published research showing toxicity of NDs or NPs in animals. In fact, *in vitro* studies have shown that NDs are non-toxic to a variety of cell types (55). In the current study, DPV576-C-treated animals were monitored to observe potential toxic side effects. Results demonstrated that DPV576-C-treated mice had normal animal behavior. In addition, different organs of these mice had no pathology detected up to 30 days post-treatment with DPV576-C.

Conclusion

DPV576-C caused phenotypic correction of age-associated functional decline in T lymphocytes of mice *in vivo*. Future studies may reveal a potential role for DPV576-C in reversing immune dysfunction in elderly humans using this safe and non-toxic cloth.

Acknowledgements

We thank Venex Company, Japan, for providing DPV576-C.

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Received November 19, 2009
Accepted February 25, 2010