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Fluorescent nanodiamonds engage innate immune effector cells: A potential vehicle for targeted anti-tumor immunotherapy

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Abstract

Fluorescent nanodiamonds (FNDs) are nontoxic, infinitely photostable, and emit fluorescence in the near infrared region. Natural killer (NK) cells and monocytes are part of the innate immune system and are crucial to the control of carcinogenesis. FND-mediated stimulation of these cells may serve as a strategy to enhance anti-tumor activity. FNDs were fabricated with a diameter of 70 ± 28 nm. Innate immune cell FND uptake, viability, surface marker expression, and cytokine production were evaluated *in vitro*. Evaluation of fluorescence emission from the FNDs was conducted in an animal model. *In vitro* results demonstrated that treatment of immune cells with FNDs resulted in significant dose-dependent FND uptake, no compromise in cell viability, and immune cell activation. FNDs were visualized in an animal model. Hence, FNDs may serve as novel agents with "track and trace" capabilities to stimulate innate immune cell anti-tumor responses, especially as FNDs are amenable to surface-conjugation with immunomodulatory molecules.

Key words: Fluorescence; Nanodiamonds; Immunotherapy; Natural killer cells; Monocytes

Abbreviations: Ab, antibody; APC, allphycocyanin; ELISA, enzyme-linked immunosorbent assay; FND(s), fluorescent nanodiamond(s); FS, forward laser light scatter; HAB, human AB serum; HLA, human leukocyte antigen; IFN-γ, interferon gamma; mAb, monoclonal-antibody; MFI, mean fluorescence intensity; MIP, maximum intensity projections; NIR, near-infrared; NK, natural killer; NV, nitrogen-vacancy; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; SEM, scanning electron microscopy; SS, side laser light scatter; TNF-α, tumor necrosis factor alpha.

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Fluorescent nanodiamonds (FNDs) are unique, multifunctional reagents that may be employed in cancer immunotherapy and other biomedical applications. The generation of nitrogen vacancy (NV)-centers within the diamond lattice endows nanodiamonds with fluorescent properties.[1–3] FNDs containing NV-centers are nontoxic, biocompatible nanomaterials that emit strong fluorescence in the near-infrared (NIR) region, remain photostable in the presence of intense laser excitation (>1 GW/cm^2), and exhibit excellent *in vivo* stability.[4–7] Additionally, FNDs present an extensive, modifiable surface area owing to their faceted architecture.[6,8]

The versatile surface of FNDs may be functionally modified. Some functional modification techniques include coating FNDs with biocompatible molecules, such as polyethylene glycol, glycidol or cellobiose, which may be further conjugated for targeted drug delivery applications.[9–11] Importantly, FND surface functionalization may be used as a potential therapeutic anti-tumor strategy. Emerging therapeutic delivery platforms displaying peptides, proteins, and nucleic acids are being developed and provide FNDs with a broad range of future therapeutic options.[12–15] Therefore, FNDs have the potential to be coated with biocompatible chemicals, conjugated to antibodies or other immunomodulatory agents, and thereby targeted to innate immune cells to promote anti-tumor activity.[9–12,16–18]

Innate immune cells contribute to cancer immunosurveillance through recognition and elimination of developing tumors. Monocytes and natural killer (NK) cells are key contributors to this line of defense. Monocytes are phagocytic cells that target and kill opsonized cells.[19–21] NK cells are non-MHC-restricted cytotoxic lymphocytes that have the ability to lyse transformed cells without prior sensitization.[22,23] Upon activation, these cells secrete several immune stimulatory cytokines (e.g., TNF-α and IL-12 by monocytes/macrophages and IFN-γ by NK cells) and upregulate expression of surface molecules indicative of activation (e.g., HLA-DR and CD86 on monocytes and NKG2D and CD69 on NK cells).[24–27] These responses not only mediate direct anti-tumor activity but also promote the recruitment of adaptive immune cells, which further enhances the anti-tumor immune response.[20–22,28–30] Thus, immunotherapeutic strategies that target monocytes and NK cells to promote their effector functions may provide a mechanism to modulate the tumor microenvironment and inhibit tumorigenesis.

With the knowledge that the immune system shapes the course of tumor progression, scientists in the growing field of cancer immunotherapy have aspired to identify immunomodulatory agents to harness the power of the immune system for the treatment of cancer. In the present study, it was hypothesized that FNDs may serve as vectors for targeted immune cell activation to promote anti-tumor activity. To address this question, it was important to first evaluate direct immune cell uptake, biocompatibility, and immunostimulation mediated by unconjugated FNDs prior to examining further biomedical applications. Therefore, we have characterized the behavior of monocytes and NK cells following exposure to unconjugated FNDs through evaluation of cellular uptake, FND localization, cell viability and effects on immune cell activation. The findings presented herein support future investigation into novel therapeutic applications utilizing FNDs. Notably, this study confirms that innate immune cells will take up FNDs with no compromise in cell viability and result in stimulation of pro-inflammatory responses. Hence, these results support the notion that FNDs may serve as novel agents to stimulate innate immune cell anti-tumor responses.

Methods

Reagents and cell lines

Murine macrophage cell line, RAW264.7, was obtained from American Type Culture Collection (Manassas, VA). Human natural killer cell (NKL) cell line was provided by Dr. Michael A. Caligiuri at The Ohio State University.

FND preparation

Columbus Nanoworks, Inc. (Columbus, OH) generated FNDs from micron-sized, high pressure, high temperature diamonds that were then milled to nanoscale size and cleaned with concentrated sulfuric acid and nitric acid as previously described.[31] The FNDs generated in this study were uncoated, non-functionalized, and not activated.

FND characterization

Samples were prepared by depositing 0.0005% (w/w) FNDs on a coverslip and drying in a vacuum oven for 2 h. Scanning electron microscopy (SEM) images were acquired on a field emission scanning electron microscope (Zeiss Ultra 55) operating at 5 kV and a working distance of 7.9 mm. Size characterization was performed from the SEM images (n = 168) using Image J. The fluorescence emission spectrum was obtained using a Horiba ARAMIS Raman upright microscope (50× objective) and a 532 nm excitation laser. Confocal microscopy images were collected on an Olympus FV1000-Filter Confocal System using a 543 nm excitation laser and corresponding 655/55 nm barrier filters.

Isolation of human NK cells and monocytes

Peripheral blood mononuclear cells (PBMCs) and NK cells were isolated from healthy donor leukopacks (American Red Cross, Columbus, OH) as previously described.[32] For NK cell isolation, PBMCs were incubated for 30 min with RosetteSep Human NK Cell Enrichment Cocktail (Immunodensity Negative Selection Cocktail, Stem Cell Technologies, Vancouver, BC). Ficoll hypaque density gradient centrifugation was performed and PBMC and NK cells were collected. NK cells procured in this manner were confirmed to be greater than 95% pure by flow cytometric analysis according to their CD56 surface marker expression. For monocyte isolation, PBMCs were added to 6-well plastic plates and allowed to adhere for 24 h prior to washing and harvesting the monolayer. Monocytes procured in this manner were confirmed to be greater than 94% pure by flow cytometric analysis according to their CD14 surface marker expression. Immune cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated pooled human AB serum (C-six Diagnostics; Germantown, WI), 100 U/ml of