Antibody Conjugation of Fluorescent Nanodiamonds for Targeted Innate Immune Cell Activation

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ABSTRACT: Background: fluorescent nanodiamonds (FND) are nontoxic, infinitely photostable nanoparticles that emit near-infrared fluorescence and have a modifiable surface allowing for the generation of protein–FND conjugates. FND-mediated immune cell targeting may serve as a strategy to visualize immune cells and promote immune cell activation. Methods: uncoated-FND (uFND) were fabricated, coated with glycidol (gFND), and conjugated with immunoglobulin G (IgG–gFND). In vitro studies were performed using a breast cancer/natural killer/monocyte co-culture system, and in vivo studies were performed using a breast cancer mouse model. Results: in vitro studies demonstrated the targeted immune cell uptake of IgG–gFND, resulting in significant immune cell activation and no compromise in immune cell viability. IgG–gFND remained at the tumor site following intratumoral injection compared to uFND which migrated to the liver and kidneys. Conclusion: antibody-conjugated FND may serve as immune drug delivery vehicles with “track and trace capabilities” to promote directed antitumor activity and minimize systemic toxicities.

KEYWORDS: fluorescent nanodiamonds, antibody conjugation, immunotherapy, natural killer cells, monocytes

INTRODUCTION

Fluorescent nanodiamonds (FND) are carbon-based nanomaterials that contain nitrogen-vacancy (NV) centers and emit bright, photostable fluorescence centered at ~700 nm in the near-infrared (NIR) region. They are chemically inert and display minimal cytotoxicity in vivo. Additionally, FND do not show photobleaching after continuous excitation or fluorescence blinking and are brighter than organic dyes on a molar basis, making them preferable to most commercial fluorophores and fluorescent proteins. NV-center nanodiamonds exhibit a longer lifetime, higher quantum yield, and finite photostability when compared to organic dyes, such as indocyanine green, and are at least comparable to quantum dots in physical properties. Because of these favorable aspects, FND are being investigated as potential fluorescent biomarkers and as therapeutic agents.

As with non-FND, the biological applications of FND are enhanced by their high surface area-to-volume ratio and modifiable surface chemistry, allowing them to be conjugated to a variety of different molecules. Molecules can be conjugated to nanodiamonds using surface oxygen-terminating groups on the nanodiamonds or coating them with biocompatible molecules, such as silica, or polymers, such as polyethylene glycol (PEG) or glycidol. Glycidol is a three-carbon epoxy alcohol that creates a dense hydrophilic coating around FND. It is an alternative to PEG and silica coating and minimizes nonspecific interactions, mitigates the tendency to aggregate in physiological conditions, and provides functional groups for conjugation with DNA, proteins, or therapeutic drugs.

FND can be utilized in cancer immunotherapy for targeting and modulating immune cells. The tumor microenvironment is made up of complex interactions between various immune cell populations and tumor cells. Innate immune cells, such as monocytes and natural killer (NK) cells, play a major role in tumor immune surveillance. Monocytes participate in phagocytosis and recognize damage-associated molecular patterns released by tumor cells through specific toll-like receptors (TLRs), leading to immune activation. NK cells are cytotoxic lymphocytes that recognize and lyse cells under...
significant stress. In response to stress, cells downregulate MHC-I expression, leaving them sensitive to lysis by NK cells. These stressed cells may also upregulate the expression of stress-induced ligands that lead to NK cell activation and target

Figure 1. FTIR spectroscopy and SEM analyses of FND. (a) FTIR spectra of uFND (I) and gFND (II) collected from a Nicolet 6700 spectrometer with a Smart Orbit ATR module. The gFND show an increase in alcohol (−OH) stretch between 3500 and 3200 cm$^{-1}$ and ether linkage’s (C−O−C) asymmetric stretch between 1300 and 1000 cm$^{-1}$. Weak symmetric vibrations are also visible in the 890−820 region of the spectra. The carboxylic acid carbonyl (C=O) stretch is present in the spectral range 1850−1650; however, the contribution of carboxylic acid hydroxyl (−OH) stretch (3300−2500) is reduced in the gFND. The corresponding spectra of glycidol show similar signature peaks as coated FND (III). (b) SEM images of fluorescent NV-center nanodiamonds dispersed on a carbon-adhesive tab. Images shown in (I−III) were collected on a Thermo Fisher Apreo LoVac SEM whereas images shown in (IV) were collected on a JEOL-7200F FE-SEM. Image (I) shows a high-resolution secondary electron image of agglomerated nanodiamonds, whereas image (II) shows the corresponding red cathodoluminescent signal generated by the features visible in image (I), and image (III) shows an enlarged image of the region circled in image (I).
cell lysis. In contrast, immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), promote tumorigenesis and inhibit the immune response against tumor cells. The application of nanodiamonds in cancer treatment is in tumor targeting and as drug delivery vehicles that evade the innate immune system and deliver their payload to tumor cells without causing toxicity to nonmalignant cells. Polyglycerol (PG)-coated nanodiamonds have been conjugated with arginine-glycine-aspartic acid (RGD) to deliver the chemotherapy drug doxorubicin to cancer cells expressing RGD receptor on their surface. Nanodiamonds have also been coupled with cetuximab, targeting epidermal growth factor receptor-expressing cancers cells, to deliver paclitaxel and cisplatin drug payloads.

Few studies have explored the effect of nanodiamonds on immune cells in the tumor microenvironment. Nanodiamonds activate monocytes and dendritic cells in vitro, which indicates their potential in cancer immunotherapy. Innate immune cells, including monocytes and NK cells, contain a fragment crystallizable gamma receptor (FcγR) that is specific for and activated by IgG. Conjugation of IgG molecules on the surface of FND could expose the Fc regions of the IgG for FcγR binding and thereby activate FcR-expressing innate immune cells and/or the uptake of the FND. Understanding how FND conjugated with IgG can target and activate innate immune cells is a necessary step for future studies to use FND, conjugated to more specific antibodies, for targeting tumor or immune cells.

In this study, we investigated the effects of a glycidol-coated FND (gFND) conjugated with immunoglobulin G (IgG–gFND) on immune cells in the tumor microenvironment. We compare uncoated (uFND), gFND, and IgG–gFND regarding their in vitro FND uptake, immune cell viability, and activation. We also evaluate the use of IgG–gFND in vivo using a murine breast cancer model. This study evaluates the potential of antibody-conjugated FND as novel agents for enhanced cancer immunotherapy.

Figure 2. FND characterization. (A) FND were coupled to antibodies using disuccinimidyl carbonate (DSC), and the conjugates were characterized. (B) Fluorescence emission spectra of uFND, gFND, and IgG–gFND at 535 and 637 nm. (C) Hydrodynamic size data (DLS) of all three diamond types, indicating that IgG–gFND show an appreciable size increase post-conjugation.
immunotherapy and targeted real-time innate immune cell visualization.

**RESULTS**

**FND Characterization.** FND were generated from synthetic high-pressure high-temperature diamonds containing nitrogen impurities, following a previously described electron irradiation process.32,33 Following subsequent annealing to create NV centers and extensive cleaning, the uFND were then reacted with glycidol, a bio compatible, epoxy alcohol compound, to create gFND. Figure 1a shows the FTIR spectroscopy results for both uFND and gFND and shows evidence for the surface carboxyl and alcohol groups on both uFND and gFND. The glycidol coat introduced more alcohol groups on the surface than the uncoated nanodiamonds.

Figure 3. Assessment of FND-antibody conjugation. (A) ELISA results show that FND coated with rabbit IgG were detected by GaR-IgG and FND coated with human IgG were detected by GaH-IgG, whereas gFND were unreactive. (B) IgG-coated FND were detected by GaR-IgG and GaH-IgG linked to HRP. For A and B, the means ± standard errors for n = 3 for all panels. * Represents p < 0.05 compared to gFND controls and + with underlying bracket represents p < 0.05 for comparisons across groups. (C) ELISA results to estimate the amount of human IgG captured by a polyclonal IgG antibody-gFND conjugate. Shown are the average of three separate experiments in which no FND (0 μg FND), gFND, or variable amounts of anti-human IgG-gFND were added to approximately 10 ng/mL human IgG. Supernatants were recovered from these incubations and tested by standard ELISA, as described in the Methods section. The inset shows a standard curve of known amounts of IgG, ranging from 0 to 10 ng/mL ($r^2 = 0.92$) and was used to estimate IgG protein amounts.
esters for conjugation with antibodies to create immunoglobulin G-conjugated FND (IgG–gFND). Our overall strategy for creating IgG–FND is illustrated in Figure 2a.

Figure 2B shows the fluorescence emission spectrum for uFND, gFND, and IgG–FND, following excitation with a green LED. All samples show a broad band between 550 and 800 nm, with the maximum emission around 700 nm. Two zero-phonon lines (ZPLs) at 535 and 637 nm, representing the NV− and NV+ charge states, are the characteristic features of NV centers.1,2,3,4,5,6 No significant differences were noted between the different FND preparations following excitation, with both ZPLs readily visible in glycidol-coated and antibody-conjugated FND.

FND size characterization was performed by dynamic light scattering (DLS) (Figure 2c). Three separate samples of each FND type were evaluated, and the mean hydrodynamic diameter of each analysis was then used to calculate the overall FND size. The uFND hydrodynamic size ranged between 76 and 98 nm with an overall mean diameter of 86.3 ± 0.4 nm (mean ± standard error). As expected, the FND hydrodynamic size increased following glycidol coating to a mean diameter of 98.7 ± 0.2 nm and further increased after IgG antibody conjugation to a mean hydrodynamic diameter of 148.9 ± 0.4 nm. However, all FND conjugates remained under 200 nm in hydrodynamic size, which is crucial for maintaining their important biological nanoparticle properties.

The diamond powder manufacturer does not produce an absolute diamond size, rather they provide a range of sizes between 79 and 187 nm, of which 100 nm represents the average size. This shows a discrepancy in the data presented in Figure 2c, where these nanodiamonds were shown to have a distribution between 76 and 98 nm with an average hydrodynamic size of ~86 nm. Extensive processing (centrifugation, irradiation, etc.) could have produced losses at both ends of the size class. Differences between the manufacturer (Horiba LA-910) and the instrument (Brookhaven) used in this study could have also led to the differences in size data.

**Functional Assessment of FND–Antibody Conjugation.** **Modified Enzyme-Linked Immunosorbent Assay.** A modified enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the functionality of IgG–gFND and its binding specificity to antibody-coated wells. ELISA plates were coated with either goat anti-rabbit (GaR)–IgG or goat anti-human (GaH)–IgG and treated with gFND (as negative control), rabbit IgG–gFND (R-IgG–gFND), or human IgG–H-IgG–gFND fluorescence (Figure 3a). No significant binding of gFND to the antibody-coated wells was observed. R-IgG–gFND demonstrated no significant binding to the GaH–IgG-coated wells compared to the gFND control (0.16 ± 0.02 vs 0.11 ± 0.01 μg, p = 0.983). However, significant R-IgG–gFND binding was seen to the GaR–IgG-coated wells compared to the GaH–IgG-coated wells (1.33 ± 0.11 vs 0.08 ± 0.01 μg, p < 0.001). Contrarily, H-IgG–gFND demonstrated no significant binding to the GaR–IgG-coated wells compared to gFND (0.08 ± 0.01 vs 0.09 ± 0.01 μg, p = 0.603), but there was significant binding to the GaH–IgG-coated wells compared to GaR–IgG-coated wells (0.71 ± 0.10 vs 0.16 ± 0.02 μg, p < 0.001).

As polyclonal antibodies are a pool of different antibodies targeting different epitopes within the same antigen, it was difficult to estimate the antibody affinity following FND conjugation. We therefore aimed at testing a defined polyclonal antibody–antigen pair that could generally reflect the affinity of an antibody–FND conjugate. We chose GaH and its antigen human IgG. In this approach, we created GaH–IgG–gFND conjugates and tested if they could deplete a fixed amount of human IgG in solution. Following 2 h of incubation, reactants were centrifuged to pellet the FND conjugate, and the supernatant was evaluated for any remaining IgG using standard ELISA. Figure 3C shows the average of three separate experiments using no FND, gFND, or variable amounts of GaH–IgG–gFND to deplete a fixed amount of human IgG. The results show that GaH–IgG–gFND but not gFND could remove a significant amount of human IgG from solution. Based on these results, we estimate that the antibody-binding range for a polyclonal IgG–gFND conjugate can be between 1.5 and 3 ng/mL per μg FND.

**Horseradish Peroxidase Assay.** Horseradish peroxidase (HRP) assay was performed to evaluate the IgG–gFND binding specificity to secondary IgG–HRP conjugates. gFND, R-IgG–gFND, and H-IgG–gFND were incubated with either goat anti-rabbit biotinylated HRP–IgG (GaR–HRP) or goat anti-human biotinylated HRP–IgG (GaH–HRP) conjugates. After incubation, the HRP catalytic activity for these immune conjugates captured by the FND was evaluated (Figure 3b). These results confirmed the ELISA data. After incubation with the GaR–HRP conjugates, the R-IgG–gFND demonstrated significant HRP activity compared to the GaH–HRP incubation (4.1 ± 1.1 vs 0.02 ± 0.05 mU/mL, p = 0.001). Correspondingly, H-IgG–gFND demonstrated significant HRP activity after incubation with GaH–HRP compared to the GaR–HRP incubation (5.59 ± 0.35 vs 0.02 ± 0.05 mU/mL, p < 0.001).

**Immunoblot Probing with Fc-Specific GaH–IgG–gFND.** Immunoblot analysis was performed to confirm the presence of Fc-containing IgG molecules on the surface of conjugated FND. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with known concentrations of human IgG (Figure 4A, lanes 3–5, containing 7, 5, and 2.5 μg of human IgG, respectively). Lane 1 contains BSA and lane 2 is blank. The gels were electrophoretically transferred to nitrocellulose membranes, probed with GaH-(Fc)–gFND, and evaluated for fluorescence using a Maestro imaging system (Figure 4A right panel). The GaH–IgG–gFND is specific to the Fc region of heavy chains (HCs) and thus fluorescent bands were visible over the HCs (Figure 4A, lanes 7–9) after probing with GaH(Fc)–gFND, whereas light chain (LC) bands were not seen.

**SDS-PAGE and Chemiluminescence.** The stabilities of FND bioconjugates were evaluated by SDS-PAGE. In this approach, proteins that are not covalently bound will be released by the SDS detergent into the gel. SDS-PAGE gels were run with human IgG alone (Figure 4B, lane 2), uFND that were incubated with human IgG and rinsed (Figure 4B, lane 3), gFND that were incubated with human IgG and rinsed (Figure 4B, lane 4), and H-IgG–gFND (Figure 4B, lane 5). The uFND sample demonstrated the migration pattern of IgG, indicating the noncovalent binding of IgG to uFND during incubation and the subsequent release of IgG by the SDS detergent (Figure 4B lane 3). The gFND sample did not show any IgG pattern, indicating a decrease in the nonspecific binding of IgG to gFND as compared with uFND (Figure 4B lane 4). As expected, the IgG–gFND sample did not demonstrate any IgG pattern as IgG is covalently bonded to
gFND and cannot be released by the SDS detergent (Figure 4B lane 5).

SDS–PAGE gels were then used to evaluate the antibody function of IgG–gFND conjugates. The gels were run with GaH–IgG alone (Figure 4C, lane 2), gFND that were incubated with both human IgG and GaH–IgG and rinsed (Figure 4C, lane 3), and H-IgG–gFND that were incubated with GaH–IgG and rinsed (Figure 4C, lane 4). The GaH–IgG sample in lane 2 demonstrated the expected IgG migration pattern. The gFND sample revealed a faint IgG migration pattern, suggesting a minimal noncovalent binding of IgG to gFND, similar to the previous SDS–PAGE gel. The H-IgG–gFND sample revealed a more pronounced IgG migration pattern. To identify which antibody was released from the H-IgG–gFND sample incubated with GaH–IgG (lane 4), immunoblot analysis and chemiluminescence of this gel were performed (Figure 4C, right panel). After immunoblot processing, the membrane was probed using rabbit anti-goat/HRP antibody, and chemiluminescence was observed in both the GaH–IgG control (Figure 4C lane 6) and the H-IgG–gFND sample (Figure 4C lane 8), identifying the released antibody as GaH–IgG. This indicates the noncovalent binding of H-IgG–gFND to GaH–IgG, suggesting antibody capture as GaH–IgG recognizes epitopes on the H-IgG–gFND and subsequent release upon introduction into the SDS loading buffer. These results demonstrate that antibodies can be covalently conjugated to FND and that they retain their important biological functions within these conjugates.

**Evaluation of Cellular FND Uptake.** Glycidol Coating Decreases Nonspecific Uptake. Our preliminary studies demonstrated that uFND are nonspecifically taken up by a variety of cell types (data not shown). Therefore, the ability to decrease nonspecific FND uptake via FND polymer coating with glycidol (gFND) was first evaluated. SK-BR-3 human breast cancer cells, EMT6 mouse breast cancer cells, and human NK cells and monocytes were individually cultured and treated for 24 h with either the medium alone (untreated control, CTL) or the medium with gFND or uFND. The cells were then collected and evaluated via flow cytometry for FND uptake based on the changes in intracellular granularity (side scatter—SS) and NIR fluorescence. FND uptake changes the intracellular particle complexity and scatters light in the visible spectra. Therefore, FND uptake is indicated by an increase in both SS and NIR fluorescence. SS by NIR bivariate plots were generated from the SS by FS cytograms. FND uptake was determined based on the proportion of SS and NIR double-positive cells identified (Figure 5a). Compared to the CTL, treatment with uFND resulted in significant FND uptake by both phagocytic cells (monocytes: 3.6 ± 1.1% vs 77.5 ± 6.7%, p < 0.001) and nonphagocytic cells (SK-BR-3: 2.6 ± 0.5% vs 94.3 ± 1.9%, p < 0.001; EMT6: 1.8 ± 0.2% vs 97.3 ± 0.1%, p < 0.001; NK cells: 1.6 ± 0.2% vs 62.1 ± 4.3%, p < 0.001). Compared to uFND, gFND treatment resulted in a significant decrease in FND uptake by nonphagocytic cells (SK-BR-3: 94.3 ± 1.9% vs 15.9 ± 4.0%, p < 0.001; EMT6: 97.3 ± 0.1% vs 7.0 ± 0.6%, p < 0.001; NK cells: 62.1 ± 4.3% vs 5.6 ± 0.8%, p < 0.001). Monocyte uptake of gFND was only slightly reduced compared to uFND. Therefore, conjugation of FND to glycidol or other molecules could potentially be used to target FND uptake to specific cell populations.

**FND Antibody Conjugation Increases Uptake by Immune Cells.** Human NK cells and monocytes were individually cultured and treated for 24 h with CTL, gFND, or IgG–gFND. The cells were then collected and evaluated via flow cytometry for FND uptake, as described above (Figure 5b). Similar to the
results above, only the monocytes demonstrated significant gFND uptake compared to CTL (45.4 ± 7.9% vs 3.6 ± 1.1%, p < 0.001). IgG–gFND treatment resulted in significant FND uptake in both immune cell populations compared to CTL (NK cells: 24.1 ± 4.2 vs 1.6 ± 0.2%; monocytes: 73.9 ± 5.9% vs 3.6 ± 1.1%, p < 0.001, p < 0.001).

**FND Antibody Conjugation Results in Targeted Immune Cell Uptake in a Co-culture System.** To investigate if FND uptake can be specifically targeted to immune cells in a tumor

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**Figure 5.** FND uptake. (a) SK-BR-3 human breast cancer cells, EMT6 mouse breast cancer cells, and human monocytes and NK cells were each cultured and treated for 24 h only in the medium (untreated control, CTL), or with the addition of 100 μg of either gFND or uFND. The cells were then collected, and the percentage of FND uptake was determined by flow cytometry based on the percentage of cells showing increased SS and near-infrared fluorescence (SS(NIR)). The means ± standard errors for three independent experiments for SK-BR-3 and EMT6, four independent experiments for monocytes, and nine independent experiments for NK cells are shown. (b) Human monocytes and NK cells were individually cultured and treated for 24 h with CTL or 100 μg of either gFND or IgG–gFND. The cells were then collected and the percentage of FND uptake was determined. The means ± standard errors for four independent experiments for monocytes and nine independent experiments for NK cells are shown. (c) SK-BR-3 human breast cancer cells and primary human monocytes and NK cells were co-cultured and treated for 24 h with CTL or 100 μg of uFND, gFND, or IgG–gFND. The cells were then collected and labeled with CD14-APC mAb for monocyte identification and CD56/NKH1 RD1 mAb for NK cell identification and evaluated by flow cytometry for FND uptake based on the changes in the intracellular NIR fluorescence of each cell population. The means ± standard errors for three independent experiments are shown. (d) TAMs and MDSCs were individually cultured and treated for 24 h with CTL or with 100 μg of IgG–gFND. The cells were then collected and the percentage of FND uptake was determined. The means ± standard errors of three independent experiments for TAM and four independent experiments for MDSC are shown. * Represents p < 0.05 versus CTL and * with underlying bracket represents p < 0.05 for comparisons across groups.
microenvironment, we evaluated the amount of FND uptake in each cell type in an in vitro co-culture system. SK-BR-3 human breast cancer cells and human NK cells and monocytes were cultured together for 24 h with CTL, uFND, gFND, or IgG–gFND. The cells were then harvested and labeled with fluorescent-labeled antibodies to CD14 (allophycocyanin, APC) and CD56 (phycoerythrin, PE) for monocyte/NK cell determination by flow cytometry. Bivariate plots were created and analyzed for identification of the three distinct cell populations. FND uptake was then evaluated based on changes in the intracellular NIR fluorescence of each cell population (Figure 5c). In this co-culture system, NK cells did not demonstrate significant FND uptake with any of the FND treatments compared to CTL. Compared to CTL, the SK-BR-3 tumor cells demonstrated significant uptake of both the uFND (1.1 ± 0.1% vs 48.1%, p < 0.001) and gFND (1.1 ± 0.1% vs 23.2 ± 5.2%, p = 0.011), but the uptake of IgG–gFND was not significant (1.1 ± 0.1% vs 17.0 ± 2.4%, p = 0.059). Monocytes demonstrated significant uptake for all the FND treatment groups compared to CTL (uFND: 96.4 ± 0.9%; gFND: 94.7 ± 0.3%; IgG–gFND: 84.9 ± 6.9%, all conditions with p < 0.001). Notably, a high level of monocyte IgG–gFND uptake was still observed within the context of the co-culture system.

In monoculture, monocytes and NK cells show a high nonspecific uptake of uFND, a decrease in the nonspecific uptake of gFND, and an increase in the specific uptake of IgG–gFND. However, this pattern is not observed in the co-culture system. In addition, it should be noted that the co-culture system, while providing for multicell conditions, may not be representative of the actual tumor microenvironment. The monoculture data are robust and indicative of the ability of IgG to enhance FND uptake by FcR-bearing cells. We speculate about the reasons for our results in the Discussion section.

**FND Antibody Conjugation Results in FND Uptake by Immune Suppressor Cells.** After demonstrating that antibody-conjugated FND can be targeted to innate immune cells, the potential to target immune suppressor cells with antibody-conjugated FND was evaluated. TAMs generated in vitro with tumor-conditioned media and MDSCs isolated from stage IV cancer patients were treated with CTL or IgG–gFND. Following 24 h of incubation, the cells were collected and evaluated via flow cytometry for FND uptake, as described above (Figure 5d). Both TAMs and MDSCs demonstrated significant IgG–gFND uptake compared to CTL (TAMs: 76.9 ± 7.9% vs 0.9 ± 0.1%, p = 0.011; MDSC: 30.9 ± 10.5% vs 1.4 ± 0.9%, p = 0.015), indicating that FND antibody conjugation may also target immune suppressor cells. In monoculture, monocytes and NK cells show a high nonspecific uptake of uFND, a decrease in the nonspecific uptake of gFND, and an increase in the specific uptake of IgG–gFND. However, this pattern is not observed in the co-culture system.

**IgG–gFND Localize to Immune Cells over Tumor Cells.** A co-culture model was created using MSC2, a murine MDSC-like cell line, with EMT6, a murine breast cancer cell line, to mimic the tumor microenvironment. The two cell lines were co-cultured in media and incubated with CTL, uFND, gFND, or IgG–gFND for 24 h. Cells were then collected and evaluated via flow cytometry to assess the percentage of cells that had FND uptake. The mean percentage of uFND, gFND, and IgG–gFND uptake by MSC2 cells was 16.0 ± 0.3, 5.5 ± 0.3, and 23.7 ± 3.6%, respectively. The mean percentage of uFND, gFND, and IgG–gFND uptake by EMT6 cells was 18.4 ± 3.0, 9.7 ± 0.1, and 6.5 ± 1.0% (Figure 6), respectively.

Whereas the uptake of uFND was similar between immune cells and tumor cells (p = 0.51), following IgG conjugation, the IgG–gFND uptake by immune cells is significantly higher compared to that by tumor cells in co-culture (p = 0.044). Confocal Microscopy Demonstrates Intracellular FND Uptake. Confocal microscopy was used to visualize immune cell FND uptake. NK cells and monocytes were cultured with CTL, gFND, IgG–gFND, or uFND for 24 h. Nuclear staining was then performed, and the cells were imaged with confocal microscopy. Cross-sectional images were generated, demonstrating the fluorescence and interference contrast (Figure 7). These images depicted NIR fluorescence from the FND inside the cell but outside of the blue-stained nucleus, indicating FND uptake within the cell cytoplasm. Monocytes generally appeared to have greater FND uptake than NK cells, and the greatest uptake of FND occurred in monocytes incubated with IgG–gFND. These images support the notion that FND enter the cell and reside in vesicles within the cytoplasm.

**Impact of IgG–gFND on Immune Cells.** IgG–gFND Do Not Compromise Immune Cell Viability. Cell viability studies were performed to evaluate the effect of FND treatments on immune cell viability. Monocytes and NK cells were incubated with CTL, uFND, gFND, or IgG–gFND for 24 h. The cells were then collected and stained with trypan blue to assess viability. The mean percentage of viable monocytes treated with CTL, uFND, gFND, and IgG–gFND was 88.7 ± 0.1, 89.4 ± 0.7, 89.0 ± 1.6, and 92.6 ± 0.8%, respectively. The mean percentage of viable NK cells treated with CTL, uFND, gFND, and IgG–gFND was 89.6 ± 2.3, 86.8 ± 3.1, 93.3 ± 1.3, and 92.2 ± 1.1%, respectively.

**IgG–gFND Stimulate Immune Cell Function.** Monocyte and NK cell activation by IgG–gFND was evaluated through the differences in cell surface marker expression. CD86 and HLA-DR, as well as CD69 and NKG2D, were the two cell surface markers used to evaluate monocyte and NK cell expression, respectively. Monocytes and NK cells were individually cultured for 24 h with CTL, free IgG (as a positive control), or IgG–gFND. Monocytes treated with
the FND within the NK cells. Scale bar = 10 μm.

Figure 7. FND localize within the cytoplasm of immune cells. Human monocytes and NK cells were cultured for 24 h in medium alone (CTL) or with 100 μg/mL gFND, IgG–gFND, or uFND. Cells were collected and stained using PureBlu Hoechst 33342 to show nuclei in blue. FNDs are visualized in red. White arrows additionally highlight the FND within the NK cells. Scale bar = 10 μm.

IgG–gFND showed no significant differences in CD86 or HLA-DR expression compared to CTL or IgG (Figure 8a). NK cells treated with IgG–gFND showed increased expression of both CD69 and NKG2D compared to IgG (CD69, p < 0.001; NKG2D, p = 0.016) and CTL (CD69, p < 0.001; NKG2D, p = 0.013) (Figure 8b). IgG–gFND immune cell activation was further evaluated via the production of TNF-α and IFN-γ by monocytes and NK cells, respectively, in a coculture system (Figure 8c). Monocytes and SK-BR-3 tumor cells were cultured for 48 h with CTL, free IgG, or IgG–gFND. Supernatants were collected, and ELISA was performed. In this co-culture system, IgG–gFND treatment resulted in both increased TNF-α and IFN-γ production compared to IgG (TNF-α, p = 0.028; IFN-γ, p = 0.011) and CTL (TNF-α, p = 0.015; IFN-γ, p = 0.036).

Notably, we have previously demonstrated that uFND are able to activate innate immune cells as well. To further differentiate the activating effect of antibody conjugation from the FND vehicle itself, we incubated Raji, a human leukocytic leukemia cell line, with CTL, uFND, gFND, or IgG–gFND for 24 h, and then evaluated the resultant TNF-α production. Here, we saw a significant increase in TNF-α production in the IgG–gFND group compared to gFND (p = 0.011) and uFND (p = 0.0045) (Figure 9). These results suggest that antibody conjugation results in greater activation than free IgG or the FND vehicle itself.

Ex Vivo IgG–gFND Fluorescence Imaging. To evaluate the imaging depth of FND, 150 μg of IgG–gFND and uFND was placed beneath chicken breast tissues of different thicknesses and fluorescence images were collected (Figure 10a). Fluorescence from IgG–gFND and uFND was detected at 0.5 and 1 cm, following a 10 s exposure time. There was increased scattering at thicker depths, which could not be resolved at a fixed exposure time of 10 s. At a depth of 1.5 cm, the FND fluorescence became diffuse, and at 2 cm, the FND could not be visualized for either IgG–gFND or uFND. These results demonstrate that antibody-conjugated FND can be used for in vivo imaging experiments, with the sharpest images obtained when the FND are 1 cm or less from the skin surface. However, longer exposures may be required for imaging of FND in deeper tissues.

In Vivo IgG–gFND Studies Utilizing a Murine Breast Cancer Model. To evaluate the use of IgG–gFND in vivo, an EMT6 mouse breast cancer model was employed. Intratumoral injections were performed with non-FND as negative control, uFND as positive control, or mouse IgG–gFND. Five minutes after injection, in vivo imaging demonstrated the presence of intratumoral fluorescence in both uFND- and mouse IgG–gFND-treated mice (Figure 10b).

uFND Accumulate in the Liver and Kidneys, Whereas IgG–gFND Do Not. As FND survive harsh treatments that otherwise would damage biological tissues, examination of FND biodistribution is possible. After 24 h of intratumoral injections of non-FND, uFND, or mouse IgG–gFND, mice were euthanized as per protocol and necropsies were performed. The liver, kidneys, and spleen were processed by oxidation and acid treatment protocols, and the samples were dried on glass slides. The samples were analyzed for NV center fluorescence emission spectra utilizing a confocal spectrometer (Figure 10c). No fluorescence spectra indicative of NV centers were observed in any of the digested organs from the mice injected with non-FND or mouse IgG–gFND. However, fluorescence spectra indicative of NV centers were observed in the digested liver and kidneys of mice injected with uFND, indicating the presence of FND. No fluorescence spectra were observed in the spleen sample. These findings suggest that the uFND were localized in the liver and kidneys, whereas the mouse IgG–gFND were not. These results correlate with the in vivo images obtained prior to organ collection.

Discussion

In this study, we demonstrate that antibody-conjugated FND (IgG–gFND) remain biocompatible, maintain their bright photostable NIR fluorescence, and retain the biological functions of antibodies. The glycidol coating of FND (gFND) resulted in a significant decrease of nonspecific cellular FND uptake, whereas the IgG antibody conjugation of gFND permitted a significant increase in FND uptake by both monocytes and NK cells. FND immune cell targeting was validated in a tumor model and an immune cell co-culture system demonstrating preferential IgG–gFND uptake by...
immune cells. TAMs and MDSCs also demonstrate significant IgG−gFND uptake, indicating that FND antibody conjugation may be used for the targeting of both innate immune cells and immune suppressor cells. In addition to immune cell targeting, this study also demonstrates that antibody-conjugated FND can stimulate innate immune cells, as demonstrated by the increased NK cell surface marker expression (CD69 and NKG2D) and increased production of TNF-α and IFN-γ by monocytes and NK cells, respectively. In vivo imaging studies performed in a murine breast cancer model demonstrated that antibody-conjugated FND were retained within the tumor, whereas uFND did not. Furthermore, necropsy studies identified the presence of FND in the liver and kidney of mice treated with uFND but not in mice treated with antibody-conjugated FND. Ultimately, this study demonstrates that FND can be conjugated to antibodies and that this modification impacts their uptake by immune cells.

There have been many proposed mechanisms for the reduced nonspecific uptake of nanocarriers when coated with polymers. The best studied polymer is PEG, and it has been shown that PEG coating on nanodiamonds enables further modification, as well as stabilizes its dispersion in physiological conditions. This results in a prolonged circulation time when introduced in the bloodstream. The flexible and hydrophilic nature of PEG leads to steric stabilization that decreases the adsorption of opsonins and allows for the evasion of the reticuloendothelial system. Similarly, the PG coating formed by reacting FND with glycidol is flexible and hydrophilic, and many studies have shown a similar stealth effect. Therefore, the decrease in the nonspecific uptake of gFND by innate immune cells is likely due to the hydrophilic properties of the glycidol coating causing a change in opsonin adhesion and protein adsorption.

As discussed in our previous study, FND may be endocytosed by certain cell types through a clathrin-dependent

Figure 8. IgG−gFND promotes immune cell activation. Human monocytes and NK cells were cultured for 24 h in CTRL or with 100 μg/mL of either free IgG or IgG−gFND in media. Cells were then collected. (a) Monocytes were stained with Abs for HLA-DR (left) or CD86 (right) and evaluated via flow cytometry. Fold changes in the mean fluorescence intensity (MFI) compared to CTRL were calculated. The means ± standard errors of three independent experiments are shown. (b) NK cells were stained with Abs for NKG2D (left) or CD69 (right) and evaluated via flow cytometry. Fold changes in MFI compared to CTRL were calculated. The means ± standard errors for five independent experiments are shown. (c) Monocytes and SK-BR-3 tumor cells (left) or NK cells and SK-BR-3 tumor cells (right) were cultured for 48 h with CTRL, free IgG, or IgG−gFND. Supernatants were collected and evaluated for TNF-α and IFN-γ production via ELISA. Fold changes in cytokine production compared to CTRL were calculated. The means ± standard errors for three independent experiments are shown. * Represents p < 0.05 compared to CTRL and with underlying bracket represents p < 0.05 for comparisons across groups.
process or may diffuse across the cell membrane. These are the likely mechanisms of uptake for uFND and gFND. Although IgG–gFND may also be taken up by these mechanisms, it is possible that the antibody coating could lead to antibody-dependent cellular phagocytosis. Such a process would require the Fc regions of IgG (bound to the FND) to cross-link FcRs on monocytes and NK cells to initiate phagocytosis.

How FND activate innate immune cells is unknown. It has been hypothesized that either the internalization of nanoparticles or the recognition of nanoparticles by TLRs triggers the activation of innate immune cells. Another mechanism may involve FcγR as it is specific for and activated by the Fc region of IgG, and many innate immune cells including monocytes and NK cells express FcγR on their surface. An FcγR-mediated process could lead to increased activation as measured by cell surface marker expression and cytokine production. An interesting observation of our study was that in co-culture, monocytes were activated by FND, as measured by TNF-α production, but not in monoculture, as measured by the expression of activation markers CD86 and HLA-DR. This leads us to speculate that SK-BR-3 cancer cells contribute to the activation of monocytes by FND. A possible candidate is that damage-associated molecular patterns (DAMPs) are released by these cancer cells. In monoculture, the monocytes would not be exposed to DAMPs and would therefore not show as much activation.

FND are chemically inert with low cytotoxicity. When combined with their high photostability, fluorescence from FND can be observed, in vivo, for long periods of time. Detailed biocompatibility studies, including safety, pharmacokinetics, and biodistribution, in mice and rats show that although FND can persist in the parenchymal cells of the liver, the liver function remains normal without abnormal histopathology or vascular lesions. The absence of significant tissue damage from these in vivo studies suggests that nanodiamonds may be less detrimental than larger, more abrasive diamonds.

Several studies have described methods of coating the surface of nanodiamonds with biocompatible molecules containing functional cross-linkers to create durable covalent attachments of proteins or therapeutic agents. Similar to our findings with glycidol coating, other studies have demonstrated that in addition to improved conjugation, coating of nanodiamonds with poly-L-lysine, polydopamine, silica, PEG, or PG decreases nonspecific nanoparticle uptake and allows nanodiamond conjugates to evade macrophage clearance. Mechanistically, the decrease in the nonspecific uptake of gFND by innate immune cells could be due to the hydrophilic properties of glycine coating causing a change in opsonin adhesion and protein adsorption.

Possible explanations for the localization of IgG–gFND at the tumor site include specific and nonspecific binding of IgG–gFND to cells at the tumor site. As we did not observe uFND at the tumor site, localization would appear to require IgG. Aggregation of IgG could be a nonspecific event and could slow down the diffusion of individual FND. This is plausible as IgG antibodies aggregate under acidic conditions and the tumor microenvironment typically has a pH below that of normal tissue. Alternatively, the tumor microenvironment contains different immune cells with Fc receptors, including dendritic cells, NK cells, and tumor-associated macrophages. Thus, specific binding of IgG–gFND to Fc receptors and uptake into immune cells could also account for the prolonged localization of IgG–gFND at the tumor site. The uFND migrated to the liver and kidneys following intratumoral injection, and this is consistent with previous animal studies that also show accumulation in the liver, kidneys, spleen, and lungs.

Several studies have described the theranostic potential of FND via individual cell labeling and tracking. However, for FND to have a true biomedical application, they must be able to target specific cells of interest. Studies have shown specific FND cell labeling via conjugation with viral envelope proteins for the targeting of glycaminoglycans or a β-lactamase tag for targeting the plasma membrane of embryonic kidney cells. Additionally, FND cancer cell labeling and targeting have been demonstrated by the conjugation of FND with vascular endothelial growth factor receptor ligands, RGD, transferrin, and FA. Although there have been many reports of FND tumor cell targeting, there is not a great deal of literature on FND immune cell targeting or the effects of non-FND or FND on immune cells within the tumor microenvironment. Non-FND conjugation with cytotoxic–phosphate–guanine oligonucleotides has demonstrated in vitro and in vivo immunomodulating effects with increased TNF-α and IL-6 production by RAW264.7 macrophage-like cells in vitro and increased IL-12 secretion, decreased IL-6 production, and decreased tumor growth in vivo. Another study reported the use of trimeric hemagglutinin non-FND bioconjugates in vaccineology demonstrating an increased antigen size and the resultant enhanced antigen-specific IgG response. Similarly, another study has demonstrated increased RAW 264.7 cell TNF-α and IL-6 production in vitro. We previously reported the immunomodulatory effects of uFND on monocytes and NK cells. Similar to the IgG–gFND results reported here, in the presence of uFND, innate immune cells were activated and the uFND were able to be visualized in an animal model, unlike previous studies with non-FND.

FND have the potential for a broad variety of biomedical applications. In this study, we demonstrate that FND antibody conjugation may be used to target immune cells and can modulate their function within a recreated in vitro tumor microenvironment. Various techniques such as upconverting nanoparticles, quantum dots, or organic dyes have been
utilized for cell labeling, but each of these has limitations with either fluorescence quenching or toxicity.\textsuperscript{60} FND have none of these limitations and thus offer the potential for long-term cell tracking with increased signal-to-noise sensitivity in the NIR region. This property potentially opens the door for in vivo cell tracking of immune cells labeled with antibody–FND conjugates and tracing of antibody–FND-labeled immune cell populations during immunotherapy treatment.

Figure 10. \textit{In vivo} and \textit{ex vivo} FND imaging results. (a) Fluorescence images of 150 μg of IgG–gFND or uFND that were imaged through 0.5, 1.0, 1.5, or 2.0 cm thick chicken breast meat. The IgG–gFND or uFND were dried on a blotting paper and placed underneath the chicken breast, and the FND were imaged using a 532 nm excitation laser and a 660 nm glass filter. (b) Intratumoral injections of 50 μg of non-FND, uFND, or mouse IgG–gFND were administered to tumor-bearing mice. \textit{In vivo} fluorescence images were collected at 5 min and 60 min after injection, utilizing an IVIS Lumina II optical imaging system (Caliper Life Science Co., USA). The tumor area is indicated by the dashed circle. (c) Fluorescence emission spectra of thermally treated, acid-digested liver, kidneys, and spleen of mice in Figure 10b. The digested tissue samples were imaged for the presence of FND using a custom-built confocal spectrometer utilizing a 532 nm laser. The spectra of the liver and kidney samples are characteristic of NV-center FND.
CONCLUSIONS

This study suggests that FND have the potential to be used as theranostic immune drug delivery vehicles to promote directed antitumor activity, minimize systemic toxicities, and provide “track and trace” capabilities. Although systemic delivery of drugs, toxins, and other payloads might be problematic due to the uptake by FcR-expressing cells, intratumoral administration is available in several tumor types including melanoma and breast cancer. It is notable that nanodiamonds, taken up into cells, do not produce damaging reactive oxygen species (ROS). However, nanodiamonds that are coated with a metal such as copper can accumulate in cells and subsequently release cytotoxic Cu²⁺ ions that elevate intracellular ROS levels. Such a process could be used as an image-guided photodynamic therapy. Future studies will also investigate the utility of FND conjugated with antigen-specific therapeutic mAbs in targeting tumor cells and activating or deactivating specific immune cell populations within the tumor microenvironment.

METHODS

FND Preparation. All chemicals used for nanodiamond processing were purchased from GFS Chemicals Inc. (Columbus, OH). FND were generated by photolysis of nanodiamonds (SJK-5 0.0-0.1, 140 ppm N) purchased from Hyperion Materials and Technology (Columbus, OH). Approximately 400 g of nanodiamond was spread on a water-cooled aluminum surface (1.2 m x 2 cm) and irradiated twice, on two different dates, at E-Beam Services (Lebanon OH) for a calculated delivered dose of 2 x 10¹⁸ e/µm² for each irradiation. Between irradiation and after the last irradiation, the nanodiamonds were annealed at 800 °C in a nitrogen atmosphere. During annealing, vacancies (V) created by electron irradiation migrate within the diamond lattice and associate with substitutional nitrogen atoms (Nᵥ) and thereby create NV color centers. To clean the nanodiamonds, they were submerged for 72 h in concentrated H₂SO₄/HNO₃ (9:1, by volume) at 75 °C, followed by exhaustive rinsing with deionized water until the pH of the rinse water was neutral. They were then treated for 2 h with 0.1 M NaOH at 90 °C, washed with water again, and treated for another 2 h in 0.1 M HCl solution at 90 °C. The final FND were designated uFND and stored at room temperature in deionized water until their use. The nanodiamonds remained colloidal for at least 8 months and based on FTIR results contain both alcohol and carboxyl groups on the surface (Figure 1a–i).

FND Conjugation. Glycidol, chemicals, and organic solvents were purchased from MilliporeSigma, (St. Louis MO). Cleaned FND were suspended from synthetic HPHT nanodiamonds (SJK-5 0.0-0.1, 140 ppm N) purchased from Hyperion Materials and Technology (Columbus, OH). Approximately 400 g of nanodiamond was spread on a water-cooled aluminum surface (1.2 m x 2 cm) and irradiated twice, on two different dates, at E-Beam Services (Lebanon OH) for a calculated delivered dose of 2 x 10¹⁸ e/µm² for each irradiation. Between irradiation and after the last irradiation, the nanodiamonds were annealed at 800 °C in a nitrogen atmosphere. During annealing, vacancies (V) created by electron irradiation migrate within the diamond lattice and associate with substitutional nitrogen atoms (Nᵥ) and thereby create NV color centers. To clean the nanodiamonds, they were submerged for 72 h in concentrated H₂SO₄/HNO₃ (9:1, by volume) at 75 °C, followed by exhaustive rinsing with deionized water until the pH of the rinse water was neutral. They were then treated for 2 h with 0.1 M NaOH at 90 °C, washed with water again, and treated for another 2 h in 0.1 M HCl solution at 90 °C. The final FND were designated uFND and stored at room temperature in deionized water until their use. The nanodiamonds remained colloidal for at least 8 months and based on FTIR results contain both alcohol and carboxyl groups on the surface (Figure 1a–i).

FND Characterization. Fluorescence emission spectra of FND were collected on a custom-built confocal spectrophotometer, using a 532 nm laser diode as the light source. The excitation laser was focused through a long working distance NIR microscope (100X objective) with NA = 0.7 (Mitutoyo Plan Infinity Corrected Objective). The fluorescence from the FND was filtered from the reflected laser light using a 532 nm notch filter, then passed through a grating, collected on a CCD camera, and analyzed with customized spectral software. An Olympus FV1000-Filter Confocal System was used to collect microscopy images. The excitation laser wavelength was 543 nm and that for emission detection was 655–755 nm. Images were also collected on a Nikon Ti-S epifluorescence microscope with a Texas Red filter cube and analyzed with NIS Elements imaging software. The size of FND was determined by DLS using a Brookhaven 90Plus nanoparticle size analyzer (BIC, Holtsville, NY). From SEM images (Figure 1b), the diamonds could be characterized as having an irregular, blocky appearance.

Modified ELISA. ELISA was performed on 96-well ELISA plates (Nunc, Rochester, NY) that were coated with 10 µg of either GaR-IgG or GaH-IgG (American Qualex, San Clemente, CA) in 0.05 M carbonate buffer at pH 9.5 for 12 h. Unreacted sites in the wells were then blocked with 1% bovine serum albumin (Fisher Scientific, Hampton, NH) in PBS (137 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, and 2 mM KH₂PO₄, at pH 7.5) for 1 h and then incubated with 10 µg of g-FND, rabbit IgG-FND, or human IgG-FND in PBST (PBS +0.05% Tween 20, at pH 7.5) for 2 h while shaking. Plates were then washed three times with PBST, and FND conjugate binding to the wells was analyzed by measuring the fluorescence using a TECAN Geniosplus plate reader (Mannndorf, Switzerland). The filters used were a 535 nm bandpass filter with 35 nm fmwh for excitation (Tecan) and a 650 nm bandpass filter with 80 nm fmwh for emission (Edmund Optics, Barrington, NJ). A standard curve of known amounts of FND, ranging from 0 to 10 µg/well, was added to the plate before reading to allow for the quantification of the diamonds bound to the plate.

HRP Assays. Immune complexes formed between antibody-conjugated FND and species-specific antibody horse radish peroxidase (HRP) conjugates were tested for HRP activity using pyrogallol and H₂O₂ as substrates. Approximately 100 µg of fFND, rabbit IgG-conjugated FND (R-IgG–gFND), or human IgG-conjugated FND (H-IgG–gFND) was mixed with 100 µg of either goat anti-rabbit (GaR–HRP) or goat anti-human biotinylated HRP IgG (GaH–HRP) conjugate (American Qualex, San Clemente, CA) in a 0.05 M HEPEs, 0.005% Triton X100, pH 7.1 (NPT) buffer. The reactants were gently mixed and placed on a rocking platform at room temperature for 1 h. The samples were subsequently centrifuged in a microcentrifuge, rinsed with NPT, and resuspended in 100 µL of the same buffer. HRP activity in the immunocomplexes was measured by the enzymatic oxidation of pyrogallol (Sigma-Aldrich, St. Louis, MO) to purpurugallin. Assays were conducted in plastic cuvettes containing 2.4 mL of 0.1 M KH₂PO₄ pH 6.0, 0.3 mL pyrogallol (5.33%, 3 mL), and 0.2 mL dilute H₂O₂ (330 µL of 30% H₂O₂ to 25 mL of distilled water). Reactions were started by the addition of 25 µL of the appropriate FND–IgG–HRP complexes to the cuvette. The increase in absorbance at 420 nm was recorded relative to a reaction blank on a dual-beam UV/vis spectrophotometer (Shimadzu UV-2101PC scanning spectrophotometer). Enzyme activity was calculated from the linear plot of A₄₂₀ versus time (120 s), where one enzyme unit is the amount of enzyme that produces 1 mg of purpurugallin from pyrogallol in 20 s at 20 °C and at pH 6.0. The absorbance of a 1 mg/mL solution of purpurugallin at 420 nm is 12.
Immunoblot Analysis with GaH(Fc)–fGND. Western blots were performed using a Mini-PROTEAN Tetra Vertical Electrophoresis System (Bio-Rad, CA) according to the manufacturer’s protocol. Briefly, a 4–20% TGX SDS-PAGE gel was run with varying amounts of 2.5, 5, and 7 μg of human IgH until the bromophenol blue tracking dye ran off the gel. The gel was removed from the cassette and carefully placed in the immunoblot sandwich. A prewetted nitrocellulose membrane was used to capture the proteins during the transfer. Transfer was completed in ice-cold Tris–glycine buffer with 20% methanol. The entire apparatus was stored at 4 °C and run with 60 V constant voltage for a period of 1 h. Post-transfer, capture was confirmed by soaking the membrane in 3 mL of Ponceau S staining solution (results not shown). The Ponceau S solution was rinsed from the membrane and then blocked with 3% BSA in 0.1× PBST for a period of 1 h at 4 °C. Afterward, it was probed with Fc-specific goat anti-human IgG-conjugated FND [GaH(Fc)–fGND] for an hour with steady rocking. The membrane was then rinsed three times with 0.1× PBST and imaged using a Maestro imaging system equipped with a 660 nm glass filter to capture the NV center emission.

SDS-PAGE and Chemiluminescence Analyses. Evaluation of the targeted antibody capture of human IgG-conjugated FND (H-IgG–fGND) by GaH–IgG was done according to a modified pull-down procedure. Briefly, 0.2 mg of H-IgG–fGND was incubated with 5 μg of GaH, Fc-specific, IgG for 2 h in an orbital shaker. The capture complex was then rinsed thrice with HEPES-T and then resuspended in 20 μL of 2× SDS–PAGE-denaturing sample buffer (BioRAD, Hercules, CA). The samples were then heated at 90 °C for 10 min and then loaded directly into a TGX–PAGE gel equilibrated in TGS buffer. Each SDS-PAGE was performed using a Mini-PROTEAN Tetra Vertical Electrophoresis System (Bio-Rad, Hercules, CA) and 4–20% SDS-PAGE TGX precast gel (BioRAD) in duplicate, one for imaging and one for western transfer. Electrophoresis was performed at a constant voltage of 100 V until the bromophenol blue tracking dye ran off the bottom of the gel (about 1.5 h). For each experiment, one gel was stained with Coomassie R-250 (BioRAD, Hercules, CA) for a period of 1 h and then destained with a destaining solution (20% methanol, 10% acetic acid). This gel was then imaged on a Maestro imaging system set for grayscale imaging to track the expected band migrations. The other gel was transferred to a 0.45 μm nitrocellulose membrane (MilliporeSigma, Burlington, MA) via the western blot technique. The sponges, membrane, and blotting paper were preincubated in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 10 min before assembling the sandwich. The apparatus was stored at 4 °C for the duration of the run. A constant voltage of 60 V was applied for a period of 1 h 15 min before stopping the run. The membrane was removed and stained with Ponceau S to confirm the successful transfer of protein to the blot.

Post-transfer, the membrane was blocked for 4 h with 0.5% fish gelatin in 50 mM HEPES, pH 7.4 (Sigma-Aldrich, St. Louis, MO). The blot was then probed overnight at 4 °C with a 1:1000 dilution of rabbit anti-goat-HRP (American Qualex, San Clemente, CA) in 0.1% fish gelatin in HEPES (pH 7.4) buffer with gentle rocking. The blot was rinsed twice with the rinse buffer (0.1× PBST), once with D2O, and finally twice again with the rinse buffer. Membrane-bound antibodies were illuminated using a Super Signal West-Pico Plus Chemiluminescent Substrate kit (ThermoScientific, Waltham, MA), a luminol-based chemiluminescent substrate for the detection of HRP. The blot was then incubated for 5 min in a working solution, that is, equal parts of luminol and peroxide. The substrate solution was drained and the blot was sealed in a sandwich bag. Imaging was completed using a Chemidoc MP imaging system (Bio-RAD, Hercules, CA).

Cytokines and Antibodies. Recombinant human IL-12 was provided by Genetics Institute Inc. (Cambridge, MA). Additional cytokines employed for the generation of TAM include recombinant human IL-4 (Shenandoah, 100-09), recombinant human IL-10 (Peprotech, 200-10), and recombinant human M-CSF (Shenandoah, 100-03). Polyclonal human IgG was purchased from Equitech-Bio Inc. (Kerrville, TX) and was a mixture of subtypes as it appears in normal human bloodstream (IgG1, IgG2, IgG3, and IgG4). Secondary antibody–HRP conjugates, GaR–IgG–HRP and GaH–IgG–HRP, were obtained from American Qualex (San Clemente, CA). Polyclonal mouse IgG, rabbit IgG, and goat anti-human IgG were obtained from Southern Biotech (Birmingham, AL).

Cell Lines. Human SK-BR-3 breast adenocarcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). EMT6-Her2 mouse breast adenocarcinoma cell line was provided by Dr. Seung-Uon Shin at the University of Miami Health System. Cells were maintained in RPMI-1640 (SK-BR-3) or Iscove (EMT6-Her2) medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies Inc., Rockville, MD).

Isolation of Human NK Cells and Monocytes. Peripheral blood mononuclear cells (PBMC) and NK cells were isolated from healthy donor leukopacks (American Red Cross, Columbus, OH), as previously described.66 For monocyte isolation, CD14+ monocytes were isolated from PBMC using anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA). The purity of NK cells and monocytes was confirmed using CD56 and CD14 expression, respectively, via flow cytometry. Immune cells were then cultured in a medium, as previously described by our group.66

Isolation of MDSCs from the Peripheral Blood of Metastatic Cancer Patients. MDSCs were isolated from blood samples provided by patients with metastatic cancer. Blood was acquired under an IRB-approved protocol for human subject research (IRB protocol 1999C0348). Isolation was accomplished by processing the blood samples first using the RosetteSep HLA-myeloid cell enrichment kit (StemCell Technologies, Vancouver, BC), followed by anti-HLA-DR MicroBeads (Miltenyi Biotec, Auburn, CA). The resultant cells are enriched for CD33 and negative for HLA-DR.

In Vitro Generation of Human TAMs. The TAMs employed in these experiments were generated in vitro by culturing human CD14+ monocytes in a tumor-conditioned medium with IL-4, IL-10, and M-CSF, as previously described by our group.67 Tumor culture supernatants were obtained by growing approximately 3 × 10^5 cancer cells and incubated for 24 h in a low serum (0.2% FBS) medium. The tumor conditioned medium was then collected, removing cells via centrifugation. FBS (10%) was then added to the supernatant. Freshly isolated healthy human CD14+ monocytes were plated in a six-well, flat-bottom plate (USA Scientific) at a concentration of 1 × 10^6 cells/mL. Monocytes were cultured in a medium containing equal volumes of 10% HAB medium and tumor-conditioned medium plus the addition of 1 μg/mL each of IL-4, IL-10, and M-CSF. Every 48 h, fresh medium and cytokines were added. Cells were harvested on day 7 using a nonenzymatic cell dissociation solution (Sigma-Aldrich). In vitro-generated TAMs were identified by the dual expression of CD163 and CD206.59

In Vitro FND Treatment Assays. SK-BR-3 cells, EMT6 cells, monocytes, and NK cells were cultured in 24-well flat-bottom plates at a concentration of 1 × 10^5 cells/well in a medium with or without 100 μg/mL of FND. NK cell functional analysis was performed, as described previously by our group.5

In Vitro Co-culture Treatment Assays. SK-BR-3 cells were cultured in 24-well plates for 4 h. Immune cells were then added to the wells (2 × 10^5 NK cells/well and 1 × 10^5 monocytes/well) in 10% HAB medium with 1 μg/mL of IL-12 alone (negative control), 1 μg/mL of IL-12 and 100 μg/mL of IgG (positive control), or 1 μg/mL of IL-12 and 100 μg/mL of FND.

Flow Cytometry. Flow cytometry was performed on an LSR II flow cytometer (BD Biosciences, San Jose, CA). Monocytes were labeled with an APC anti-CD14 monoclonal antibody (mAb) (Beckman Coulter, Brea, CA); NK cells were labeled with a PE anti-CD56/NKH1-RD1 mAb (Beckman Coulter, Brea, CA), and TAMs were labeled with PE-CFS94 anti-CD163 (BD Biosciences, 562670) and APC anti-CD206 (BD Biosciences, 561783) mAbs. Flow cytometric analysis of FND uptake and activated monocyte and NK cell surface marker expression were performed as previously described by our group.66
Trypan Blue Cell Viability Staining. Cells were harvested after overnight treatment and incubation and analyzed by a hemocytometer after trypan blue staining, as previously described.5

Fluorescence Microscopy Analysis of FND Uptake. Following 24 h treatments, the cells were stained, and FND uptake and distribution were visualized. Image capture was performed using confocal microscopy with light settings, as previously described.6

TNF-α and IFN-γ ELISA. Following 48 h treatments, cell-free supernatants were collected, and the levels of TNF-α and IFN-γ were determined by ELISA (R&D Systems, Minneapolis, MN), as previously described.7

Imaging of FND Through Tissues. Raw chicken breast was purchased from a local grocer, placed in the freezer for 3 h, then cut into four different thicknesses: 5, 10, 15, and 20 mm, based on a digital caliper. A 10 μL of a 15 mg/mL solution (150 μg) of 100 nm uFND or IgG-gFND was spotted and dried on a blotting paper and then taped to the bottom platform of a Maestro in vivo fluorescence imaging system (CRI Inc, Woburn, MA). Each chicken breast was thawed, placed over the diamond sample, and imaged, as described above. FND were imaged on the Maestro system using a Hamamatsu Opto-spectrum generator (OSG) to generate 614 and 532 nm excitation light and a 660 nm glass long-pass filter (Schott Glass). Spectral deconvolution was performed using Maestro software. For imaging in the chicken breast of different thicknesses, the exposure time was approximately 10 s. Images were obtained at emission wavelengths between 660 and 775 nm with a 5 nm bandpass per step.

Murine Breast Cancer Model and In Vivo Imaging. Female 4–6 week old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in all studies. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was conducted under a protocol approved by Ohio State University’s Institutional Animal Care and Use Committee. Approximately 1 × 106 EMT6 mouse breast adenocarcinoma cells were injected into the mammary fat pad to produce tumors. Intrauterine injections were performed with 5 mg/kg of non-FND, uFND, or mouse IgG-gFND.

In vivo imaging was performed using an IVIS Lumina II optical imaging system (Caliper Life Science Co., USA) at 5 min, 1 h, and 24 h after treatment. After 24 h of intratumoral injections, the mice were euthanized as per protocol and necropsies were performed.

Necropsy Organ Fluorescence. Organs (liver, kidneys, and spleen) were excised from each euthanized animal, minced into fine pieces, then placed in a furnace and oxidized for 1 h at 100 °C, followed by 1 h at 200 °C, and then 6 h at 450 °C. The resulting ash was scraped into tubes containing 10 mL of concentrated sulfuric acid and nitric acid (9:1 ratio) and heated to 90 °C in a sand bath for 2 h. After cooling, the samples were carefully diluted in water and centrifuged at 3000 rpm for 30 min. The resulting pellets were extensively washed with water, with centrifugation increased to 14,000 rpm. Each pellet was resuspended in 20 μL of water, and approximately 10 μL was dried on a glass slide. The fluorescence emission spectra of the dried spots were collected on a custom-built confocal spectrometer or on a PMA-12 photonic multichannel analyzer (Hamamatsu Photonics, Japan) with a 532 nm excitation from an OSG (Hamamatsu Photonics, Japan).

Statistical Analysis. Analyses of the HRP catalytic rates, modified ELISA, FND uptake, cell viability, and innate immune cell activation were performed using linear mixed-effect models and t tests for treatment comparisons. Multiple comparisons were adjusted by Holm’s method.70 Statistical analysis was performed using SAS 9.4 software, and the level of statistical significance was controlled at 0.05.

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Author Contributions
L.P.-K. and S.H.S. contributed equally to the manuscript. Authors L.P.-K., S.H.S., A.A.R., and W.E.C. were involved in study conception and design. Experiments and collection and assembly of data were conducted by L.P.-K., S.H.S., C.R., I.V.R., D.A., M.C.D., T.N., N.C., N.J.B., and C.M. All authors were involved in data analysis and interpretation. Administrative support was provided by L.P.-K., S.H.S., and W.E.C. J.P.B., S.T., A.A.R., and W.E.C. were involved in the provision of study materials. W.E.C. was involved in the provision of patient samples. L.P.-K., S.H.S., C.R., I.V.R., D.A., C.M,
A.R., and W.E.C. were involved in manuscript writing. Final approval of the manuscript was provided by all authors.

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Notes
The authors declare the following competing financial interest(s): The authors from Columbus NanoWorks commercially develop nanodiamond imaging technologies. All other authors declare no competing interests.

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