Supporting Information

Self-Assembled DNA Immuno-Nanoflowers as Multivalent CpG Nanoagents

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Supplemental Figures

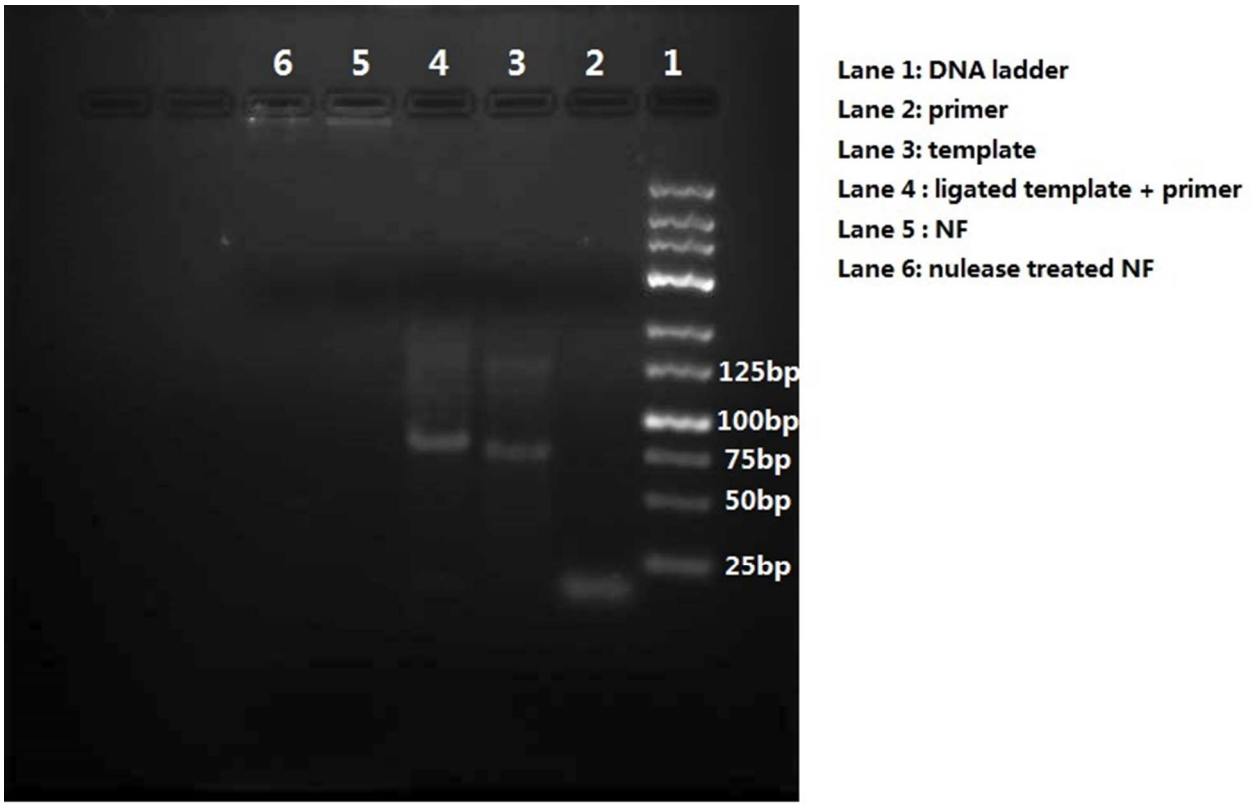

Figure S1. Agarose gel electrophoresis images confirming rolling circle replication (RCR) of CpG-NF self-assembly. The size of elongated DNA in NFs was too large to migrate in the gel.

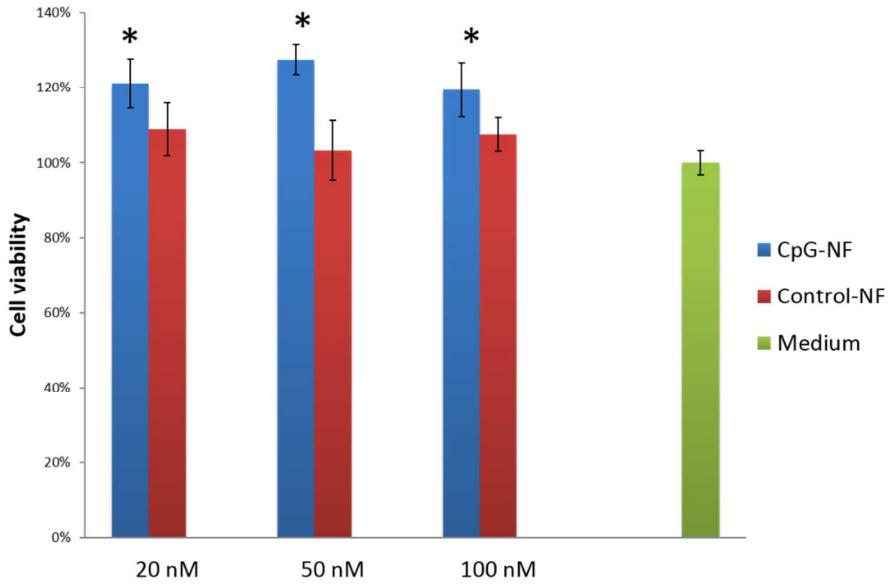

Figure S2. MTS assay data showing that 1) CpG-NFs did not inhibit cell proliferation and are, therefore, biocompatible under our experimental conditions and 2) proliferation of these immune cells was specifically stimulated by CpG-NFs, as indicated by enhanced cell viability. In contrast, control NFs not containing CpG motifs failed to induce significant enhancement of cell proliferation. Error bars represent standard deviation (SD) of at least three independent measurements.

* $P < 0.05$ significantly different from medium control group.

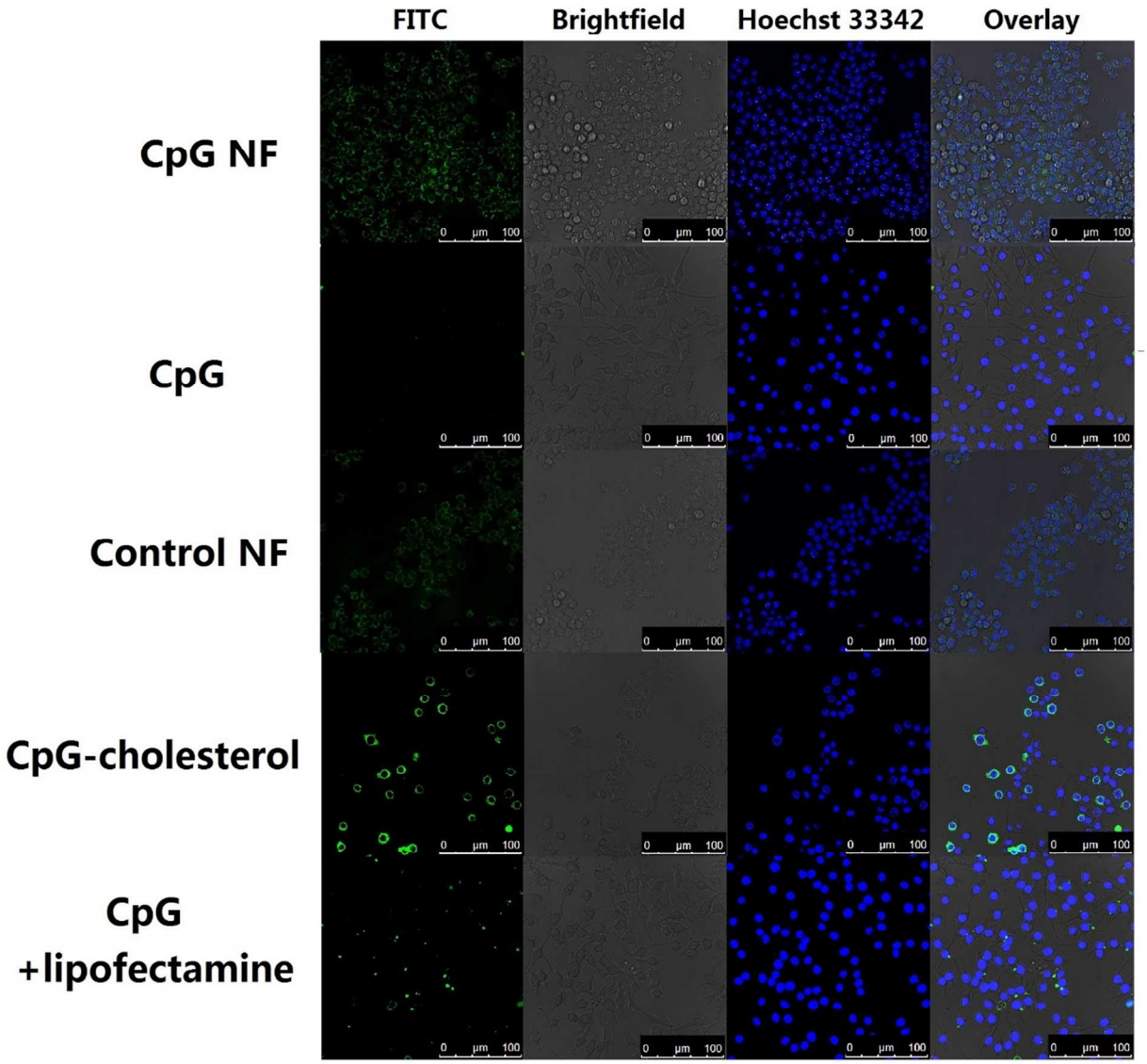

Figure S3. Confocal microscopy images demonstrating the internalization of CpG-NFs into macrophage RAW264.7 cells. NFs were labeled with FITC (green) by modifying FITC on the end of primers used in RCR. Cells were stained with Hoechst 33342 to identify nuclei.

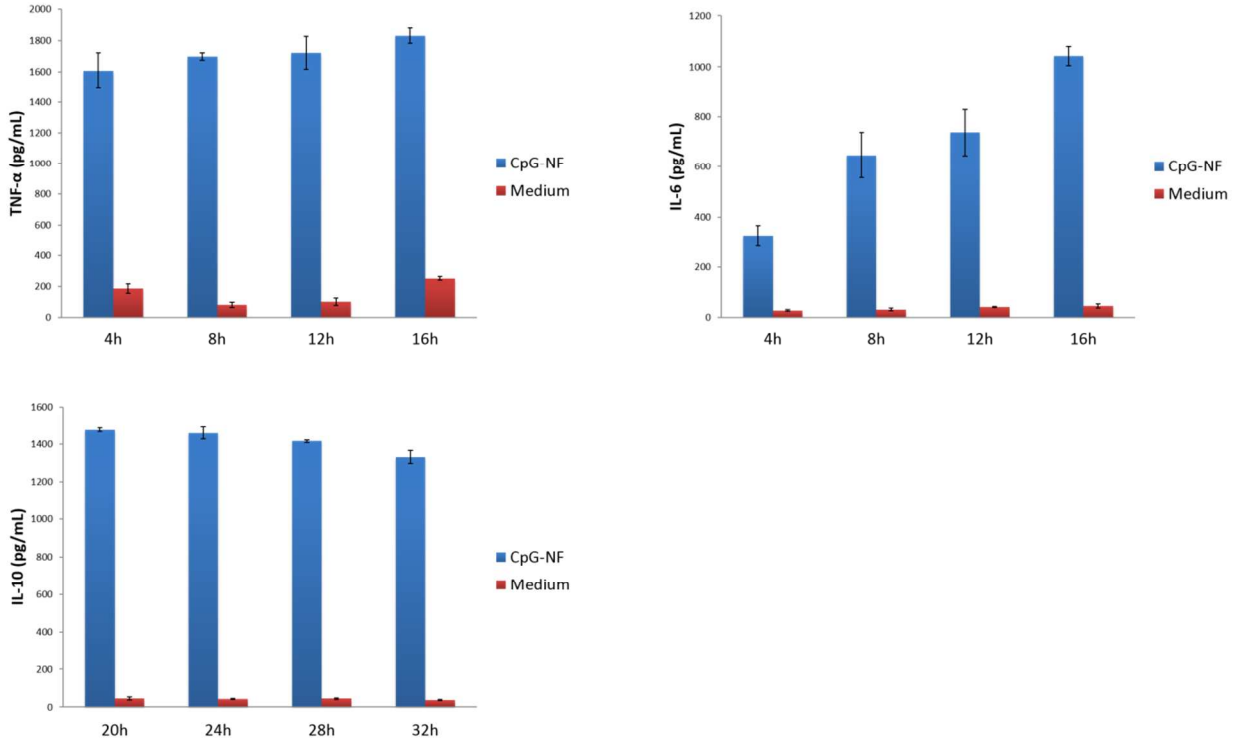

Figure S4 Time course experiment of CpG-NF immunostimulation. Preliminary test of stimulatory effect of CpG-NFs on RAW 264.7 cells shows significant secretion of each cytokine observed over a wide range of time points: 8h for TNF-α and IL-6 and 24h for IL-10 were chosen for the further comparison with other CpG ODNs.

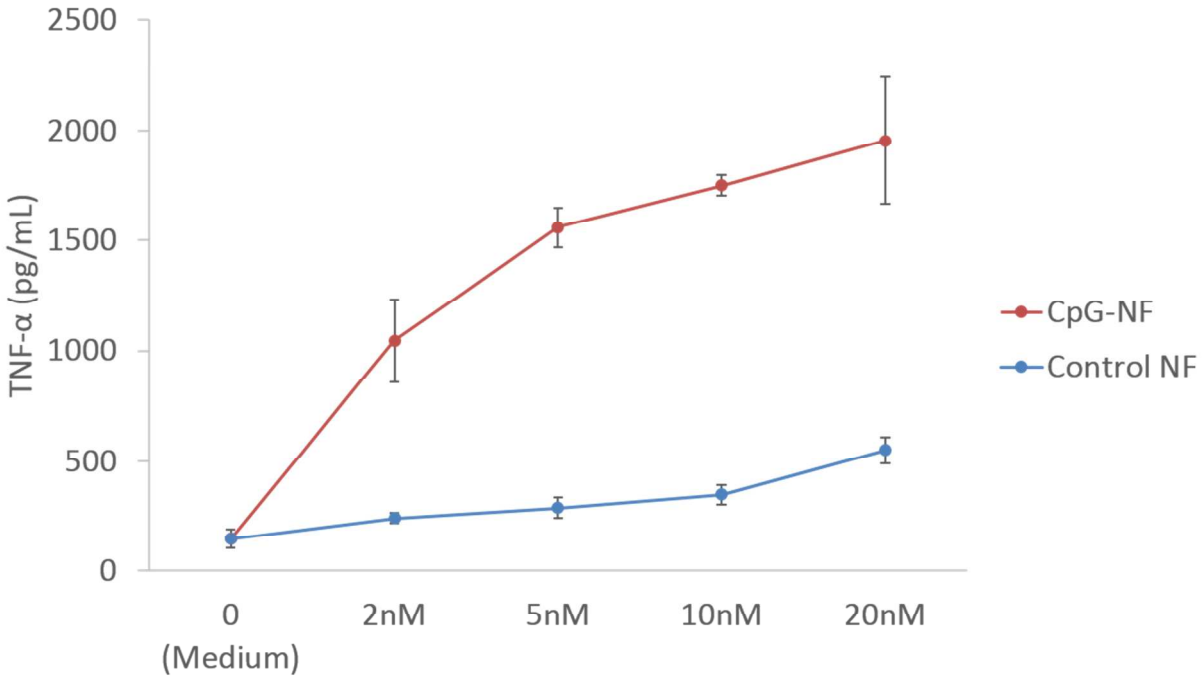

Figure S5 Concentration dependent experiment of CpG-NF immunostimulation stimulation. A series of concentrations (0-20nM) of CpG-NF and control NF treated RAW 264.7 cells show different secretion level of TNF-α.

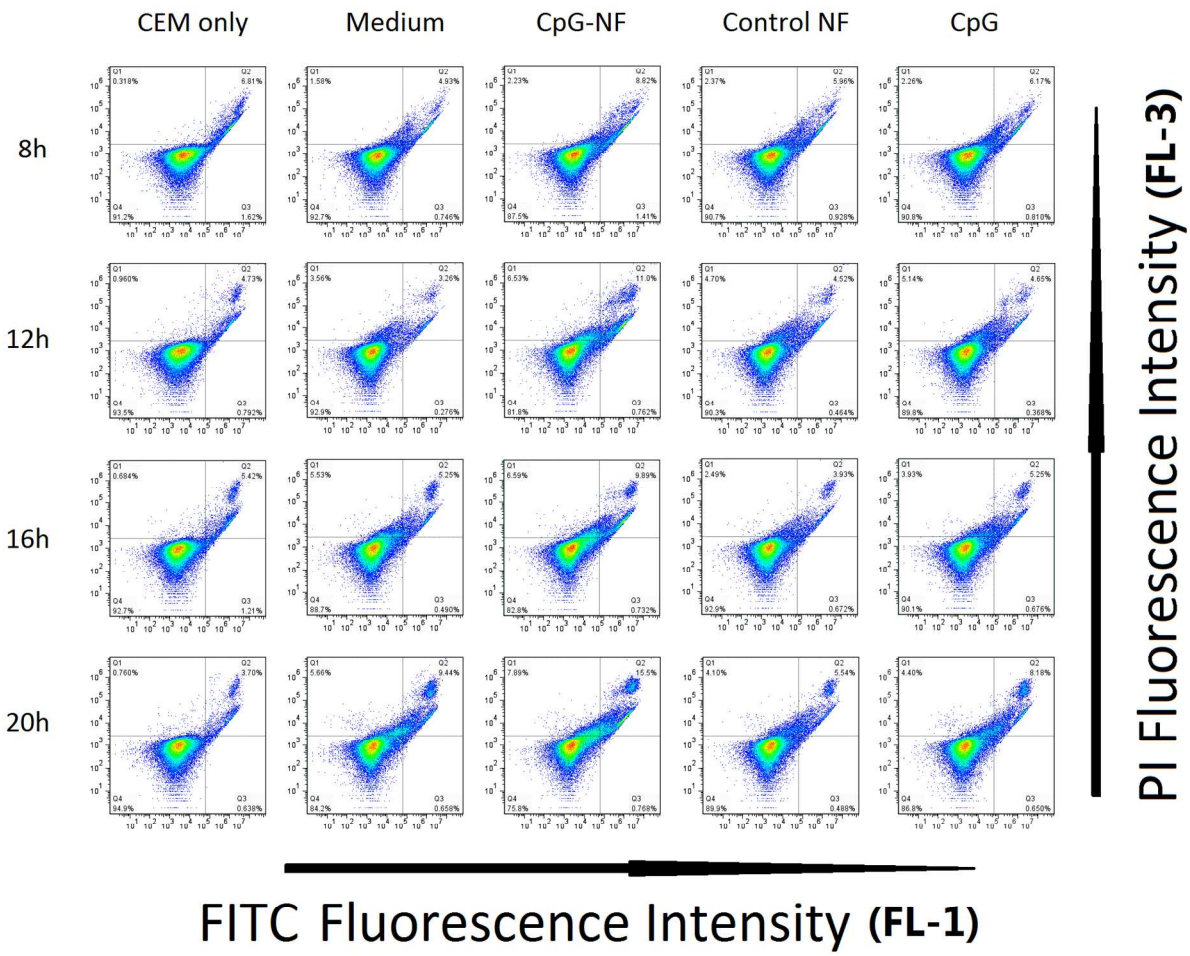

Figure S6 Evaluation of therapeutic effect of CpG-NFs (expanded data of Figure 5).

Supplemental Tables

Table S1. DNA sequences. FITC, cholesterol, or phosphate was labeled at the 5'-ends, if applicable.

	Sequences (5'-3')
CpG 1826	TCCATGACGTTCCTGACGTT
Primer for CpG-NFs	TTTCAGTTCCAGAATGACTTGACTTT
Template for CpG-NFs	TCTGGAACTGAAAAACGTCAGGAACGTCATGGAAAAAACGTCAGGAACGTCATGGAAAAACGTCAGGAACGTCATGGAAAGTCAAGTCAT
Primer for control NFs	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGATGCTGCTGC
Template for control NFs	TTCCCGGCGGCGCAGCAGTTAGAGCTGCAGCGATACGCGTATCGCTATGGCATATCGTACGATATGCCGCAGCTCTAACCGTACAGTATT

MATERIALS AND METHOD

Preparation of nanoflowers. A mixture of 0.6 μM 5'-phosphorylated linear template and 1.2 μM primer (either 5'-labelled with FITC or unlabelled) in T4 ligase buffer (5 mM Tris-HCl, 1 mM MgCl2, 0.1 mM ATP, 1 mM dithiothreitol) was heated at 95 ˚C for 5 min, followed by gradual cooling to room temperature over 3 h for annealing. The annealed product was incubated with T4 DNA ligase (10 U/μL) and Bovine Serum Albumin (BSA, 1X) at room temperature for 2-3 h. For RCR reaction, the annealed and ligated primer-template complex (0.3 μM) was incubated with Φ29 DNA polymerase (2 U/μL), dNTP (2 mM/μL), and BSA (1×) in Φ29 buffer solution (50 mM Tris-HCl, 10 mM (NH4)2SO4, 10 mM MgCl2, 4 mM dithiothreitol) at 30 °C for 6h. Reactions were terminated by heating at 65°C for 10 min. The NFs were then washed twice with double-distilled H2O (diH2O), gathered by centrifuge at 16,400 rpm for 10 min, and stored at 4 °C for future use. All reagents used above for NF preparation were purchased from New England Biolabs, Ipswich, MA.

SEM analysis for structure confirmation and nuclease resistance study. To examine NFs using scanning electron microscopy, the products were deposited on silicone matrices, dried, washed with diH2O and coated with Au by spray, followed by observation on an S-4800 scanning electron microscope (HITACHI, Japan) at the Micro- and Nanotechnology Research Center at Hunan University. Dynamic light scattering (DLS) on a Nano-zs90 Zetasizer (Malvern Instruments Ltd, UK) was used for size determination, and polarized light microscopy was performed using Optiphot-2 polarizing microscope (Nikon, Japan). Bright field images of NFs were obtained under a DM6000 B fluorescence microscope (Leica Microsystems, Germany). The NFs were treated with 5U/mL of DNase I for 24h at 37˚C and examined again by SEM.

Concentration of nanoflowers. The concentration of CpG-NFs was measured based on the number of CpG copies involved in all NFs in the treatment group. Since dNTP added into the reaction system was 2mM for each dNTP, one circular template contained 91 nucleotides and 3 copies of CpG. If Abs is the absorbance of dNTP at 260 nm and ε is the extinction coefficient of dNTP at 260 nm, then the consumed dNTP in the RCR reaction could be measured and total copies of CpG calculated by the following equation: (2mM *4 – Abs/ ε*1,000,000)/(91*3). The concentration of control CpG was measured as the same number of DNA copies, but with random DNA sequences.

Dynamic light scattering analysis. The apparent hydrodynamic sizes of the CpG-NFs were estimated by dynamic light scattering on a Nano-zs90 Zetasizer instrument (Malvern Instruments Ltd., UK).

Cell culture. Murine macrophage RAW264.7 cells (ATCC® TIB-71™) and human T-cell leukemia CCRF-CEM cells (ATCC® CCL-119™) were purchased from ATCC (Manassas, VA). RAW264.7 cells were grown at 37 °C with 5% CO2 in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (heat inactivated, Gibco), 1.5 g/L NaHCO3, and 100 IU/mL penicillin-streptomycin (Cellgro). CCRF-CEM cells were cultured in the same conditions, but using RPMI 1640 medium (Sigma-Aldrich) supplemented with FBS and penicillin-streptomycin. Cell density was determined using a hemocytometer prior to each experiment.

Cytotoxicity analysis. The cytotoxicity of CpG-NFs was evaluated by CellTiter 96 cell proliferation assay (Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well plates at a concentration of 4×104 cells per well and cultured for 24h. Fresh media containing CpG-NFs

or control NFs at 20 nM, 50 nM, and 100 nM were incubated with cells for 24h at 37 °C. Then media were removed, and CellTiter reagent (20 μL) diluted in fresh FBS-free medium (100 μL) was added to each well and incubated for 1-2 h. The absorbance (490 nm) was recorded using a microplate reader (Tecan Safire microplate reader, AG, Switzerland). Cell viability was determined using the absorbance of treatment group divided by absorbance of the medium group.

Uptake of CpG-NFs by macrophage cells. Cellular uptake of FITC-labeled CpG-NFs was studied using confocal microscopy. RAW264.7 cells were seeded on confocal dishes and incubated at 37 °C. 24 h later, cells were washed with PBS and exposed to fresh culture medium containing either FITC-CpG-NFs or free FITC-CpG for 1.5 h. Hoechst 33258 (3μg/ml) was added to the system and incubated for another 0.5 h to stain the nuclei. Bioimaging was performed using confocal laser scanning microscopy (CLSM) on a Leica TCS SP5 confocal microscope (Leica Microsystems Inc., Exton, PA) in DIC mode. An Ar laser was used for excitation of FITC.

Cytokine secretion from macrophage cells. RAW264.7 cells were seeded on 24-well culture plates at a density of 2×105 cells per well (0.5 mL) in fresh medium and incubated for 24h. Cells were then washed twice with 0.5 ml PBS before treatment with the indicated conditions and incubated at 37 °C for the indicated time. The media were collected afterwards and centrifuged at 12000 rpm for 20 min at 4 °C. The supernatants were stored at -80 °C for further testing. The levels of TNF-α, IL-6 and IL-10 were measured using ELISA kits (Life Technologies, Carlsbad, CA) following protocols recommended by the manufacturer.

Cancer therapeutic effect. RAW264.7 cells were seeded on 24-well culture plates at a density of 2×105 cells per well (0.5 mL) in fresh medium and incubated for 24h. After washing twice

with PBS, CCRF-CEM cells at a density of 2×105 cells per well mixed with the indicated therapeutics in fresh DMEM medium were incubated with the RAW264.7 cells for the indicated time. The cells in supernatant medium were collected and washed twice with PBS. Cell apoptosis was evaluated using FITC Annexin V/ Dead Cell Apoptosis Kit (Life Technologies, Carlsbad, CA) by following the procedure recommended by the manufacturer and using on an Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA). FITC and PI were exited at 488 nm; FITC was detected at FL1 (533/30 nm) while PI was detected at FL3 (>670 nm).

Statistical Analysis. Statistical differences of data were evaluated by Student's t-test. A p value of less than 0.05 was considered to be statistically significant.