Supplementary Information

Metal-Coordination-Driven Self-Assembly DNA Nanohybrids for Robust Intracellular Anti-Tuberculosis Therapy

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Experimental procedures

Reagents. The primary commercial chemicals utilized in the synthesis of CIF NP including $FeCl_2 \cdot 4H_2O$ and Isoniazid (INH), were purchased from Sigma Aldrich (St. Louis, MI, USA). The HPLC purified CpG ODN was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Hoechst 33342 staining solution and Triton X-100 were obtained from Solarbio Biotech Co., Ltd (Beijing, China). TNF-α, and IL-6 ELISA kits were obtained from Thermo Fisher Scientific Co., Ltd. (Shanghai, China). Resazurin staining solution, Glycerol and Tween 80 were bought from Sigma Aldrich (St. Louis, MI, USA). Difco™ Middlebrook 7H9 medium, 7H10 medium were purchased from BD Biosciences (Rutherford, NJ, US). Cell culture-associated reagents, including fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, PBS and trypsin-EDTA were obtained from Wisent Bio Co., Ltd. (Nanjing, China). Cell Counting Kit-8 (CCK-8) was bought from Sangon Biotechnology Co., Ltd (Shanghai, China). All the reagents were used as received without further purification. Millipore water (18.2 MΩ) was used throughout the experiments.

Synthesis of CIF NP. CIF NP was synthesized through a simple one step method. In detail, 50 μL $FeCl_2 \cdot 4H_2O$ (20 mmol/L) aqueous solution was added to 494 μL aqueous solution containing 6 nmol CpG ODN (6 μL, 1 mmol/L). After shaking for 30 s, 100 μL of INH (20 mmol/L) was added into the solution followed 10 s vortex. Subsequently, the mixture was incubated at 95^{o}C for 1.0 h. After cooling to room temperature, the resultant CIF NP was collected via centrifugation and washed with Milli-Q water for three times, then redispersed in Milli-Q water, finally.

Characterizations and Instrumentations. The transmission electron microscopic (TEM) images and the scanning electron microscope (SEM) images were collected using Hitachi HT7700 (Hitachi Co. Ltd., Japan) and Hitachi SU8200 (Hitachi Co. Ltd., Japan) transmission electron microscope, respectively. The high-angle annular dark-field scanning TEM (HAADF-STEM) images and elemental analysis were performed

by JEM200CX TEM (JEOL Ltd., Japan). The hydrodynamic diameter and ζ-potentials of nanoparticles were conducted using Malvern Nano ZS90 (Malvern Instrument Ltd., UK). The UV-Vis spectrum was obtained from an Edinburgh DS5 spectrophotometer (Edinburgh Instruments Ltd., UK). The FTIR spectrum was measured by infrared spectrometer (Thermo Fisher, Inc, USA). Fluorescence spectra were collected using a Hitachi F-4600 fluorimeter (Hitachi Co. Ltd., Japan). HPLC assays were conducted by Agilent 1260 Series Liquid Chromatogram (Agilent Technology Co. Ltd., China). Cell viability data were determined using a Thermo MULTISCAN GO microreader (Thermo, USA). Confocal laser scanning microscopy (CLSM) images were captured using an Olympus FluoView FV1000 confocal microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). The quantitative of cellular uptake NPs was recorded using a Guava easyCyte flow cytometer 6 (Guava Technologies Inc., Hayward, CA, USA).

Drug Loading. The free INH in supernatant was quantified using HPLC (injection volume: 40 μL, Eclipse Plus C18 columns of length 4.6×150 mm, Agilent 1260 Series). Acetonitrile and water containing 0.1% TFA were utilized as solvents A and B of the mobile phase, respectively. An isocratic elution at a flow rate of 0.5 mL/min was performed using a ratio of A to B (10:90), and UV absorbance at 264 nm was employed for the detection of INH. The drug loading efficiency (DLE) of INH was determined using the following equations:

$$DLE\% = \frac{INH\ added - free\ INH}{INH\ added} \times 100\%$$

Relative drug release efficiency. The obtained CIF was dispersed in culture medium. The mixture was kept at 37°C with gently continuous shaking. The buffer containing released INH or Cy5-CpG was separated by Amicon at different time points for quantification by HPLC or fluorescence spectrophotometer, respectively.

Investigation of stability of CIF in storage conditions. CIF was synthesized in accordance with the procedures mentioned above. The stability was evaluated in water, and TEM and DLS were used to monitor the dissociation at indicated timepoints.

Bacterial strains and cell lines. *Mycobacterium smegmatis* (*M. smegmatis*) mc^2-155

(ATCC700084) was used in this study. Such mycobacteria were grown in Middlebrook 7H9 broth medium supplemented with 0.02% Tween 80 and 0.5% glycerol. The experiments involving *Mycobacterium* were carried out in a biosafety level II laboratory (BSL-2). The mouse macrophage cell line (RAW 264.7) was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences, and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (0.1 mg/mL) and L-glutamine (4 mmol/L) at 37°C in a 5% CO_2 sterile humidified incubator.

Cell viability study of CIF using Cell Counting Kit-8 (CCK-8) assay. The cytotoxicity of the as-synthesized CIF was determined against RAW 264.7 murine macrophages. Briefly, ~2 × 10^4 cells were seeded into a flat-bottom 96-well plates beforehand, and cultured to 70-80% confluence. Then, they were treated to CIF NP at varying concentrations and maintained at 37°C for 24 h. Thereafter, cells were supplemented with 100 μL of FBS-free DMEM medium containing 10% CCK-8 1 h after incubation, and absorbance at 450 nm was measured with the microplate reader.

Qualification of cell uptake of CIF. RAW264.7 macrophage cells were plated in 35-mm confocal dishes for CLSM imaging or in a 6-well plate for flow cytometry, at a density of 1 × 10^5 cells per well overnight. Afterwards, cells were washed twice with PBS and replaced with Opti-minimum essential medium (Opti-MEM) containing the CIF (CpG was labelled with Cy5). After incubation for various durations (0, 1, 2, 4, 8, 12 h), the cells were rinsed twice with PBS, stained with Hoechst 33342, and then imaged using CLSM. For flow cytometry analysis, the treatment conditions were the same as those used for CLSM imaging. The cells were washed twice with PBS, detached with 200 μL of 0.25% trypsin-EDTA solution, and subsequently resuspended in 300 μL of PBS supplemented with 1% FBS for flow cytometry assay.

Assessment of immunostimulatory activity by ELISA. RAW264.7 macrophages were cultured in 6 well plates at a density of 1 × 10^5 cells/well overnight. Thereafter, CIF NP or free CpG were added incubated for 2 h maintained at 37 °C. Then the culture medium was collected and centrifuged for 15 min at 4°C. The levels of IL-6 and TNF-α were analyzed with a standard enzyme-linked immunosorbent assay (ELISA) using

protocols recommended by the manufacturer, on a Thermo MULTISCAN GO microreader.

Determination of *in vitro* MIC of CIF against *M. smegmatis.* The *M. smegmatis* suspensions was diluted to 1×10^5 CFU/mL using Middlebrook 7H9 broth medium. 100 μL of above suspensions were seeded in a sterile, polystyrene 96-well flat-bottom plate, followed with 100 μL of different concentrations of CIF or INH, then incubated at 37°C for 48 h in sterile humidified incubator. Afterwards, additional 30 μL of a freshly prepared resazurin dye (0.01%) was added to the wells and incubated in the dark for 6 h at 37°C. Fluorescence intensity was recorded at the absorbance of 600 nm using the Thermo MULTISCAN GO microplate reader. The color change from blue (oxidized state) to pink (reduced state) indicates growth of the bacteria. The concentration, which shows a greater than 95% prevention in color change, has been considered as minimum inhibitory concentration (MIC).

Mycobacterial CFU assay. The *M. smegmatis* suspensions was diluted to 1×10^5 CFU/mL using Middlebrook 7H9 broth medium, and treated with various conditions as the same with those for MIC determination. After incubation at 37°C for 48 h, 100 μL of the bacterial suspension was taken from each group and diluted 10^3 times. Thereafter, 100 μL of the diluted solutions was dropped evenly onto 7H10 plates with 0.2% (v/v) glycerol. After 2 days of culturing at 37°C in an aerophilic environment, the bacterial number of colonies was counted and recorded.

Cell culture, infections, and imaging. RAW264.7 cells were cultured overnight in complete DMEM medium at a density of 1×10^5 cells per glass-bottom petri dish or in a 6-well plate to facilitate adhesion. Infections were carried out by adding Cy3-NO_2-tre dye-labeled *M. smegmatis* to the plated cells at a multiplicity of infection (MOI) of 10:1 one day after seeding. 3 h post-infection, the infected RAW264.7 cells were cultured in fresh medium for an additional 2 hours. To remove extracellular bacteria, the cells were rinsed three times with PBS, stained with Hoechst 33342, and subsequently subjected for CLSM imaging by Olympus laser scanning confocal microscope.

Inhibition of *M. smegmatis* proliferation in macrophage. The *M. smegmatis* were co-cultured with adherent RAW264.7 cells for 3 h, followed with treatment of PBS twice to wash away the extracellular bacteria. Then, exposed in various conditions, including CIF NP at different concentrations, INH, free CpG, or Fe^{2+}, respectively. Afterwards, the cells were lysed with 0.25% Triton X-100 to release the intracellular bacteria, and the cell lysis was diluted at 1:1000, and 100 μL of such dilution was evenly spread onto a 7H10 plates with 0.2% (v/v) glycerol for 2 days at 37°C. The bacterial CFU assay was performed to make a count of bacteria colonies.

Investigation of immune responses. The cellular response of *M. smegmatis* infected macrophages to CIF or CpG was investigated by analyzing the expression of pro-inflammatory cytokines, including TNF-α and IL-6. The treatment of cell as the same with those for inhibition of *M. smegmatis* proliferation in macrophage, and the cytokines measurement procedure was the same as mentioned above.

Data analysis. All experiments were carried out using at least three biological replicates. Quantitative data in this study were presented as mean ± standard deviation (SD). Two-sided unpaired *t*-tests was employed to assess difference between two groups, and statistical analysis among multiple comparisons was calculated using ne-way ANOVA with post-hoc correction. A *p-value* less than 0.05 ($P < 0.05$) was considered statistically significant. GraphPad Prism software or Origin software was used to perform Statistical analyses.

Supporting figures

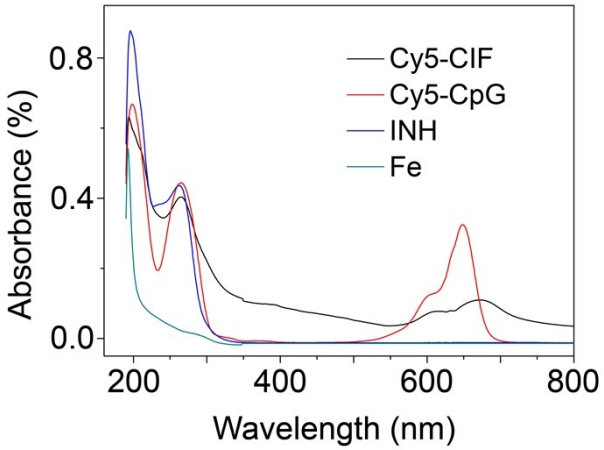

Figure S1. UV−Vis absorption spectrum of the CIF NPs. The appearance of absorbance at 260 cm and 640 nm indicates the presence of the DNA and INH load in the CIF NPs.

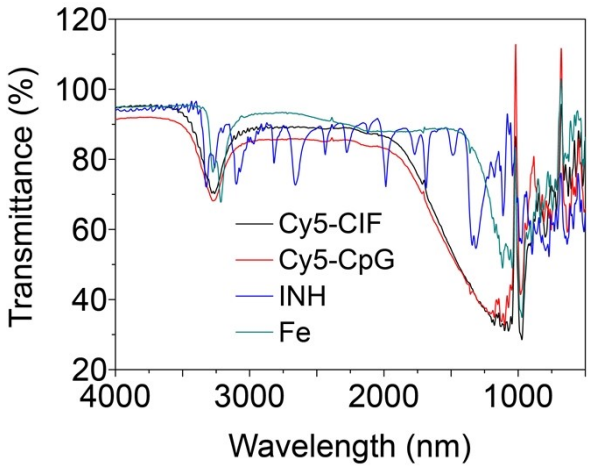

Figure S2. FTIR characterization of on-step synthesis of CIF NPs.

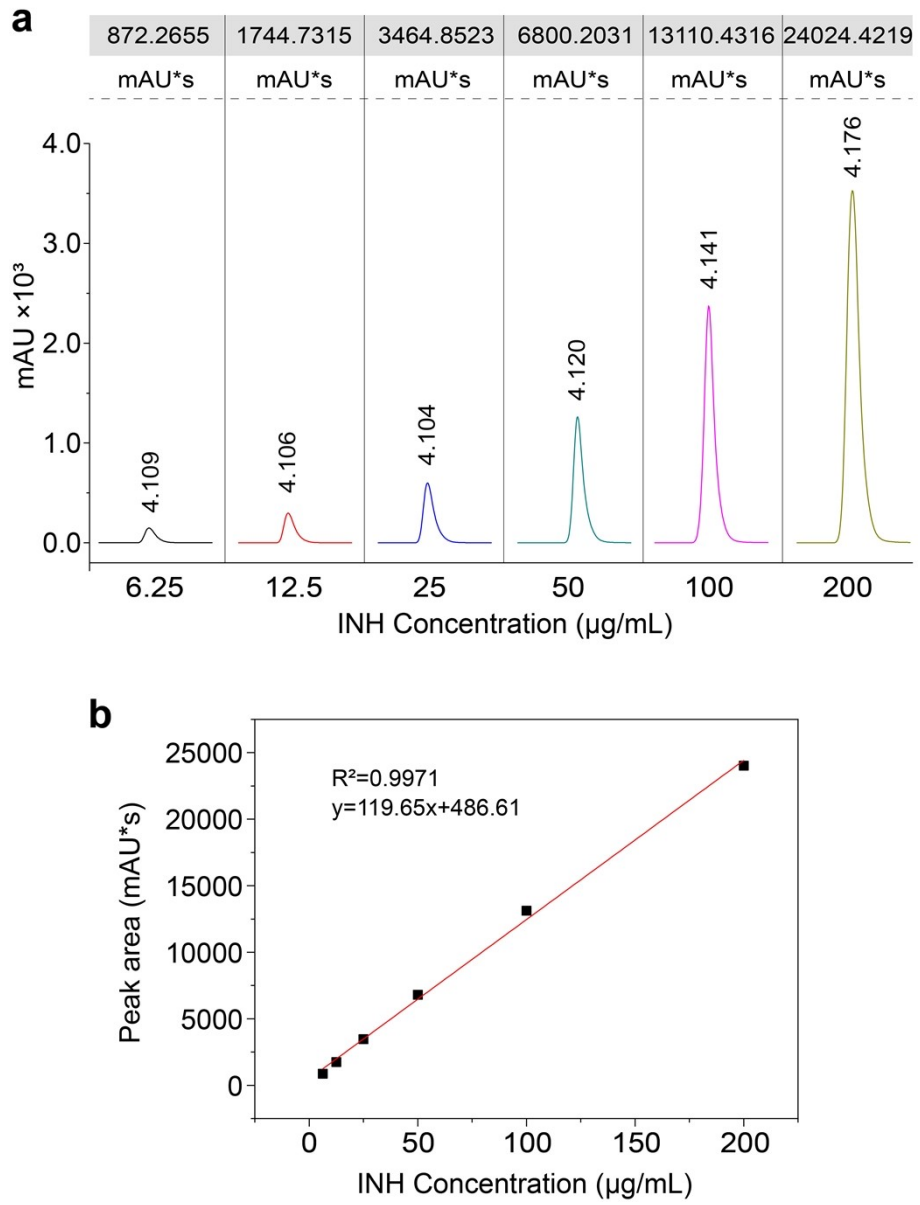

Figure S3. (a) HPLC analysis of INH with different concentrations. (b) Standard curve of INH.

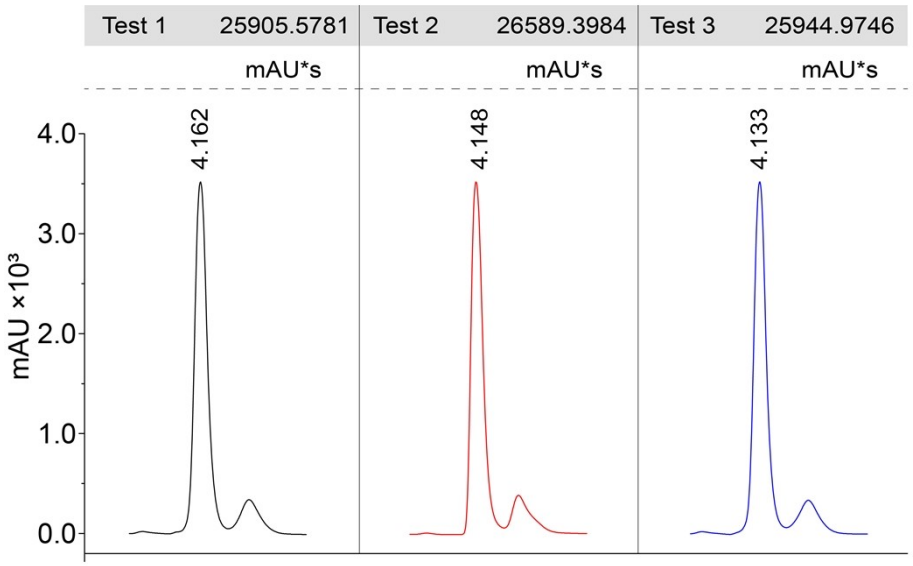

Figure S4. Quantification of INH by HPLC for evaluation of drug loading efficiency.

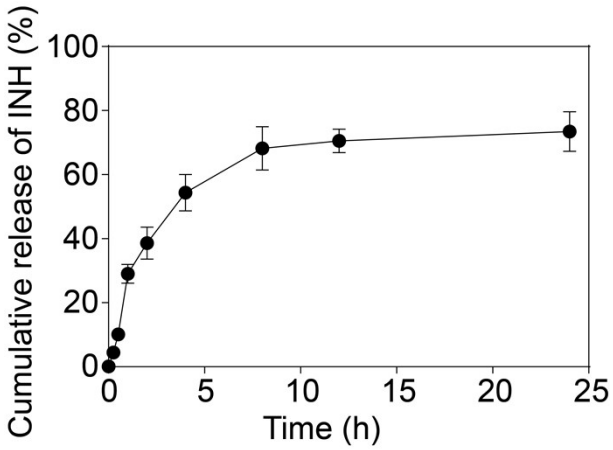

Figure S5. Cumulative INH release efficiency of CIF in culture medium at 37°C.

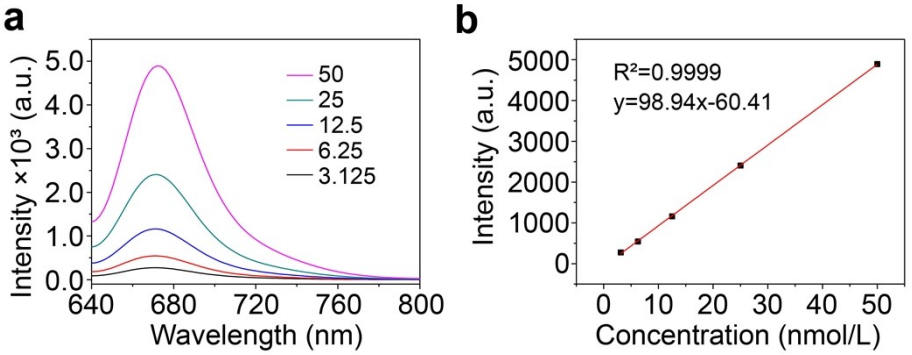

Figure S6. (a) Fluorescence analysis of Cy5-CpG with different concentrations. (b) Standard curve of Cy5-CpG.

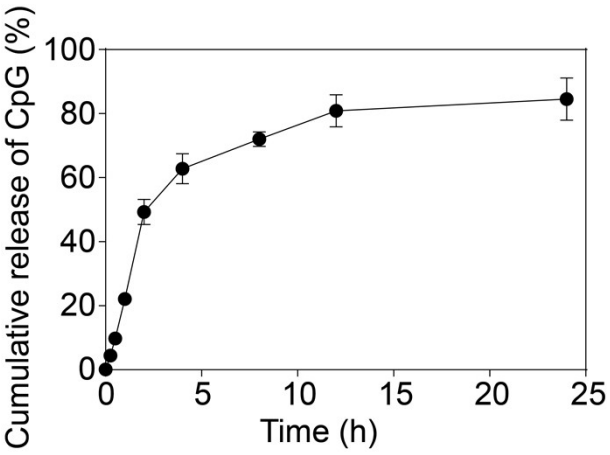

Figure S7. Cumulative Cy5-CpG release efficiency of CIF in culture medium at 37°C.

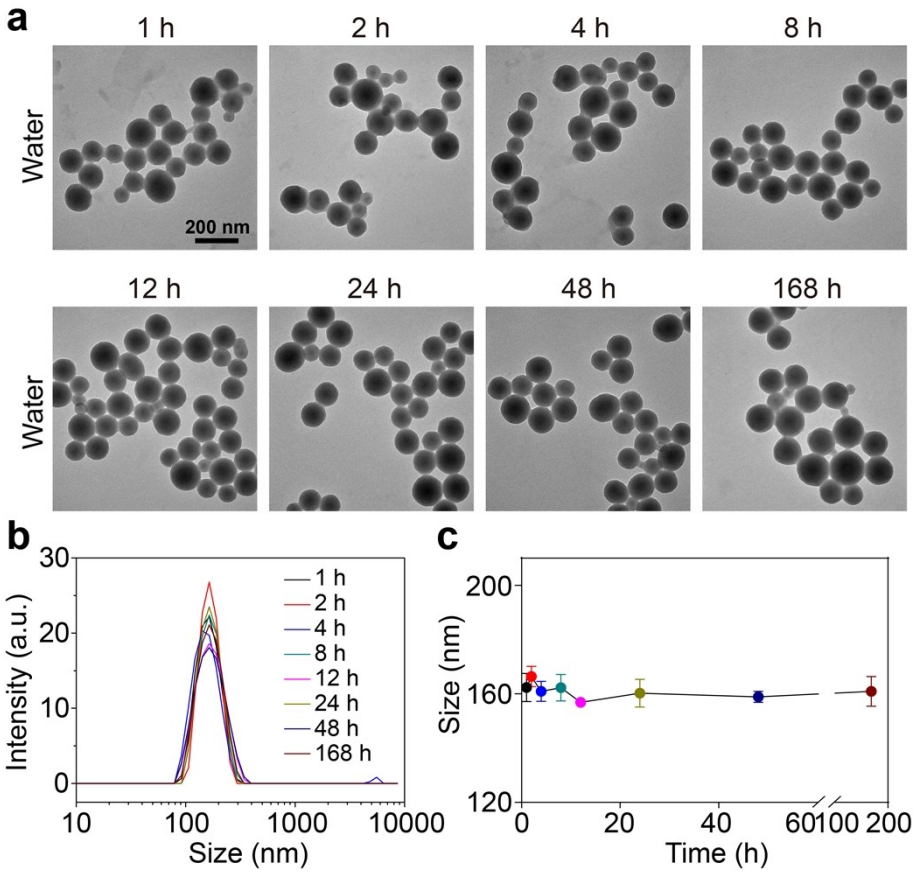

Figure S8. (a) TEM images of CIF in water in different time points. (b) DLS measurement of CIF incubated in water conditions at various times, and (e) the corresponding Z-average size distributions.

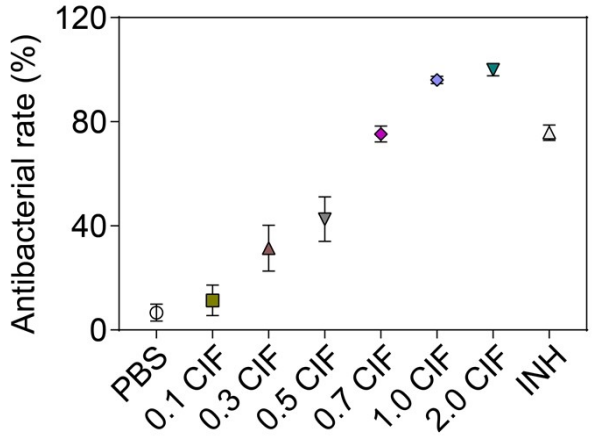

Figure S9. Bacterial suppression results for the OD600 tests.

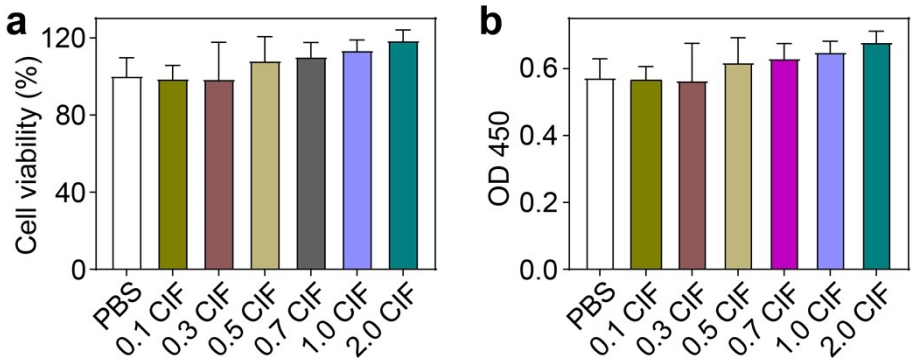

Figure S10. (a) Cytotoxicity assessment of CIF NPs. Cell viability of RAW264.7 cell after 24 h incubation with CIF NPs at concentration of 0.1, 0.3, 0.5, 0.7, 1.0 and 2.0 times for 24 h, respectively. The cell viability remains more than 80% in each case, indicating a good cellular compatibility of CIF NPs. (b) The absorbance at 450 nm measured by microplate reader.

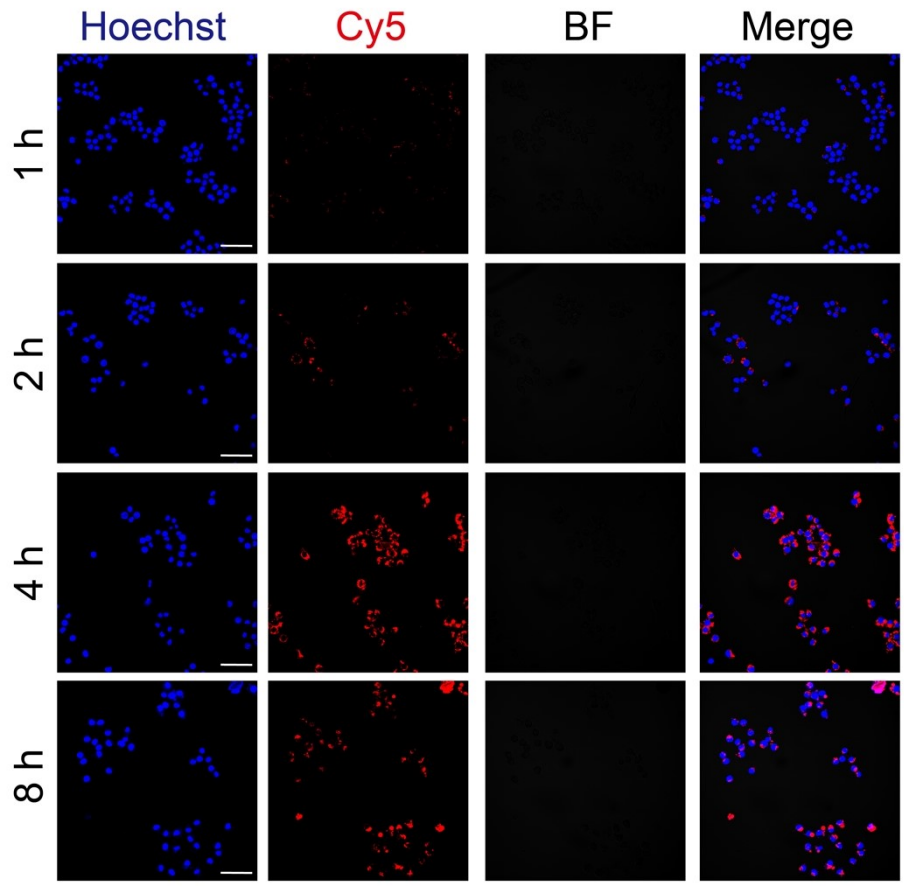

Figure S11. Confocal microscopy images of RAW264.7 cell treated with CIF NPs for different times. Scale bar: 50 μm.

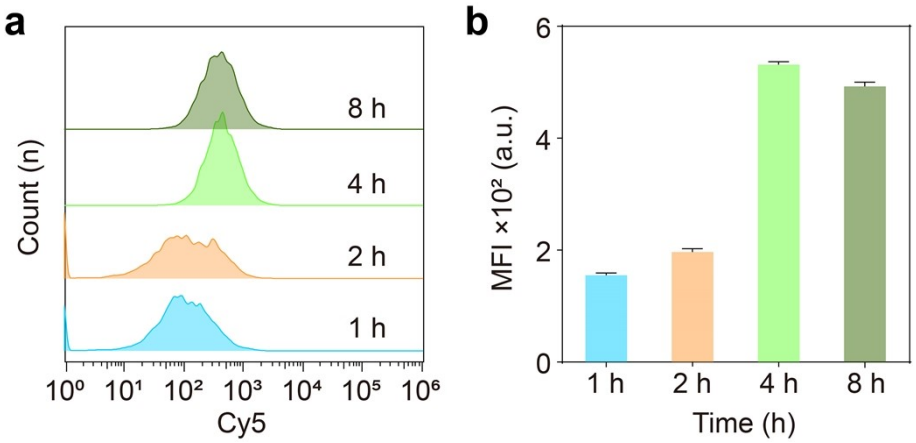

Figure S12. (a) Flow cytometric analysis of the cellular uptake of CIF NPs along with different times. (b) Flow cytometry quantification of cellular uptake of CIF NPs upon different times, obtained from the cell in (a). Data are represented as means ± SD (n = 5).

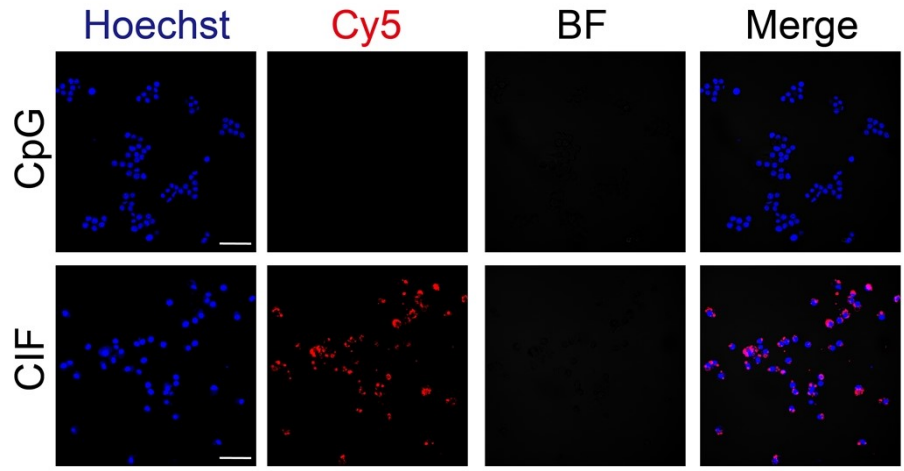

Figure S13. Confocal microscopy images of RAW264.7 cell treated with free CpG or CIF NPs for 4 h. Scale bar: 50 μm.

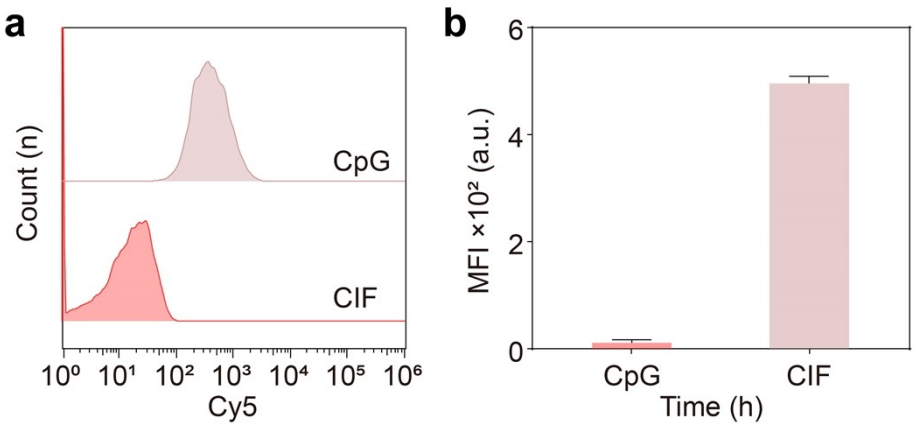

Figure S14. (a) Flow cytometric analysis of the cellular uptake of free CpG or CIF NPs for 4 h. (b) Flow cytometric quantification of cellular uptake of free CpG or CIF NPs upon 4 h, obtained from the cell in (a). Data are represented as means ± SD (n = 5).

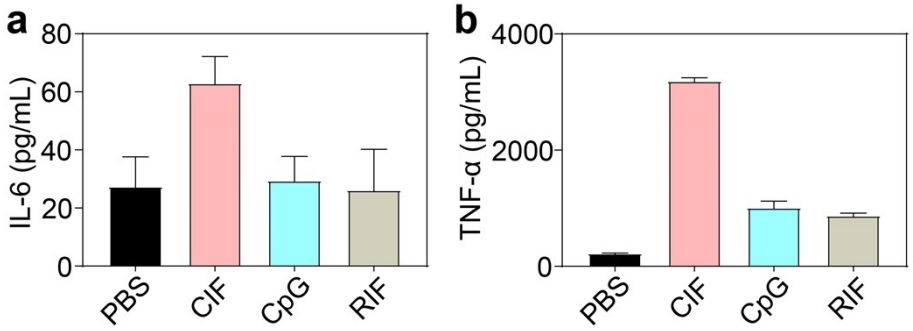

Figure S15. Cytokine secretion by RAW264.7 cells upon treatment with indicated materials at a DNA concentration of 5×10^{-7} M. The generation of (a) IL-6 and (b) TNF-α were measured at 24 h after the initial 8 h incubation. Data are represented as means ± SD, n = 4.

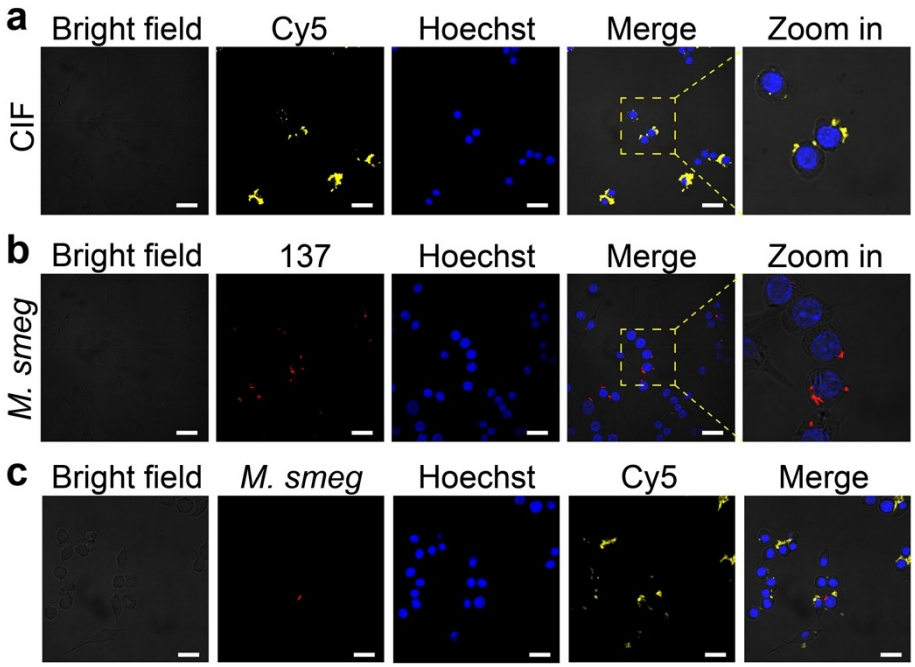

Figure S16. (a) Confocal microscopy images of RAW264.7 cell treated with CIF NPs. (b) Confocal images of RAW264.7 macrophages infected with *M. smegmatis*. (c) CLSM image of RAW 264.7 cell infected with *M. smegmatis* and treated with CIF NPs. Scale bar: 20 μm.