Supporting Information

Metal-Organic Framworks supported immunostimulatory oligonucleotides for enhanced immune response and imaging

Yan Zhang, Chaoqun Liu, Faming Wang, Zhen Liu, Jinsong Ren and Xiaogang Qu**

Materials and Instrumentation

Iron chloride hexahydrate ($FeCl_3$•$6H_2O$) and terephthalic acid (1, 4-BDC) were purchased from Aladdin (Shanghai, China). TNF-α standard, purified anti-mouse TNF-α, biotin conjugated anti-mouse TNF-α, IL-6 standard, purified anti-mouse IL-6, biotin conjugated anti-mouse IL-6 and avidin-HRP were obtained from eBioscience. The synthetic CpG oligonucleotides were ordered from Shanghai Sangon Biological Engineering Technology (China) and the sequence was as follow: 5'-TCC ATG ACG TTC CTG ACG TT-3'. All others chemicals used in this study were of commercially available analytical grade and used without further purification. Nanopure water (18.2 MΩ; Millpore Co., USA) was used throughout the experiment. Transmission electron microscope (TEM) measurements were carried out on a JEOLJEM-2010EX transmission electron microscope with a tungsten filament at an accelerating voltage of 200 kV. Scanning electron microscope (SEM) images were obtained with a Hitachi S-4800 FE-SEM. FT-IR analyses were carried out on a Bruker Vertex 70 FT-IR Spectrometer. Fluorescence measurements were carried out using a JASCO FP-6500 spectrofluorometer (Jasco International Co., Japan). UV-Vis spectroscopy was carried out with Cary 300 UV-Vis spectrometer. Fluorescence images were captured using an Olympus BX-51 optical equiped with a CCD camera.

Experimental Section

Preparation of MIL-101(Fe): 1.350 g (4.90 mmol) of $FeCl_3$•$6H_2O$ and 0.412 g (2.48 mmol) of 1, 4-BDC were dissolved in 30 mL DMF. Then the mixture was transferred to Teflon-lined stainless steel bomb and heated at 110 °C for 20 h. The resulting product was centrifuged for

10 min at 10 000 rpm and purified by a double treatment in ethanol at 60 °C for 3 h. Finally, the MIL-101(Fe) nanoparticles were activated by drying under vacuum at 120 °C for 4 h.

Preparation of MIL-101(Fe)-CpG: MIL-101(Fe) nanoparticles (50 μg mL^{-1}) were mixed with CpG ODNs (4 μM) in nanopure water. The complexes could be formed after being incubated at room temperature for 30 min. The product was obtained by centrifugation. The supernatant of the dispersions was analyzed by UV–Vis spectrophotometry at 260 nm. The amount of CpG ODN attached on the nanoparticles can be calculated.

Cell culture: The RAW264.7 cells were incubated with Dulbecco's modified Eagle's medium (DMEM) complemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units mL^{-1}), $NaHCO_3$ (1.5 g L^{-1}), streptomycin (100 mg mL^{-1}), glutamine (4 mmol L^{-1}) and glucose (4.5 g L^{-1}) at 37 °C, 5% (v/v) CO_2 in air. The media was changed every two days, and the cells were digested by trypsin and resuspended in fresh complete medium before plating.

Cytotoxicity Analysis: RAW264.7 cells were seeded into 96-well plates at a density of 5000 cells per well (100 μL) and maintained for 24 h. Then, MIL-101(Fe) or MIL-101(Fe)-CpG with various concentrations were added to the cell culture medium and incubated with cells for 24 h in the CO_2 incubator. After that, the media was removed and the cells were washed with PBS twice. To determine toxicity, 10 μL of MTT solution was added to each well of the microtiter plate to a final volume of 100 μL and the plate was incubated in the CO_2 incubator for an additional 4 h. The media was removed and DMSO (100 μL) was added into each well. Absorbance values of formazan were determined at 490/630 nm with a Bio-Rad model-680 microplate reader.

Analysis of the cellular uptake efficiency by flow cytometry: The cellular uptake efficiency was analysized by flow cytometric assay. RAW264.7 cells were seeded in a 6-well plate and maintained for 24 h. Then the cells were incubated with PBS, FAM-CpG or MIL-101(Fe)-FAM-CpG for 4 h at 37 °C, respectively. After that, the cells were washed three times with PBS to remove the residual materials and analyzed by flow cytometry.

Endolysosome/CpG colocalization fluorescent imaging analysis: RAW264.7 cells were seeded into 12 mm sterile coverslips in a 24-well plate and maintained for 24 h. Then the cells were incubated with FAM-CpG or MIL-101(Fe)-FAM-CpG for 4 h at 37 °C. After that, the cells were washed several times with PBS to remove the residual materials. And then cells were stained with Lysotracker Red for 20 min. Cell imaging was then carried out after washing cells with PBS. The images were captured using an Olympus BX-51 optical equipped with a CCD camera.

Cytokine assays: RAW264.7 cells were seeded into 24-well plates for 24 h, then cells were washed several times with PBS before treatment with indicated conditions for 8 h (TNF-α) or 24 h (IL-6). The cells were incubated with PBS, 2 μM CpG, 100 μg mL^{-1} MIL-101(Fe) and 100 μg mL^{-1} MIL-101(Fe)-CpG, respectively. The media were collected and centrifuged at 4 °C with the speed of 8,000 rpm for 15 min. The supernatants were stored at -80 °C before assay. The levels of TNF-α (IL-6) in the supernatants were determined by enzyme-linked immune sorbent assay (ELISA) using antibody pairs specific to these cytokines following protocols recommended by the manufacturer.

In vivo immunostimulatory experiments: Balb/c mice (18-22 g) were purchased from Laboratory Animal Center of Jilin University (Changchun, China) and all animal procedures were in accord with the guidelines of the Institutional Animal Care and Use Committee. The mice were divided into different groups with five mice in each group. Mice were immunized with PBS, 2.5 nmol of CpG or 0.125 mg of MIL-101(Fe)-CpG, respectively, through tail-vein-injection. The blood samples were taken from retro-orbital plexus of mice at 3 h after injection and the sera stored at −80 °C until assays.

In Vitro and In Vivo Magnetic resonance imaging:

For in vitro experiment, MIL-101(Fe)-CpG nanoconjugates with expected different concentrations as contrast agent were placed in a series of Eppendorf tubes for T_2-weighted MR imaging. The Eppendorf tubes were scanned in a 1.5 T Magnetom Espree MIR system

(Siemens). RAW264.7 cells were seed into culture plates and then cultured for 24 h at 37°C under 5% CO_2. Then, the media was removed and replaced with fresh media containing 250 μg mL^{-1} MIL-101(Fe)-CpG nanoconjugates. After co-incubation for 4 h, the cells were washed three times with PBS and trypsinized. Finally, the cells were resuspended in 1 mL PBS and centrifuged at 3,000 rpm for 15 min to obtain cell pellets. The MRI testes were performed with a Huantong 1.2 T MR scanner (HT-MSRI50-50KY, shanghai huantong kejiao).

For in vivo T_2-weighted MR imaging, the Balb/c mice were first anesthetized by intraperitoneal injection of chloral hydrate solution (10 wt%), and then 200 μL of 250 μg mL^{-1} MIL-101(Fe)-CpG dispersion in PBS buffer solution was administrated subcutaneously into a mouse. Then, the T_2-weighted images were acquired. To establish the tumor-bearing mouse model, a subcutaneous model of liver cancer was established by injecting 1×10^6 mouse H22 hepatocellular carcinoma cells in 100 μL of PBS (pH 7.4) subcutaneously at the right axillary fossa of female Balb/c mice. The MRI experiments were performed on anesthetized tumor-bearing Balb/c mice. The solution of MIL-101(Fe)-CpG nanoconjugates (250 μg mL^{-1} 200 μL) was injected into the tumor of the mouse. The mouse was scanned before and after the administration.

Biodistribution of MIL-101(Fe)-CpG: Kumming mice (n=5 each time point) were intravenous injected with 0.25 mL of 0.9% NaCl solution containing MIL-101(Fe)-CpG (1 mg mL^{-1}). At different timed intervals the content of Fe ions in the major organs of injected mice were analyzed using inductively coupled plasma–mass spectrometry (ICP-MS). In detail, above tissues were harvested and melted via heat treatment (80 °C) in aqua regia.

Histology analysis and liver damage-associated serum biochemical assay: Mice were sacrificed 2 days after intravanous administration of 0.25 mL 0.9% NaCl solution containing MIL-101(Fe)-CpG (1 mg mL^{-1}) and 0.25 mL of 0.9% NaCl solution. Main organs including heart, liver, spleen, lung and kidney were collected. Above organs were then fixed in 10%

neutral buffered formalin. After careful preparation, collected organs were embedded in paraffin, sectioned (4 mm thick), as well as stained with hematoxylin and eosin (H&E). The histological sections were observed under an optical microscope. Blood of above mice was collected to perform blood biochemical assay different periods after injection of MIL-101(Fe)-CpG nanoconjugates and 0.9% NaCl solution.

Statistical analysis

All data were expressed in this article as mean result ± standard deviation (SD). All figures shown in this article were obtained from three independent experiments with similar results. The statistical analysis was performed by using Origin 8.0 software.

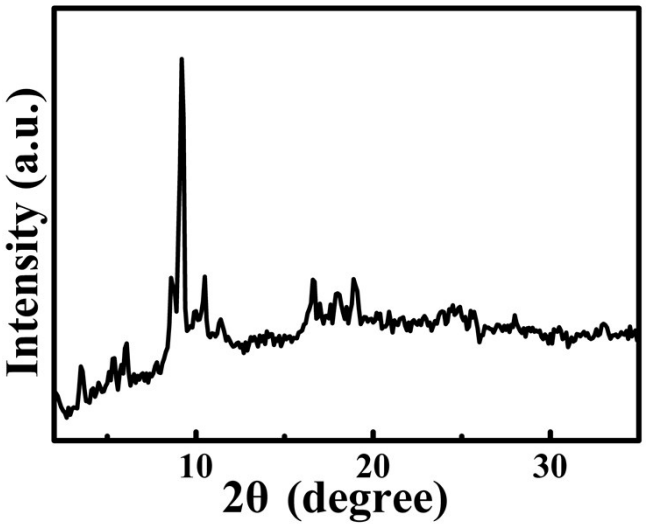

Figure S1. XRD pattern of MIL-101(Fe).

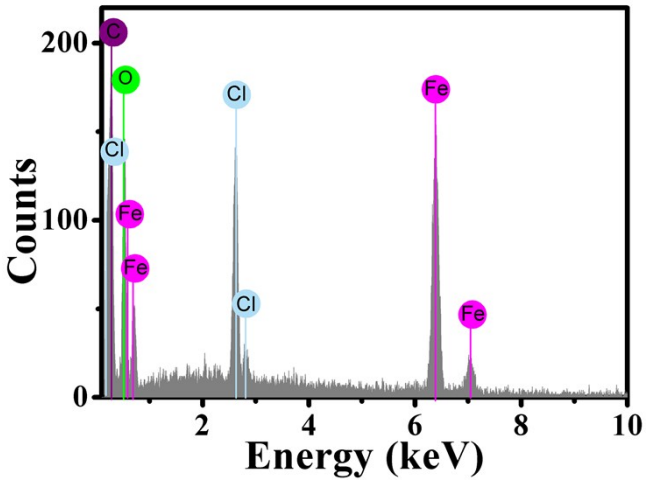

Figure S2. EDS spectrum of MIL-101(Fe).

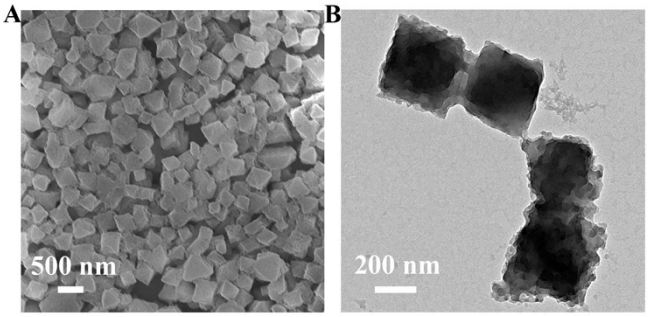

Figure S3. SEM image (A) and TEM image (B) of MIL-101(Fe)-CpG nanoconjugates.

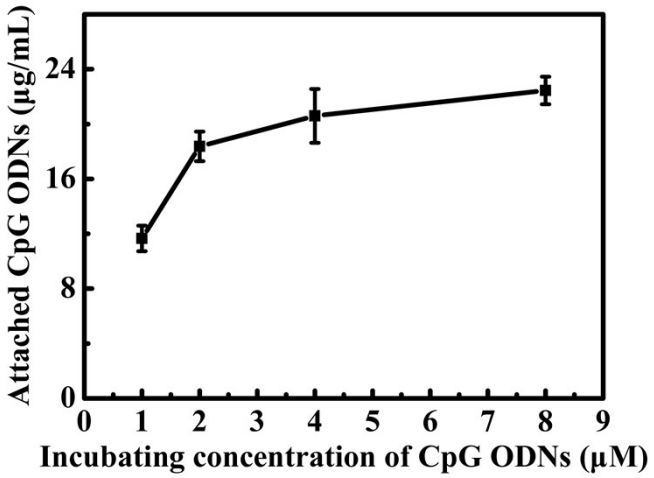

Figure S4. The amount of attached CpG ODNs on MIL-101(Fe) at different conditions. MIL-101(Fe) nanoparticles (50 μg mL^{-1}) were mixed with different concentrations of CpG ODNs (1 μM, 2 μM, 4 μM, 8 μM) in nanopure water at room temperature for 30 min. With the increase of the incubating concentration, the amount of attached CpG ODNs increased and gradually reached the maximum adsorption capacity. So we chose 4 μM as the incubation concentration for the formation MIL-101(Fe)-CpG nanoconjugates.

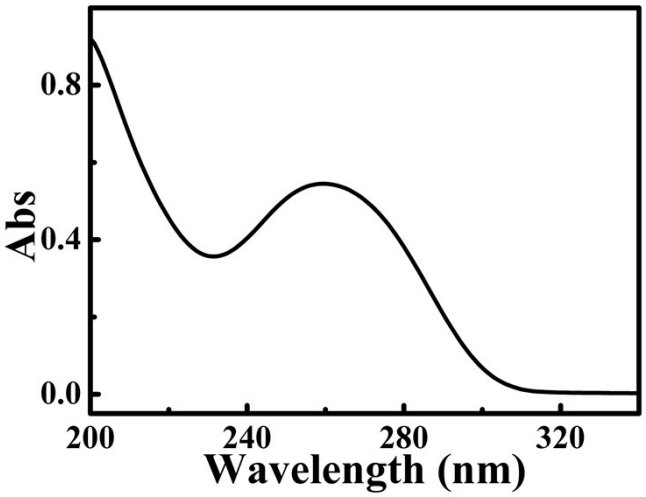

Figure S5. UV absorption spectrum of the supernatant of CpG ODNs after being adsorbed. The amount of CpG ODNs in the supernatant can be calculated by the following equation:

$$n_{CpG} = \frac{A_{260} - A_{340}}{\varepsilon * l} * V$$

Where A_{260} (A_{340})is the UV absorbance of the CpG ODNs in the supernatant solution at 260 nm (340 nm); ε is the molar extinction coefficient of CpG ODNs, which is 181100 L $(mol \cdot cm)^{-1}$; l is the length of quartz cuvette, which is 1 cm; V is the volume of solution.

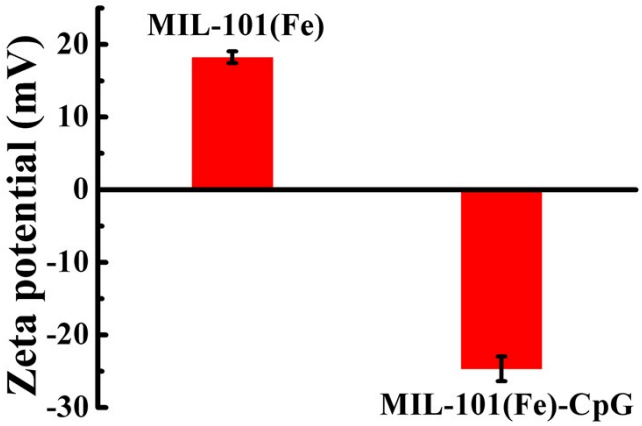

Figure S6. Zeta potentials of MIL-101(Fe) and MIL-101(Fe)-CpG.

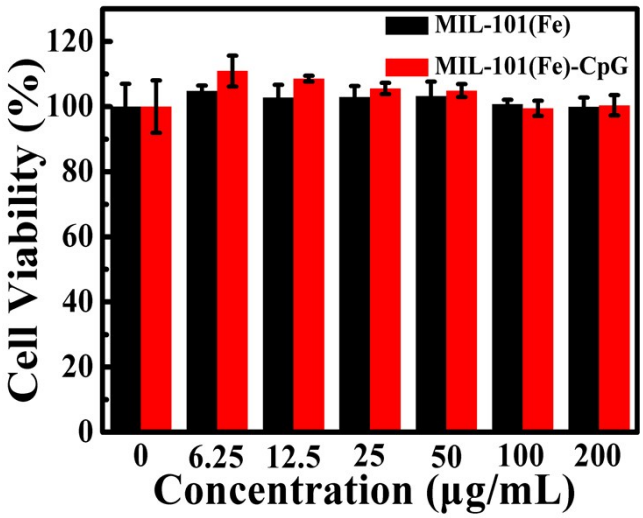

Figure S7. In vitro viability of RAW264.7 cells in the presence of MIL-101(Fe) and MIL-101(Fe)-CpG.

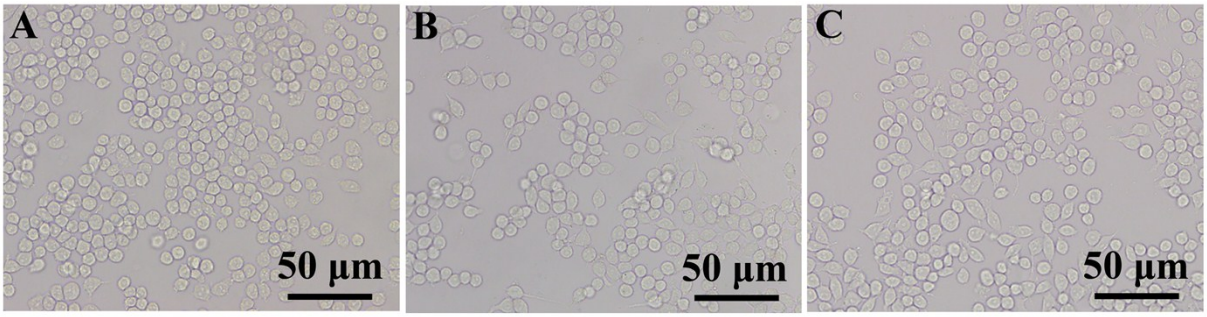

Figure S8. Microscope images of RAW264.7 cells incubated with cell medium (A), MIL-101(Fe) (B) and MIL-101(Fe)-CpG nanoconjugates (C) for 24 h.

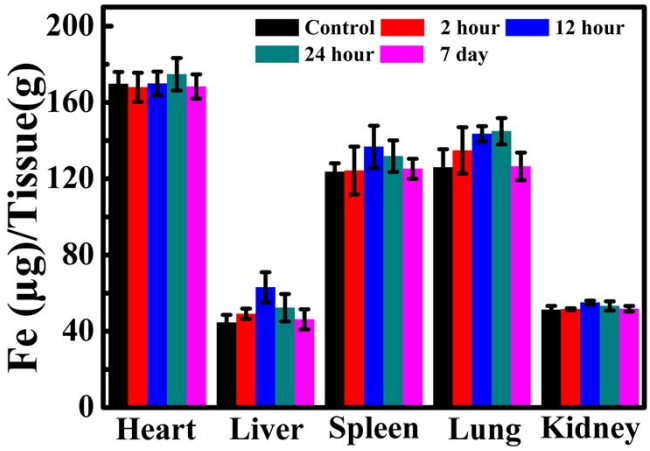

Figure S9. The biodistribution of MIL-101(Fe)-CpG nanoconjugates in major organs at different time points post-injection.

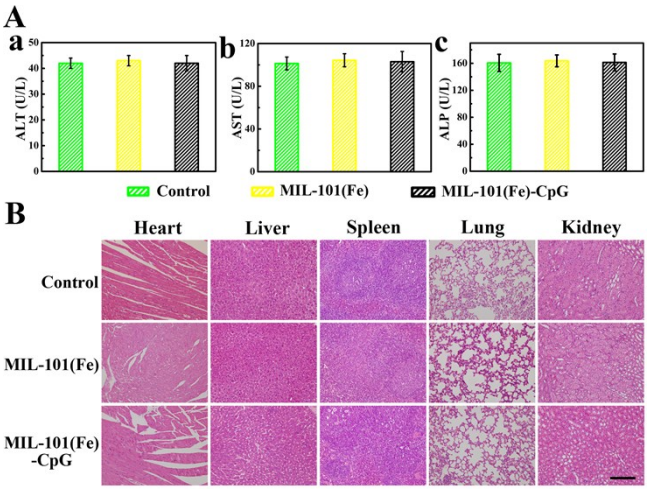

Figure S10. A) Blood biochemical assay of mice after intravenous administration of 0.9% NaCl solution, MIL-101(Fe) and MIL-101(Fe)-CpG nanoconjugates: (a) alanine aminotransferase (ALT), (b) aspartate aminotransferase (AST), (c) alkaline phosphatase (ALP). B) Histological analysis of mice after intravenous administration of 0.9% NaCl solution, MIL-101(Fe) and MIL-101(Fe)-CpG nanoconjugates.

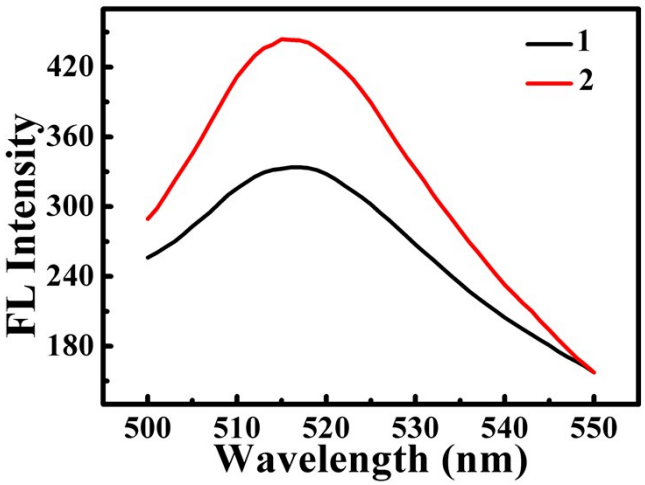

Figure S11. Fluorescence spectra of FAM-CpG and MIL-101(Fe)-FAM-CpG under the excitation wavelength of 488 nm.

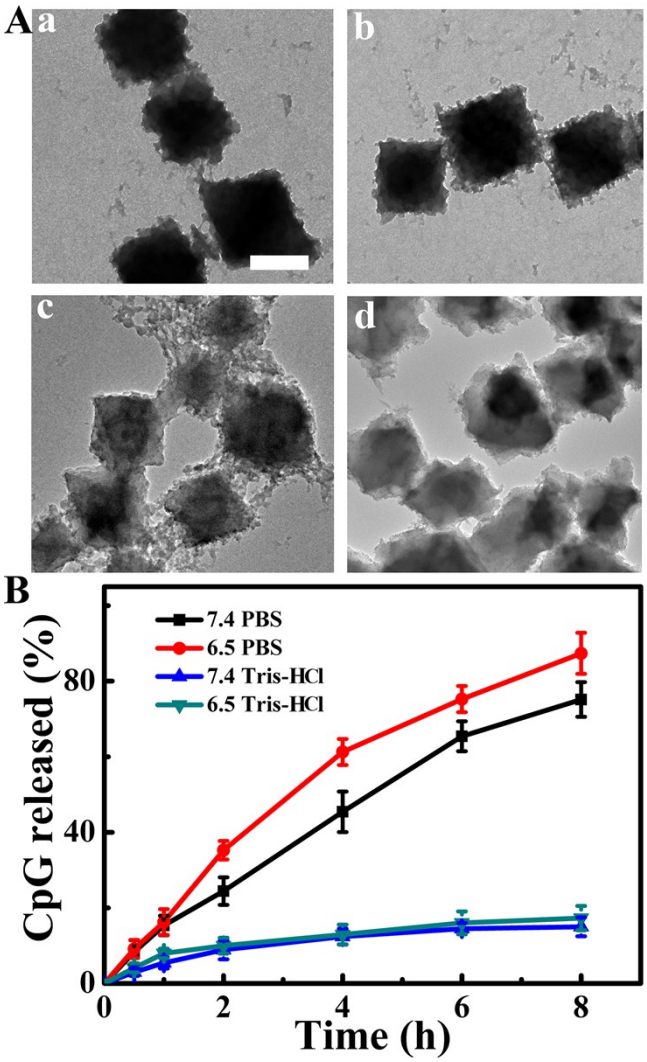

Figure S12. A) TEM images of MIL-101(Fe)-CpG nanoconjugates after incubating in 2 mM pH 7.4 Tris buffer (a), pH 6.5 Tris buffer (b), pH 7.4 PBS buffer (c) and pH 6.5 PBS buffer (d) for 4 h. Scale bar was 200 nm. B) The release of CpG ODNs from MIL-101(Fe)-CpG ODNs in different conditions. The FAM-CpG attached MIL-101(Fe) nanoparticles were used and the released CpG ODNs was calculated by fluorescence spectra.

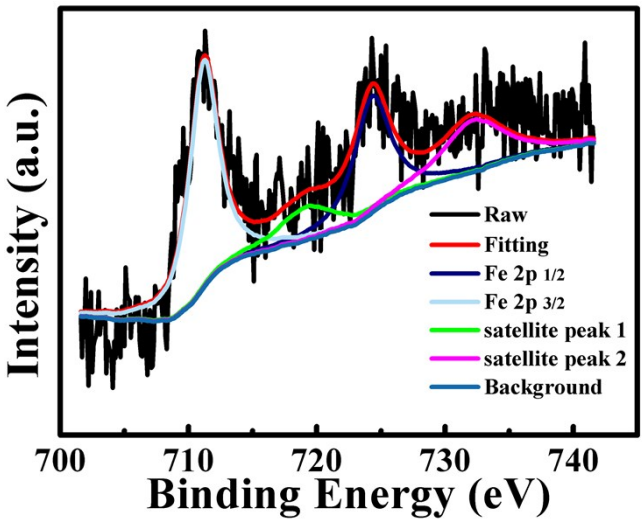

Figure S13. The XPS Fe 2p analysis of MIL-101(Fe).

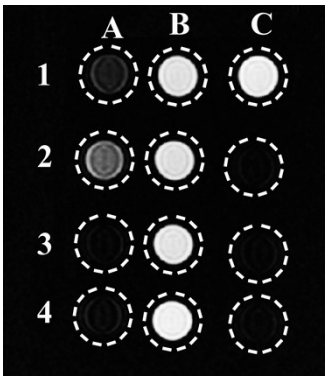

Figure S14. A: T_2-MRI images of MIL-101(Fe)-CpG nanoconjugates in pH 7.4 PBS buffer (1), pH 6.5 PBS buffer (2), pH 7.4 Tris-HCl buffer (3) and pH 6.5 Tris-HCl buffer (4), respectively. B: T_2-MRI images of pH 7.4 PBS buffer (1), pH 6.5 PBS buffer (2), pH 7.4 Tris-HCl buffer (3) and pH 6.5 Tris-HCl buffer (4), respectively. C: T_2-MRI images of water (1), MIL-101(Fe)-CpG nanoconjugates containing different amount of CpG ODNs (0 μmol g^{-1} (2), 12 μmol g^{-1} (3) and 20 μmol g^{-1} (4)).

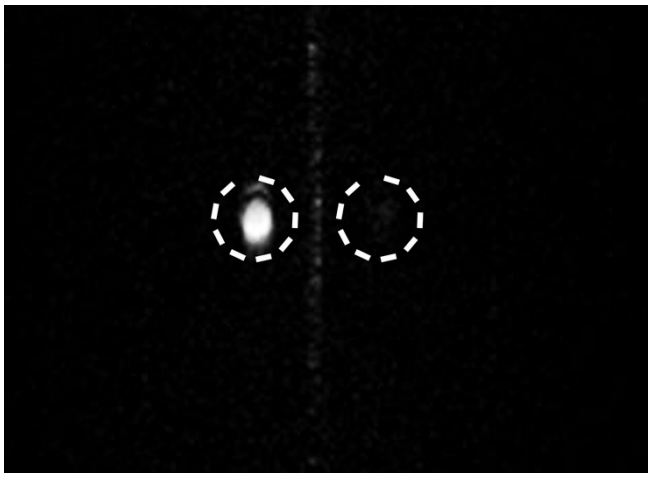

Figure S15. T_2-MRI images of RAW264.7 cell pellets incubated without (left) and with (right) MIL-101(Fe)-CpG nanoconjugates.

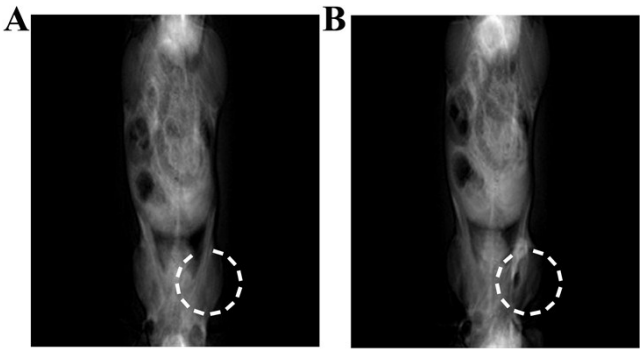

Figure S16. In vivo coronal view MRI images of a normal Balb/C mouse before (A) and after (B) subcutaneous injection of MIL-101(Fe)-CpG nanoconjugates.